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Biochemical and Cellular Response of *Catharanthus roseus* Callus Cells to Cadmium Toxicity

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Abstract

Catharanthus roseus is a medicinal and ornamental plant with growing attention toward its economical value. Cell suspension from *C. roseus* were treated with 0 to 60mM of cadmium nitrate for 1, 3 and 6 days, then cell viability was determined using trypan blue and MTT assay. Cell morphology was investigated using 0, 10, 30 and 50 mM of cadmium nitrate as selected dose for 3 days. Callus of *C.roseus* was treated with selected doses for same period to estimate the hydrogen peroxide content, level of lipid peroxidation, proline content and the activity of superoxide dismutase, catalase and peroxidase as well as protein profile. In addition, the production of total alkaloid, flavonoid and phenolic compounds was estimated. Data was analyzed using one-way ANOVA, Duncan test and $p < 0.05$ was taken as the level of significant. Trypan blue and MTT assay showed the significant ($p < 0.05$) dose dependent differences in viability as compared to control. In addition a dose dependent increase of hydrogen peroxide, total alkaloid, total flavonoid, and total phenolic compounds was observed. Also the activity of superoxide dismutase, catalase and peroxidase as well as malondialdehyde level as an indicator of lipid peroxidation increased significantly. Cadmium nitrate induced protein profile changes as well as proline content elevation. As conclusion; cadmium nitrate caused cellular membrane damage and viability reduction, but an increase in the production of secondary metabolites such as total alkaloids was observed. Therefore cadmium treatment might be a useful way to increase production of alkaloids at cellular level.

Key words: antioxidant enzymes; cadmium nitrate; *Catharanthus roseus* callus; cellular response

Introduction

Reactive oxygen species (ROS) which damage the cell membrane of the plants, are produced at low levels in chloroplasts and mitochondria of non-stressed plant cells, such as lignin formation in the plant cell walls which naturally generate ROS (Lee *et al.*, 2013). Large amount of ROS are produced under stressful conditions such as pathogen attacks, wounding, herbivore feeding, UV light and heavy metals (Sharma *et al.*, 2012). As ROS are more reactive than molecular oxygen (O_2), they react with almost every organic constituent of the living cell, therefore, plant's cells keep the ROS concentration at the possible low level (Sharma *et al.*, 2010). It is well known that ROS damage cellular membranes by inducing lipid peroxidation and also can damage DNA, proteins, and chlorophyll via oxidation of these molecules (Mittova *et al.*, 2000). The most popular ROS

are superoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) originating from one, two or three electron transfers to molecular oxygen (O_2) (Michalak, 2006). The metals such as Fe, Cu, Cd and Mn which have unpaired electrons in their orbital, accept and donate single electrons, thus promoting mono electron transfers to O_2 and forms ROS in the cell. These metals disturb metabolic pathways, especially in the thylakoid membrane, which also results in increased formation of free radicals and reactive oxygen species. It has been reported that the presence of cadmium in the plant cell results in the change of enzyme activity, imbalance of micro and macro elements distribution and produce lipid peroxidation in the plant cells (Smeets *et al.*, 2008).

Secondary metabolites are essential for the fitness of plants and play important function as chemical defense against herbivores, microbes, competing plants or stress oxidative

(War *et al.*, 2012). *Catharanthus roseus* L (*C. roseus*) (Family: *Apocynaceae*) is a very important medicinal plant which produce divers variety of secondary metabolites (about 130 difference type of secondary metabolites) which are synthesized in various plants organs. *C. roseus* produce secondary metabolites such as indole alkaloids and phenolic compounds like: flavonoids, isoflavonoids, terpenoids, tannins and saponins (Hisiger and Jolicoeur, 2007). *C. roseus* is most famous for alkaloids such as vinblastine and vincristine which are well known antitumor agents (Kumar *et al.*, 2013).

Investigations have shown that the cadmium causes reduction of cell viability, cell death and chromatin condensation as well as DNA breakage in the callus cell of tobacco (Fojtová *et al.*, 2002; Ma *et al.*, 2010; JinFen *et al.*, 2012). In addition, Wajda *et al.* showed that the cadmium toxicity caused viability reduction of the *C. roseus* callus cells (Wajda *et al.*, 1989). The exact mechanism and cellular response of the plant to cadmium toxicity is not well understood. Although the cadmium ability to generated ROS is the main culprit to damage the cell, but in the present study the oxidative effect of cadmium nitrate $\text{Cd}(\text{NO}_3)_2$ in the callus of *C. roseus* was investigated to study the anti-oxidative response of this plant at cellular level as well as to estimate the production of secondary metabolites, as a defense strategy, under influence of the cadmium stress.

Materials and methods

Plant material and callus culture

Seeds of *C. roseus* were purchased from Esfahan seed pakan society and then the sterilized seeds were immersed in water for 24h, and grown in green house for 12 days. Explants ($0.5\text{-}1\text{cm}^2$) for callus-induction were taken from leaves of 12 days old seedlings. Explants surface was sterilized with sodium hypochlorite 5% (vol.vol) for 5 min, rinsed five times with sterile distilled water, and cultured in MS (Murashige and Skoog) medium supplemented with 1.5 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.5mg l^{-1} kinetin, 30 g l^{-1} sucrose and 7 g l^{-1} agar (Sigma). Cultures were maintained at $22 \pm 3^\circ\text{C}$ under a 16hrs photoperiod with a photosynthetic photon flux density of 45 mol

$\text{m}^{-2}\text{s}^{-1}$ provided by cool white fluorescent lamps. The callus was then separated from the initial explants and was sub cultured every 18 - 20 d.

Suspension cell culture

Friable calli was on MS medium without agar and were incubated on a shaker at 100 rpm and 25°C under darkness. Samples were examined with a microscope regularly on a daily basis to ensure the formation of cell. The cells were maintained on a rotary shaker at $25 \pm 2^\circ\text{C}$ in the dark and sub-cultured weekly by 1:1 dilution with fresh medium. Ultimately increased cells were used for viability and morphology tests (Saifullah and Saifullah 2011).

Cell viability Assay

Performing MTT assay, 80000 cells were cultured in 24 well plates and after treatment with 0, 10, 20, 30, 40,50 and 60 mM of cadmium nitrate for 1, 3 and 6 days the cells were collected and washed with PBS followed by addition of fresh media to the plates. Then $10\text{ }\mu\text{l}$ of MTT (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg ml^{-1} in PBS) was added to each wells and plate was incubated for 4hrs in an incubator. After incubation period, to extract crystals of formazan, $100\text{ }\mu\text{l}$ of DMSO was added to each well and the plate was incubated for 30min. The extracted solutions were transferred to another well and absorbance was taken using an automated microplate reader (SCO diagnostic, Germany) at 505 nm. The viability was reported as percentage of the viable cell (Enikeev *et al.*, 1995).

In trypan blue staining assay, 80000 cells were cultured in 24 well plates and after treatment with 0, 10, 20, 30, 40,50 and 60 mM of cadmium nitrate for 1, 3 and 6 days the cells were collected and washed with PBS, then the cells were stained with trypan blue solution (0.4 g in 100ml PBS) for 2min in 37°C . The total number of the cells, number of the live (transparent) and dead (blue in color) cells were estimated using hemocytometer chamber and the percentage of the live cell was reported.

Fluorescence staining

The cells were suspended in a 24 well plates and treated with 0, 10, 30 and 50 mM of cadmium nitrate then after 3 days the nuclear morphology of the cells was studied using Hoechst 33342 (1mg ml⁻¹ in PBS) following 5 min of incubation in the dark. The diameters of the cells nuclei were also measured in μm with Motic Image software (Micro optical group company, version 1.2). Hoechst is a fluorescent dye which penetrates the cells through the intact plasma membrane and stain the DNA where the changes in nuclear morphology such as chromatin condensation and fragmentation can be investigated. The morphology of the cell cytoplasm was investigated using acridine orange solution (1mg ml⁻¹ in PBS) which stains the nuclei green and the cytoplasm orange. The stained cells were washed twice with PBS, examined, and immediately photographed under an inverted fluorescence microscope (Olympus, IX70) equipped with a camera (DP72) using 40X magnification.

Protein profile study

The callus were treated with 0, 10, 30 and 50 mM of cadmium nitrate for 3 days, then the total soluble proteins were extracted with Tris-HCl buffer pH=7.4 follow by protein estimation using Lowry method (Lowry *et al.*, 1951). The protein profile was determined on 12% SDS-PAGE in presence of molecular weight marker (Fermentas). The protein electrophoresis of the control and treated cells was run under constant voltage.

Proline content

The proline content was estimated by the method of Bates (Bates *et al.*, 1973). Briefly, the callus was treated with 0, 10, 30 and 50 mM of cadmium nitrate for 3 days and homogenized in 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 10000 rpm. The supernatant was used for the estimation of the proline content and added to the reaction medium containing sulfosalicylic acid, glacial acetic acid and ninhydrin solution. The mixture was kept at 95 °C for 60 min, and then the reaction was stopped quickly by an ice bath. Toluene was added to the mixture and the organic phase was extracted

and monitored at 520 nm by spectrophotometer. The standard curve was drawn and proline content of samples were measured using the formula $Y=0.123X+0.304$ with $R^2=0.995$, where Y is the absorption and X is the concentration of proline.

Lipid peroxidation

Malondialdehyde (MDA) was determined as an indication of lipid peroxidation. 0.5 g of callus treated with 0, 10, 30 and 50 mM of cadmium nitrate for 3 days was homogenized in 1 ml 0.1% trichloroacetic acid (TCA) following centrifugation at rpm 4000 for 30 min. The supernatant was treated with 0.5% thiobarbituric acid (TBA) at 95°C and cooled quickly. The absorbance was measured at 530 nm wavelength and the non-specific turbidity was corrected by subtracting A_{600} from A_{530} value. The concentration of MDA was calculated using the extinction coefficient (155 $\mu\text{M cm}^{-1}$) (Metwally *et al.*, 2003).

Measurement of antioxidant enzyme activities

The callus tissue treated with 0, 10, 30 and 50 mM of cadmium nitrate for 3 days was pulverized in 1 ml of potassium phosphate buffer, 50 mM (pH =7) containing 1 mM EDTA and then homogenate was centrifuged at 13000 rpm for 20 min at 4°C. The clear supernatant of the solution containing the enzyme extract was used to estimate the total protein content using lowery method. To determine the antioxidant enzyme activity of superoxide dismutase (SOD), guaiacol peroxidase (G-POD) and catalase (CAT) equal concentration of protein in the treated samples and control was used.

To determine the activity of SOD (Giannopolitis and Ries, 1977), the following reaction mixture was prepared, 1 mM riboflavin, 12 mM L-methionine, 0.1 mM EDTA, 50 mM Na₂CO₃, 75 mM nitrobluetetrazolium (NBT) and 25 mM sodium phosphate buffer (pH 6.8) was mixed with 200 μl of crude enzyme extract in a final volume of 3 ml. Then the activity of enzyme was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT. Glass test tubes containing the mixture were illuminated with a

fluorescent lamp (120 W); identical tubes that were not illuminated served as blanks. After illumination for 15 min, the absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme activity that was able to inhibit by 50% the photoreduction of NBT to blue formazan. The SOD activity of the extract was expressed as SOD units $\text{min}^{-1} \text{mg}^{-1}$ protein.

CAT activity was assayed in the reaction mixture contained crude enzyme extract, 10 mM H_2O_2 and 25 mM sodium phosphate buffer. The decrease in the absorbance at 240 nm was recorded for 1 min by spectrophotometer with the extinction coefficient of $39.4 \text{ mM}^{-1}\text{cm}^{-1}$. CAT activity of the extract was expressed as units $\text{min}^{-1} \text{mg}^{-1}$ protein (Cakmak and Horst, 1991).

POX activity was assayed by the oxidation of guaiacol in the presence of H_2O_2 . The increase in absorbance was recorded at 436 nm (Polle *et al.*, 1994). The reaction mixture contained 50 μL of enzyme extract, 100 μL H_2O_2 , 100 μL guaiacol 18 mM and 100 μL potassium phosphate buffer 100 mM (pH=7). POX activity of the extract was calculated using extinction coefficient of $26.6 \text{ mM}^{-1}\text{cm}^{-1}$ and expressed as units $\text{min}^{-1} \text{mg}^{-1}$ protein.

H_2O_2 content

To estimate the hydrogen peroxide content, 0.5 g of Callus was treated with 0, 10, 30 and 50 mM of cadmium nitrate for 3 days and homogenized in an ice bath with 1ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 rpm for 15 min, and 700 μL of the supernatant was added to 700 μL of 10 mM potassium phosphate buffer (pH 7.0) and 700 μL of 1 M potassium iodide (KI). The absorbance of the supernatant was measured at 390 nm in a spectrophotometer (Sergiv and Karanov, 1997). The standard calibration curve was plotted using different concentrations of H_2O_2 and the content of H_2O_2 was calculated using linear formula $Y=0.212X$ with $R^2=0.99$ where (Y) is the absorption and (X) is the concentration of H_2O_2 .

Estimation of total alkaloids

Extraction of alkaloids was according to the Ataei-Azimi, description with some modification. In brief, 3 g of callus treated

with 0, 10, 30 and 50 mM of cadmium nitrate for 3 days was abraded with 10 mL of 96% methanol in the presence of liquid nitrogen and transferred to incubator for 24 hours at temperature 25 °C. The homogenate was centrifuged at 12000 rpm for 10 min and transferred to 80 °C oven for 2 hours to reduce the volume to 1 mL, then 10 mL of 0.5N sulphuric acid was added to the solution. Using 10N NaOH, the pH of the solution was adjusted to 10, then 10 mL of chloroform was added, mixed and using separating funnel the two phases consist of acidic phase on top and chloroformic phase at bottom was made. Chloroformic phase was brought to 10 ml with 96% ethanol and used for further estimation (Ataei-Azimi *et al.*, 2008).

Total alkaloids are estimated according to Sreevidya (Sreevidya and Mehrotra, 2003); in brief, 5 mL of the cell extract was taken and the pH was maintained at 2-2.5 with dilute HCl. 2 mL of Dragendroff Reagent (DR) was added, and the precipitate formed was centrifuged. The precipitate was further washed with alcohol and the residue was then treated with 2 mL disodium sulfide solution. The brownish black precipitate formed using centrifuged was dissolved in 2 mL concentrated nitric acid and the solution was diluted to 10 mL in a standard flask with distilled water. Then 1 mL of the solution was taken and 5 mL of thiourea solution (3 % in distilled water) was added to it. The absorbance was measured at 435 nm against the blank containing nitric acid and thiourea. The standard calibration curve was plotted using different concentrations of bismuth in presence of 3% thiourea. The total content of alkaloids was calculated using linear formula $Y=0.291X+0.030$ with $R^2=0.998$ where (Y) is the absorption and (X) is the concentration of total alkaloids.

Preparation of DR.: solution of 0.8g bismuth nitrate pentahydrate in 40 mL distilled water and 10 mL glacial acetic acid was mixed with solution of 8 g potassium iodide in 20 mL distilled water.

Estimation of total phenolic compounds

Total phenolic compounds measurement was performed by Folin-Ciocalteu reagent method and gallic acid as standard (Singleton *et al.*, 1999). Briefly, 0.5g of callus treated with 0, 10, 30 and 50 mM of cadmium nitrate for 3

days was homogenized in 1 ml 80% methanol. After filtration, 30 μ l of supernatant solution, 470 μ l distilled water and 500 μ l of solution 1% (w/v) Na_2CO_3 was added and mixed. After 3 min 500 μ l Folin-Ciocalteu reagent was added and then tubes were incubated in dark for 10 min and absorbance was measured at 765 nm. The standard calibration curve was plotted using different concentrations of gallic acid and the total phenolic compounds was calculated using linear formula $Y=0.0213X+0.0016$ with $R^2=0.989$ where (Y) is the absorption and (X) is the concentration of total phenolic compounds base on mg of gallic acid.

Estimation of total flavonoids

Total flavonoids were estimated according to Marinova (Marinova 2005), briefly, 0.5 g of callus treated with 0, 10, 30 and 50 mM of cadmium nitrate for 3 days was extracted with 1 mL of 80% methanol (Merck, Germany). The mixtures were then ultrasonicated for 20 minutes followed by centrifugation at 12,000 rpm. 50 μ L of the supernatant was collected into a test tube, and 1245 μ l of deionized water was added. After that, 75 μ l of 5% (w/v) NaNO_2 (Sigma, USA) were added and tubes were left to react for 6 min. Then, 150 μ l of 10% (w/v) AlCl_3 (Sigma, USA) were added and the mixture was incubated for further 5 min. Finally, 500 μ L of 1M NaOH (Sigma, USA) was added and a total of 2 mL of the mixtures were transferred to a cuvette, and the absorbance values were measured using spectrophotometer (PG instrument, England) at 510 nm. The standard calibration curve was plotted using different concentrations of Catechin (Sigma, USA) and the total flavonoids were calculated using linear formula $Y=0.0002X+0.0411$ with $R^2=0.99$ where (Y) is the absorption and (X) is the concentration of total flavonoids base on mg of catechin.

Statistical analysis

Data were analyzed by SPSS using one-way analysis of variance (ANOVA), Duncan test and taking $P < 0.05$ as the level of significant. Values are means \pm standard deviation (SD) of three different experiments with at least three replication.

Results

Effect of $\text{Cd}(\text{NO}_3)_2$ on cell viability

Treatment of the cells with cadmium nitrate for 1, 3 and 6 days showed a significant reduction in viability ($p < 0.05$) from 10 mM upwards. Cadmium nitrate caused time dependent reduction of viability, with the lowest mortality in the 1 day and highest in the 6 days of treatment with respect to each concentration. Results of trypan blue staining (Table 1) and MTT assay (Table 1) together confirmed that the treatment of the cells with 30 mM of cadmium nitrate caused approximately 50 percent of the cells to die after 3 days. In addition, 10 and 50 mM cadmium caused approximately 25 and 75 percent mortality in 3 days, respectively. Therefore concentrations of 10, 30 and 50 mM of cadmium nitrate were chosen to carry out the further investigation in 3 days.

Cadmium induced morphological changes

Morphological study of the nuclei of the cells treated with 10, 30 and 50 mM of cadmium nitrate after 3 days showed chromatin condensation and nuclear breakage (Fig 1). Also a highly significant reduction ($p < 0.001$) in mean nuclei diameter of the cells treated with 10, 30 and 50 mM was observed as compared to control cells (table 2). It can be also noticed that cadmium nitrate at these concentrations caused remarkable changes in the morphology of the cytoplasm (Fig 2) such as cytoplasm shrinkage and in some cells complete disappearance of the cytoplasm content compared to the control group of cells.

Effect of $\text{Cd}(\text{NO}_3)_2$ on H_2O_2 and MDA content

Data analysis showed that the treatment of callus cells with different (10, 30 and 50 mM) concentrations of cadmium nitrate for 3 days caused significant increase ($p < 0.05$) in the H_2O_2 content when compared to the control group (Table 3). It was also observed that the increase in the H_2O_2 content was dose dependent and the content of the internal hydrogen peroxide was reached to maximum level in the group treated with 50 mM. In addition treatment of callus cells with the same concentrations of $\text{Cd}(\text{NO}_3)_2$ lead to highly

significant ($p < 0.001$) increase of malonyldialdehyde (MDA) content with a dose dependent manner (Table 3).

A

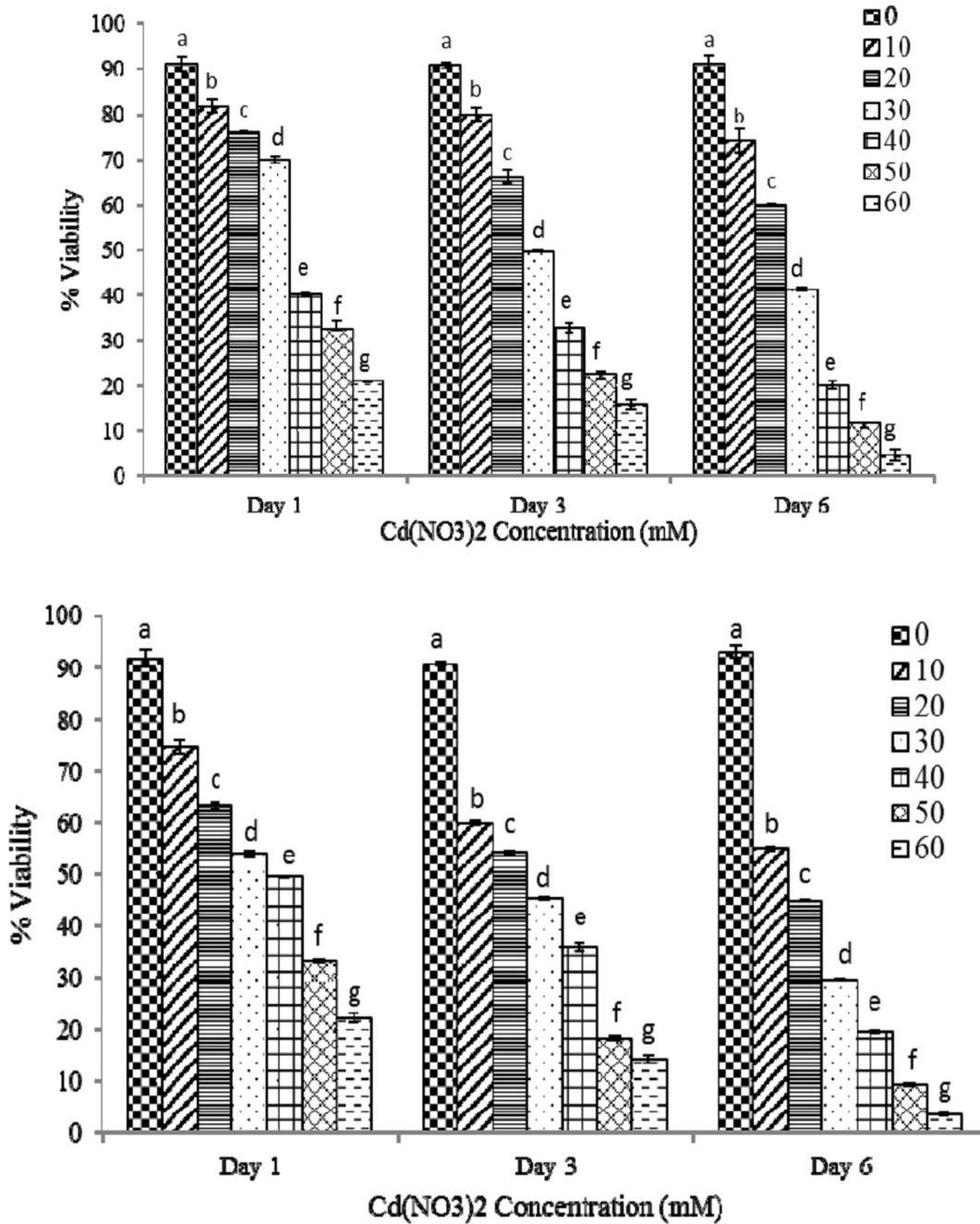


Fig. 1. Viability of the *C. roseus* callus cells after 1, 3 and 6 days of treatment with different concentrations of $\text{Cd}(\text{NO}_3)_2$ based on (A) Trypan blue staining and (B) MTT assay. Values are means \pm SD. Means with different letter code differ significantly from each other in the same period of treatment (ANOVA, Duncan test, $p < 0.05$).

Table 1. Viability of the *C. roseus* callus cells after 1, 3 and 6 days of treatment with different concentrations of Cd(NO₃)₂ based on Trypan blue staining and MTT assay.

day (mM)	1		3		6	
	Trypan blue	MTT	Trypan blue	MTT	Trypan blue	MTT
0	91.10 ^a ±1.66	91.73 ^a ±1.73	90.77 ^a ±0.68	90.77 ^a ±0.49	91.06 ^a ±1.77	93.10 ^a ±1.05
10	81.80 ^b ±1.33	74.50 ^b ±1.32	79.93 ^b ±1.48	59.81 ^b ±0.41	74.01 ^b ±2.63	55.04 ^b ±0.37
20	75.70 ^c ±0.26	63.18 ^c ±0.63	66.17 ^c ±1.55	54.31 ^c ±0.25	59.84 ^c ±0.30	44.99 ^c ±0.20
30	69.83 ^d ±0.77	54.02 ^d ±0.65	49.54 ^d ±0.17	45.50 ^d ±0.26	41.38 ^d ±0.42	29.47 ^d ±0.32
40	40.07 ^e ±0.53	49.37 ^e ±0.24	32.77 ^e ±0.99	35.86 ^e ±0.77	20.07 ^e ±0.86	19.66 ^e ±0.24
50	32.48 ^f ±1.81	33.25 ^f ±0.33	22.59 ^f ±0.39	18.39 ^f ±0.27	11.85 ^f ±0.05	9.33 ^f ±0.20
60	21.02 ^g ±0.12	22.21 ^g ±0.87	15.75 ^g ±1.11	14.26 ^g ±0.77	4.67 ^g ±1.19	3.50 ^g ±0.34

Values are means ± SD. Means with different letter code in each column differ significantly from each other (ANOVA, Duncan test, p<0.05).

Table 2. Nuclei diameter of *C. roseus* callus cells after 3 days of treatment with different concentrations of Cd(NO₃)₂.

Cd(NO ₃) ₂ (mM)	Diameter of the cell nuclei (μm)
0	33.17 ^a ±4.19
10	21.92 ^b ±1.95
30	14.32 ^c ±3.07
50	7.27 ^d ±1.43

Values are means ± SD. Means with different letter code in each column differ significantly from each other (ANOVA, Duncan test, p<0.05).

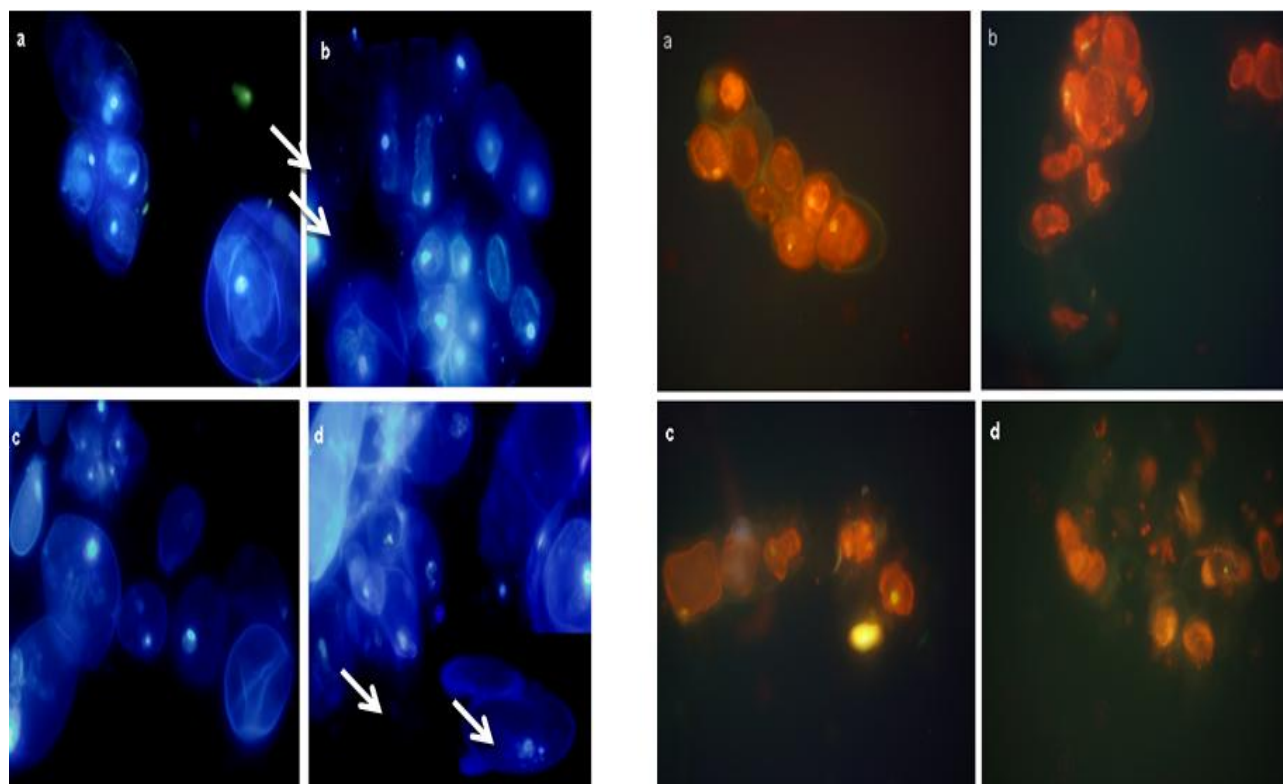


Fig. 2. Morphology of the cells cytoplasm stained with acridine orange a) controls, a distinct cytoplasm was observed and appeared round (arrows) b) the cells treated with 10 mM of Cd(NO₃)₂ c) the cells treated with 30 mM of Cd(NO₃)₂ d) the cells treated with 50 mM of Cd(NO₃)₂ (20X magnification).

Effect of Cd(NO₃)₂ on Proline and total proteins content

In the present study, a significant ($p < 0.05$) and dose dependent increase in proline accumulation in *C. roseus* callus, under Cd (NO₃)₂ treatment was observed when compared to control group (table 3). While the content of total protein showed significant decrease ($p < 0.05$) in a dose dependent manner (Table 3). The changes in the protein content of the treated cells were also observed in the denatured-polyacrylamide gel electrophoresis of the callus cells (Fig. 3). The protein profile of the treated cells showed that there are changes in the intensity of several polypeptides with molecular weight of 92, 87.5, 35, 16.25 and 12.8 KD. More prominent changes were observed in the callus cells treated with 50 mM of cadmium nitrate. Also the polypeptide band with molecular weight of 92 KD appeared in the profile of treated cell which could not be observed in the control group.

Effect of Cd(NO₃)₂ on the activity of ROS scavenger enzymes

The activity of superoxide dismutase (SOD), increased significantly ($p < 0.05$) in *C. roseus* callus cells after 3 days of treated with different concentrations of Cd(NO₃)₂ in a dose dependent manner (table 4). While the concentration of 10 mM Cd(NO₃)₂ did not change the activity of peroxidase (POX) and catalase (CAT) enzymes after 3 days of treatment. The significant increase ($p < 0.05$) in the activity of these enzymes were observed only after treatment with 30 and 50 mM of cadmium nitrate (Table 4).

Effect of Cd(NO₃)₂ on Total phenol, flavonoids and alkaloids

The total phenol and alkaloid contents of *C. roseus* callus after treatment with 10, 30 and 50 mM Cd(NO₃)₂ increased significantly ($p < 0.05$) when compared to control in a dose dependent manner. While the level of total flavonoids increased significantly but not dose dependently. The concentration of 30 and 50 mM of cadmium nitrate caused significant increase in the content of total flavonoids compared to control but no significant changes was observed in these two group (Table 4).

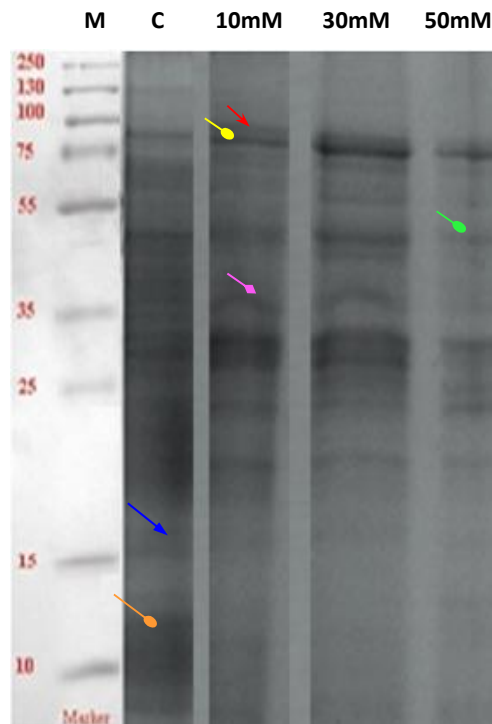


Fig. 3. Polypeptide bands on gel electrophoresis (SDS-PAGE 12%). Control group (C) and Cd(NO₃)₂ treated cell (10, 30 and 50 mM) for 3 days, as well as molecular weight markers (M). Arrows mark the change in the polypeptide bands.

Discussion

Following cadmium exposure several biochemical changes take place within the plant cells, which can lead to numerous damages in the structure and functions. The study of such changes may increase our knowledge about the effect of chemical toxicity on plant growth and development, which is the basis to develop new strategies for the improvement of plant tolerance to chemical stress. For several plant species, chemical toxicity to cadmium have been studied using cell culture and whole plants (Wajda *et al.*, 1989; Bao *et al.*, 2011; JinFen *et al.*, 2012) and negative correlation was found between the responses of these plants to cadmium stress. Cell culture method is a valuable quick and easy screening test for the evaluation of chemical tolerance in several plant species and to study the effect of cadmium at the microscopic, physiological, and biochemical levels (Wajda *et al.*, 1989; Fojtová *et al.*, 2002; JinFen *et al.*, 2012). Thus, the present study aims to investigate the effects of cadmium on *C. roseus* cell

biochemistry and morphology, for which limited data are available in literature.

In comparison to the control, treatment with cadmium nitrate caused the viability of *C. roseus* cells to reduce, dose dependently (analyzed with one way ANOVA) as well as time dependently (the data of two way ANOVA not shown) which demonstrating sensitivity of the species to this stress. In addition the morphological changes were observed in the treated cells. The reduction in viability and morphological changes was probably due to the alteration in several metabolic activities and induction of oxidative stress induced by cadmium nitrate.

Cell death triggered by biotic and abiotic stresses in plants can be classified as programmed cell death (PCD) or necrosis. PCD is an active pattern of cell death which is controlled by genes and its characteristic features are cell shrinkage, chromatin condensation, DNA strand breaks and DNA fragmentation (Ma *et al.*, 2010). Recently, several studies revealed that upon exposure to Cd²⁺, some species of plant suspension cultures undergo PCD. Fojtova, *et al.*, reported that 50 mM CdSO₄ induced a rapid decrease in cell viability and distinct changes in cell morphology and DNA integrity in tobacco suspension cultures (Fojtová *et al.*, 2002). Similar results also have been reported in tomato suspension cells (Iakimova *et al.*, 2008). Although, the mechanism of cell death

and viability reduction in *C. roseus* caused by cadmium is to be evaluated, but the previous results confirm our results which indicated the level of cell sensitivity to this chemical. The chromatin condensation and morphological changes might be a reason of program cell death but still there might be other mechanism such as oxidative stress involve in the viability reeducation, therefore we try to justify that too.

Lipid peroxidation (LPO) has been associated with damages provoked by a variety of environmental stresses. Poly-unsaturated fatty acids (PUFA) are the main membrane lipid components susceptible to peroxidation and degradation (Bidar *et al.*, 2008). The increase in LPO can be correlated with the accumulation of ions and production of reactive oxygen species (ROS) under stresses (Rucińska-Sobkowiak, 2010). In the present study, we showed that the cadmium nitrate treatment of *C. roseus* callus caused significant elevation of H₂O₂ (one of the species of ROS) and malondialdehyde (MDA) which is the end product of LPO. LPO cause disruptions of the cell membrane integrity which as a result the internal/external balance of the cells is affected. Therefore in addition to PCD, the induction of LPO via ROS generation due to Cadmium nitrate might be another reason of significant reduction of viability.

Table 3. Effect of different concentrations of Cd(NO₃)₂ on total protein (mg ml⁻¹), proline (μM g⁻¹FW), MDA (μM g⁻¹FW) and H₂O₂ (μM g⁻¹FW) content of *C. roseus* callus cells after treatment for 3 days.

Cd(NO ₃) ₂ (mM)	MDA	Proline	protein	H ₂ O ₂
0	5.07 ^a ±0.67	17.89 ^a ±4.01	0.42 ^a ±0.10	0.88 ^a ±0.05
10	19.53 ^b ±1.01	22.89 ^b ±2.08	0.29 ^b ±0.34	0.93 ^b ±0.05
30	28.03 ^c ±1.80	30.54 ^c ±1.91	0.13 ^c ±0.64	1.18 ^c ±0.03
50	43.19 ^d ±0.90	42.00 ^d ±0.23	0.09 ^d ±0.07	1.86 ^d ±0.05

Values are means ± SD. Means with different letter code in each column differ significantly from each other (ANOVA, Duncan test, p<0.05). "FW" stands for fresh weight.

Table 4. Effect of different concentrations of Cd(NO₃)₂ on the activity of ROS scavenger enzymes (SOD, POX and CAT), total alkaloids, flavonoids and phenolic compounds of *C. roseus* callus cells after 3 days of treatment.

Cd(NO ₃) ₂ (mM)	SOD (Umin ⁻¹ mg ⁻¹ protein)	POX (unit min ⁻¹ mg ⁻¹ protein)	CAT (unit min ⁻¹ mg ⁻¹ protein)	Total phenol (mg ⁻¹ FW)	Alkaloids (mg g ⁻¹ FW)	Flavonoids (mg g ⁻¹ FW)
0	20.17 ^a ±0.17	0.46 ^a ±0.04	0.78 ^a ±0.03	0.70 ^a ±0.04	5.72 ^a ±0.73	96.67 ^a ±7.21
10	23.75 ^b ±1.10	0.48 ^a ±0.07	0.84 ^a ±0.05	1.52 ^b ±0.45	6.85 ^b ±0.23	93.33 ^b ±9.71
30	29.22 ^c ±1.03	1.27 ^b ±0.15	1.45 ^b ±0.11	3.25 ^c ±0.45	13.86 ^c ±0.68	151.02 ^c ±4.03
50	34.70 ^d ±0.20	2.37 ^c ±0.55	2.06 ^c ±0.09	4.53 ^d ±0.30	18.23 ^d ±1.47	149.52 ^c ±4.50

Values are means ± SD. Means with different letter code in each column differ significantly from each other (ANOVA, Duncan test, p<0.05). "FW" stands for fresh weight.

A dose dependent increase in the activity of scavenging enzymes such as POX, CAT and SOD in *C. roseus* cells was found, which is in accordance with previous works studying the effects of cadmium stress on cell suspension (Iakimova *et al.*, 2008) and whole plants (Bao *et al.*, 2011). The high activity of these enzymes compared to control was probably due to an internal increase in the level of hydrogen peroxide and other ROS (Kim *et al.*, 2005). One of the most popular ROS is superoxide radical (O₂⁻), which can be converted to H₂O₂ due to action of SOD (Mittler, 2002). The H₂O₂ is formed in the cell as a natural product of the metabolic processes (Iannone *et al.*, 2013), and CAT reduces H₂O₂ into water, therefore this enzyme is considered as a first line of defense with respect to H₂O₂. In case of oxidative stress, when the production of hydrogen peroxide is increased, simultaneously the activity of CAT is elevated too. In some situation, when the level of hydrogen peroxide is beyond the expected CAT activity the other line of defense such as POX is activated. POX reduces H₂O₂ into water using ascorbate as the electron donor (Apel and Hirt, 2004), therefore, POX protects the cell against oxidative damage by detoxifying H₂O₂. An increase in POX activity under Cd(NO₃)₂ stress suggests its role in the detoxification of H₂O₂ in *C. roseus* callus. Therefore the elevation of ROS scavenging enzymes are expected due to cadmium toxicity. Similar response was observed in the callus of *Scbania drummondii* and *Cuscuta reflexa* with respect to cadmium toxicity (Srivastava *et al.*, 2004; Israr *et al.*, 2006) which confirm our results. Also it was observed that the ROS interfere with

protein polymerization and change in protein structure function relationship (Livanos *et al.*, 2012) which in our results, might be the main culprit of morphological changes due to significant reduction in total protein content as well as protein profile changes caused by cadmium.

A dose dependent proline accumulation was observed in the present study, the accumulation of proline has been observed in a wide variety of species grown under abiotic stress (Sayin *et al.*, 2011; Theriappan *et al.*, 2011). Therefore, increase in proline content might be involved in adaptive mechanisms to overcome the cadmium toxicity. Proline plays an important role in osmoregulation, protects plants against enzyme denaturation, acts as a reservoir of carbon and nitrogen, regulates protein synthesis as well as cytosolic acidity and/or scavenging the hydroxyl radicals (Alia and Saradhi, 1991). Thus the increase in the level of proline might be a part of the cellular response to compensate the oxidative induction of cadmium.

In addition to aforementioned cellular responses, production of other biochemical factors have been observed, which mainly was in the form of secondary metabolite productions. *C. roseus* produce secondary metabolites such as indole alkaloids and phenolic compounds like: flavonoids, isoflavonoids, terpenoids, tannins and saponins (Ohadoma and HU, 2011). This plant is most famous for production of alkaloids such as vinblastine and vincristine which are well known antitumor agents (Kumar *et al.*, 2013). In the present study, we observed a significant increase in the total alkaloids content of *C. roseus* callus

cells. Zheng and Wu, showed that the cadmium treatment of *C. roseus* cells suspension caused increase in the ajmalicine content (Zheng and Wu, 2004), their results along with ours might indicate the impotence of abiotic stresses in production of alkaloids.

In addition, we observed a significant increase in the total phenol and flavonoids content of the *C. roseus* callus cells in response to cadmium treatment. Same results also were reported by Keilig and Ludwig-Müller, where they have treated *Arabidopsis* with cadmium (Keilig and Ludwig-Müller, 2009) which confirm our results. Phenolic compounds, especially flavonoids are oxidized in presence of ROS, which results in the inhibition of these species. And also, accumulation of phenolic compounds in oxidative stress can prevent cellular destruction and increases the cell tolerance. Thus, over production of phenolic compounds can be considered as an indicator of cellular response to ameliorate the oxidative stresses.

Conclusion

We observed a strong cellular response with respect to cadmium nitrate toxicity, at which the cellular content of proline, secondary metabolites such as total alkaloids, flavonoids and total phenol were increase. It might be postulated that, this cellular response occurred to compensate the oxidative effect of this chemical. It was also shown that the cadmium toxicity can increase the production of total alkaloids; as the *C. roseus* is a good source of vinblastine and vincristine, therefore cadmium treatment might be a way to increase the production of this therapeutically important alkaloids.

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