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Full Length Research Paper

Cadmium-induced changes in mitotic index and genotoxicity on *Vigna unguiculata* (Linn.) Walp

Thangaraja Amirthalingam[1]*, Ganesan Velusamy[2] and Raja Pandian[2]

[1]Department of Biotechnology, St. Peter's University, Avadi, Tamilnadu, India.
[2]Centre for Research and P.G. Studies in Botany, Ayya Nadar Janaki Ammal College (Autonomous) Sivakasi, Tamilnadu, India.

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The present study is aimed to investigate the effect of Cadmium (Cd) on mitotic index (MI) and DNA damage of *Vigna unguiculata*. The seedlings were exposed to 2, 4, 6, 8 and 10 mM concentrations of cadmium chloride ($CdCl_2$) for 15 days. The drastic reduction in MI was observed due to Cd toxicity because the mitotic divisions had been with holding when the Cd stress increases. Cd-induced genotoxicity was measured through comet assay. This suggests that increased production of reactive oxygen species (ROS) under Cd toxicity serves as a major source of DNA damage leading to strand breakage, removal of nucleotides and variety of modifications in organic bases of nucleotides.

Key words: Genotoxicity, comet assay, mitotic index, Cadmium stress, deoxyribonucleic acid (DNA) damage.

INTRODUCTION

Cadmium (Cd) is a potent heavy metal pollutant of the environment. It is a heavy metal of widespread occurrence when released into the environment by power stations, heating systems, metal working industries, waste incinerators, urban traffic, and cement factories a by-product of phosphate fertilizers (Nolan et al., 2003). Cd released to the environment enters biogeochemical cycle and tends to accumulate in soils and sediments, where it is potentially available to rooted plants. The degree to which higher plants take Cd depends on its concentration in the soil and its bioavailability which are modulated by the presence of organic matter, pH, redox potential, temperature and concentration of other elements (Panda and Panda, 2002).

The presence of Cd at higher concentrations in the soil damages root tips, reduces nutrient and water uptake, impairs photosynthesis and inhibits growth of the plants (Sharma and Dubey, 2006). Furthermore, Cd directly or indirectly induces reactive oxygen species (ROS), which affect the redox status of the cell and cause oxidative damage to proteins, lipids, and other biomolecules (Stohs et al., 2000; Schutzendubel et al., 2001). Cd damages the nucleoli in cells of root tip (Nolan et al., 2003), alters the synthesis of RNA, inhibits ribonuclease activity (Shah and Dubey, 1995) and inhibits the DNA repair mechanism (Rossman et al., 1992). Even though the toxic effects of Cd compounds in plants have been studied over many years in consistent and results have been obtained with respect to their toxic properties (Koppen and Verschaeve, 1996; Steinkellner et al., 1998; Panda and Panda, 2002). The possible pathways of Cd-induced genotoxicity are still unknown, but may involve the interaction of the metal with DNA and damage the DNA, either directly or indirectly (Valvende et al., 2001). The present study is therefore focused on the toxic effect of Cd on cell division and genotoxicity in *Vigna unguiculata* through analyzing the mitotic index (MI), formation of micronuclei and comet assay.

MATERIALS AND METHODS

Chemicals and media

Cadmium chloride ($CdCl_2$) hemi (pentahydrate) (Cd^{2+}, CAS No. 7790-78-5), the plant growth medium, reagents for electrophoresis, normal and low melting point agarose (NMP and LMP) and general laboratory reagents were purchased from Sigma Aldrich Chemical

*Corresponding author. E-mail: athangaraja@gmail.com.

Co., India. Cd solutions were prepared by using ultra pure water (pH 6.4).

Plant materials

Cow pea seeds [*V. unguiculata* (Linn.) Walp.] were collected from Regional Research Station, Tamilnadu Agricultural University, Srivilliputhur, Virudhunagar Dt, Tamilnadu, India. Cow pea seeds were germinated using sawdust as support media in polystyrene seedlings trays at 22 to 26°C with 16 h photoperiod each day in a growth chamber, and add half strength of Hoagland's solution for their growth.

Mutagenic treatment

A stock solution of Cd (100 mM) was prepared by dissolving 2.1 mg of $CdCl_2$ salt in 100 ml of ultra pure water. Required dilutions (2 to 10 mM) of this solution were used. For control treatment, 50% Hoagland's solution was applied up to 15 days. For mutagenic treatment the seedlings were treated with respective concentrations of Cd (2, 4, 6, 8, and 10 Mm) dissolved in 50% Hoagland's solution, and was applied up to 15 days with a 16 h photoperiod at 22 to 26°C.

Cytogenetic assay

After chemical treatment of the plants, excised roots of cow pea seedlings were placed for 5 min on Carnoy's (3:1 ethanol: glacial acetic acid) fixative for scoring MI and Micro Nuclei formation (MN). In brief, fixed root tips were hydrolyzed in 1 N HCl and ethanol (1:3 ratio) for 3 min and immersed in 70% ethanol for 5 min, properly washed 3 to 4 times with distilled water and stained in acetocarmine. Stained root tips were squashed in 45% acetic acid and analyzed under light microscope (Olympus).

MI was calculated by number of cell which undergone mitotic divisions divided by total cells in a microscopic field into 100. Similarly, micro-nucleated interphase cells were scored from same number of non-dividing cells. Both the endpoints, MI and MN were expressed in percentage as mean ±standard deviation (SD).

Comet assay

After chemical treatment, the roots were excised from cow pea seedlings and were placed for 2 min on ice to keep them rigid (Navarrete et al., 1997). For isolation of nuclei, roots were cut in 5 mm from their ends and about 20 mg of root tips, treated or untreated as appropriate (Pfosser et al., 1995), were placed in a Petri dish kept on ice cold 200 mM Tris buffer, pH 7.5. Using a fresh razor blade, roots were gently sliced. The plate was kept tilted in the ice so that the isolated nuclei would collect in the buffer. The nuclei were filtered through 50 µm nylon mesh, and centrifugation was carried out 200 g for 5 min (4°C) in a swinging bucket rotor. The pellet was then resuspended in 200 µl of Tris-Mg Cl_2 buffer. The integrity of the nuclei was checked under a fluorescent microscope after staining with ethidium bromide (10 mg/ml) using an excitation filter of 515 to 560 nm.

The normal microscope slide was pre-coated with a layer of 1% normal melting point agarose and dried at room temperature and add 100 µl of 0.8% low melting point agarose at 37°C, and was mixed with 20 µl of the nuclear suspension and dropped on top of the first layer. The agarose layers on slides were allowed to solidify for 2 min on an ice cold tray and were then immersed in ice cold lysing solution (1 M NaCl; 30 mM NaOH, 0.5% w/v SDS, pH 12.3) for 1 h (Pfosser et al., 1995).

After lysis, the slides were placed in a horizontal gel electrophoresis chamber and the DNA was allowed to unwind for 1 h in the electrophoretic buffer [30 mM NaOH, 1.5 mM ethylene diamine tetraacetic acid (EDTA) pH 12.3] electrophoresis was conducted for 20 min at 1.0 cm^{-1} (25 V, 300 mA) at 8°C. Then the slides were washed twice with water, tried for 1 h at room temperature, and stained with ethidium bromide (20 mg/ml) for 5 min, dipped in ice cold water to remove the excess ethidium bromide. The gels were fixed for 10 min in a fixative solution (15% w/v trichloro acetic acid, 5% w/v zinc sulphate, 5% v/v glycerol). Then the slides were washed 3 times in deionized water and dried overnight at room temperature.

For each slide, 25 randomly chosen nuclei were analyzed using a fluorescence microscope with an excitation filter of BP 546/10 nm and at barrier filter of 590 nm. The extent of DNA migration was determined and analyzed by Autocomet Image analysis system (The Tritek Corporation, Virginia, USA). The tail DNA, tail length (measured from the right edge of comet head) and tail movement (the integrated value of tail density multiplied by the migration distance) parameters were analyzed from comet assay (Lovely et al., 1999). Generally, 100 comets were scored per treatment (20 comets were randomly from 5 replicated slides).

Statistics

All data presented are the mean values ± SD. Statistical analysis was performed by students t-test to test the significant difference between means at $p<0.05$ level.

RESULTS

The inhibition of MI and MN formation observed with the $CdCl_2$ exposure in cow pea root cells is summarized in Table 1. The MI in the root tip cells of *V. unguiculata* was found to decrease ($P \leq 0.05$) significantly than control. Treatment of $CdCl_2$ (2 to 10 mM) inhibited the MI in a concentration dependent manner. The root meristem cells exposed to Cd showed a concentration related decrease in MI ranging from 0.54 to 0.09. The Cd treated root meristem cell kept in water after treatment increase in MI ranging from 1.60 to 41.46. MN formation was also found with treatment of higher concentration of Cd (10 mM) which persist even after recovery period and significant level ($P \leq 0.05$) was found at 6 mM onwards.

Table 2 illustrates the chromosomal aberrations (CA) and mitotic aberrations (MA) observed in the root meristem cells exposed to Cd and recovery from the Cd effect. All the treatments induced CA and MA in concentration dependent pattern though significant level ($P \leq 0.05$) CA found at 8 mM and 10 mM. The CA was decreased significantly after post exposure recovery period ranging from 7.22 to 40.65%. Induction of MA due to exposure of Cd is significantly ($P \leq 0.05$) and concentration dependently. The post exposure of treatment revealed reduces the frequencies of MA ranging from 10.34 to 49.02%.

After 15 days of treatment of cow pea with Cd, nuclei were isolated and a concentration response analysis in the comet assay was performed (Table 3). In roots of cow

Table 1. Inhibition of MI and induction of micronuclei formation in the root meristem cells of *V. unguigulata* exposed to different concentrations of $CdCl_2$.

S/N	Cd concentration (mM)	Mitotic index		Formation of micronuclei	
		Treatment	Recovery (%)	Treatment	Recovery (%)
1	Control	0.54 ± 0.04	1.6 ± 0.20	1.6 ± 0.05	12.11 ± 1.28
2	2	0.33 ± 0.05	7.82 ± 1.23	4.1 ± 0.03	16.78 ± 2.37
3	4	0.29 ± 0.03	18.71 ± 3.29	6.6 ± 0.04	28.73 ± 3.54
4	6	0.23 ± 0.05*	27.25 ± 2.33	10.0 ± 0.02*	37.45 ± 3.11
5	8	0.18 ± 0.02	35.56 ± 3.98	15.0 ± 0.04	43.02 ± 2.99
6	10	0.09 ± 0.04*	41.46 ± 2.61	16.6 ± 0.05	52.76 ± 4.65

Table 2. Chromosome and MA in the root meristem cells of *V. unguiculata* exposed to different concentration of $CdCl_2$.

S/N	Cd concentration (mM)	Chromosome aberrations				Mitotic aberrations	
		Breaks (%)	Fragments (%)	Aberration (%)	Recovery (%)	Aberrant cells (%)	Recovery (%)
1	Control	0.43 ± 0.12	0.21 ± 0.04	1.54 ± 0.12	7.22 ± 1.25	0.87 ± 0.38	10.34 ± 2.34
2	2	1.20 ± 0.39	0.67 ± 0.05	2.99 ± 0.26	12.45 ± 2.65	8.25 ± 1.98	19.45 ± 3.45
3	4	2.93 ± 0.28	1.02 ± 0.07	3.56 ± 0.58	15.78 ± 2.11	16.98 ± 4.36	28.65 ± 3.67
4	6	4.25 ± 0.47	1.98 ± 0.11	5.98 ± 0.77	24.22 ± 3.90	29.76 ± 5.45	35.43 ± 3.75
5	8	5.67 ± 0.28	2.36 ± 0.27	7.45 ± 0.32	31.78 ± 3.14	37.87 ± 4.98	42.23 ± 5.12
6	10	7.23 ± 0.34	3.04 ± 0.33	8.45 ± 0.87	40.65 ± 2.39	43.76 ± 4.65	49.02 ± 2.35

Table 3. Concentration dependent genotoxicity induced by $CdCl_2$, in isolated root nuclei of *V. unguiculata* by comet assay.

S/N	Level of Cd treatment (mM)	Comet assay		
		Tail length (µm)	Tail moment (µm)	Tail DNA (%)
1	Control	72.13 ± 23.45	1.42 ± 0.81	1.14 ± 0.44
2	2	150.34 ± 39.11	8.33 ± 1.65	5.60 ± 1.06
3	4	210.24 ± 25.99**	13.64 ± 2.54*	6.23 ± 0.95
4	6	264.54 ± 48.51	23.29 ± 4.79	8.86 ± 1.20
5	8	468.11 ± 53.20*	29.37 ± 2.88	10.29 ± 0.77
6	10	576.19 ± 49.82	30.57 ± 1.50	11.12 ± 0.65

pea with increased concentration of Cd, the percentage of tail DNA increased significantly from 1.14 (control) to 11.12 (10 mM) Cd treatment.

As demonstrated in Table 3 after 15 days continuous treatment, significant ($P \leq 0.05$) increase in the tail length was observed at 6 mM Cd and above. In control, the root meristamatic cells of the tail length was found to be 72.13 µm, whereas cells exposed to 10 mM of Cd showed 576 µm. Exposure of Cd-induced concentration dependent increase in both the tail length and tail movement. In control treatment, the TM was observed to be 1.42 m, whereas seedling exposed to 10 mM showed 30.57 µm.

Figure 1 shows the mitotic phases in root apical cells of control seedlings of *V. unguiculata* and those exposed to various concentrations of Cd. The normal mitotic divisions and phases like metaphase, anaphase and telophase are found in the root apical cells of control seedlings (Figure 1). In the seedlings exposed to various concentrations of Cd, very few cells have undergone mitotic phases, instead, more number of micronucleus are found in all the cases except control.

Comet assay were performed through alkaline single-cell gel electrophoresis followed by ethidium bromide staining are given in Figure 2. It shows the isolated nucleus comprising a head and tail forming a comet. The head corresponds to the amount of DNA that still remains in the region of the nuclear matrix, whereas the tail visualizes the fragments of DNA migrating from the nuclei.

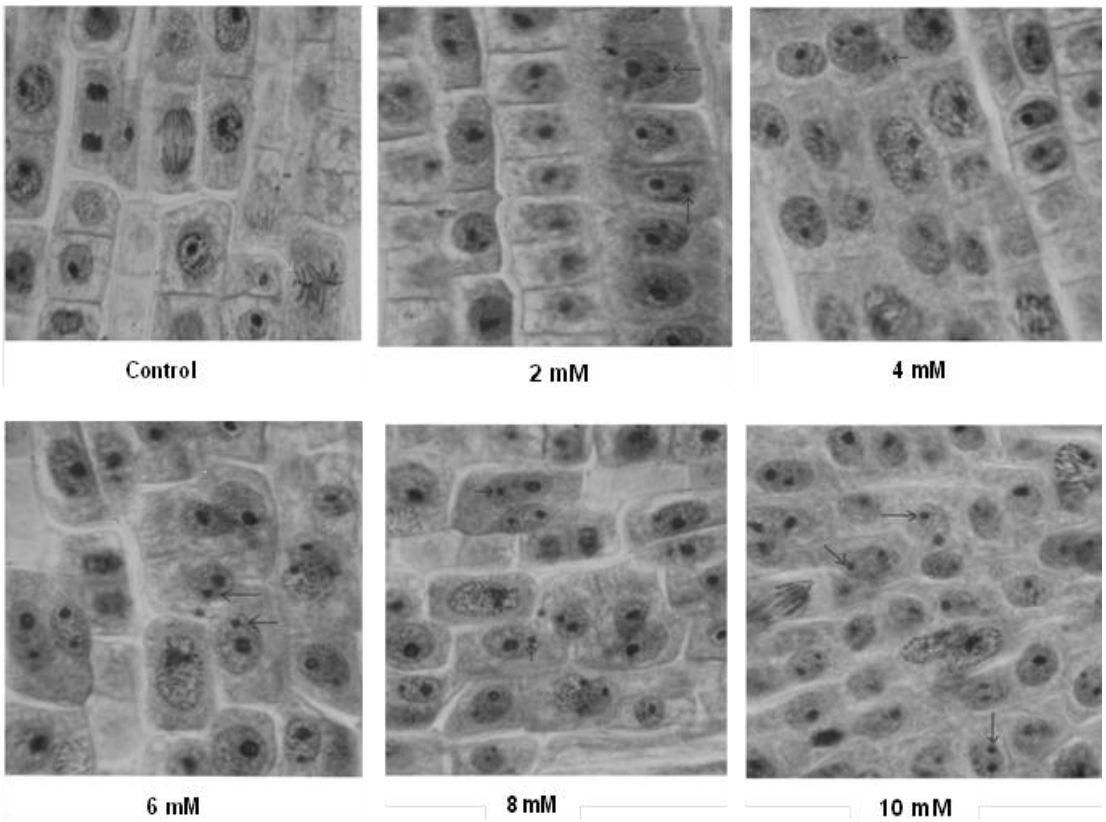

Figure 1. Mitotic phases in root apical cells of control seedlings of *V. unguiculata* and those exposed to various concentrations of Cd. Red arrow marks indicate the micronuclei; Green arrow marks indicate normal mitotic phases.

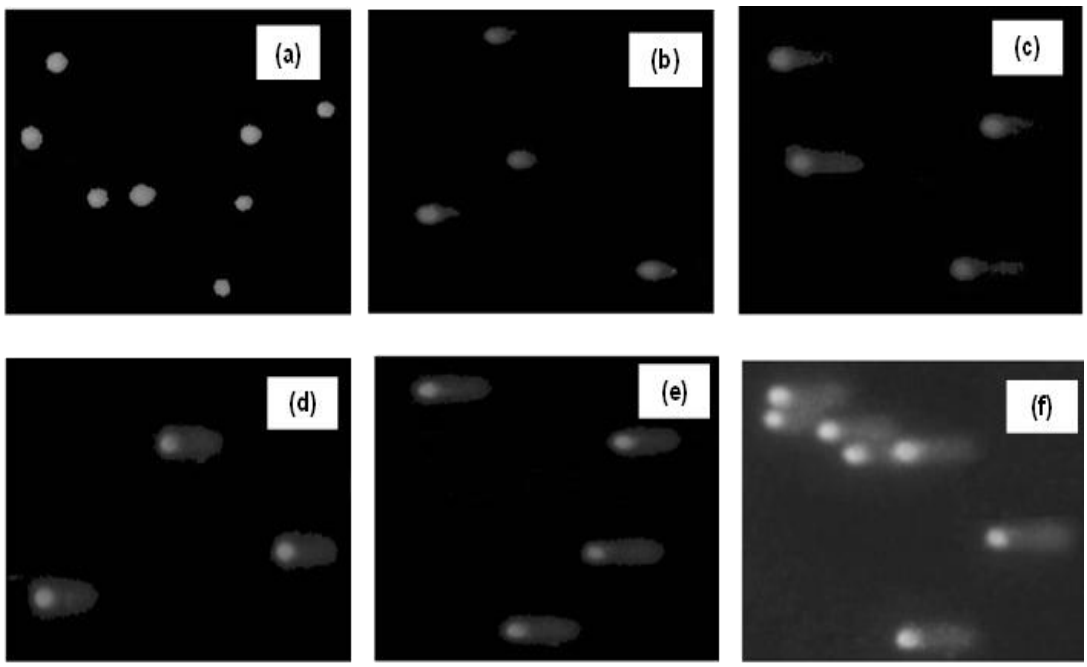

Figure 2. Comet images after alkaline single-cell gel electrophoresis followed by ethidium bromide staining. The cells were isolated from the leaves of *V. unguiculata* of: (2a), control seedlings; (2b), or those exposed to 2 mM; (2c), 4 mM; (2d), 6 mM; (2e), 8 mM; (2f), 10 mM of $CdCl_2$.

The formation of comet showing different levels of damage of DNA and it was observed through visual scoring. Control shows undamaged DNA (Figure 2a). The various degrees of damage increased from minor (Figure 2b) to severe (Figure 2f) as the seedlings exposed to different concentration of $CdCl_2$ (2 to 10 mM). The increasing trend was noticed in tail length when the Cd stress increased from 2 to 10 mM (Table 1) and the increase in tail length was found to be significant ($P < 0.05$).

DISCUSSION

In general, Cd causes toxic effects in turn leads to the inhibition of plant growth. It reveals through the investigations made in the present study. Shah and Dubey (1997) noticed that Cd toxicity adversely affected germination of seeds and seedling vigour in rice by limiting water transport to growing tissues.

Inhibition of cell growth due to Cd appears to be as a result of increased cross-linking of pectin in the middle lamellae. This cross-linking might be responsible for inhibition of cell expansion and its further growth (Huang et al., 1974; Poschenrieder et al., 1989). The inhibition of cell expansion and its growth might also be due to direct or indirect effect of Cd on auxin metabolism and auxin carriers (Barcelo and Poschenreider, 1990). Even at moderate concentration levels Cd adversely affects the growth, survival and yield of several crops (Bingham et al., 1976; Shah and Dubey, 1995).

Inhibition of DNA repair is an important genotoxicity mechanism of Cd. This genotoxicity is revealed in the present investigation through comet analysis and MI. It has been suggested that this may occur when Cd competes Zinc for a common binding site on enzymes involved in DNA synthesis (Rossman et al., 1992). The various plant genotoxic tests demonstrated that Cd strongly induce DNA damage in a concentration dependent manner (Gichner et al., 2004).

The results obtained for *V. unguiculata* leaves with the application of the alkaline comet assay and based on tail length and tail moment are consistent with previous observation (Gichner et al., 2004). In general, long thin tails are induced at the lower concentrations of Cd, while longer and thick tails with DNA fragments in the vicinity of the comet head are visible at the higher concentration. This phenomenon has been explained by the formation of chromosome bridges and stickiness of chromosomes observed and same type of observation was also noticed in barley by Koppen and Verschareva (1996).

Cd is a non-redox metal, unable to perform single electron, reactions, however, it can cause oxidative stress by reducing the antioxidant glutathione (GSH) pool, activating calcium-dependent system and affecting iron-mediated processes (Shah et al., 2001). Cd can disrupt the photosynthetic electron chain, leading to increased production of $O_2^{\cdot -}$ and O2 ('Ag) (Asada and Takahashi, 1987). Cd-induced production of ROS within plants depends on the intensity of the stress, repeated stress periods and age of the plants (Singh and Tewari, 2003; Milone et al., 2003).

Rice plants grown for 20 days in presence of 500 μm Cd showed about 0.8 to 1.7 times increase in superoxide anion generation and about 1.6 times increase in lipid peroxidation products as measured in terms of malondialdehyde (MDA) levels indicating thereby that Cd induces oxidative stress in rice plants (Shah et al., 2001). Lipid peroxidation is regarded as an indicator of oxidative damage involving oxidative degradation of polyunsaturated fatty acyl residues of membranes (Girotti, 1990; Ptacek et al., 2002). On Cd exposure, the level of lipid peroxidation was elevated in *Phaseolus aureus* (Shah and Dubey, 1995), *Phaseolus vulgaris* (Chaoui et al., 1997) and *Pisum sativum* (Lozano-Rodriguez et al., 1997). Like all aerobic organisms, plants possess the antioxidative mechanism comprising of antioxidant molecules and enzymes to protect themselves from the oxidative damage caused due to harmful oxygen species. The Cd exposure causes the generation of ROS in various plants (Sharma and Dubey, 2006). Increased activity of the antioxidative enzyme superoxide dismutase and peroxidase is observed when plants are exposed to Cd (Shah et al., 2001).

The increased activity of antioxidative enzymes in metal exposed plants appears to serve as an important component of antioxidant defense mechanism of plants to combat metal-induced oxidative injury (Shah et al., 2001). The activity of another antioxidative enzyme catalase increased in rice seedlings grown at moderately toxic Cd (100 μM) level, whereas with highly toxic Cd (500 μm) level, a marked inhibition in catalase activity was noted (Shah et al., 2001). Decline in catalase activity in plants growing under higher levels of Cd appears to be supposedly due to inhibition of enzymes synthesis on a change in assembly for enzyme submits (Shah et al., 2001).

Moreover, the drastic reduction in MI due to Cd toxicity explains that the mitotic divisions have been withheld when the Cd stress increases. DNA damage in the cells of *V. unguiculata* suggests that production of ROS under Cd toxicity leading to strand breakage, removal of nucleotides and variety of modifications in organic bases of nucleotides. The comet assay may be more suitable for monitoring genotoxic effects of environmental chemical pollutants, where the induced DNA damage may persist for a longer period (Patki et al., 2002).

The following events may occur due to Cd and ultimately resulted into retarded growth of a plant (Sharma and Dubey, 2006).

1) Cadmium → Interaction with cell wall and middle lamellae → Reduced cell expansion and growth,
2) Cadmium → Interaction with nucleophilic groups →

Altered the membrane permeability → Reduced mineral and water uptake,
3) Cadmium → Decline in protein content → Disturbed metabolic process → premature senescence,
4) Cadmium → Replacement of Zinc is Zinc fingers of DNA and Zinc - enzymes → Disturbed transcriptional mechanism DNA repair.

Thus, Cd has arrest the cell division, formation of micronuclei which causes DNA damage to seedlings of *V. unguiculata*. To conclude the present investigation reveals that Cd stress has shown drastic toxic effect in the growth of *V. unguiculata* and has induced the genotoxicity.

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