BIOCHEMICAL AND DNA DAMAGE RESPONSES OF HYDROPONICALLY GROWN ELANDS SOURFIG (*CARPOBROTUS ACINACIFORMIS* L.) LEAVES TO CADMIUM STRESS CONDITIONS

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Abstract. Biochemical responses, DNA damages, and cadmium (Cd) accumulation capacity of Elands sourfig (*Carpobrotus acinaciformis* L.), a halophyte plant, were investigated under a variety of $CdNO_3$ concentrations in hydroponic conditions for 30 days. Total chlorophyll/carotenoid and proline contents of the plants under differing Cd concentrations (0-, 2.5-, 5-, 10-, 20-, 40-, 60-, 80 and 100 ppm) gradually increased up to 20 and 40 ppm, respectively, which was followed by a decrease, *P≤0.05*. Accumulation of malondialdehyde (MDA, 3.22 to 22.87 nmol g^{-1} fresh weight, Fwt) and H_2O_2 (2.93-24.14 µmol g^{-1} Fwt) contents showed an increasing trend along with higher Cd concentrations, *P≤*0.05. On the other hand, antioxidant enzymes such as peroxidase (POX) and catalase (CAT) exhibited increasing trends only up to 40 ppm of Cd concentrations and the following drastic decline was evident, *P≤*0.05. The content of Cd levels in those leaves increased from 0.01 to 65.77 mg kg^{-1} with respect to Cd concentrations. DNA in leaves was not fragmented up to 5 ppm Cd level. When DNA damage was assayed in a single cell via comet assay, 80- and 100 ppm of Cd toxicity resulted in DNA breaks. Measuring DNA damage via comet assay could be a very useful approach to find out what level of Cd toxicity could be tolerated if a particular halophyte plant is used to remediate the Cd-contaminated media.
Keywords: *heavy metals, cadmium toxicity, DNA fragmentation, halophytes, comet assay*

Introduction

Heavy metals are one of the most significant environmental pollutants in nature. Their toxicity has now become a global issue since ecology, nutritional and humans' health are very much affected. Almost all heavy metals are non-essential and toxic to human and to the environment. Among heavy metals, cadmium has received much attention in soil science and plant nutritional studies due to its potential toxicity and its mobility in the soil-plant system (Tran and Popova, 2013; Shanmugaraj et al., 2019). Cadmium can be taken up by plant roots and enter the food chain resulting in a serious health issue for human beings. Even very low concentrations of Cd can cause significant toxicity and hamper the growth and development of plants (Al-Qurainy et al., 2017).

Cadmium has a long biological half-life originating mainly from industrial effluents, power stations, metal working industries, heating systems, batteries, urban traffic, etc. as well as application of fertilizers containing phosphates (Gill et al., 2013). Although it is non-essential, it is taken up very quickly by the plants and negatively affects the plant metabolism such as growth and development. Cd has a large solubility in water and has

been ranked number seven among the top 20 toxins, therefore, Cd has been widely studied for its impacts on plant at various levels including metabolism (Bagheri et al., 2014).

In plants, the symptoms of Cd toxicity can be observed between slight injury and lethality. The main known mechanisms of Cd toxicity include its affinity for sulfhydryl groups in proteins and its ability to replace some essential metals in active sites of enzymes, thus causing inhibition of enzyme activities and protein denaturation (Garg and Bhandari, 2013; Tamás et al., 2014). It also alters the enzyme pathway and indirectly induces oxidative stress by generating reactive oxygen species (ROS) (Romero-Puertas et al., 2004; Xie et al., 2019). The ROS react with lipids, proteins, pigments and nucleic acids and cause oxidative damage including lipid peroxidation leading to membrane and DNA damages (Chien et al., 2001; Dutta et al., 2018).

Once absorbed by plants, Cd causes several metabolic and molecular changes in cells that leads to reduction in plant growth and increased leaf chlorosis and senescence (Dalcorso et al., 2008). Plants employ different defense mechanisms to prevent these biochemical and molecular damages including plant chelates and antioxidative defense, and low molecular weight substances such as thiols and metal-binding polypeptides. The plants have various antioxidant molecules such as ascorbate, glutathione, α-tocopherol and enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), which protect the host from oxidative damage (Ahmad et al., 2017). Although many studies have shown that the effects of Cd on plants via decrease in photosynthesis and growth (Andresen and Küpper, 2013; Dias et al., 2013), disturbed nutrient uptake (López-Millán et al., 2009; Solti et al., 2011), and increases ROS accumulation (Jaskulak et al., 2018) have been evident. However, the mechanisms involved in toxicity are still need to be elucidated.

Cadmium inhibits the growth of lateral root formation in plants while the main root becomes brown, rigid, and twisted (Yadav, 2010; Xie et al., 2019). It has been shown that Cd interacts with various nutrient elements in terms of availability and transport from the soil (Nazar et al., 2012). For example, the mutagenic and cytotoxic nature of Cd causes DNA damage by producing ROS (Fodor, 2002; Dutta et al., 2018). Moreover, Cd binds to DNA bases and inhibits DNA mismatch repair (Al-Qurainy et al., 2017). Genotoxic disturbances could vary among plant species. For example, the genotoxic effect was varied among the plant organs of lettuce and tobacco as more effects were observed in the root while no changes were noticed in the leaf (Gichner et al., 2004; Monteiro et al., 2007). Similarly, the genotoxic effects of Cd in different concentrations were reported on *Nicotiana tabacum*, the effect was more pronounced in roots (Gichner et al., 2004). The oxidative DNA damage, chromosomal aberrations, DNA strand breaks, and the induction of micronuclei have all been observed *in vivo* and *in vitro* under Cd stress (Beyersmann and Hartwig, 2008). Different molecular markers such as microsatellite (simple sequence repeat, SSR) (Monteiro et al., 2007), random amplified polymorphic DNA (RAPD) (Al-Qurainy et al., 2010), and inter-simple sequence repeat (ISSR) (Al-Qurainy, 2010) have all been used to assess the genotoxicity of heavy metals in plants. In this study, genotoxicity was measured via DNA fragmentation along with the single cell gel electrophoresis (SCGE) methods to find out what level of Cd actually disrupts and permanently damages DNA of the host cell (Surapu et al., 2014; Dikilitas et al., 2015).

The response of antioxidant enzymes in general to heavy metals and Cd can also vary in various tissues and among plant species (Zhang et al., 2009; Ovecka et al., 2014). The ROS caused by Cd toxicity can be detoxified via antioxidant system which plays an important role in the removal of ROS and provides tolerance to plants under abiotic

stresses. Measuring antioxidant enzymes along with the metabolites could give crucial findings in phytoremediation studies.

Previous studies showed that *Carpobrotus* species showed great tolerance to a mixture of heavy metals including Cd, Cr, Cu, Mn, Ni, Pb and Zn and was able to accumulate Cd in its shoots (Zhang et al., 2014, 2015). Halophyte species *C. acinaciformis* L. (Elands sourfig) is a fast growing, succulent perennial plant belonging to Azioaceae family. It is commonly inhabited in the Mediterranean area. It is resistant to harsh conditions such as salt and drought and multiple heavy metals (Zhang et al., 2016). We are in the search of finding new plant species showing high degree of tolerance to heavy metal stress such as Cd. We established a novel test system to measure the level of tolerance system via measurement of DNA integrity and DNA health. The present study focused on Cd tolerance in *C. acinaciformis* plants via measuring biochemical and genetic parameters such as proline, protein, enzymes, DNA integrity, etc. to elucidate the toxicity of Cd.

Materials and Methods

C. acinaciformis L. was selected as a model halophyte plant to observe the effects of Cd toxicity. The plants with visually similar biomass and equal length were carefully collected from the fields of Agricultural Faculty of Harran University, Turkey. The plants were exposed to eight different Cd concentrations (0-, 2.5-, 5-, 10-, 20-, 40-, 80- and 100 ppm) in hydroponic conditions. Treatments in each group were replicated four times. To maintain constant volume and concentration of the solution, Cd was added in proper amounts every other day along with the modified Arnon and Hoagland's nutrient solutions (Jasoni et al., 2002). The solutions during this period were changed twice every two weeks. At the end of the trial period, leaf samples were collected and washed with deionized water to remove excess dirt and the samples were stored at -20 ºC in a freezer followed by removal of excess water via filter paper. The samples were preserved there until used.

Biochemical analysis methods

Total chlorophyll (Chl-*a*+Chl-*b*) and carotenoid contents of *C. acinaciformis* were determined based on the method of Arnon (1949) with slight modifications (Dikilitas, 2003). Fresh leaf samples (0.5 g) were homogenized in a 10 mL acetone:water (80:20, v:v) mixture and filtered through Whatman No.2 filter paper then placed in the dark tubes. Chl-*a* and Chl-*b* of the plant samples were read at a UV microplate spectrophotometer (Epoch, SN: 1611187, made in USA) at 663 and 645 nm, carotenoid contents of the leaves were measured at 480 and 510 nm, respectively against 80% acetone blank. Total chlorophyll was calculated as mg L^{-1} and expressed as mg g^{-1} Fwt (*Eq. 1*).

$$\text{Total chlorophyll (mg g}^{-1}) = \frac{20.2 \times (A480) - 8.02 \times (A510) \times V}{1000} \, xW \qquad \text{(Eq.1)}$$

where, A: Absorbance at specific wavelengths, W: Fresh weight to tissue extracted.

Proline (Pro) measurement was conducted according to the method of Bates et al. (1973) with slight modifications (Karakas et al., 2019). Leaf material (0.5 g) was homogenized in 3% w/v sulphosalicylic acid using a mortar and a pestle. The homogenate was filtered through Whatman No. 2 filter paper. Then, 2 mL of filtrate was mixed in a test tube with 2 mL of acid-ninhydrin reagent (1.25 g of ninhydrin in 30 mL of glacial

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acetic acid and 20 mL of 6 mol L^{-1} phosphoric acid) and boiled at 100 ºC for one hour. The reaction was terminated in an ice bath. The reaction mixture was then extracted using 5 mL of toluene. The tubes were thoroughly shaken for 15 - 20 seconds and left for 20 min at room temperature in order to achieve separation for two layers. The chromophore containing toluene was removed and allowed to warm to room temperature, and the absorbance of the solution was measured at 515 nm using a toluene blank. Proline concentration was determined using a calibration curve made with L-proline (Sigma-Aldrich 81202-06-4) and the results were expressed as µmol g^{-1} Fwt.

The malondialdehyde (MDA) content was determined according to the method of Sairam and Saxena (2000) with slight modifications (Karakas et al., 2019). Leaf material (0.5 g) was homogenized in 10 ml of 0.1% (w/v) trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 10,000 *g* for five minutes. Four mL of 20% v/v TCA containing 0.5% v/v thiobarbituric acid (TBA) was added to one mililiter of the supernatant. The mixture was incubated in boiling water for 30 min, and the reaction was stopped by placing the reaction tubes in an ice bath. The mixture was centrifuged again at 10,000 *g* for 5 min and the absorbance of the supernatant was read at 532 and 600 nm. Here, the MDA content of leaves is expressed as nmol g^{-1} Fwt, (*Eq. 2*).

$$MDA\ (nmol\ g^{-1}) = \frac{Extract\ volume\ (ml)x\left[\frac{A_{532}-A_{600}}{155\ mM^{-1}\ cm^{-1}}\right]}{W(g)}x10^3 \qquad \text{(Eq.2)}$$

where, A: Absorbance at specific wavelengths, W: samples weight.

Hydrogen peroxide levels (H$_2$O$_2$) were determined according to Velikova et al. (2000) with slight modifications (Karakas et al., 2019). Fresh plant tissue (0.5 g) was homogenized in an ice bath with 5 mL 0.1% (w:v) TCA. The homogenate was centrifuged at 12,000 *g* for 15 min at 4 ºC and 0.5 mL of the supernatant were added to 0.5 mL 10 mmol L^{-1} potassium phosphate buffer (pH 7.0) and 1 mL of 1 mol L^{-1} potassium iodide. The absorbance was read at 390 nm using a UV microplate spectrophotometer (Epoch, SN: 1611187, made in USA). The H$_2$O$_2$ content was expressed as µmol g^{-1} Fwt.

Catalase enzyme activity (CAT, E.C. 1.11.1.6) was determined by monitoring the decomposition of H$_2$O$_2$ according to the method of Milosevic and Slusarenko (1996) with slight modifications (Karakas et al., 2019). Fresh leaf tissue (0.5 g) was homogenized in 10 mL of 50 mmol L^{-1} Na-phosphate buffer solution, then 50 µL of plant extract was added to a 2.95 mL (10 mmol L^{-1} H$_2$O$_2$, 50 mmol L^{-1} Na-phosphate buffer and 4 mmol L^{-1} Na$_2$EDTA) reaction mixture and measured for 30 seconds at 240 nm with a UV microplate spectrophotometer (Epoch, SN: 1611187, made in USA). One CAT activity unit (U) is defined as a change of 0.1 absorbance unit per minute. Activity is expressed as enzyme units per gram of Fwt.

Peroxidase enzyme activity (E.C.1.11.1.7) was determined according to the method of Cvikrova et al. (1994) with slight modifications (Karakas et al., 2019). For the analysis, 100 µL of extract (obtained as above) was added to 3 mL of the reaction mixture (13 mmol L^{-1} guaiacol, 5 mmol L^{-1} H$_2$O$_2$, and 50 mmol L^{-1} Na-phosphate, pH 6.5). The reaction was measured at 470 nm using a UV microplate spectrophotometer (Epoch, SN: 1611187, made in USA) at one-minute interval until 3rd minute. One unit (U) of POX activity was defined as a change of 0.1 absorbance unit per minute at 470 nm. Activity is expressed as enzyme units per gram of Fwt.

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Cd ion content

Cd content of leaves was determined according to the procedure of Walinga et al. (1989) with slight modifications. The homogenate obtained following filtration was analyzed by Inductively Coupled Plasma (ICP, Perkin Elmer).

DNA fragmentation

Genomic DNA was isolated from fresh leaves of *C. acinaciformis* according to the method of Ahrens and Seemüller (1992) and Surapu et al. (2014). Fresh leaf tissue (1 g) were homogenized in 4 mL of CTAB buffer (2% w/v cetyltrimethylammonium bromide, 1.4 mol L^{-1} NaCl, 0.2% 2-β-mercaptoethanol, 20 mol L^{-1} EDTA, 100 mol L^{-1} Tris-HCl, 2% polyvinylpyrrolidone, pH 8.0) and incubated at 65 °C for 30 min. An equal volume of chloroform-isoamyl alcohol (24:1) was added to the lysis buffer (CTAB), vigorously mixed for 1 min and centrifuged at 12,000 g for 10 min. This step was repeated twice. The aqueous nucleic acid layer was transferred to a new tube and 2/3 volumes of ice-cold isopropanol (-20 ºC) was added and centrifuged for 10 min at 8,000 *g*. The supernatant was discarded, and the pellet containing DNA was washed with 70% ethanol, vacuum-dried and suspended in 50 µl of Tris-EDTA (TE) buffer (10 mmol L^{-1}-HCl and 0.1 mmol L^{-1} EDTA, pH 7.5). DNA fragmentation was analyzed following electrophoresis on a 1% agarose gel (Sigma, Aldrich) using 1xTris Acetate EDTA (TAE) buffer and a constant voltage of 85 V for 75 min.

DNA was visualized by UV fluorescence after staining with ethidium bromide (1 µg mL^{-1}) and the images of DNA bands were captured using an imaging system. The damaged DNA appeared as a ladder consisting of DNA fragments whereas intact DNA had high molecular weight and did not migrate very much in the gel (Karuppanapandian and Kim, 2013; Surapu et al., 2014).

DNA damage in leaves

The method of single cell gel electrophoresis (SCGE) which is also known as "Comet assay" was used to detect DNA damage in a single cell in leaves of *C. acinaciformis* induced by Cd. The leaves were washed three times with deionized water, blotted dry with fitler paper and used in the comet assay immediatelly. All operations were conducted under dim or yellow light to avoid DNA damage induced by light. The leaves were placed in a 60-mm Petri dish on ice and leaf was gently chooped into pieces in 250 µl of cold phosphate buffered saline (PBS) (130 mmol L^{-1} NaCl, 7 mmol L^{-1} Na₂HPO₄, 3 mmol L^{-1} NaH₂PO₄, 50 mmol L^{-1} EDTA, pH 7.5) in just 20 s. The pieces were washed in the buffer by repeated pipetting using a micropipette. The suspended solution (0.15 mL) containing nuclei was mixed with 0.2 mL of a solution containing 1% low melting point agarose (LMPA) (Sigma, Aldrich) in PBS at 37 ºC. A drop of the nuclei/agarose mixture was placed onto a pre-coated glass slide with normal melting point agarose (NMPA) and a coverslip was placed over the suspension. The slides were kept at 4 ºC in the fridge for 5 min, after gel formation, the coverslip was removed and the slides were put in freshly prepared cold alkaline buffer (300 mmol L^{-1} NaOH, 1 mmol L^{-1} Na₂EDTA, pH>13) in darkness at 4 ºC to allow the DNA to denature for 15 minutes (Kassaye et al., 2013). The total time from the beginning of slicing to transfer of slides was about 6-7 min. After this stage, electrophoresis was conducted at 4 ºC in the alkaline buffer for 15 min at 25 V, 300 mA. After electrophoresis, the slides were neutralized with a cold neutralization buffer (0.4 mol L^{-1} Tris-HCl, pH 7.5) at 4 ºC for 5 min then dipped in 95% ethanol to

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facilitate drying. After drying at room temperature, each slide was stained with 70 µl ethidium bromide (2 µg mL^{-1} in distilled water H$_2$O). The stained DNA was viewed using a fluorescent microscope (Olympus, Japan) at 400 x magnificitation provided with epifluorescence and equipped with a rhodamine filter (excitation wavelength 546 nm; barrier 580 nm). The damaged DNA has a similar structure to that of the comet. Randomly chosen comets were scored using "comet assay software program" (CASP) (Konca et al., 2003). Fifty comets representing each cells were scored per plant and the level of DNA damage was expressed as the percentage of DNA in the tail (% tail DNA) (Dikilitas et. al., 2009).

Statistical analysis

Data were statistically analysed by one-way analysis of variance (ANOVA) using the SPSS software program (Version 22.0). Duncan's Multiple Range Test was performed at a significance level of $P \leq 0.05$ to separate treatment means for each measured parameter.

Result

Biochemical responses such as total chlorophyll, carotenoid content, proline, MDA, H$_2$O$_2$, POX and CAT in leaves of *C. acinaciformis* were determined under differing concentrations of cadmium (CdNO$_3$).

Increased Cd concentrations adversely affected the pigment formation, the pigment contents such as chlorophyll and carotenoid significantly declined after 40 ppm cadmium dose. With respect to the carotenoid content, increased Cd concentrations led to increased carotenoid content up to 40 ppm dose level indicating a defensive strategy of the halophyte *C. acinaciformis*. Therefore, Cd doses up to 40 ppm were not suppressive to chlorophyll and carotenoid. However, after 40 ppm Cd doses, a remarkable decline in both chlorophyll and carotenoid contents were evident (*Figure 1A,B, Table 1*).

Again, proline content of *C. acinaciformis* gradually increased up to 40 ppm CdNO$_3$ dose (3.53 µmol g^{-1} Fwt, *Figure 2, Table 1*), then, the proline content of *C. acinaciformis* showed a gradual decline. Although proline level declined at 60 ppm CdNO$_3$ concentration, this level, however, was not statistically different from those of proline levels at 20 and 40 ppm CdNO$_3$. Proline contents at 80 and 100 ppm were remarkably low as compared to that of 40 ppm, which was the threshold level for proline content. It is important to note that Cd was not suppressive to proline synthesis up to 40 ppm indicating that synthesis of proline was responsive against Cd toxicity. However, a gradual decline was observed thereafter and the synthesis of proline was minimal at 80 and 100 ppm Cd concentrations. This clearly showed that *C. acinaciformis* plant was not successful to synthesize proline at 80 ppm Cd level to reduce the toxic effects of Cd.

When the stress markers were examined, both MDA and H$_2$O$_2$ accumulation in leaves of *C. acinaciformis* showed similar trends in which the stress markers, MDA, H$_2$O$_2$, were evident at 10 ppm CdNO$_3$ concentrations. Increasing concentrations led to further increases of MDA and H$_2$O$_2$ accumulation, (*Figure 3a,b, Table 1*). Accumulation of MDA and H$_2$O$_2$ showed plateau between 60 and 100 ppm CdNO$_3$ indicating that halophyte *C. acinaciformis* tolerated 100 ppm Cd as it tolerated 60 ppm CdNO$_3$. This was negatively corelated with the accumulation of proline as *C. acinaciformis* exhibited decreasing proline accumulation between 60 and 100 ppm Cd doses.
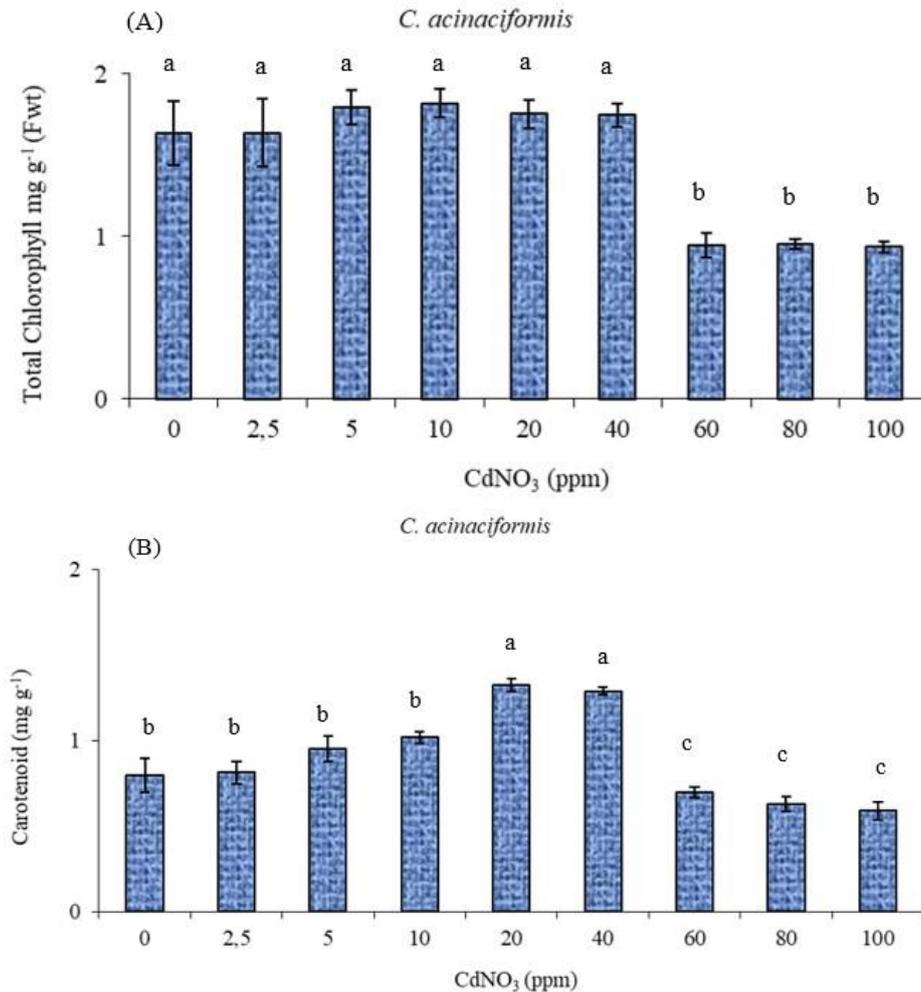
Figure 1. *Total Chlorophyll (A) and Carotenoid (B) contents in leaves of C. acinaciformis at different CdNO₃ stress. Bars indicate the means of the four replicates ± standard error. Letters indicate significant differences from one another according to Duncan's Multiple Range Test at P≤0.05*
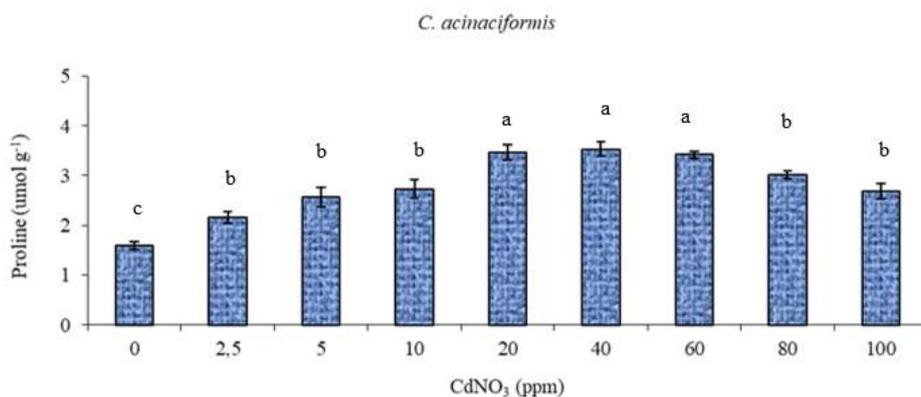

Figure 2. *Proline contents in leaves of C. acinaciformis at different CdNO₃ stress. Letters indicate the means of the four replicates ± standard error. Bars indicate significant differences from one another according to Duncan's Multiple Range Test at P≤0.05*

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Table 1. *Statistically analyses of Data using ANOVA*

Parameters	CdNO$_3$ (ppm)	n	Mean±S.E	F	p
Total Chlorpohyll	0	4	1.63±0.19a	47.89	0.00
	2.5	4	1.64±0.20 a		
	5	4	1.79±0.10 a		
	10	4	1.82±0.09 a		
	20	4	1.75±0.08 a		
	40	4	1.74±0.07 a		
	60	4	0.95±0.07 b		
	80	4	0.95±0.03 b		
	100	4	0.94±0.03 b		
	Total	36	1.47±0.07		
Carotenoid	0	4	0.79±0.09 b	23.08	0.00
	2.5	4	0.81±0.07 b		
	5	4	0.95±0.03 b		
	10	4	1.02±0.04 b		
	20	4	1.33±0.02 a		
	40	4	1.29±0.02 a		
	60	4	0.70±0.03 c		
	80	4	0.63±0.05 c		
	100	4	0.59±0.05 c		
	Total	36	0.90±0.05		
Proline	0	4	1.60±0.08 c	20.60	0.00
	2.5	4	2.17±0.12 b		
	5	4	2.57±0.19 b		
	10	4	2.74±0.18 b		
	20	4	3.46±0.16 a		
	40	4	3.53±0.15 a		
	60	4	3.42±0.06 a		
	80	4	3.01±0.08 b		
	100	4	2.69±0.16 b		
	Total	36	2.80±0.11		
MDA	0	4	3.22±0.24 c	38.41	0.00
	2.5	4	4.12±0.32 c		
	5	4	5.82±0.36 c		
	10	4	15.82±2.11 b		
	20	4	17.14±1.42 b		
	40	4	17.69±1.36 b		
	60	4	20.85±1.74 a		
	80	4	22.53±1.12 a		
	100	4	22.87±1.36 a		
	Total	36	14.45±1.32		
H$_2$O$_2$	0	4	2.93±0.27 c	66.22	0.00
	2.5	4	4.05±0.13 c		
	5	4	5.49±0.13 c		
	10	4	16.62±1.07 b		
	20	4	17.98±1.26 b		
	40	4	19.54±2.05 a		
	60	4	22.23±1.24 a		
	80	4	22.35±1.02 a		
	100	4	24.14±0.44 a		
	Total	36	15.04±1.39		
POX	0	4	0.98±0.12 c	63.96	0.00
	2.5	4	1.10±0.07 c		
	5	4	1.26±0.06 c		
	10	4	1.55±0.18 c		
	20	4	3.43±0.13 a		
	40	4	3.74±0.17 a		
	60	4	1.93±0.14 b		

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Parameters	CdNO$_3$ (ppm)	n	Mean±S.E	F	p
	80	4	2.11±0.08 b		
	100	4	1.90±0.09 b		
	Total	36	1.99±0.16		
CAT	0	4	0.43±0.03 c	33.48	0.00
	2.5	4	0.46±0.03 c		
	5	4	0.61±0.05 c		
	10	4	0.73±0.11 c		
	20	4	1.95±0.20 a		
	40	4	1.81±0.14 a		
	60	4	1.22±0.03 b		
	80	4	1.20±0.01 b		
	100	4	1.02±0.06 b		
	Total	36	1.05±0.09		
Leaf Cd contents	0	4	0.01±0.01 f	765.16	0.00
	2.5	4	1.25±0.11 f		
	5	4	2.15±0.15 f		
	10	4	4.78±0.19 e		
	20	4	13.14±0.85 d		
	40	4	29.84±0.64 c		
	60	4	38.24±1.10 b		
	80	4	40.12±1.15 b		
	100	4	65.77±1.29 a		
	Total	36	21.70±3.68		
DNA Damage	0	4	7.22±0.08 c	40.25	0.00
	2.5	4	7.46±0.12 c		
	5	4	7.68±0.16 c		
	10	4	7.73±0.19 c		
	20	4	8.26±0.37 c		
	40	4	9.11±0.01 c		
	60	4	12.24±1.21 b		
	80	4	13.68±0.97 b		
	100	4	17.95±0.68 a		
	Total	36	10.15±0.62		

*Different letters indicate significant differences from one another according to Duncan's Multiple Range Test at P ≤0.05

When antioxidant enzymes such as POX and CAT were evaluated, a similar trend to those of proline and carotenoid was observed in that both POX and CAT gave increased responses both at 20 and 40 ppm CdNO$_3$ doses, (*Figure 4a,b, Table 1*). However, declining trends in both enzymes were more apparent.

Impact of enzymes between 0-10 ppm or 60-100 ppm was low when compared to those of concentrations between 20-40 ppm CdNO$_3$ levels. The results showed that the antioxidant mechanism gave the highest response between 20 and 40 ppm CdNO$_3$ level, thereafter, the antioxidant response including enzymatic and non-enzymatic responses were decreased. This clearly showed that the defence mechanisms of *C. acinaciformis* plant broke down and did not function properly. It is important to note that following 60 ppm Cd toxicity in which the defence mechanism was minimized in the following section.

Apart from biochemical responses, molecular responses along with the accumulation of Cd in leaves of *C. acinaciformis* were evaluated upon exposure to various CdNO$_3$ concentrations. As the concentration of CdNO$_3$ increased, *C. acinaciformis* leaves accumulated increasing amount of Cd in their leaves.
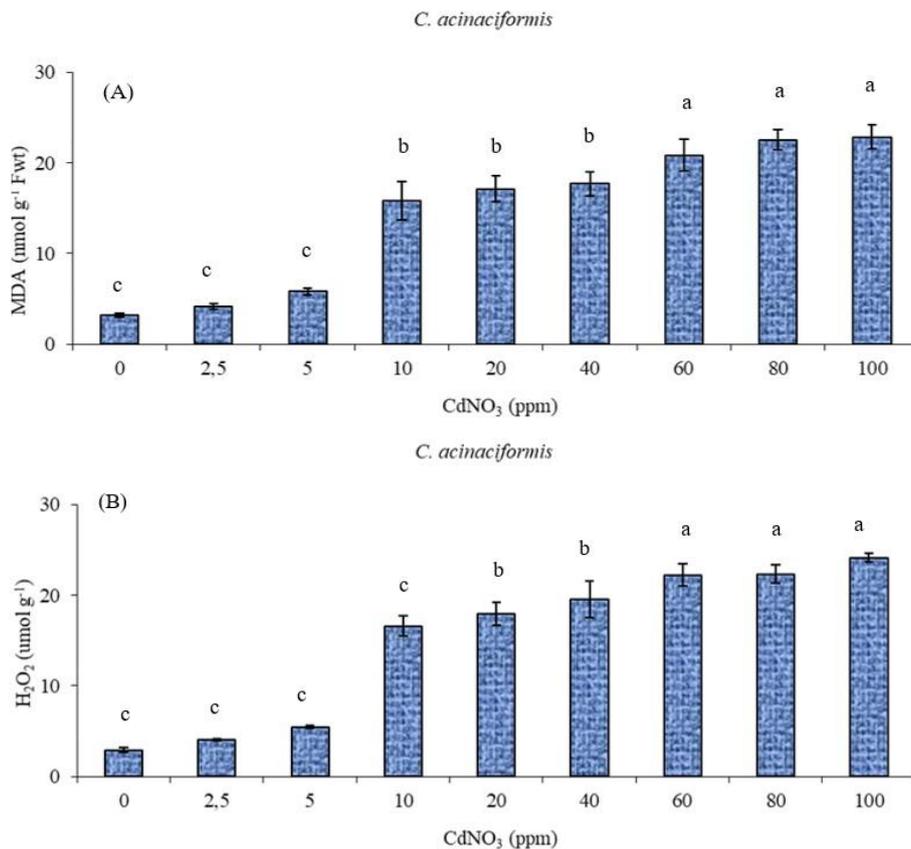
Figure 3. *MDA (A) and H_2O_2 (B) contents in leaves of C. acinaciformis at different $CdNO_3$ stress. Letters indicate the means of the four replicates ± standard error. Bars indicate significant differences from one another according to Duncan's Multiple Range Test at P≤0.05*

The higher the concentration of $CdNO_3$ led to more accumulation of Cd in the leaves of *C. acinaciformis.* When DNA fragmentation was analysed through 1% agarose gel, the first sign of DNA fragmentation was evident at 5 ppm $CdNO_3$ concentrations. Then, increasing concentration of Cd led to increased DNA fragmentation (*Figure 5*). At 5 ppm and above Cd concentrations, DNA fragmentations were evident both at 250 and 2000 bp. However, more fragments were visible at 250 bp DNA molecule. It is evident that at 80 and 100 ppm Cd concentrations, DNA fragments were more pronounced being at 250 and 500 bp. Since DNA fragmentation was performed on total DNA of leaves, it is possible that highly damaged DNA in a cell of leaves might contribute to increase of DNA damage in leaf samples. Therefore, DNA damage was also assessed by performing in a single cell to determine the condition of DNA. The results showed that the damage caused by $CdNO_3$ was insignificant up to 40 ppm dose, then a slightly increased DNA damage was evident at 60 ppm dose, then 80 and 100 ppm doses of $CdNO_3$ led to highly significant DNA damages.

It is important to note that the tolerance of $CdNO_3$ toxicity by *C. acinaciformis* was found consistent with the antioxidant enzyme responses in which the halophyte plant gave remarkable responses at 40 ppm $CdNO_3$. However, 60 ppm and above concentrations of $CdNO_3$ significantly reduced chlorophyll and carotenoid contents and other enzymatic and non-enzymatic responses and the stress metabolites such as MDA and H_2O_2 were remarkably accumulated at these concentrations.
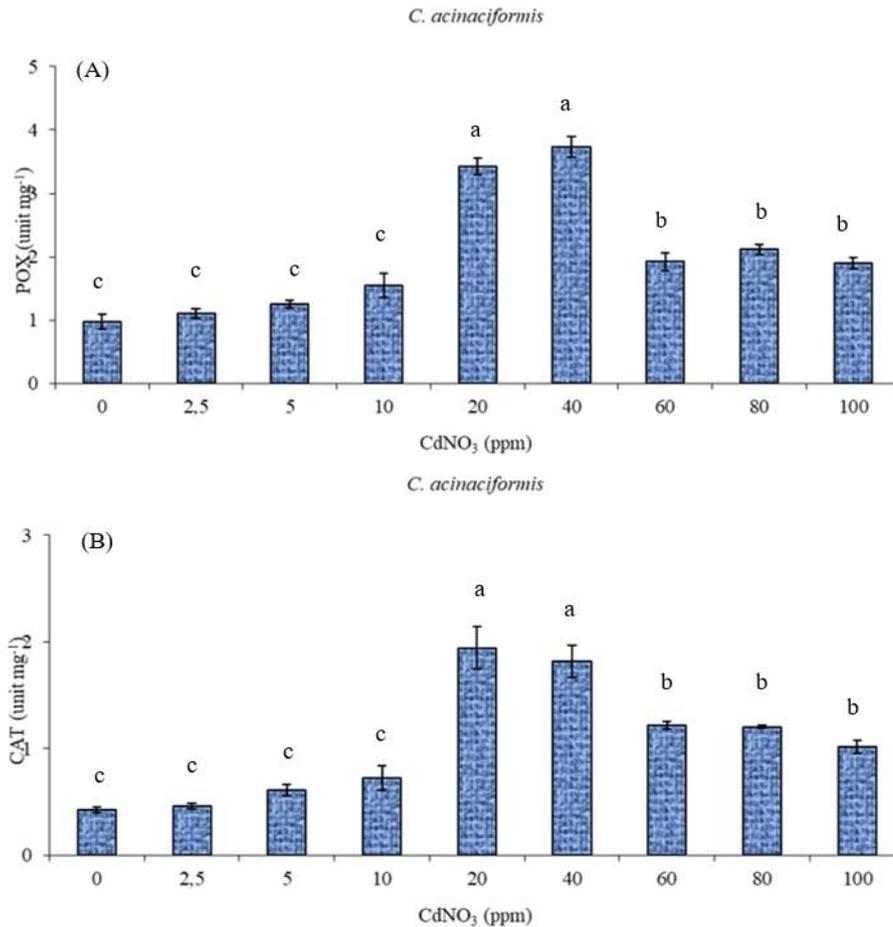
Figure 4. POX (A) and CAT (B) contents in leaves of C. acinaciformis at different CdNO₃ stress. Letters indicate the means of the four replicates ± standard error. Letters indicate significant differences from one another according to Duncan's Multiple Range Test at P≤0.05
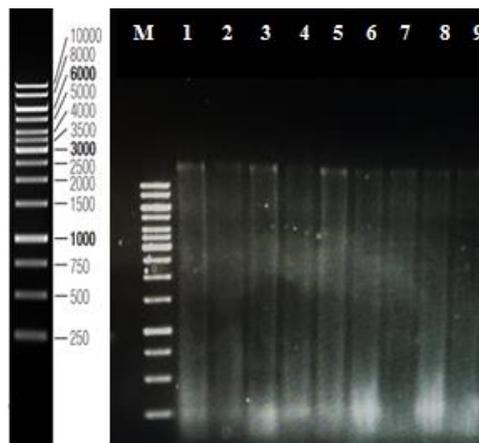

Figure 5. DNA fragmentation analysis of C. acinaciformis under differing CdNO₃ concentrations, Lanes (M-9): M: marker 10 kb standard molecular weight marker, 1: 0 ppm, 2: 2.5 ppm, 3: 5 ppm, 4: 10 ppm, 5: 20 ppm, 6:40 ppm, 7: 60 ppm, 8: 80 ppm, 9: 100 ppm CdNO₃ stress. DNA of C. acinaciformis was separated through 1% agarose gel and visualized under UV fluorescence using ethidium bromide. Arrows show fragmented DNA molecules at different band sizes

Upon exposure to Cd toxicity, *C. acinaciformis* leaves accumulated significant amount of Cd ions. The highest Cd accumulation was 65.77 mg kg^{-1} at 100 ppm Cd levels. Assessment of DNA damage caused by Cd showed that *C. acinaciformis* did not show any dose-response increased DNA damage up to 60 ppm Cd levels (*Figure 6A,B, Table 1*).
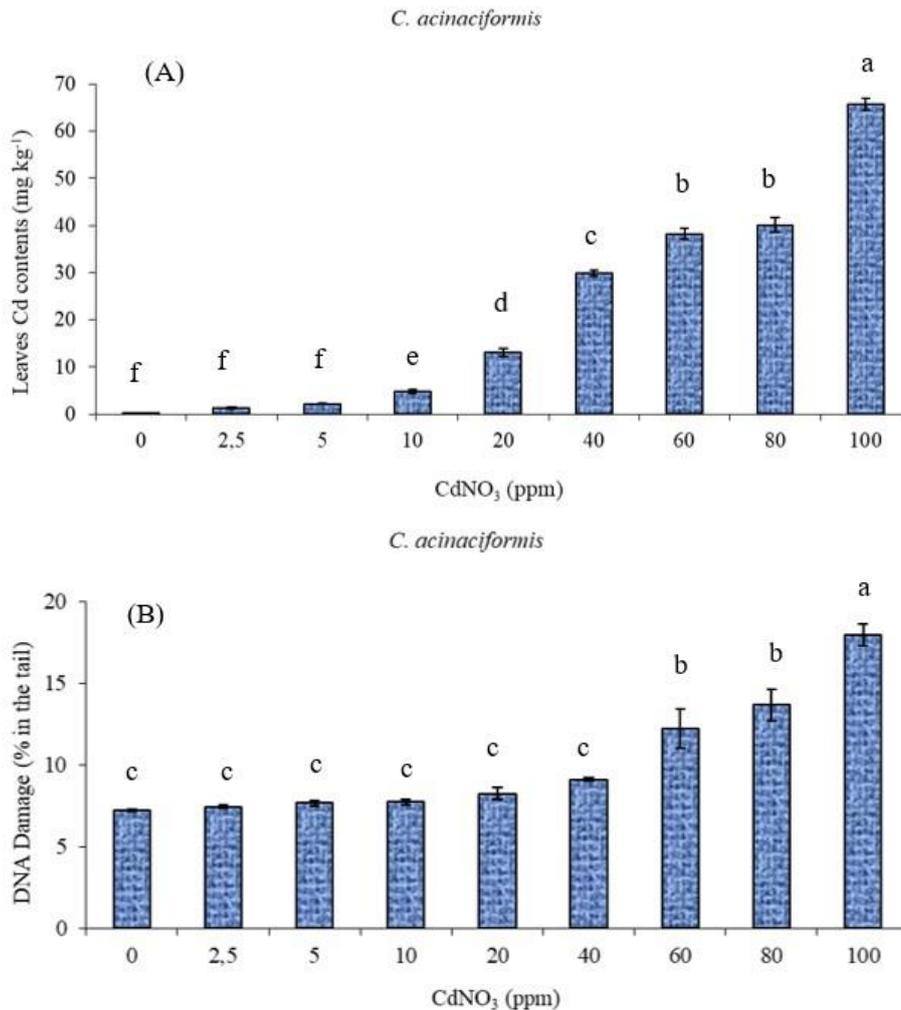

Figure 6. Cd ion contets (a) and DNA damage (b) in leaves of C. acinaciformis at different CdNO₃ stress. Bars indicate the means of the four replicates ± standard error. Letters indicate significant differences from one another according to Duncan's Multiple Range Test at P≤0.05

It is clear that a significant amount of CdNO₃ accumulation in leaves led to not only biochemical disturbances but also molecular mechanisms were disturbed as shown by DNA fragmentation through gel electrophoresis and DNA damages via comet assay. It could be pointed out that the tolerance capacity of DNA of cells positively correlated with the biochemical responses of the cell. However, DNA molecule was found much stronger and resistant to Cd toxicity in terms of tail DNA damage % and DNA fragment analysis. Therefore, it could be said that recovery of a cell in plants could totally depend on the integrity and health status of DNA. If DNA is not highly damaged, tolerance to stress could well be achieved.

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Discussion

A great number of studies have shown that the major sites of action for Cd are photosynthetic pigments, particularly the biosynthesis of chlorophyll (Guo et al., 2016; Paunov et al., 2018) and carotenoids (Zhang et al., 2020). Cd ions have been known to affect the structure and function of chloroplasts in many plant species such as *Pisum sativum* L. (Sandalio et al., 2001), *Spinacea oleracea* (Fagioni et al., 2009), *Beta vulgaris* (Basa et al., 2014), *Triticum aestivum* (Abedi and Mojiri, 2020). It is important to note that the halophyte still synthesized a remarkable content of carotenoids and chlorophyll between 60-100 ppm Cd dose levels in which the most glycophytes or tolerant plants such as tomato, pepper, wheat, vegetables show sensitivity. At higher doses, it could have serious effects and lead to death of crop plants. Although halophytes are more tolerant to abiotic stresses than those of glycophytes, however, heavy metal stress could have serious adverse effects on halophytes. Determination of level of tolerance of halophytes to heavy metal stress is important if those plants are aimed to be used for the purpose of cleaning up soils from heavy metals or used as companion plants with the crop plants. We determined that *C. acinaciformis* plants showed great declines at 60 ppm and above Cd concentrations. the synthesized pigments were still enough and enabled the plant green colour. Although the halophyte plant was not able to synthesize sufficient amounts of chlorophyll and carotenoids as those of plants grown in control conditions, however, it was noticed that the synthesized pigments were still enough and enabled the plant green colour.

Proline is an adaptive response in plants exposed to a stressful environment. Proline accumulation appeared to be a suitable indicator of Cd stress. In this study, proline level in *C. acinaciformis* increased significantly following exposure to 2.5-, 5-,10-, 20-, 40-, 60-, 80-, and 100 ppm $CdNO_3$ toxicity. Although the synthesis of proline started to decline at 60 ppm Cd doses, the remarkable reduction was evident at 80 ppm Cd dose indicating that the build-up of defence metabolites was still active at 80 ppm Cd dose level. A similar study reported that tobacco cells exposed to Cd treatment accumulated high levels of proline and proline accumulation alleviated the inhibitory effects of Cd in tobacco cell (Islam et al., 2009; Zouari et al., 2016). For maintaining cell turgidity, plants are able to accumulate osmolytes such as proline, soluble protein, and ions under Cd stress (Cao et al., 2014). Proline stability of the sub-cellular structures by scavenging free radicals and modulating cellular homeostasis (Kour and Asthir, 2015).

Cd is a non-redox metal unable to perform single electron transfer reactions, and does not produce ROS such as the superoxide anion (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), but generates oxidative stress by interfering with the antioxidant defence system (Benavides et al., 2005; Smeets et al., 2008; Das and Roychoudhury, 2014). Accumulation of stress metabolites started as early as at 2.5 ppm Cd dose and increased thereafter. The remarkable build-up of stress metabolites started at 10 ppm Cd dose. At 60 ppm Cd dose, a significant accumulation of stress metabolites was evident, *Figure 3*. Increasing concentrations of Cd further up to 100 ppm Cd dose did not increase the build-up of stress metabolites. This was clearly reflected with the fact that the activities of antioxidant enzymes were minimal at 60 ppm Cd dose and increasing concentrations of Cd further up to 100 ppm did not increase the level of antioxidant enzymes (POX and CAT), *Figure 4*. In our findings, POX and CAT antioxidant enzymes increased up to 40 ppm Cd concentration, then slightly decreased. A similar finding was made by Yilmaz and Parlak (2011) who reported that the tolerance of *Groenlandia densa* to Cd stress was partially due to the high activity of CAT. It is important to note that here

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the accumulation of stress metabolites was in parallel with the synthesis of antioxidant enzymes. Reduction in the synthesis of antioxidant enzymes at and above 60 ppm Cd dose was accordance with the accumulation of stress metabolites such as MDA and H_2O_2.

Although the concentration of Cd increased and the higher amount of concentration of Cd increased and the higher amount of Cd was taken up by the halophyte plants, *Figure 6*, and resulted in increased DNA damages via shown by DNA fragment analysis and Comet assay, the halophyte plant *C. acinaciformis* was not able to synthesize antioxidant enzymes higher than that of 60 ppm Cd dose at 80 and 100 ppm Cd doses. At those concentrations, no more stress metabolites were accumulated either. We suggest that the accumulation of stress metabolites totally depends on the defence metabolism available. It has been known that ROS damages in plants involve lipid peroxidation, protein oxidation, and DNA damage. Cd produced an enhancement of lipid peroxidation in bean (Chaoui et al., 1997), sunflower (Gallego et al., 1996), and maize and pea plants (Lozano-Rodriguez et al., 1997). DNA damage caused by Cd involved the destruction of nucleic acids, cell membrane, lipids, and proteins; reduction of protein synthesis; and damage of photosynthetic proteins, which affects growth and development of the whole organism. DNA damage has also been defined via the determination of the frequency of abnormalities such as fragments, single and double bridges, and stickiness (Gill and Tuteja, 2010; Noor et al., 2018). It is clear that DNA breaks occurred much faster and at higher impact at 60 and above concentrations of Cd without accumulating stress metabolites and defence enzymes. So, we could conclude that level of Cd dose that causes significant DNA damages is the threshold level for plants that no recovery should be expected unless additional help is enabled.

Conclusion

C. acinaciformis plant could well be used to remediate Cd-contaminated sites. To determine the removal Cd capacity of *C. acinaciformis,* we measured the accumulation of Cd in leaves. *C. acinaciformis* plants accumulated significant amount of Cd up to 60 ppm without exerting significant stress symptoms. At high Cd concentrations, significant amounts of pigment loss along with the increase of oxidant molecules and decrease of antioxidant enzymes were evident. Cd toxicity results in chromosomal aberrations, DNA stand breaks, sister chromatid exchange in DNA molecules. Its toxicity involves depletion of antioxidant molecules and enhances ROS. However, at high doses, it could cause direct DNA damages without exerting oxidative stress as shown in this study. DNA integrity and health also proved that the nuclear elements were more resistant to the toxicity of Cd as compared to those of metabolites. Measuring metabolites is important to determine the tolerance level of the halophyte, however, molecular approaches to determine the possible highest level of tolerance of the halophyte to Cd toxicity is of great significance. We conclude that the roots of halophyte *C. acinaciformis* plant has ability to remove Cd from contaminated sites and are a strong candidate to clean up the soils contaminated with heavy metals.

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