HIGHER CADMIUM AND ZINC ACCUMULATION IN PARSLEY (PETROSELINUM CRISPUM) ROOTS ACTIVATES ITS ANTIOXIDANTS DEFENSE SYSTEM

Toxic Effects of Zinc and Cadmium and the Role of Enzymatic and Non-enzymatic Antioxidants to Alleviate the Stress in Parsley (*Petroselinum crispum*)

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Abstract

Heavy metals have become a prominent pollutant of the present era. Zinc (Zn) and cadmium (Cd) are heavy metals whose minerals often occur in combination and accumulate into the soil, greatly damaging plant growth. Plant counteracts these challenges by showing enzymatic and non-enzymatic responses. Parsley (*Petroselinum crispum*) is a biennial herb that exhibits various biological and medicinal activities. The present study focuses on investigating the effects of Zn and Cd on *P. crispum* physiological parameters, enzymatic profile in root and leaves, and metal uptake in shoot and root. For this, *P. crispum* was exposed to Zn at 0, 50, 100, 150, and 200 µM, and Cd at 0, 10, 20, 40, and 80 µM, in soil less Hoagland's solution. The results showed that Zn and Cd stress decreased the growth of *P. crispum* significantly and increased the contents of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and electrolyte leakage (EL). Still, the Zn-induced oxidative damage was more pronounced than Cd stress, as showed by higher H_2O_2 and EL. Various concentrations of Zn and Cd significantly increased the uptake and accumulation of metals in the *P. crispum*. However, the accumulation was significantly higher in roots compared to shoots. Non-enzymatic antioxidant activities, like total phenolic contents (TPC) and ferric reducing antioxidant power (FRAP), were induced significantly strongly in leaves of *P. crispum* only upon Cd stress. In contrast, total flavonoid contents (TFC) were decreased upon Zn and Cd stress of *P. crispum* leaves and roots. Moreover, Zn and Cd stress-induced higher antioxidant enzymes activities for superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), Glutathione peroxidase (GPx), as compared to control conditions. SOD and APX activities were more strongly induced in leaves of *P. crispum* upon Zn stress than Cd stress. Glutathione S-transferase (GST) was the only antioxidant enzyme whose activity was decreased upon Zn and Cd stress. These findings revealed that *P. crispum* has more serious oxidative damage upon Zn stress but exhibited a better tolerance due to an efficient enzymatic antioxidant mechanism than Cd stress. *P. crispum* tried to keep the metals in roots to avoid damage to leaves cellular machinery. This research study provides information about Zn and Cd phytotoxic effects and tolerance in *P. crispum*, which is used as an essential medicinal plant in many parts of the world.

Keywords: Parsley, zinc, cadmium, heavy metals, plant enzyme, metal uptake

Introduction

Heavy metals are a severe threat to the ecosystem in the present era. Zinc (Zn) and cadmium (Cd) are heavy metals whose minerals frequently accompany each other in the earth's crust and enter the ecosystem through anthropogenic activities. Cd is the most toxic and non-essential metal for the plant, whereas Zn is an essential microelement (kabata-Pendias, 2011). Still, a higher concentration of both metals can have phytotoxic effects on plants. Zn and Cd accumulate in plant tissues, which ultimately affects human health due to the consumption of contaminated food through the food chain (soil-plant-human) (Hussain et al., 2021a). Plants mostly uptake Zn and Cd as a divalent cations. Apoplastic and symplastic pathways are responsible for the transport of Cd through the roots to leaves (Lux et al., 2011). Cd affects the plant's growth decreases the chlorophyll contents and photosynthetic activity resulting in necrotic and chlorotic leaves (Li et al., 2008; Rizwan et al., 2016). Due to chemical similarity, Cd also interferes with plant uptake of Zn, which further contributes to leaf chlorosis (Xu et al., 2017). Zn plays a vital role in the catalytic activity of many enzymes and protein structural stability (Broadley et al., 2007). Zn plays a fundamental role in protecting and stabilizing the many biological membranes against oxidative and peroxidative damages (Aravind and Prasad, 2003). However, the higher concentration of Zn in the plants caused various physiological damage like reduced growth and poor root system development (Wang et al., 2009; Szopinski et al., 2019). On the cellular level, the higher concentration of both metals (Zn/Cd) triggers the production of reactive oxygen species (ROS), which causes oxidative damage such as lipid peroxidation in the plant (Jin et al., 2008; Farooq et al., 2020; Qin et al., 2020; Hussain et al., 2021a,b). Production of ROS is lethal for protein structure and damages the DNA content of the plant body. Natural antioxidants are likely to prevent the damage of cellular and sub-cellular components either by an enzymatic or non-enzymatic antioxidant mechanism. The scavenging system for ROS contains enzymes including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and glutathione s-transferase (GST). In contrast, the non-enzymatic response of the plant antioxidant system comprises the carotenoid, phenolics, and flavonoids components (Haida and Hakiman, 2019; Hussain et al., 2021a). Higher Cd accumulation with reduced growth along with hydrogen peroxide (H2O2) contents, electrolyte leakage (EL), and malondialdehyde (MDA) concentration in two cultivars of Sorghum bicolor (Hassan et al., 2020). One cultivar (e JS-2002) showed a better antioxidant response and Cd stress tolerance. Certain plant species have been reported effective antioxidants in response to Zn and Cd stress (Szopinski et al., 2019; Hussain et al., 2021a). Luyckx et al. (2021b) reported that Cd and Zn increased the overall concentration of antioxidant enzymes via the tricarboxylic acid (TCA) cycle. However, different plant species express different physiological responses to deal with Zn and Cd's external toxic concentrations (Luyckx et al. 2021; Luyckx et al. 2021b).

Parsley (*Petroselinum crispum*, Apiaceae) is an annual herb. It serves as an essential source of specific vitamins and essential metals in our diet (Zhai et al., 2015). The presence of certain phytochemicals like flavonoids, carotenoids, ascorbic acid, and tocopherol in *P. crispum* leaves is also evident (Fejes et al., 2000; Francis and Isaksen, 1989; Davey et al., 1996). The use of fresh *P. crispum* leaves as a diet supplement can decrease oxidative stress in humans (Nielsen et al., 1999). *P. crispum* essential oil showed a certain amount of antioxidants with their free radical scavenging activities (Zhang et al., 2006). Due to these valuable compounds, *P. crispum* has many medicinal usages to treat diabetes, renal diseases, constipation, jaundice, colic, flatulence edema, rheumatism, and bleeding (Jouad et al., 2001; Manderfeld and Schafer, 1997). Alamer and Fayez (2020) proposed that upon lead (Pb) stress, the *P. crispum* showed a decrease in leaf photosynthetic pigments. It was coupled with an increased concentration of MDA and lipid peroxidation, which induced the higher production of reactive oxygen species (ROS). Kripper and Schubert (2021) elevated the different levels of Zn concentration as compared to Cd in *P. crispum* tissues. They proposed that plants did not show phytotoxic symptoms due to Zn stress except the slight browning of roots of *P. crispum* upon Cd stress. They also reported Cd-induced thiol production in *P. crispum.*

Thus, scientific reports showed that *P. crispum* is a valuable plant (due to medicinal usage) that can modulate its physiological response upon different types of metal stresses. However, according to our knowledge, the reported literature regarding the toxic effects of Zn and Cd on the physiological and biochemical response of *P. crispum* is either meager or still limited. Investigation of the phytotoxic effects of Zn and Cd is also intriguing, as the combinations of both metals' minerals are pretty common in our natural environment. Therefore, the main objective of the present work was to investigate the intensity of stress injury, physiological response, activities of enzymatic antioxidants (SOD, CAT, APX, GPx, and GST), and non-enzymatic antioxidants (phenolics, flavonoids, and FRAAP) in *P. crispum*, upon exposure to the different levels of Zn and Cd stresses.

Material and Methods

Plant material and experimental procedure

The seeds of flat leave *P. crispum* were obtained from Saeed Seed Store, F-11 Markaz Islamabad, and were sown in peat moss. The plantlets (at the two-leaf stage) were transplanted to the hydroponics system of half-strength Hoagland's nutrient solution. The experiment was conducted in Greenhouse of COMSATS University Islamabad, Abbottabad Campus with photoperiod 16:8 day: nighttime at 22:25 °C. When plantlets were acclimatized (after 2 weeks), Zn stress as $ZnSO_4.7H_2O$ and Cd stress as $Cd(NO_3)_2$ were introduced incrementally in the pot. Zn's stress levels were 0-, 50-, 100-, 150-, and 200 µM, whereas Cd was managed 0-, 10-, 20-, 40-, and 80 µM with triplicate for each treatment.

Plants harvesting

Three days after the final stress was established, plants were harvested. Roots and shoots were separated, and measurements were made of respective lengths (cm) and fresh weights (g). Fresh leaf tissues were stored at -80 °C in a freezer for enzymatic and biochemical analysis. The rest of the plant material was oven-dried at 60 °C till constant weight was attained. Dried samples were used to analyze HM uptake and plants' compartmentalization. The dried samples were ground to fine powdered, and 200 mg material was digested with 10 ml of HNO_3:$HClO_4$ (4:1v/v) mixed solution at 150 °C, which was used to determine Zn and Cd concentration on Atomic Absorption Spectroscopy (Li et al., 2012). All samples were performed in biological triplicate.

Stress injury assay upon Zn and Cd stress

Chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid contents were measured spectrophotometrically as Arnon (1949) described. Leave tissue (1 g) was macerated with 20 ml of 80% acetone ml. Absorbance was taken from spectrophotometer at 645 nm, 663 nm, and 470 nm against chlorophyll a, chlorophyll b, and carotenoids, respectively (Lichtenthaler et al., 1987):

$$Chlorophyll\ a = (12.7\ \times A663) - (2.69 \times A645) \qquad (1)$$

$$Chlorophyll\ b = (22.9\ \times\ A645) - (4.68\ \times A663) \qquad (2)$$

$$Total\ chlorophyll\ content = Chlorophyll\ a + Chlorophyll\ b \qquad (3)$$

Lipid peroxidation was quantified with malondialdehyde (MDA) content, using thiobarbituric acid (TBA) and trichloroacetic acid (TCA) as described by Venkatacahalm et al. (2017). 0.1 g sample was used for extraction, and absorbance was taken at 450 nm, 532 nm, and 600 nm through a spectrophotometer. Total lipid peroxidation was determined in µM of malondialdehyde g^{-1} of fresh weight using the following equation,

$$MDA\ (\mu mol\ g^{-1}) = \frac{[6.45 \times (A532 - A600) - (0.56 \times A450)] \times Vt}{W} \qquad (4)$$

Where $V_t = 0.001$ L; W = 0.1 g.

Electrolyte leakage (EL) was determined using the methods described by Hnilickova et al. (2019) with the EC meter. Washed leaf disks were immersed into 10 ml of distilled water for 24 hours at 10 °C, and electrical conductivity (EC_1) was taken. Then the samples were incubated in the water bath for 20 min at 95 °C and allowed to cool down at 25 °C, and EC (EC_2) was taken. EL was calculated using the following formula:

$$EL = \frac{EC_1}{EC_2} \times 100 \qquad (5)$$

Non-enzymatic antioxidant assay upon Zn and Cd stress

Samples were prepared by immersing 1 g of powdered material in 25 ml of methanol. The mixture was kept for 24 hours, then centrifuged at 6000 rpm for 50 minutes. The supernatant was collected and used for further analysis. Total Phenolic Content (TPC) was determined as described by Alves et al. (2013). Methanol extracted sample (200 µl) was mixed in Folin & Ciocalteu reagent (10-fold diluted). After 5 min, 6% sodium carbonate was added and incubated for 90 min. Absorbance was measured at 725 nm against blank. Gallic acid was used as the standard for phenolic contents.

Flavonoid content was measured via the Lin et al. (2011) method with some modification. Methanol extracted sample (1 ml) was diluted and mixed with 5% Na_2CO_3. After 5 min, $AlCl_3$ (10%), NaOH (1 M), and distilled water was added to a final volume of 10 ml. Absorbance was checked at 510 nm through a spectrophotometer against the blank. RUTIN was used as a standard for flavonoid content.

Ferric Reducing Antioxidant Power (FRAP) of methanol extracted samples was measured through Huang et al. (2008). Shortly, methanol extracted solution (1 ml) was treated with 2 ml of phosphate buffer (0.2 M: pH 6.6). Afterward, 2 ml of ferricyanide (0.1%) and 2 ml of trichloroacetic acid (10%) were added and mixed. Then, 2 ml was diluted from the mixture, and 2 ml of ferric chloride

(0.01%) was added (incubation for 20 min). Absorbance was checked at 700 nm against blank via spectrophotometer. Gallic acid was used as the standard for FRAP assay.

Enzymatic antioxidant assay upon Zn and Cd stress

About 0.1 g of sample was homogenized with 1 ml of ice-cold KH_2PO_4 buffer (50 mM KH_2PO_4 and 0.5 mM EDTA with pH 7.4). The homogenized mixture was then centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was collected and stored at 4 °C, for further enzyme assays and H_2O_2 content analysis.

Superoxide dismutase (SOD) activity was measured using the method (Dhinsdsa et al., 1981). A reaction mixture was prepared with 50 mM phosphate buffer, 75 M NBT, 13 mM methionine, 2 M riboflavin, and 0.1 mM EDTA. Both mixes were placed under a 15 W fluorescent lamp for 10 min, and the reduction in NBT was measured at absorbance 560 nm.

Catalase (CAT) activity was measured by quantifying H_2O_2 content at an absorbance rate of 240 nm (Maehly, 1954) with few modifications. The enzyme extract was mixed with a reaction mixture that contained 50 mM phosphate buffer and 60 mM H_2O_2. Absorbance was taken at 240 nm, and decomposition of H_2O_2 was calculated with an extinction coefficient of 39.4 mM^{-1} cm^{-1}.

The ascorbate peroxidase (APx) activity was measured at an absorbance of 240 nm after 3 min. The protocol adopted was the work of Chen and Asada (1989). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 1.54 mM H_2O_2, 0.5 mM ascorbate mixed with enzyme extract. The extinction coefficient of 2.8 mM^{-1} cm^{-1} was used.

With few changes, Guaiacol peroxidase (GPx) activity was assayed through Upadhyaya et al. (1985). Reaction mixtures contained 50 mM phosphate buffer, 1% H_2O_2, 1% guaiacol, and 8 µl of enzyme extract. Absorbance was checked at 420 nm for 1 min. The extinction coefficient of 26.6 mM^{-1} cm^{-1} was used.

For glutathione-s-transferase **(**GST) activity, the reaction mixture contained potassium phosphate, glutathione (GSH), 1-chlro-2,4dinitrobenzene (CDNB) was mixed with enzyme extract. According to Irzyk and Fuerst (1993), GST activity was assayed with some modification. Absorbance was taken at 340 nm twice, with a time interval of 3 min. The extinction coefficient of 9.6 mM^{-1} cm^{-1} was used.

H_2O_2 quantification was carried out following the protocol of Kanwal et al. (2015) with some modifications. The reaction mixture for H_2O_2 consists of 0.05 mM potassium phosphate buffer and

0.1% Ti $(SO_4)_2$ mixed with enzyme extract. The absorbance rate was checked at 410 nm with an extinction coefficient of 0.28 μM^{-1} cm^{-1}.

Statistical analysis

The data were first analyzed using the Shapiro Wilk normality test. Once the normality was confirmed, the one-way analysis of variance (One-way ANOVA) was applied. Similarly, the principal components analysis (PCA) was performed to investigate the difference between variable data scattering upon treatment with different metals. For PCA, the principal component extraction was carried out till the Eigenvalues reached 1. The PCs having value PC values lower than 1 were considered insignificant. The variables showing higher loading values in PC 1 were colored green, while red had a higher loading value in PC2. The information related to PC extraction and cumulative variance, and component score coefficient matrix is presented in the supplementary material. Statistical analysis of the data was performed using SPSS, version 21.

Results

Plant growth parameters

After 15 days of Zn stress, plants showed stunted and restricted growth than plants in control. Leaves' color was pale green with chlorosis symptoms on most leaves. The stem's width was reduced to plants at the control level upon Zn exposure (Supplementary Fig. 1a). Cd-treated plants show a considerable toxic effect on plants with yellowing leaves, weakened stem, stunted growth, and death of leaves (Supplementary Fig. 1b.).

There is a successive decrease in shoot and root length (SL and RL, respectively) with increased Zn stress levels. After the onset of Zn stress (50 µM), SL and RL decreased by 24 % and 32%, respectively, compared to control (Table 1). Compared to control, the highest decrease was observed at 200 µM, 60%, and 70% for SL and RL (Table 1). The same declining trend in SL and RL was observed in the case of Cd stress to *P. crispum*. Decreased in SL was 14 (8.75 cm), 34 (6.73 cm), 42 (5.88 cm) and 54% (4.66 cm) upon 10, 20 40 and 80 µM of Cd stress, respectively as compared to control plants (10.20 cm). For RL, decrease was 20 (9.50), 39 (7.25), 59 (4.93) and 73% (3.26 cm) at 10, 20 40 and 80 µM of Cd, respectively as compared to control (12 cm) (Table 1).

There was a declining trend for SFW and RFW of *P. crispum* upon exposure to different Zn stress levels (Table 1). The decrease was more pronounced at 200 µM of Zn, where plants decreased their SFW to 65% of the biomass than the control. Reduction in RFW was even more apparent (85%) in plants at 200 µM of Zn than in the control plants (Table 1). Upon Cd stress, a significant decrease in SFW was observed at 10 µM with a 32% reduction compared to control. The decrease of SFW on rest Cd levels was not significantly different. RFW was initially decreased at 10 and 20 µM, with 175.8 and 145.3 mg compared to control (210.0 mg). At 40 and 80 µM, RFW was 118.3 and 80.5.5 mg, respectively (Table 1).

Different concentrations of Zn and Cd affect *P. crispum* DW (Table 1). Control plants have an SDW of 28 mg, but at 200 µM, it was 19 mg (with a 32% reduction). Upon increasing Zn stress (50, 100, and 150 µM), SDW was successively decreased (25.3 and 23 and 22.3 mg). The RDW was also negatively affected by Zn stress. Initially, there was a sudden decrease in RDW, almost 8 mg decrease at 50 µM as compared to control. Later, reduction in RDW is less pronounced. For example, 100, 150, and 200 µM have 9.33, 8.00, and 7.66 g, respectively. In the case of Cd stress, there was a decreasing trend in SDW with an increasing level of stress both in leaves and roots (Table 1).

Evaluation of Cd and Zn induced stress injury

The highest chlorophyll a and b and carotenoid contents were found at 100 µM of Zn, with 21%, 6%, and 23%, respectively, compared to control (Fig. 1a). At 200 µM, chlorophyll and carotenoid contents were decreased substantially compared to 100 µM and control. Upon Cd stress (20 µM), there was a reduction in total chlorophyll (2%), chlorophyll b (28%), and carotenoid contents (25%) as compared to control (Fig. 1b). Plants showed the highest decrease in total chlorophyll, chlorophyll a and b, and carotenoid contents with 27%, 8%, 69%, and 63%, respectively, at 40 µM of Cd compared to control (Fig. 1b). Overall, at higher Zn stress, reduction in chlorophyll contents was more evident than Cd stress, where a gradual decrease was observed (Fig. 1).

The amount of lipid peroxidation was determined in terms of malonaldehyde content µmol MDA g^{-1} of fresh weight of *P. crispum* plants (Table 2). MDA content of leaves was much higher than root and showed an increasing trend upon induction of Zn/Cd stress (Table 2). For Zn stress, leaves MDA contents were 0.012 µmol g^{-1}, 0.014 µmol g$^{-1,}$ and 0.017 µmol g^{-1} at control, 100 µM and 200 µM, respectively. Zn-induced MDA contents in roots were not significantly varied between the treatments (Table 2). Cd stress increased the MDA contents in leaves that were 0.015 µmol g^{-1} and 0.019 µmol g^{-1} at 20 µM and 40 µM, respectively in comparison with control (0.013 µmol g^{-1}), while in root the lowest content (0.005 µmol g^{-1}) was found at 20 µM, and highest (0.008

µmol g^{-1}) was at 40 µM (Table 2). Although MDA contents of *P. crispum* leaves were increased when exposed to metals, but not significantly when exposed by either Zn or Cd stress.

At 100 µM of Zn, leaves H_2O_2 contents were decreased by 12% (144.5 µmol g^{-1} FW) in comparison to control (166 µmol g^{-1} FW), whereas, in roots, the contents were increased by to 273.8 µmol g^{-1} FW as compared to control 129.0 µmol g^{-1} FW (Table 2). The highest H_2O_2 contents were at 200 µM (for both metals) was 275.8 and 251.3 µmol g^{-1} FW in leaves and roots, respectively. Cd stress did not significantly affect the H_2O_2 contents of leaves. In roots, plants showed an increase in H_2O_2 contents with 237.8 and 257.8 µmol g^{-1} FW at 20 µM and 40 µM, respectively (Table 2). Overall, there was more H_2O_2 production in leaves of *P. crispum* upon Zn stress (particularly at 200 µM) as compared to Cd stress.

EL was assessed in percentage. EL percentage at 100 µM of Zn (26%) was significantly higher as compared to control (12%) and at 200 µM (19%) (Table 2). In the case of Cd stress, the EL percentage was increased with increasing levels of Cd stress. The highest EL was at 40 µM that is 19%, whereas the lowest was found in control 14% (Table 2). EL was more pronounced upon Zn stress than Cd stress in leaves of *P. crispum*.

Non-enzymatic antioxidant assay upon Zn and Cd stress

The amount of Total Phenolic Content (TPC) in the samples was calculated from the Gallic acid calibration curve where obtained regression equation was $y = 0.0015x-0.0008$ ($R^2= 0.9944$). Upon Zn stress (100 and 200 µM), TPC was increased in leaves (688.9 and 815.6 mg GAE g^{-1} DW) and roots (319.7 and 403.9 mg GAE g^{-1} DW) as compared to respective controls (668.9-leaves and 210.6-roots; Table 2). When plants were exposed to Cd stress, TPC increased significantly in leaves with 818.5 and 864.7 mg GAE g^{-1} DW at 20 µM and 40 µM, respectively, compared to control with 562.2 mg GAE g^{-1} DW. Variation in roots TPC was insignificant between the treatment (Table 2). Overall, TPC production was much higher upon Cd stress than Zn stress.

Amount of Total Flavonoid Content (TFC) were calculated from Rutin calibration curve with regression equation $y = 0.0015x-0.0166$ ($R^2 = 0.994$). Total flavonoid contents in the roots and leaves of *P. crispum* decreased substantially with increased Zn stress (Table 2). The reduction was obvious at 200 µM of Zn concentration in leaves (50.5 mg RE g^{-1} DW) and in roots (17.1 mg RE g^{-1} DW) as compared to control (33-leaves, 10.17-roots, Table 2). Overall, TFC decrease with Cd concentration increased was more pronounced than decrease upon Zn stress (Table 2).

Ferric reducing antioxidant power **(**FRAP) was calculated from a gallic acid calibration curve with a y = 0.0048x-0.0299 (R^2=0.9974) regression equation. In leaves, FRAP decreased significantly 212.2 and 176 µM GAE g^{-1} DW at 100 µM and 200 µM of Zn as compared to control (244.1 µM GAE g^{-1} DW), whereas, in roots, the decrease was not evident (Table 2). Upon Cd stress, FRAP of *P. crispum* leaves increased significantly with 259 and 293 µM GAE g^{-1} DW at 20 and 40 µM, respectively, compared to control (254.1 µM GAE g^{-1} DW). FRAP was also increased in roots of *P. crispum* upon Cd stress. Overall, FRAP was significantly higher in leaves of *P. crispum* upon Cd stress than Zn stressed condition (Table 2).

Enzymatic antioxidant assay upon Zn and Cd stress

In leaves, SOD activity increased significantly (0.93 and 1.14) at 100 and 200 µM of Zn, respectively, compared to control (0.66; Table 3). There was a slight change in SOD activity in roots, and values ranged from 0.57 to 0.7 (Table 3). A similar trend in root and shoot of *P. crispum* was observed upon Cd stress (Table 3), where values ranged between 0.67 to 0.95-leaves and 0.56 to 0.84-roots. Overall, SOD activity was higher in leaves upon Zn stress than Cd stress.

CAT activity was highest both in leaves (0.00054) and root (0.00055) at 200 µM of Zn stress as compared to control with 0.0003 and 0.00026, respectively (Table 3). At 20 µM of Cd stress, CAT activity was increased significantly in leaves and roots (0.00051, 0.00037, respectively) compared to control (0.0003). Overall, CAT activity was more pronounced upon Cd stress, but for Zn, the highest activity was observed only at 200 µM (Table 3).

In leaves, APX activity was not significantly different among various Zn treatments; however, in root at 100 µM of Zn, APX activity was 0.0034 as compared to control 0.0021 (Table 3). In the case of Cd stress, APX activity was observed highest at 40 µM that was 0.0049 (leaves) and 0.0032 (roots), as compared to control (0.0014-leaves and 0.0021-roots; Table 3).

GPx activity was significantly higher (0.0004 and 0.0005) in leaves of *P. crispum* compared to root with 0.00008 and 0.00007 at 100 and 200 µM of Zn, respectively, compared to control (0.00015-leaves and 0.00003-roots; Table 3). The activity of GPx in leaves and roots upon Cd stress was not significantly different among various treatments. Overall, GPx activity was much higher in leaves upon Zn stress than Cd stress conditions (Table 3).

GST activity was reduced upon Zn or Cd stress, particularly in leaves (Table 3). However, the reduction was significant in Cd stress (0.038, 0.028 leaves; 0.016, 0.019 roots at 20 and 40 µM, respectively) compared with control (0.072 leaves and 0.028 roots). Zn stress decreased the GST contents of *P. crispum* leaves by 0.057 (100 µM) and 0.043 (200 µM) as compared to 0.072 (control) (Table 3).

Zn and Cd Accumulation in plants

Zn uptake and accumulation in roots were significantly higher at 100 µM (8435 mg kg^{-1}) than other stress levels (Fig. 2a). The Least Zn accumulation in root was 2507 mg kg^{-1} at 150 µM. Shoot Zn uptake and accumulation were not significantly varying between different stress levels. Zn accumulation was much higher in root than in shoot (Fig. 2a). In roots, Cd uptake and accumulation varied significantly among treatments, and the highest accumulation was at 80 µM (9630 mg kg^{-1}; Fig 1b), followed by 8257 mg kg^{-1} at 40 µM, 6746 mg kg^{-1} at 20 µM, and 4981 mg kg^{-1} at 10 µM (Fig. 2b). In shoots, the highest Cd concentration was at 80 µM is 2220 mg kg^{-1}, and is significantly higher than other stress levels. Lowest concentration was 1126 mg kg^{-1} at 10 µM (Fig. 2b). The accumulation pattern of Cd among different plant parts was much less in shoots than in the root (Fig. 2b). Overall, root accumulation of Zn and Cd was significantly higher in root than shoot.

Data scattering upon exposure to the Zn and Cd of *P. crispum*

Data scattering in *P. crispum* plant regarding Zn and Cd exposure is presented in Figures 3a and b. The scatter plot prepared after PCA clearly shows the difference in the pattern of variables scattering, which indicates that few parameters are impacted much higher than others when the plants were exposed to Zn or Cd exposure.

The Zn exposure to *P. crispum* resulted in the extraction of 3 components heaving eigenvalue higher than 1, with the cumulative variance of 92.52%, for the three principal components axis. A total variance of 63.67% was observed for PC 1. Shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, root dry weight, Zn in the shoot, Chl b, MDA in roots, CAT in leaves, CAT in roots, GST in leaves, H_2O_2 in leaves, phenolics in leaves, flavonoids in leaves, the flavonoid in roots, FRAP assay in leaves and roots, showed having higher loading values in PC 1 (Fig. 3 and Supplementary Table 1, and 2). While PC 2 contributed 25.56% in the data scattering as per the PCA, with Zn in roots, Chl a, Total Chl, carotenoids, MDA in leaves, EL, SOD in leaves, SOD in roots, APX in leaves, APX in roots, GST in roots, GPX in leaves, GPX in roots, H_2O_2 in roots, and phenolics in roots, having higher loading values in PC2 (Fig. 3 and Supplementary Table 1, and 2). The cumulative variance PC 1 and PC 2 was 89.22%.

Upon exposure to Cd, the PC 1 showed a total variance of 72.62%, with Cd in roots, Cd in shoot, Chl a, Chl b, Total Chl, carotenoids, SOD in leaves, CAT in leaves, APX in roots, GST in leaves, GST in roots, GPX in leaves, GPX in roots, H_2O_2 in leaves, H_2O_2 in roots, phenolics in leaves, phenolics in roots, and flavonoids in leaves, having higher loading values in this component (Fig. 3b, and Supplementary Table 3, and 4). The PC 2 showed a total variance of 14.30%, with shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, root dry weight, MDA in leaves, MDA in roots, EL, SOD in leaves, SOD in roots, CAT in leaves, CAT in roots, APX in leaves, APX in roots, GST in leaves, GST in roots, the flavonoid in roots, FRAP assay in leaves, FRAP assay in roots, showing higher loading values in the PC 2 (Fig. 3b, and Supplementary Table 3, and 4). The cumulative variance of these two extracted components is 86.92, which indicates that these two components define more than 86% of data variability. However, two more components having eigenvalues of higher than equal 1 were extracted, showing an additional 4.91%, and 3.37% of data variability, with which the cumulative variance was 95.20%.

Discussion

Cd has no evident advantageous role in plant metabolism; however, Zn is essential for normal plant growth. The root becomes necrotic and glutinous with necrotic leaves after Cd exposure, reducing roots and shoots plants' length (Haider et al., 2021). Cadmium higher stress condition generally decreases the mitotic division at the meristematic cells, which results in reduced root length and dry biomass with an improved diameter of roots (Gratao et al., 2009). Under Cd stress, plants showed stunted growth, which may be attributed to the reduced uptake of water and other essential bio-element, photosynthesis, assimilation of carbon and nitrogen, and antioxidant activity (Rizwan et al., 2017). Despite Zn being a crucial microelement, its excess can also bring phytotoxic effects on plants. A higher concentration of trace metal elements can have a similar undesirable influence on different plant growth parameters (Szopinski et al., 2019). Our results exhibited that the application of Zn and Cd led to a reduction in the growth of *P. crispum*, as evident from a substantial drop in plant fresh weight and plant length, with marked hazardous effects at higher concentrations of both metals. Decrease in length, fresh and dry weight of the *P. crispum* shoot and root was more pronounced upon Zn stress (Table 1).

Higher concentrations of any of the two metals showed a remarkable negative effect on photosynthetic pigment production, phytoconstituent, antioxidant enzyme production, and other physiological parameters like biomass and length (Haider et al., 2021; Szopinski et al., 2019). Our results showed that the photosynthetic pigment like chlorophyll a and b, carotenoids decreased markedly in leaves of *P. crispum* upon Zn and Cd stress, except for 100 µM of Zn (Fig. 1). Reduction in photosynthetic pigments like chlorophyll a and b and carotenoid under lead (Pb) stress as compared to control was also reported by Alamer and Fayez (2020), with evident chlorosis on leaves. It has also been stated that the synthesis of chlorophyll pigments was greatly affected due to Zn's increased concentration (Wang et al., 2009). The amount of carotenoid reduced, whereas the MDA contents increased in maize (*Zea mays*) under Zn stress (Cui et al., 2011) and in pea (*Pisum sativum*) plants under Cd stress (Dixit et al., 2001). In our experiment, *P. crispum* also showed higher lipid peroxidation in roots and leaves, leading to increased production of MDA contents under Zn and Cd stress (Table 1).

The EL is an imperative parameter in cell stress physiology which is mainly used to assess the leakage of cell components via a membrane. The results from our study revealed that Zn and Cd toxicity to *P. crispum* significantly enhanced the percentage of EL, particularly at 100 µM of Zn (Table 1), along with boosted ROS production. It is most likely that heavy metals toxicity induces ROS production (e.g., O_2-, H_2O_2, OH-, and O_2) in different plant cell organelles, leading to obvious oxidative-related damages. In the present study, H_2O_2 contents in *P. crispum* were increased, particularly in roots upon Zn and Cd stress. However, H_2O_2 contents were highest at 200 µM of Zn in leaves (Table 1). The indirect role of Cd in ROS production through disruption in the chloroplasts has been reported by Gallego et al. (2012). Another study on alfalfa showed a rapid deposition of peroxides and reduction of homoglutathione and glutathione upon Cd exposure, leading to a redox imbalance (Gutsch et al., 2019).

Our results showed that various concentrations of Zn and Cd significantly increased the uptake and accumulation of metals in the *P. crispum*; however, the accumulation was significantly higher in roots than in shoots. In a study conducted by Krippner and Schubert (2021) on *P. crispum*, Zn translocation to the shoot was decreased at 10 µM of Zn treatment compared to control plants. In *P. crispum*, one cultivar, Cd was chiefly retained in roots with < 1 shoot: root ratio.

A range of amino acids and proteins play a role in acting as scavengers of H_2O_2 and superoxide. These include, but are not limited to enzymatic scavengers, i.e., CAT, SOD, glutathione reductase (GR), peroxidase (POD), ascorbate peroxidase (APX), and non-enzymatic scavengers, i.e., phenolic, flavonoids, carotenoids, ascorbic acid (ASA), FRAP, glutathione (GSH), and tocopherols (Haider et al., 2021).

Zn and Cd stress enhanced the production of phenolic contents in leaves of *P. crispum* as they play a crucial role in minimizing oxidative stress injuries. Flavonoids contents showed a declining trend in leaves and roots of *P. crispum* (Table 2). In leaves of *P. crispum*, total phenolic contents were significantly increased by 38.1 and 48.1%, respectively, in response to 1 and 2 mM Pb stress, compared to the control (Alamer and Fayez, 2020). Lachman et al. (2005) have also documented the same pattern of decline in flavonoid contents in barley plants. FRAP intensity in plants exposed to oxidative stress highlights the tissue antioxidant power and is considered the best indicator of the plant's potential for antioxidant capacity to cope with stresses (Szollosi et al., 2011). In this experiment, *P. crispum* exhibited a differential pattern for FRAP level. FRAP decreased as Zn stress level increased in hydroponic culture, while the opposite effect was observed in Cd-induced stress. Ion leakage causes oxidative cell death by impairing the transport of specific ions vital for performing cellular functions. In *Brassica juncea*, a similar effect of Cd stress has been reported (Mobin and Khan, 2007).

The plant shows an effective enzymatic response to oxidative stress by scavenging reactive oxygen species (ROS). As the concentration of Zn/Cd increases, antioxidation enzyme production was also enhanced in *P. crispum*. Zn and Cd-induced increased activity of SOD in the leaves and roots of *P. crispum* were observed. SOD is a borderline defense enzyme that reduces ROS production. More SOD production indicates more tolerance of plants towards oxidative stress in metal accumulator species like a mustard plant (Mobin and Khan, 2007). CAT and GPx degrade the H_2O_2 produced during oxidative stress efficiently from the plant body. The increased concentration of Cd stress causes the high production of CAT in metal accumulator plants (Wang et al., 2008). CAT activity was also enhanced upon Zn/Cd stress in *P. crispum*, mainly leaves. APx activity was exceptionally high in roots of *P. crispum* upon Zn stress and leaves when exposed to Cd stress. GPX acts as a stress enzyme involved in quenching the reactive form of the O_2 radical during stress. GPx activity increases at an increased concentration of Zn and Cd in leaves of *P. crispum* while its activity reduces in plant roots. The same finding was observed in *Pfaffila glomerata* at a higher concentration of Cd (Cristina et al., 2011). It may be due to the formation of more hydrogen peroxide production in leaves irrespective of ionic stress or the translocation of Zn and Cd ion from the root to the aerial part of the plant. GST level also increases in both root and leaves of *P.*

crispum at higher Zn and Cd stress concentrations. A similar finding has been observed in roots and leaves of pea plants at higher concentrations of Cd (Dixit et al., 2001). The PCA analysis for the studied parameters of *P. crispum* regarding Zn and Cd exposure (Figure 3) indicated the versatility in the plant's response toward it. Therefore, the current study establishes a better understanding of Zn and Cd's phytotoxicity effects and tolerance mechanisms in *P. crispum*. However, rigorous work is still needed to explain the molecular mechanisms of Zn and Cd toxic role in *P. crispum* or other plant species.

Conclusion

The current study has provided pieces of evidence that Zn and Cd-induced stress show remarkable stress injuries in *P. crispum* in both root and leaves. Parsley shows an effective antioxidant response to overcome the effect of stress injuries by scavenging H_2O_2, free O_2 radicals formed during ROS production by increased antioxidant enzyme synthesis. High levels of an antioxidant enzyme like SOD, CAT, APX, GST, and GPX are found in leaves compared to the roots of the *P. crispum* plant, indicating that Zn and Cd ions translocate to the aerial part of the plant, causing more oxidative damage in roots.

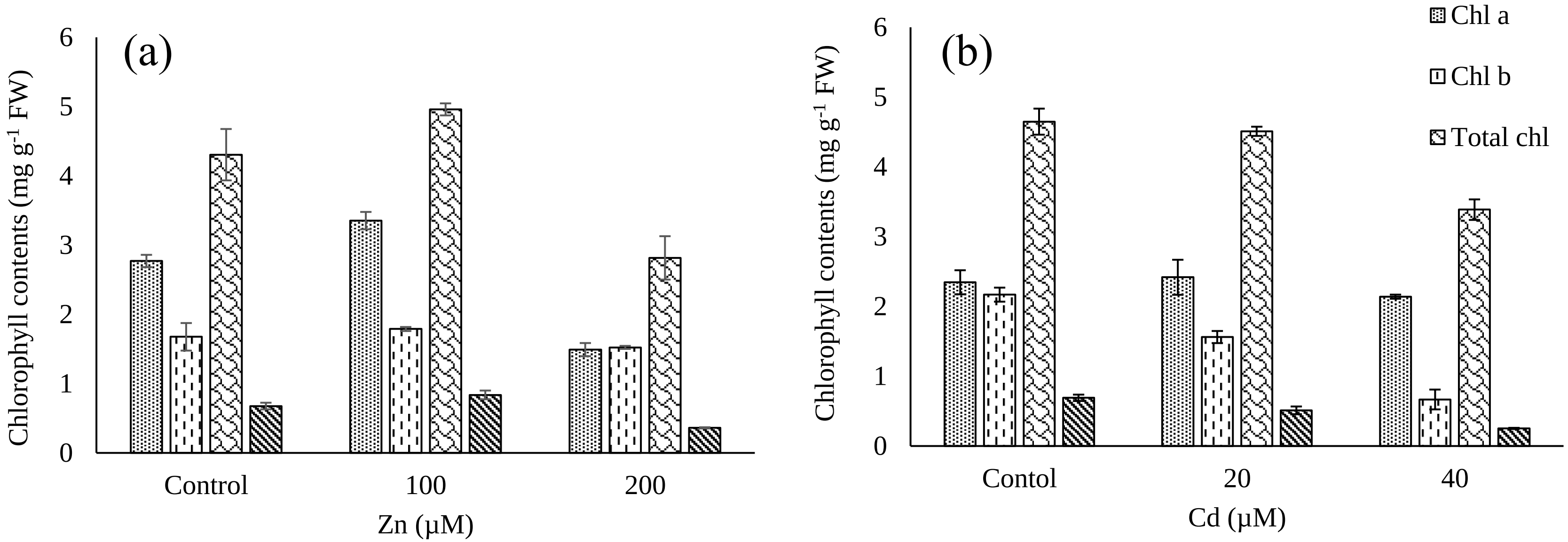

Figure 1. Effect of Zn (a) and Cd (b) on photosynthetic pigmnets of *P. crispum* leaves after 7 days of exposure. Data is mean (*n* = 3) and error bars are ± SD.

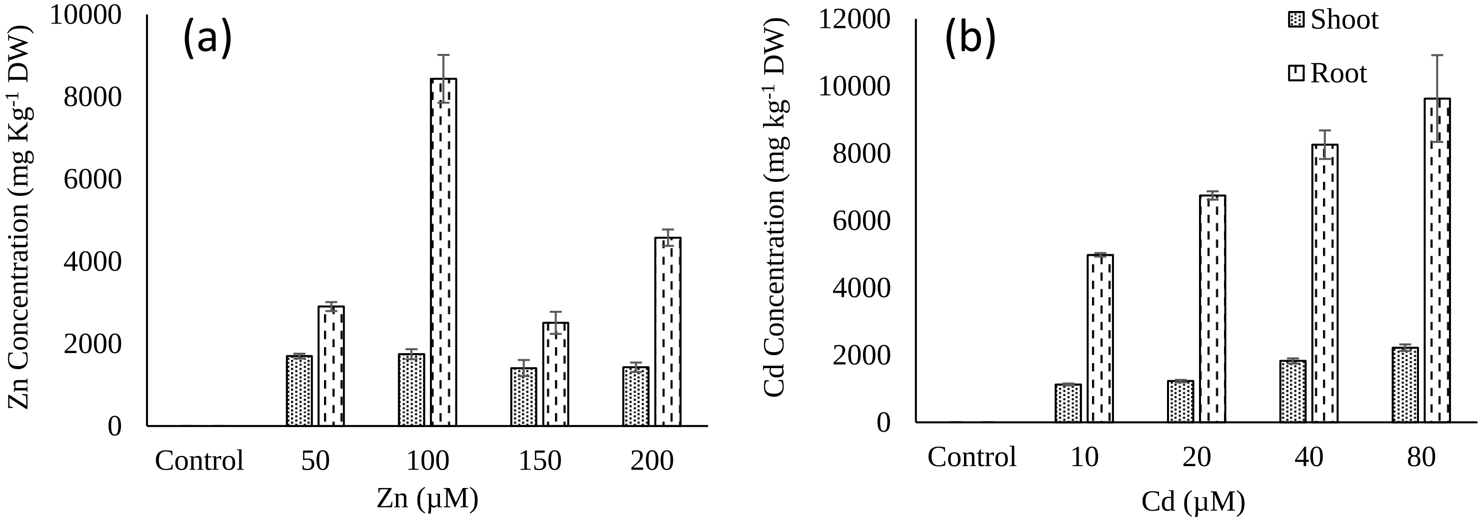

Figure 2. Effect of Zn (a) and Cd (b) on metal accumulation of *P. crispum* shoots and roots after 7 days of exposure. Data is mean (*n* = 3) and error bars are ± SD.

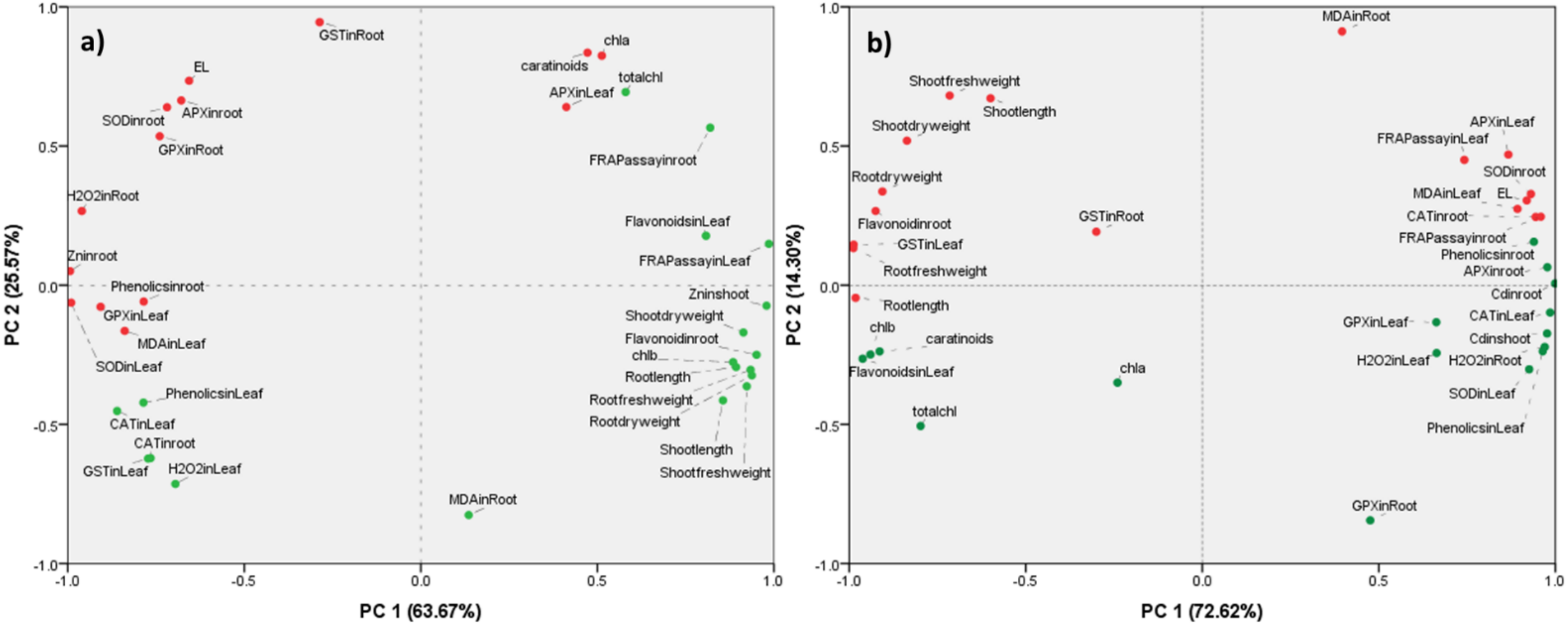

Figure 3. Principle component analysis (PCA) plots for the studied parameters of *P. crispum* with reference to a) Zn exposure, and b) Cd exposure. In PC plots, numerical values followed by PC indicate the percentage contribution in the variability. Green dot have higher loading value in PC 1, while orange have higher loading value in PC 2

Table 1. Physiological parameters of *P. crispum* upon exposure to different levels of Zn and Cd stress. Data is mean (*n* = 3) with ± SD.

Treatments (µM)		SL (cm)	RL (cm)	SFW (g)	RFW (g)	SDW (g)	RDW (g)
Zn	Control	11.66 ± 0.33	12.33 ± 0.76	666.66 ± 28.71	186.66 ± 14.98	28.50 ± 0.86	19.66 ± 0.88
	50	8.83 ± 0.30	8.31 ± 0.43	430.00 ± 20.49	160.66 ± 4.75	25.33 ± 0.88	11.00 ± 0.57
	100	7.58 ± 0.37	7.48 ± 0.43	343.66 ± 13.90	141.33 ± 11.09	23.00 ± 1.52	9.33 ± 0.88
	150	6.63 ± 0.83	5.75 ± 0.46	268.00 ± 24.00	66.00 ± 5.53	22.33 ± 1.85	8.00 ± 0.57
	200	4.66 ± 0.52	3.75 ± 0.54	233.00 ± 7.27	28.5 ± 2.77	19.00 ± 0.57	7.66 ± 1.20
Cd	Control	10.20 ± 0.60	12.00 ± 0.57	683.33 ± 21.40	210.00 ± 12.11	39.83 ± 2.70	14.33 ± 0.88
	10	8.75 ± 0.72	9.50 ± 0.61	467.00 ± 12.96	175.83 ± 8.08	22.50 ± 0.28	10.50 ± 0.29
	20	6.73 ± 0.33	7.25 ± 0.17	315.66 ± 36.56	145.33 ± 9.39	30.50 ± 2.35	9.00 ± 0.58
	40	5.88 ± 0.25	4.93 ± 0.18	381.50 ± 31.03	118.00 ± 6.85	27.83 ± 1.50	8.50. 0.87
	80	4.66 ± 0.24	3.26 ± 0.16	279.00 ± 9.32	80.50 ± 9.32	17.00 ± 1.00	7.66 ± 0.67

SL (Shoot Length), RL (Root Length), SFW (Shoot Fresh Weight), RFW (Root Fresh Weight), SDW (Shoot Dried Weight), RDW (Root Dried Weight).

Old Table

Treatments (µM)	SL (cm)	RL (cm)	SFW (g)	RFW (g)	SDW (g)	RDW (g)
Zn Control	11.66 ± 0.33	12.33 ± 0.76	666.66 ± 28.71	186.66 ± 14.98	28.50 ± 0.86	19.66 ± 0.88
Zn (50)	8.83 ± 0.30	8.31 ± 0.43	430.00 ± 20.49	136.16 ± 11.72	25.33 ± 0.88	11.00 ± 0.57
Zn (100)	7.58 ± 0.37	6.50 ± 0.61	227.00 ± 26.12	158.00 ± 18.03	23.00 ± 1.52	9.33 ± 0.88
Zn (150)	10.13 ± 0.84	10.50 ± 0.42	288.00 ± 26.88	66.00 ± 5.53	22.33 ± 1.85	8.00 ± 0.57
Zn (200)	8.00 ± 0.44	5.83 ± 0.60	233.00 ± 7.27	28.5 ± 2.77	19.00 ± 0.57	7.66 ± 1.20
Cd Control	10.20 ± 0.60	12.00 ± 0.57	683.33 ± 21.40	193.33 ± 22.16	39.83 ± 2.70	14.33 ± 0.88
Cd (10)	8.75 ± 0.72	8.25 ± 0.30	239.16 ± 21.12	57.50 ± 6.02	22.50 ± 0.28	10.50 ± 0.29
Cd (20)	8.58 ± 0.71	7.66 ± 0.49	315.66 ± 36.56	65.33 ± 5.74	30.50 ± 2.35	9.00 ± 0.58
Cd (40)	8.33 ± 0.55	7.66 ± 0.33	381.50 ± 31.03	162.33 ± 12.06	27.83 ± 1.50	8.50. 0.87
Cd (80)	8.33 ± 0.21	8.50 ± 0.34	320.66 ± 21.44	119.50 ± 21.44	17.00 ± 1.00	7.66 ± 0.67

Table 2. MDA, H_2O_2, TPC, TFC and FRAP contents of *P. crispum* upon exposure to different levels of Zn and Cd stress. Data is mean ($n = 3$) with ± SD.

Treatments (μM)		**MDA (μmol g^{-1} FW)**		**H_2O_2 (μmol g^{-1} FW)**		**EL (% age)**	**TPC (mg GAE g^{-1} DW)**		**TFC (mg RE g^{-1} DW)**		**FRAP (μM GAE g^{-1} DW)**	
		leaves	roots	leaves	roots	leaves	leaves	roots	leaves	roots	leaves	roots
Zn	Control	0.012 ± 0.0004	0.009 ± 0.0005	166.00 ± 2.65	129.07 ± 0.716	12.98 ± 0.447	668.95 ± 29.37	210.62 ± 11.92	43.91 ± 2.83	17.79 ± 0.401	244.07 ± 1.83	156.84 ± 1.526
	100	0.014 ± 0.0008	0.007 ± 0.0002	144.58 ± 0.358	273.86 ± 1.0525	26.96 ± 0.912	688.95 ± 24.33	319.79 ± 9.61	40.04 ± 2.74	11.10 ± 0.550	212.25 ± 1.602	155.20 ± 1.062
	200	0.017 ± 0.0014	0.009 ± 0.0001	275.86 ± 1.835	251.36 ± 1.074	19.92 ± 0.999	815.62 ± 15.15	403.95 ± 16.93	33.00 ± 1.59	10.17 ± 0.195	176.12 ± 1.52	111.73 ± 1.313
Cd	Control	0.013 ± 0.0002	0.007 ± 0.00009	159.78 ± 0.802	126.49 ± 0.7224	14.37 ± 0.355	562.29 ± 23.18	207.29 ± 11.50	50.23 ± 0.347	17.11 ± 1.069	254.07 ± 7.58	156.84 ± 1.526
	20	0.015 ± 0.0007	0.005 ± 0.00007	169.38 ± 3.224	237.87 ± 2.712	16.09 ± 0.444	818.54 ± 12.99	218.95 ± 43.57	41.32 ± 1.793	9.75 ± 0.469	259.69 ± 4.72	174.01 ± 2.31
	40	0.019 ± 0.0005	0.008 ± 0.0001	170.49 ± 4.855	257.86 ± 5.782	19.24 ± 0.343	864.79 ± 5.87	272.29 ± 25.62	30.42 ± 2.111	9.45 ± 0.217	293.64 ± 8.71	198.17 ± 1.49

Malondialdehyde (MDA), Total Phenolics Contents (TPC), Total Flavonoid Contents (TFC), Ferric Reducing Antioxidant Power (FRAP).

Table 3. SOD, CAT, APX, GPx and GST activities in fresh *P. crispum* leaves/roots samples upon exposure to different levels of Zn and Cd stress. Data is mean (*n* = 3) with ± SD.

Treatments (μM)		**SOD (Unit g^{-1} FW)**		**CAT (Unit g^{-1} FW)**		**APX (Unit g^{-1} FW)**		**GPx (Unit g^{-1} FW)**		**GST (Unit g^{-1} FW)**	
		leaves	roots	leaves	roots	leaves	roots	leaves	roots	leaves	roots
Zn	Control	0.667 ± 0.002	0.562 ± 0.0133	0.0003 ± 0.0000001	0.00026 ± 0.000009	0.0013 ± 0.00003	0.0012 ± 0.00003	0.00014 ± 0.00002	0.00003 ± 0.000002	0.072 ± 0.001	0.028 ± 0.0015
	100	0.930 ± 0.013	0.755 ± 0.023	0.0003 ± 0.00003	0.00025 ± 0.00003	0.0014 ± 0.00006	0.0034 ± 0.0001	0.00030 ± 0.00044	0.00008 ± 0.000010	0.057 ± 0.003	0.043 ± 0.0012
	200	1.142 ± 0.015	0.638 ± 0.012	0.0005 ± 0.000006	0.00050 ± 0.000006	0.0012 ± 0.00002	0.0028 ± 0.0001	0.00050 ± 0.00060	0.00006 ± 0.000005	0.043 ± 0.003	0.028 ± 0.0002
Cd	Control	0.667 ± 0.002	0.562 ± 0.013	0.0003 ± 0.000001	0.00024 ± 0.00001	0.0013 ± 0.00002	0.002 ± 0.00003	0.00010 ± 0.000006	0.00003 ± 0.000002	0.072 ± 0.001	0.028 ± 0.0016
	20	0.938 ± 0.032	0.654 ± 0.0113	0.0005 ± 0.00001	0.00037 ± 0.00003	0.0019 ± 0.00001	0.0027 ± 0.0001	0.00014 ± 0.000012	0.00011 ± 0.000010	0.038 ± 0.001	0.016 ± 0.0013
	40	0.954 ± 0.013	0.835 ± 0.023	0.00059 ± 0.00001	0.00055 ± 0.000009	0.0049 ± 0.0002	0.0003 ± 0.00005	0.00014 ± 0.000004	0.0006 ± 0.000004	0.028 ± 0.002	0.019 ± 0.0015

Superoxide Dimutase (SOD), Catalase (CAT), Ascorbate peroxidase (APX), Glutathione peroxidase (GPx), Glutathione S-transferase (GST).