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Cadmium Toxicity in Wheat: Impacts on Element Contents, Antioxidant Enzyme Activities, Oxidative Stress, and Genotoxicity

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Abstract

Cadmium (Cd) pollution is constantly increasing in agricultural systems due to anthropogenic activities and causes significant reductions in the yield of crop species. In this study, we aimed to determine the effect of Cd stress on growth, element contents, oxidative damage, antioxidant enzyme activities, and genotoxicity in wheat (*Triticum aestivum* L.). To achieve this goal, 7-day-old wheat seedlings were subjected to different concentrations of $Cd(NO_3)_2{\cdot}4H_2O$ (250, 500, and 1000 µM) for 4 days. The results show that Cd stress induces growth inhibition, oxidative injury, and genotoxicity in wheat seedlings. Moreover, the highest concentration of Cd treatment led to a significant increase in the activities of antioxidant enzymes, except for catalase. In addition, a dramatic decrease was observed in K and Ca contents in response to Cd treatments. Overall, our findings suggest that even short-term exposure to Cd can impair key physiological processes influencing growth, oxidative homeostasis, and genomic stability in wheat.

Keywords Cadmium toxicity · Oxidative stress · Genotoxicity · Antioxidant enzymes · Element contents · Wheat

Cadmium is a non-essential heavy metal that can be highly toxic to living organisms (Andresen and Küpper 2013; Shanmugaraj et al. 2019). Anthropogenic sources like industrial wastes, sewage sludge, and phosphate fertilizers lead to an increase in Cd pollution in agricultural systems, resulting in a significant reduction in crop yield (Satarug et al. 2003; Wu et al. 2007; Rady 2011). The most common symptoms of Cd toxicity in plants include growth inhibition and chlorosis (Das et al. 1997; Gallego et al. 2012). In addition, it has been shown that Cd toxicity can (i) inhibit the uptake and translocation of mineral nutrients (Yang et al. 1996), (ii) cause morphological changes in the cell nucleus (Souza et al. 2011), (iii) decrease CO_2 assimilation (Popova et al. 2009), (iv) increase lipid peroxidation (Ahmad et al. 2016), and (v) alter antioxidant enzyme activities (Mostofa et al. 2015).

Reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radical, singlet oxygen, and superoxide anion are constantly produced as by-products of aerobic metabolic processes in different cellular compartments (Apel and Hirt 2004). It is thought that ROS play a dual role in plant biology depending on their concentrations, either functioning in cells as signaling molecules or causing oxidative stress (Mittler 2017). Therefore, the amount of generated ROS is maintained in a physiological range by enzymatic and non-enzymatic antioxidants (e.g., catalase, peroxidase, ascorbate, and glutathione) under steady-state conditions (Sharma et al. 2012). However, a broad range of stress conditions, including drought, salinity, and metal toxicity can lead to a significant increase in the levels of ROS through disrupting oxidative homeostasis (Mittler 2002; Gill and Tuteja 2010). High concentrations of ROS are known to cause damage to biomolecules like membrane lipids, proteins, and nucleic acids (Demidchik 2015).

Genotoxicity describes the potency of a physical or chemical agent to induce DNA damage (Deutschle et al. 2006). Comet and micronucleus assays are commonly used to determine the genotoxic effects of toxicants on organisms (Seth et al. 2008; Khadra et al. 2012). In addition to these, the random amplified polymorphic DNA (RAPD) technique, applied for the estimation of genetic diversity (Gajera et al. 2010), is also frequently employed in genotoxicity studies (Liu et al. 2005; Çatav et al. 2018). The changes observed in RAPD profiles between individuals exposed and unexposed to a genotoxic compound are considered to be related to

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DNA damage and mutations (De Wolf et al. 2004; Atienzar and Jha 2006).

Wheat is one of the most important crop species contributing to almost 26% of global cereal production in the world (Daryanto et al. 2016). Even though a number of studies have been carried out to evaluate the physiological characteristics of wheat under Cd stress (reviewed by Rizwan et al. 2016), very little is known about the genotoxic potential of Cd on wheat (but see Mutlu and Mutlu 2015). Furthermore, a better understanding of Cd-related responses in wheat may provide valuable insights for agronomic practices, such as the selection of more tolerant cultivars and limitation of Cd transfer to the food chain. Therefore, in this study, we aimed to examine the physiological, biochemical, and genotoxic effects of Cd on wheat. In order to achieve this goal, wheat seedlings were subjected to different concentrations of Cd for 4 days, and growth and oxidative stress parameters, antioxidant enzyme activities, element contents, and RAPD profiles were assessed for each treatment group.

Materials and Methods

Wheat (cultivar: Bayraktar-2000) seeds were sterilized with 5% (v/v) sodium hypochlorite and 0.1% (w/v) sodium dodecyl sulfate for 10 min and rinsed with dH_2O several times. Surface-sterilized seeds were put on autoclaved paper towels saturated with dH_2O and incubated at 22 ± 1°C in darkness for 4 days. After that, similar-sized seedlings were placed on Styrofoam discs and grown hydroponically at 22 ± 1°C under 16/8 h photoperiod with light intensity of 100 µmol m^{-2} s^{-1} for 3 days. A 2000 mL of modified nutrient solution (pH 5.60) containing 0.65 mM KNO_3, 0.40 mM $CaCl_2$, 0.25 mM $MgCl_2$, and 0.08 mM NH_4NO_3 was used in order to supply essential nutrients (Rincón and Gonzales 1992). Cadmium treatments were started by adding different concentrations of $Cd(NO_3)_2{\cdot}4H_2O$ (250, 500, and 1000 µM) to the nutrient solutions. Control and Cd-treated seedlings were grown under the same growth conditions for another 4 days. Nutrient solutions were changed every 2–3 days and aerated continuously. Five replicates of 15 seedlings were used for each treatment, and the experiment was repeated 4 times.

At the end of each experiment, 30 seedlings from each treatment were immediately frozen with liquid nitrogen and stored at − 80°C until used for DNA extraction and biochemical analyses. Digital photographs of the rest of the seedlings were taken, and root and shoot lengths were then measured using ImageJ analysis software (version 1.48). Root and shoot dry weights were determined by drying samples in an electric oven at 70°C for 48 h.

Approximately 100 to 200 mg of dried root and shoot samples were put into Teflon vessels containing 7 mL of HNO_3 (65%) and 3 mL of H_2O_2 (30%). After standing for 10 min at room temperature, samples were digested in a microwave system (Berghof Speedwave MWS-3, Germany) as described by Genç et al. (2015). Digested samples were then filtered and diluted with ultrapure water. The concentrations of Ca, Cd, K, and Mg were determined by inductively coupled plasma optical emission spectrometry (ICP-OES).

Malondialdehyde (MDA), a by-product of lipid peroxidation, was measured by the thiobarbituric acid (TBA) method. Briefly, 0.5 g of shoot sample was homogenized with 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 13,000 rpm for 15 min at 4°C. Following this, 1 mL of supernatant was mixed with 4 mL of 20% (w/v) TCA and 0.5% (w/v) TBA and incubated at 95°C for 30 min. The reaction was terminated in an ice bath, and the absorbance of each mixture was read at 440, 532, and 600 nm. MDA content was estimated using the formula of Du and Bramlage (1992). H_2O_2 content of shoot samples was determined spectrophotometrically at 390 nm based on the oxidation of potassium iodide by H_2O_2 (Velikova et al. 2000). Proline content of shoot samples was quantified by the method of Shabnam et al. (2016) using L-proline as a standard.

Approximately 0.5 g of shoot samples were homogenized in 5 mL of ice-cold sodium phosphate buffer (0.05 M, pH 7.0) containing 1 mM disodium EDTA and 2% (w/v) polyvinylpyrrolidone. The homogenates were centrifuged at 4°C for 15 min at 13,000 rpm. Subsequently, the supernatants were used for determination of protein content and antioxidant enzyme activities. The protein content of the samples was estimated according to Bradford (1976) using bovine serum albumin as a standard. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured by monitoring the decrease in absorbance at 290 nm due to the oxidation of ascorbate as described by Nakano and Asada (1981). Catalase (CAT, EC 1.11.1.6) activity was determined by following the decrease in absorbance at 240 nm caused by the decomposition of H_2O_2 (Aebi 1984). Glutathione reductase (GR, EC 1.6.4.2) activity was assayed according to Foyer and Halliwell (1976) by monitoring glutathione-dependent oxidation of NADPH at 340 nm. Peroxidase (POD, EC 1.11.1.7) activity was determined by the method of Chance and Maehly (1955) by following the formation of tetraguaiacol at 470 nm. Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by its ability to inhibit photochemical reduction of nitro blue tetrazolium at 560 nm (Beauchamp and Fridovich 1971).

Genomic DNA was extracted from 0.1 g of shoot samples using a plant/fungi DNA isolation kit (Norgen, catalog number: E5038) according to the manufacturer's instructions. PCR reactions were performed in a total volume of 25 µL consisting of 20 ng DNA, 2 µM primer, 0.33 mM dNTPs, 3 mM $MgCl_2$, and 2 U Taq DNA polymerase (Thermo). The sequences, 5′ -3′, of primers used in this study are as follows: AATCGGGCTG (OPA-4), GTCCAC

ACGG (OPB-8), CTGACCAGCC (OPH-19), GGCGGA TAAG (OPW-5), CTGGACGTCA (OPW-7), TTCAGG GCAC (OPW-18), AGGCAGAGCA (OPY-8), AGTCGC CCTT (OPY-15), GGGCCAATGT (OPY-16), and ACCTTT GCGG (Primer 5) (Çatav et al. 2018). Amplification was conducted in a thermal cycler (TurboCycler Lite, Blue-Ray Biotech) programmed for 1 cycle of initial denaturation at 95°C for 4 min; 39 cycles of denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, and extension at 72°C for 1 min; and 1 cycle of final extension at 72°C for 10 min (Atienzar and Jha 2006). This procedure was repeated 3 times for each sample, and negative controls were used to avoid cross-contamination. PCR products were electrophoresed on 1.5% agarose gels supplemented with ethidium bromide in 1X TAE buffer at 70 V for 3 h. Images of the gels were taken using the Bio-Print gel documentation system (VILBER) and analyzed by Bio-Vision software (version 17.06). Genomic template stability [GTS (%)] was calculated as follows: $(1-(a/n))\times 100$, where "n" is the number of total bands in control samples, and "a" is the number of polymorphic bands detected in samples exposed to different concentrations of Cd.

The data sets were analyzed by one-way ANOVA followed by Tukey's HSD test to compare differences between control and Cd treatments. Normality and homogeneity of variance were examined with Shapiro–Wilk and Bartlett's tests, respectively. Principal component analysis (PCA) of log-transformed and mean-centered data was performed using "FactoMineR" and "factoextra" packages in R software (version 3.5.1). The significance level for all analyses was $p<0.05$.

Results and Discussion

Cadmium pollution is mainly derived from anthropogenic sources and has become an important problem for agricultural systems in many parts of the world. It has been reported that Cd toxicity can adversely affect plant growth and development by altering morphological traits and reducing the mitotic index, and this, in turn, can lead to a substantial reduction in the yield of crops (Wu et al. 2007; Souza et al. 2011; Tran and Popova 2013). Consistent with these reports, we found that Cd exposure resulted in a significant decrease in all growth parameters of wheat seedlings and that the degree of Cd-induced growth inhibition showed a concentration-dependent trend (Table 1). Moreover, visible toxicity symptoms like root browning and chlorosis appeared in seedlings treated with 500 and 1000 µM of Cd.

In many plants, Cd accumulation has been shown to be greater in roots than in shoots (Das et al. 1997; Gajewska and Skłodowska 2010). The present results, demonstrating that Cd accumulation in roots constitutes 76% to 83% of total Cd content per seedling depending on the concentration of treatment, are compatible with these observations (Fig. 1a). In addition, several reports have suggested that Cd stress can interfere with the uptake and transport of mineral nutrients, such as K, P, Ca, and Mn (Yang et al. 1996; Monteiro et al. 2009). Our findings revealed that K and Ca contents in shoots of Cd-treated seedlings were remarkably lower than those of control seedlings (Fig. 1b, c). Furthermore, Cd exposure caused a dose-dependent decrease in K content of roots. On the other hand, there were no significant differences between control and Cd treatments with regard to Mg content in root and shoot tissues (Fig. 1d).

A vast amount of experimental work has indicated that redox-active metals, such as iron, copper, and chromium increase the generation of ROS at high concentrations (Panda 2007; Zhang et al. 2009; Chalmardi et al. 2014). For instance, iron is known to mediate the formation of highly reactive hydroxyl radical by catalyzing Haber–Weiss reaction, also called as superoxide-driven Fenton chemistry (Liochev and Fridovich 2002). In addition, it has been suggested that redox-inactive metals (e.g., arsenic, cadmium, lead, and mercury) can also trigger the production of ROS via inducing depletion of thiol reserves or disrupting electron transport chain (Ercal et al. 2001; Benavides et al. 2005).

Table 1 Effects of different concentrations of Cd on growth parameters of wheat (Bayraktar-2000) seedlings

Growth parameter	Cd concentration (µM)				Statistics	
	0	250	500	1000	F	*p*
Root length (mm)	102 ± 7^{a}	73 ± 3^{b}	68 ± 4^{b}	66 ± 5^{b}	11.62	< 0.0001
Shoot length (mm)	141 ± 3^{a}	110 ± 3^{b}	103 ± 2^{bc}	98 ± 1^{c}	51.25	< 0.0001
Total seedling length (mm)	243 ± 10^{a}	184 ± 5^{b}	171 ± 5^{b}	164 ± 5^{b}	31.01	< 0.0001
Root dry weight (mg)	5.06 ± 0.16^{a}	4.32 ± 0.09^{b}	3.89 ± 0.15^{bc}	3.43 ± 0.10^{c}	30.52	< 0.0001
Shoot dry weight (mg)	11.25 ± 0.22^{a}	10.02 ± 0.36^{b}	9.14 ± 0.27^{b}	8.13 ± 0.17^{c}	24.98	< 0.0001
Total seedling dry weight (mg)	16.31 ± 0.34^{a}	14.34 ± 0.42^{b}	13.03 ± 0.36^{c}	11.56 ± 0.21^{d}	34.80	< 0.0001

Results are presented as mean ± SE (n = 12 replicates of 15 seedlings). Values with different superscript letters in the same row are significantly different from each other ($p<0.05$; Tukey's HSD test)

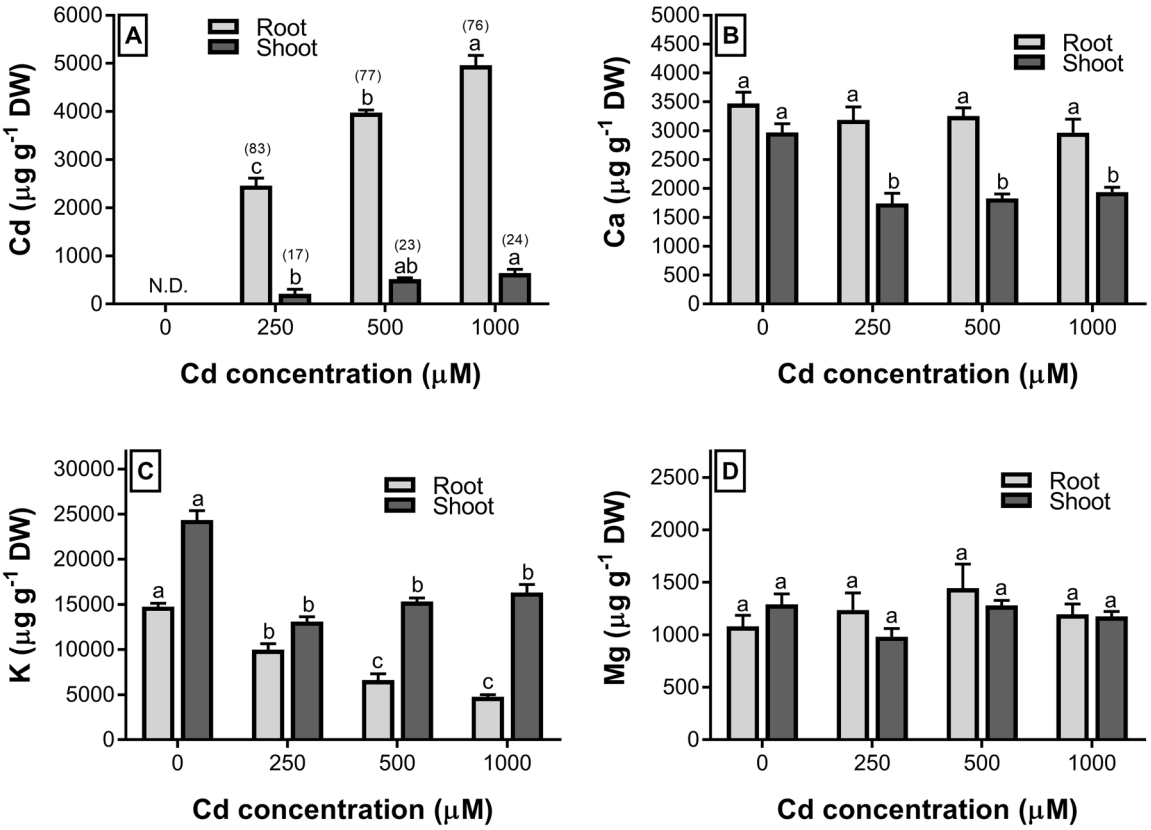

Fig. 1 **a** Cd, **b** Ca, **c** K, and **d** Mg contents of wheat (Bayraktar 2000) seedlings subjected to different concentrations of Cd. Results are presented as mean ± SE (n = 4). Bars with different letters are significantly different from each other for each tissue type ($p < 0.05$; Tukey's HSD test). Values in parentheses indicate the accumulation of Cd in tissues (% of total Cd content per seedling)

Our results showed that, as compared to the control group, Cd treatments gave rise to a notable increase in H_2O_2 content of wheat seedlings ($p < 0.05$). Moreover, a gradual rise in MDA level was observed in response to increasing concentrations of Cd (Table 2). These findings are consistent with previous studies reporting that Cd can induce ROS production and lipid peroxidation in various plants, including wheat (Wang et al. 2011; Khan et al. 2015; Mostofa et al. 2015; Ahmad et al. 2016).

Antioxidant enzymes play an important role in protecting cells against oxidative damage. For instance, SOD acts as the first line of defense converting superoxide radical into H_2O_2

Table 2 Effects of different concentrations of Cd on biochemical parameters of wheat (Bayraktar 2000) seedlings

Biochemical parameter	Cd concentration (µM)				Statistics	
	0	250	500	1000	F	*p*
H_2O_2 (µM g^{-1} FW)	23.1 ± 1.9^{b}	38.0 ± 3.6^{a}	38.7 ± 3.4^{a}	42.6 ± 4.4^{a}	6.149	0.0089
MDA (nmol g^{-1} FW)	0.61 ± 0.14^{c}	0.92 ± 0.10^{bc}	1.26 ± 0.17^{ab}	1.51 ± 0.04^{a}	10.52	0.0011
Proline (nmol g^{-1} FW)	367 ± 31^{c}	1674 ± 132^{b}	2013 ± 112^{ab}	2284 ± 170^{a}	48.10	< 0.0001
Protein (mg g^{-1} FW)	8.0 ± 0.8^{a}	10.4 ± 1.1^{a}	11.5 ± 1.5^{a}	9.8 ± 0.8^{a}	1.720	0.216
APX (nmol min^{-1} mg^{-1} protein)	227 ± 21^{b}	396 ± 40^{ab}	398 ± 27^{ab}	444 ± 27^{a}	5.809	0.011
CAT (nmol min^{-1} mg^{-1} protein)	353 ± 34^{a}	328 ± 34^{a}	317 ± 11^{a}	277 ± 73^{a}	0.515	0.680
GR (nmol min^{-1} mg^{-1} protein)	52 ± 7^{b}	71 ± 11^{ab}	73 ± 8^{ab}	104 ± 14^{a}	4.223	0.030
POD (µmol min^{-1} mg^{-1} protein)	7.9 ± 1.1^{b}	7.4 ± 0.8^{b}	8.9 ± 0.7^{b}	16.9 ± 1.3^{a}	19.46	< 0.0001
SOD (unit mg^{-1} protein)	13.3 ± 1.7^{b}	14.5 ± 0.8^{b}	19.0 ± 1.4^{ab}	28.2 ± 3.8^{a}	9.044	0.0021

Results are presented as mean ± SE (n = 4). Values with different superscript letters in the same row are significantly different from each other ($p < 0.05$; Tukey's HSD test)

Table 3 Molecular sizes (base pair) of appearing (+) and disappearing (−) bands, polymorphism ratio, and GTS value in the shoots of wheat seedlings exposed to the different concentration of Cd

Primer	Control	Concentration of Cd (μM)		
		250	500	1000
OPA-4	10	−970; +330	−970; +330; +660	−970; +330; +660
OPB-8	13	ND	+850	+750; +850
OPH-19	9	−1050	+890; +950; +1350; +1890	−1050; −1445; +765; +950; +1890
OPW-5	7	−1180	−1180; +1420	−1180; −1260; +535; +1100; +1310
OPW-7	10	−320; −1050	−320; −1050	−320; −1050
OPW-18	4	+1450	+1360; +1450; +2260	+1360; +1450; +2260
OPY-8	3	+1230	+800; +1100	+800; +1100
OPY-15	8	−2000	−2000; +630; +770; +2750	−2000; +630; +770
OPY-16	9	ND	+600; +960; +1280	−760; +600; +960; +1280
Primer-5	7	−310; +812; +1290; +1370	−160; −310; +1370; +1500	−310
Total band	80	13	28	30
Polymorphism (%)	0	16.25	35.00	37.50
GTS (%)	100	83.75	65.00	62.50

and molecular oxygen, while APX, CAT, and POD subsequently detoxify H_2O_2 (Mittler 2002; Apel and Hirt 2004). Proline, an amino acid that accumulates under stress conditions (e.g., drought and salinity), also provides protection to plants by contributing to cellular osmotic adjustment, stabilization of protein structure, scavenging of hydroxyl radicals, and regulation of cytosolic pH (Matysik et al. 2002; Hayat et al. 2012). The results of the present study showed that the highest concentration of Cd treatment resulted in a prominent increase in the activities of APX, GR, POD, and SOD (Table 2). On the other hand, a slight, insignificant decrease in CAT activity was observed in response to Cd treatments ($p > 0.05$). Furthermore, Cd treatments caused increases ranging from 3.6- to 5.2-folds in proline content of wheat seedlings within the tested concentration range (Table 2).

In this study, we used RAPD technique to determine whether Cd induces a genotoxic effect on wheat seedlings. Ten selected oligonucleotide primers gave a total of 80 bands ranging from 160 (Primer-5) to 2475 (OPY-15) bp for control samples. Cd treatments led to alterations in RAPD profiles in terms of the appearance of new bands and the disappearance of existing bands (Table 3). The number of newly appeared bands at 500 and 1000 μM Cd was significantly higher than that at 250 μM Cd. It was found that the total number of polymorphic bands produced by tested primers was 13, 28, and 30 for 250, 500, and 1000 μM Cd treatments, respectively. Overall, our results indicate that the genomic template stability of wheat seedlings is markedly decreased by increasing concentrations of Cd (Table 3). Similar patterns were also observed in *Phaseolus vulgaris* and *Pisum sativum* in response to Cd treatments (Gjorgieva et al. 2012; Surgun-Acar 2018). It has been suggested that Cd can exert a genotoxic effect by promoting ROS generation and affecting DNA repair systems, such as mismatch repair, nucleotide excision repair, and base excision repair (Bertin and Averbeck 2006). For instance, Jin et al. (2003) showed that Cd inhibited the activity of mismatch repair in human cell extracts in a concentration-dependent manner. Moreover, Risso-de Faverney et al. (2001) and Lin et al. (2007) reported that ROS might be involved in Cd-induced genotoxicity in rainbow trout and broad bean, respectively. In view of these findings and our own results, we consider that over-production of ROS resulting from Cd exposure is likely to cause genotoxicity in wheat seedlings (Tables 2, 3).

The results of the PCA analysis indicated that the first three components, which had eigenvalues greater than 1, explained 78.8% of the total variance in the data. The PCA scores plot in Fig. 2a reveals that Cd treatment groups (500 and 1000 μM) are clearly separated from the control group. The variables with the highest contribution to the PCA model are demonstrated in Fig. 2b. From this figure, it appears that changes in POD, SOD, GR, proline, MDA, K, and Ca levels are the most important determinants of group differentiation.

Taken as a whole, the findings of this study indicate that Cd treatments, especially at high concentrations, (i) induce growth inhibition, oxidative damage, and genotoxicity in wheat seedlings, (ii) increase antioxidant enzyme activities and proline accumulation, and (iii) limit the uptake of K and Ca.

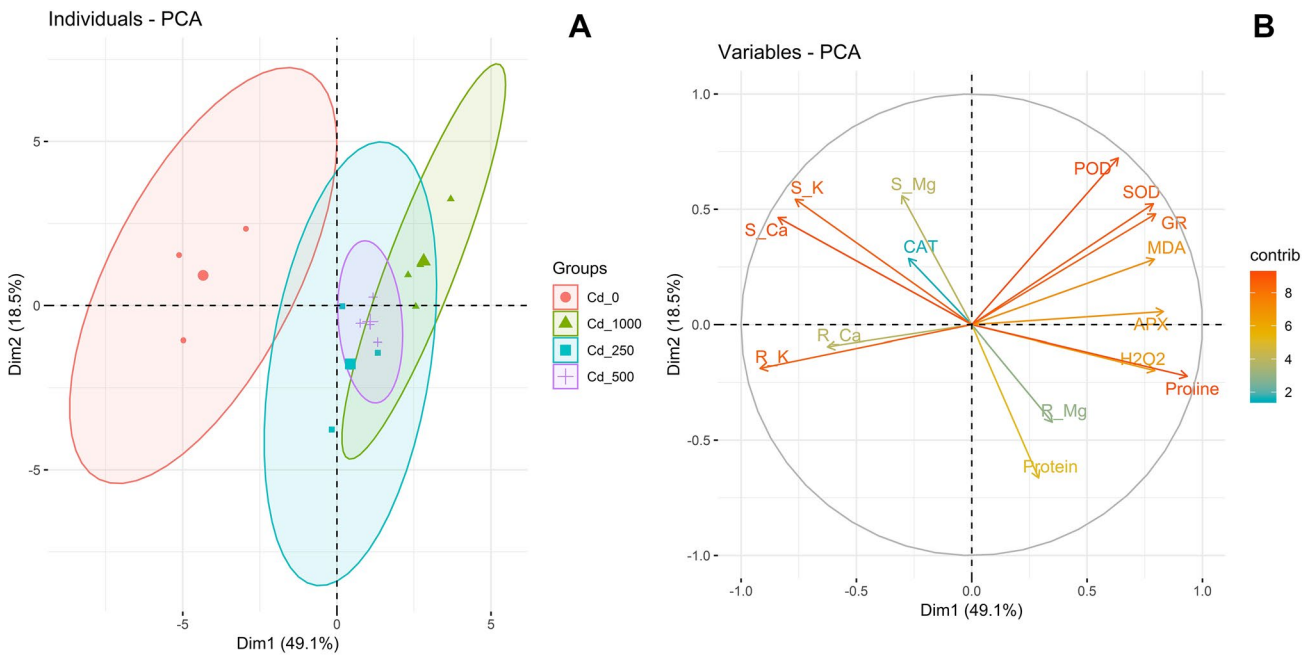

Fig. 2 **a** PCA scores plot obtained from the experimental data of wheat seedlings subjected to different concentrations of Cd. **b** The contribution of variables to the principal components (PC). *R_Ca* root Ca content, *S_Ca* shoot Ca content, *R_K* root K content, *S_K* shoot K content, *R_Mg* root Mg content, *S_mg* shoot Mg content

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