International Journal of Plant Physiology and Biochemistry Vol. 4(3), pp. 46-51, 2 February, 2012
Available online at http://www.academicjournals.org/ijppb
DOI: 10.5897/IJPPB11.051
ISSN-2141-2162 ©2012 Academic Journals

Full Length Research Paper

Genotoxicity and molecular screening of proteins in root tip cells of *Trigonella foenum- graecum* (Fenugreek var- Azad) under cadmium stress condition

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Accepted 9 December, 2011

Plants are unique in their ability to serve as *in situ* monitors for environmental genotoxins. *Trigonella* is receiving global attention due to rare medicinal properties of significance. Cadmium is one of the most toxic environmental pollutant affecting absorption and transportation of essential elements, disturb the metabolism, growth and reproduction. Present experiment was designed to investigate the genotoxic damage on root tip cells exposed to cadmium nitrate [$Cd(N0_3)_2$] solution at six different concentration 25, 50, 75, 100 and 125 ppm. We evaluated with biological tests based on micronucleus (MN), Lipid peroxidation (MDA) and protein profiling (PP) assay in *Trigonella foenum- graecum* (var-azad). The results indicate that there was a direct correlation between increased MN frequency, increased MDA content, and increased in protein bands with the increasing concentrations of Cd and maximum damage was found at 125 ppm concentrations. *Trigonella* constantly exposed to heavy metals and endogenous processes inflict damage to DNA and cause genotoxic stress, which can reduce plant genome stability, growth and productivity.

Key words: Cadmium, genotoxicity, protein profiling, MDA content, *Trigonella.*

INTRODUCTION

Several heavy metals are naturally present in the environment. Experimental pollution by heavy metals arises as a result of various industrial activities that is widespread nowadays (Arvind and Prasad, 2005). Plants make-up a large portion of our biosphere and constitute a vital link in the food chain due to highly conserved structure of genetic material, it is possible to use a broad variety of species in genotoxicity tests. Cadmium is quite genotoxic metal for plants (George, 2000; Herawati et al., 2000; Kumar and Tripathi, 2007; Kumar and Rai, 2007). It is particularly dangerous pollutant due to its high toxicity and greater solubility in water. (Duxbury, 1985; Jiang et al., 2001).It can be easily absorbed by soil and accumulated in different parts such as root stem and leaf of plants (Suzuki, 2005; Lone et al., 2006). Exposures of high Cadmium concentrations have been found to be carcinogenic and mutagenic to a large number of plant species (Degraeve, 1981). Characteristically, they inhibit root growth and cell division in such plants such as *Allium cepa* (Liu, 2004), *Tradescantia*, *Nicotiana tobacum* (Fojtova and Kovaric, 2000), *Zea mays* L. (Jiang and Liu, 2001), *Allium sativum* (Yi and Meng, 2003), *Helianthus annus* (Kumar and Srivastava, 2006).The visual symptom of heavy metal toxicity due to cadmium and lead are rapid inhibition of root growth, stunted growth of plant, reduced chlorophyll content, reduced photosynthesis and chlorophyll biosynthesis, the formation of reactive oxygen species (ROS), and induction of oxidative stress which caused protein trafficking in plants (Yun et al., 2001; John et al., 2007; Stoyanova and Doncheva, 2002).

Heavy metals are known to cause oxidative damage to plants either directly or indirectly by triggering an increase level of production of reactive oxygen species (Malecka et al., 1997). The ROS includes superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) that

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are produced as by products during membrane linked electron transport activities as well as by a number of metabolic pathways (Shah et al., 2001) and in turn causes damage to the biomolecules such as membrane lipids, proteins, chloroplast pigments, enzymes, (Mishra et al., 1992).

MN induction is one of the common genotoxicity tests for monitoring environmental toxicants (Serpil et al., 2007). MN test is an *in vitro* and *in vivo* short time screening test developed by Schmid (1975) is widely used to detect genotoxic effects in different plants (Saleh et al., 2001; Heddle et al., 1991). It is simple, reliable, less expensive and rapid screening system for both clastogenic effect such as chromosome breakage, formation of centric fragment, lagging of chromosomes and effects on spindles.

Mutation and sexual recombination may allow the evolution of new character. Each allele codes for the production of amino acids that string together to form proteins. Thus, the difference in the nucleotide sequence of alleles, results in the production of slightly different string of amino acids or variant forms of proteins. These proteins code for the development of morphological, anatomical and physiological characteristics of the organism and are also responsible for determining aspect of the behaviour, of the organism. So SDS-PAGE has been used successfully to resolve taxonomic and evolutionary problem of several plants (Rabbani et al., 2008). In this technique proteins are separated according to their molecular weights. There for it could be a reliable source for phylogenetic relationship screening of mutants, identification of varieties and cultivars and establishing genetic diversity in cultivated plants such as *Cicer arietinum* (Chowdhury et al., 2002), *Arachis hypogea* (Javaid et al., 2004). Electrophoresis of seed protein is a very effective technique for the discrimination of species (Rabbani et al., 2008).

Trigonella foenum - graecum L (fenugreek or commonly as 'methi' is cultivated as annual, winter legume in India. Both the species can be grown easily in low-input, marginal environment, and are promising sources of calories, seed proteins, B-vitamins and minerals. Major fenugreek producing countries are India, Pakistan, Egypt, France, Yemen, Spain, Turkey, Morocco and China, and India is the largest producer of fenugreek in the world (Zohary and Hopf, 2000). In India, fenugreek is extensively used as spice, leafy vegetable and in medicine as a carminative, analgesic, anti-inflammatory as well as tonic for gastric troubles, diabetes, leucorrhea and as an important source of steroidal substance, diosgenin. The seeds of *T. foenum-graecum* L. contain the most potent medicinal effects of the plant, and the role of phyto-estrogens and diosgenins to fight breast cancer and reduction of serum cholesterol has been widely recognized (Amin et al., 2009).

However, vast areas of cadmium contaminated regions in different parts of the world including Bangladesh and state of Uttar-pradesh in India have been used for cultivation of this pulse crop without any preliminary assessment of its toxic effect. The possibility of bioaccumulation of Cd in *Trigonella* therefore, may pose a serious threat to both human and cattle health. Development of safe legume crops for cultivation in cadmium contaminated soil is an important strategy to counter the detrimental effect of Cadmium accumulation in proteins and mineral rich legumes. Minimizing the uptake and translocation of Cd to edible parts would form the basis for improving crops (Tripathi et al., 2007) for which understanding of different cyto and morpho-physiological traits is extremely important from very early stage of growth and development (Bayuelo-Jiménez et al., 2002).

The present study was designed to study the effect of Cadmium on MN induction, lipid peroxidation (malondialdehyde content; MDA) in addition with change in protein bands caused due to DNA transition.

MATERIALS AND METHODS

Preparation of root tips

Dry, uniform healthy and equal sized seeds of *T. foenum graecum (Fenugreek var-azad)* were pre-soaked in distilled water for 12 h, treated with 5 different concentrations (25, 50, 75, 100 and 125 ppm) of cadmium in the form of cadmium nitrate. The solution was freshly prepared in sodium buffer/DDW (pH 6.7) for 8 h with constant intermediate shaking. The treated seeds were washed in running tap water 2 to 3 times to remove the residual effect of the metal (Pb^{++}, Cd^{++}) sticking to the seed coat. One set of the seeds were kept untreated to act as control. 50 seeds from each 3 treatment and from control were spread over moist cotton in Petri plates separately and kept in BOD incubator at 24±2°C temperature for 3 to 4 days (ISTA,1987).

Micronucleus test (MN)

The root were (washed with distilled water) excised and pre-treated with 0.02% para-dichlorobenzene for 3 h, washed with distilled water fixed in glacial acetic acid: ethanol (3:1 (v/v) for 24 h, again washed with distilled water and stored in 70% alcohol. For slide preparation and microscopic examination the rinsed root tips were hydrolyzed in 1N HCl at 60°C for 8 min. After staining with Feulgen, they were washed in ultra distilled water. An aliquot of 1 mm of mitotic zone from well stained root tips were immersed in glycerine-gelatin [(1/7/7 (v/v/v) gelatin/UPW/glycerine] on a clean slide and squashed under a cover glass.

For MN analysis 600 cells were scored for each slide to calculate MN frequency. Micro-nucleated cells were evaluated under binocular light microscope (Olympus Japan). For scoring of MN following criteria of Tolbert et al. (1991) was adapted. (1) The diameter of MN should be one third of the main nucleus, (2) Be on the same plane focus, (3) have a chromatin structure similar to that of the main nuclei, (4) Be smooth oval or round shaped and (5) Be clearly separated from the main nucleus.

Lipid peroxidation

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) equivalents according to Hodges et al. (1999). 0.5 gm of root tissue from treated and control seedling were

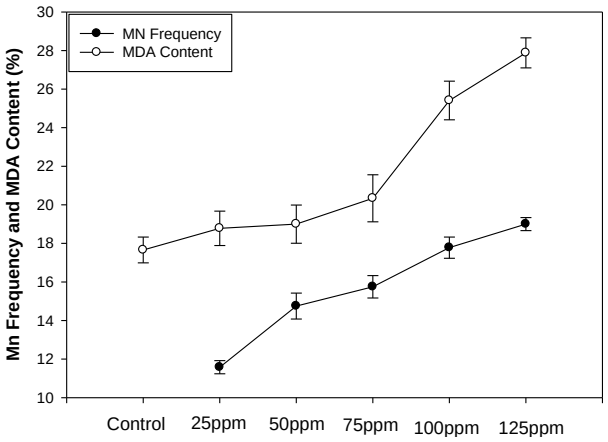

Figure 1A. Effect of different concentrations of $Cd(NO_3)_2$ on MN Frequency and MDA Content. The data is mean±SE of three replicates,Measures were calculated for 600 cells for MN frequency (P<0.01)and for MDA content p< 0.05.

cut into small pieces and homogenized in a mortar pastel with 80% ethanol. The homogenate was then transferred into tubes and centrifuged at 3000X g for 10 min at 4°C and the pellet was extracted twice with the same solvent. The supernatants were pooled and 1 ml of this sample was added to a test tube having equal volume of the solution comprised of 20% trichloroacetic acid (TCA) 0.01% butyrate hydroxyl toluene and 0.65% thiobarbutric acid (TBA). Samples were heated at 95°C for 25 min and cooled at room temperature (25±2°C). The tubes were transferred into ice bath and centrifuged for 5 min. The absorbance was recorded at 440, 532 and 660 nm.

a. [(Abs532+TBA)-Abs600+TBA] - [Abs532-TBA-Abs600-TBA] =A
b. [(Abs400 +TBA) - Abs600+TBA -0.0571] =B
c. MDA equivalent (Dmol-1) =A-B/157000)106

Protein profiling

Five mutants and one set of control seeds of *Trigonella* were collected from the experimental field and are used for electrophoretic analysis. Sodium dodecyl sulphate polyarylamide gel electrophoresis (SDS-PAGE) was used to resolve the protein pattern of seed extracts. Seeds of all 5 mutants and control plant firstly washed with distilled water then the protein were extracted from 0.01 g of seeds flour using 400 µl of extraction buffer that contained 0.05 m tris-HCL PH 8.0, 0.2% SDS, SM urea and 1% mercaptoethanol. Seeds flour was thoroughly mixed with buffer by vortexing. The extracted protein was separated by centrifugation. The seed samples were centrifuged at the rate of 15000 rpm for 10 min. Electrophoresis was carried out in a discontinuous SDS-PAGE page system of Laemmli (1970) using acryl amide gel. Electrophoresis was run at 100V, the gel was stained in the staining solution containing 44% methanol, 6% acetic acid, 500 ml distilled water and 2.25 g of coommassie brilliant blue for 45 min. De-staining was done in a solution containing 20% methanol, 5% acetic acid, and 750 ml of distilled water until the back ground color was disappeared and protein bands was clearly visible.

Statistical analysis

A total of 10 replicates for each treatment were taken. Data were statistically analyzed by using SPSS.17.0 for window (SPSS, Chicago, IL, and US4). Analysis of variance (ANOVA) was performed on the data to determined the least significance difference (LSD) between treatments means with the level of significance at P<0.05. The data of protein was analyzed by Jacquards similarly coefficient were determined and dendrogram was constructed by un-weighted pair group method with arithmetic average (UPGMA).

RESULTS

Present study was focused on screening of the effect of heavy metal on MN frequency, Lipid-peroxidaton and protein content in *T. foenum- graecum* (fenugreek Var-azad).

Effect of Cd on MN frequency

The MN frequencies were depicted in (Figure 1A). Results showed that the frequency of micro-nucleus increased more frequently in all concentrations of Cd, but not in control (Figure 1 panel B). There is a statistically significant difference between treatments and control group for MN frequency (P<0.01) and statistical analysis refers a significant increase in MN frequency with the increasing dose of metal. There was a certain dose relationship between MN frequency and metal ion. All treatment showed a higher MN frequency but highest frequency was observed at 125ppm of metal.

Effect of Cd on lipid peroxidation

Lipid peroxidation was measured as MDA content (Figure1, Panel A). MDA is the final product of lipid peroxidation and accumulate when plant are subjected to environmental stress. The MDA content in all Cd concentrations were significantly higher than control (P<0.05). All Cd concentrations significantly affected MDA production indicating altered Lipid peroxidation. However, it is determined that MDA content remarkably increased depending on Cd concentrations.

Effect of Cd on protein content

The present study revealed that treatment of cadmium nitrate showed significant morphological variations in the mutants developed in concentration dependent manner. Moreover, the correlation between various mutants developed by varied concentrations of $Cd(NO_3)_2$ were observed with the help of SDS-PAGE analysis (Figure 2). The protein profiling of total seed protein through SDS-PAGE revealed that high degree of polymorphism. Table 1 shows the Jacquard's similarity coefficient value for mutant developed. A total of 34 bands were generated in 4 mutants and one control. Out of 34 bands, 26 were found to be polymorphic which revealed significant variation among mutants and control. These results

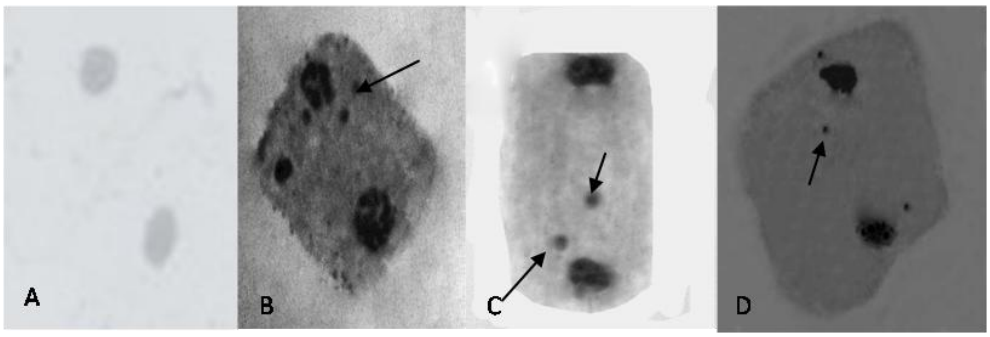

Figure 1B. Micronucleated cells of *Trigonella foenum graecum* (var-Azad) and control cells without micronucleus (A with no micronucleus and B, C, D with micronucleus).

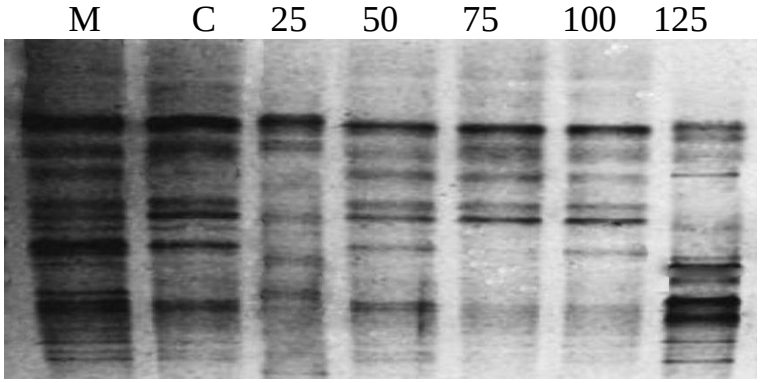

Figure 2. SDS-PAGE analysis of protein of *Trigonella foenum graecum* (var-Azad) L. Seeds at 0, 25, 50, 75, 100 and 125 ppm of Cd $(NO_3)_2$. (C-Control, M- Molecular marker).

Table 1. Jacquards similarity coefficient of protein bands of plants grown under different concentrations of cadmium.

	Control	25 ppm	50 ppm	75 ppm	100 ppm	125 ppm
Control	0					
25ppm	0.67	0				
50ppm	0.77	0.36	0			
75ppm	0.40	0.25	0.67	0		
100ppm	0.67	0.20	0.91	0.75	0	
125ppm	0.40	0.62	0.43	0.36	0.31	0

suggested that cadmium had caused DNA instability which is reflected in proteins, as protein analysis is the basis for DNA study.

From the (Figure 2) it was found that number, intensity and density of SDS-PAGE electrophoretic bands for seed protein in control generally differ with varied concentrations of $Cd(NO_3)_2$. New types of bands generated with increase in concentration of $Cd(NO_3)_2$. The response of 125 ppm of $Cd(NO_3)_2$, 2 to 5 new bands which might be related to the improvement of studied *Trigonella* traits. Dendrogram constructed from various protein bands shows much difference between the variants developed by $(CdNo_3)_2$ treatments, this is because of genomic DNA instability.

DISCUSSION

Cd is although a non-essential element and is not toxic to plants at lower concentrations and even at low concentration can inhibit some vital plant processes such as photosynthesis, mitosis and water absorption with toxic symptom on leaves, wilting of older leaves, stunted foliage and brown short roots (Mohan and Hosetti, 1997;

Patra et al., 2004).

The study revealed that Cd induce MN formation in *T. foenum- graecum* (fenugreek, Var-azad). There was a dose related increase in the frequency of MN in treated populations. The observations were in full support with results obtained by many authors so far. In most of the studies heavy metals induces MN formation which are formed as a result of chromosomal damage to the meiotic spindle apparatus in the root tip cells of the plant (Ineer et al., 2003).

According to Cavusoglu (2011) heavy metals may enter into the cell nucleus and may bind to purine and pyramidine bases or proteins. These interactions may denature spindle and may cause MN formation as a result of decrease in chromosome number in main nucleus. Wei (2004) suggested a systematical increase in MN frequency and chromosomal aberrations with increasing concentrations of CrO3 in *Vicia faba*. The study of Rosa et al. (2003) indicates that MN induction may be interpreted as a consequence of oxidative stress.

Lipid peroxidation is a biochemical marker for the free radical mediated injury. Present study showed an increase in the level of lipid peroxidation with increasing concentrations of heavy metals, indicating that these induce oxidative stress in *Trigonella.* Our results are in conformity with the observations of Malecka et al. (2001) Unyayar et al. (2006).

In many plant species heavy metals have been reported to cause oxidative damage in growing plant parts due to enhance production of ROS (Reactive oxygen species). Kasai et al. (1992) and Radetski et al. (2004) suggested non-direct formation of ROS due to the genotoxicity of metals in eukaryotes. ROS can convert fatty acid to toxic lipid peroxides, destroying biochemical membrane permeability (Zang et al., 2005). ROS over production is coupled with deficiency of antioxidant defense mechanism; this may be an important factor contributing to the increase in micronuclei. MDA formation is used as a general indicator of the extent of lipid peroxidation resulting from oxidative stress.

Present study showed that MDA content was greatly affected by highest concentration of Cd in *T. foenum graecum (Var azad)*. This shows that *Trigonella* seedlings have capability to adopt at lower concentrations of metal and may be related with the low degree of lipid peroxidation. MDA content increases after Cd exposure in *Trigonella*. This suggested that heavy metal leads to excessive generation of superoxide radicals by deficient antioxidant defenses resulting in increase lipid peroxidation and oxidative stress in *Trigonella*. The above results confirm the observation of Unyayar (2008).

Heavy metals in soil, water and atmosphere, where plants are growing are seen to demonstrate interactions between these heavy metals. Small molecular weight organic acid can chelate heavy metals (Yang et al., 2001), and metal-binding proteins or metallothionein are perfect for chelating heavy metals (Zhang et al., 1999). He et al., (1999) extracted Cd binding protein in maize; the ratio of proteins to Cd was 1:3. Yang and Wang (1985) reported metal binding protein could combine 40 to 50% of Cd, which entered into the cell. The formation of Cd binding proteins limited the behaviour of Cd and abated the injury of Cd to plant. Ma et al. (1997) identified metallothionein-like protein and its gene in *Festuca rubra*. These metal binding proteins were also identified and/or purified from ice, bean, broccoli, tobacco, with the molecular weights being from 3.1 to 33.1 KD (Li and Yu, 1990). In our finding, molecular screening of proteins in root tip cells of *Trigonella* under cadmium stress condition, new protein bands of different molecular weight have been identified on 125 ppm concentration, this may be due to transition of DNA, as DNA mutation reflects in proteins Thus difference in the nucleotide sequence of alleles, result in the production of slightly different types of amino acids or variant forms of proteins. These proteins code for the development of varied morphological, anatomical and physiological characteristics in the organism.

ACKNOWLEDGEMENT

Authors are thankful to the Chairman Department of Botany Aligarh Muslim University Aligarh-India for providing lab facilities for our experiments.

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