# Cytogenetic Responses of Plant Root Meristematic Cells to the Arsenic (III) Contaminated Groundwater in Eastern Parts of Burdwan District, W.B., India

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## *Abstract*

Both geogenic and anthropogenic activities have resulted in arsenic pollution of groundwater especially in the Eastern region of Burdwan district in West Bengal. Response of *Allium cepa* L. genetic material to the presence of potential cytotoxic and genotoxic substances in aquatic environment was used to evaluate the arsenic contaminated groundwater-induced genotoxicity and hormesis. For *in situ* monitoring of the cytotoxicity level, the inhibition of mitosis in root meristematic cells was assayed. To study genotoxicity, micronucleus assay and DNA purity assay (by $A_{260/280}$ ratio) were used. Treatment for 4 days of newly developed roots of *Allium cepa* L. with water samples with arsenic content 50 µg $L^{-1}$ exhibited stimulation in mitotic activity whereas samples with arsenic 1000 µg $L^{-1}$ showed inhibition of mitotic activity apparently indicating hormesis. Inhibition of mitosis was compared with the concentration dependent increase in generation of reactive oxygen species (ROS), cell death, decrease in activity of antioxidative enzymes (catalase, superoxide dismutase), the absorbance ratio ($A_{260/280}$) of DNA, or micronuclei in root cells. These findings indicated that contaminated groundwater depending on the magnitude of the arsenic concentration, might either be mitogenic or mitostatic/toxic, which in turn has obvious implications in agriculture and human health.

***Key words:*** Micronucleus, DNA purity assay, Hormesis, Genotoxicity.

## 1. Introduction

Arsenic contamination in groundwater and its impact on health of the people living in eastern region of Burdwan district in West Bengal is an undeniable fact today (Elangovan and Chalakh, 2006). Several researches from NGOs and Government organizations have reported this at various levels. Not only blocks beside bank of river Ganga (Purbasthali I and II) but adjacent areas viz. Kalna and Katwa blocks have shown the arsenic contamination in groundwater. Though the level of contamination is not same all over the area but in most cases the arsenic level is above the National Standard i.e. 50 µg $L^{-1}$. Chronic exposure to such high level of arsenic via agricultural product, daily uses of contaminated water for drinking, cooking, washing, bathing etc. has severe consequences as far as health is concerned. Lots of field based studies, few clinical and laboratory based assessment of the toxic effect of this arsenic contaminated groundwater were done. Arsenic may affect cells by increasing directly or indirectly the reactive oxygen species (ROS) (Hei and Filipic, 2004; Liu *et al*., 2005). ROS in turn affects cellular DNA and cell organelles. Irrepairable genome damage leads to apoptosis or programmed cell death. Among plant bioassays that are usually employed in toxicological studies, *Allium cepa* assay with several endpoints (viz. mitotic index, cells with micronucleus, cell death etc.) has been proved to be a sensitive and reliable one to evaluate toxicity of environmental pollutants (Fiskesjö, 1985; Rank and Nielsen, 1994; Leme and Marin-Morales, 2009; Panda *et al*., 2011). Sometimes low doses of environmental toxins can increase cellular division and growth in organisms and this has led to the origin of hormesis theory (Stebbing, 1982; Belz *et al*., 2011). Hormesis is characterized by low dose stimulation and high dose suppression (Calabrese and Blain, 2009). The mechanism behind such effect is poorly understood (Cedergreen *et al*., 2007; Agutter, 2008). Majority of research on metals showing hormesis has been published with cadmium, lead, zinc, mercury but information available on arsenic is scarce. Using the *Allium* bioassay, the present study was focused on *in situ* monitoring of the arsenic toxicity at cellular level and biochemical estimation of level of antioxidant enzymes with increased dose of arsenic (and hormetic effect, if any) after four days of exposure in onion root meristematic cells. The micronucleus test in *Allium cepa* root cells is widely used to evaluate the genotoxic potential of environmental pollutants. This assay is a good and sensitive method for monitoring clastogenic effects (Ma *et al*., 1995). The mitostatic/toxic effect was measured by decrease in mitotic index (MI). The purity of a solution of DNA can be determined using a comparison of the optical density values of the solution at various wavelengths (Clark and

Christopher, 2000). For pure DNA, the observed 260/280 nm ratio will be near 1.8. Elevated ratios usually indicate the presence of RNA, and below 1.8 often signal the presence of a contaminating protein or phenol. Here DNA purity assay ($A_{260/280}$) was also done especially to test the stability of DNA-protein complex.

## 2. Materials and Methods

Water samples were collected from tube wells in rural areas of Katwa blocks I and II, Purbasthali blocks I and II on a random basis. Arsenic content was measured at first via field kit (Merck, Germany) and then by visible spectrophotometric method (Narayana *et al*., 2006). Samples were grouped into four sets viz. Treated group I (50 µg $L^{-1}$), Treated group II (100 µg $L^{-1}$), Treated group III (500 µg $L^{-1}$), and Treated group IV (1000 µg $L^{-1}$).

### *2.1. Measuring cytotoxic and genotoxic potential*

*Allium cepa* bioassay was performed for measuring mitostatic/toxic effect considering mitotic index as endpoint. Bulbs of *Allium cepa* L. were germinated in glass vials using tap water. After germinating and reaching ~2 cm in length, root tips of a set of four onions per treated and for control were used and the duration of exposure was four days. After exposure, root tips were collected and fixed with Carnoy's (3:1 methanol : acetic acid) fixative for 24 hr. After fixation root tips were stained with acetocarmin stain (2%), with acid hydrolysis (for 10 min) in 1(N) hydrochloric acid at 60ºC. Root tips were sectioned to remove the meristem, which was then lightly pressed between slide and coverslip. Coverslips were sealed with nail polish. Four slides were prepared for each concentration and the control. 250 cells per slide were analyzed under light microscope (Olympus cH20i) at 11X, 450X and 1000X magnification for induction of micronucleus. The mitotic index was calculated as the percentage of number of dividing cells per 1000 observed cells (Fiskesjö, 1997).

Genotoxic effect was measured by both micronucleus assay and DNA purity assay ($A_{260/280}$) (Clark and Christopher, 2000). From the squash preparation of root tips, micronuclei were scored an extra small nuclear material in interpahse cells besides the intact nuclei. DNA of the root meristematic cells from each treated groups as well as from control was extracted following the protocol of Dellaporta *et al*. (1983) with slight modification. The extracted DNA samples were quantified using the spectrophotometric analysis at wavelengths 260 and 280 nm. The ratio of absorbance at 260 nm to that of 280 nm indicated the purity of DNA.

### *2.2. Estimation of ROS generation*

Cellular generation of $O_2^-$ (Kiba *et al*., 1997), $H_2O_2$ (Loreto and Velikova, 2001) and $OH^-$ (Halliwell *et al*., 1987) were measured using an UV-visible spectrophotometer. For measurement of $O_2^-$, ten excised root tips of equal length (1 cm) weighing ~50 mg were incubated in 3 ml of a reaction mixture [50 mM Tris-HCl buffer (pH 6.5), 0.2 mM nitroblue tetrazolium (NBT), 0.2 mM NADH and 250 mM sucrose] for 24 h at room temperature in the dark under aseptic conditions. The absorbance of the blue monoformazan thus formed was measured at 530 nm, and its concentration was calculated using an extinction coefficient (€ = 12.8 $mM^{-1}$ $cm^{-1}$), which was an indirect measure of $O_2^-$ generation.

For measurement of $H_2O_2$, freshly weighed 1.5 g root samples were homogenyzed at 4ºC in 4 ml of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12,000 x g for 15 min and 1 ml of the supernatant was mixed with 1 ml of 10 mM sodium phosphate buffer pH 7.0 and 2 ml of 1 M potassium iodide. The content of $H_2O_2$ in the supernatant was measured by comparing its absorbance at 390 nm with a standard calibration curve using solutions with known $H_2O_2$ concentrations and expressed in nmoles $g^{-1}$ fresh weight (FW).

For estimation of the amount of cellular $OH^-$, root samples weighing 1 g were homogenized in 2 ml of 10 mM sodium phosphate buffer pH 7.4 containing 15 mM 2-deoxy-D-ribose (w/v) and centrifuged at 12,000 x g for 15 min. The supernatants were incubated at 37ºC for 2 h. Aliquots of 500 µl of above supernatant were added to reaction mixture containing 3 ml of 0.5% (w/v) thiobarbituric acid (TBA, 1% stock solution made in 5 mM NaOH) and 1 ml glacial acetic acid (v/v), heated at 100ºC in a water bath for 30 min and cooled down to 4ºC for 10 min before the measurement. The absorbance of malondialdehyde (MDA) was measured at 532 nm and concentration was calculated using an extinction coefficient (ε = 155 $mM^{-1}$ $cm^{-1}$) and expressed as nmoles $g^{-1}$ FW.

### *2.3. Measurement of cell death*

Evans blue staining method was followed to determine the cell death (Baker and Mock, 1994). Elongated roots were immersed in 0.25% (w/v) aqueous solution of Evans blue for 15 min. After washing with distilled water for 30 min, 10 root tips (~1 cm) were transferred to dye-extraction solution [50% (v/v) ethanol in 1% (w/v) sodium dodecyl sulphate] for 45 min at 50ºC. The absorbance of Evans blue released was measured at 600 nm.

### *2.4. Estimation of antioxidant enzyme activity*

Antioxidant enzymes, namely catalase (CAT), and superoxide dismutase (SOD) were analyzed by spectrophotometer. Root samples weighing 1 g from each treatment were homogenized in chilled mortar and pestle in 2 ml of 50 mM sodium phosphate buffer, pH 7.2, containing 0.1 mM ethylenediamine-tetra acetic acid (EDTA), and 1% (w/v) polyvinylpolypyrrolidone at 4ºC. The homogenates

were centrifuged at 10,000 x g for 15 min at 4ºC and the resultant crude supernatant was collected and stored at –20ºC for estimation of enzyme activities.

CAT activity (Aebi, 1984) was determined by measuring the decrease in absorbance at 240 nm as a result of degradation of $H_2O_2$ ($\varepsilon$ = 39.4 $mM^{-1}$ $cm^{-1}$), which was followed in the reaction mixture containing 50 mM sodium phosphate buffer, pH 7.0 and 10 mM of $H_2O_2$. CAT activity was expressed as nmoles $H_2O_2$ utilized $mg^{-1}$ protein $min^{-1}$.

SOD activity (Beauchamp and Fridovich, 1971) was determined by measuring the inhibition of the photochemical reduction of NBT by the enzyme in the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.8), 0.1 mM of EDTA, 75 µM of NBT (w/v), 13 mM methionine (w/v), 0.3% (v/v) triton X-100 and 2 µM riboflavin (w/v). The reaction was initiated at room temperature by switching on the light for 8 min, and stopped by switching the light off. The absorbance of formazan so formed was recorded at 560 nm. One unit of SOD activity was defined as the amount of the enzyme that inhibited NBT reduction by 50% and expressed as $mg^{-1}$ protein $min^{-1}$.

### *2.5. Statistical analysis*

Values in each study were represented as mean ±SD. Obtained data were statistically verified by regression analysis, where level of significance was set at ≤0.05. Pair wise comparison was done by two tailed t test ($p \leq 0.05$). All statistical analyses were made in MS Excel 2013.

## 3. Results

The result of cytotoxicity study was shown in Table 1. In the Treated group I and II there were no significant decrease in mitotic index in comparison with control. Rather in group I, a significant increase in MI than that of control was noticed. The significant mitotic suppression was only recorded in case of Treated group III and IV.

Micronucleus assay carried out on roots after 4 day treatment with arsenic indicated a dose dependent increase from 500 to 1000 $\mu gL^{-1}$ ($p \leq 0.05$) only (Table 1, Fig. 1). Though arsenic at concentrations 50 and 100 µg $L^{-1}$ showed no significance increase in MN% than that in control but at higher concentrations (Table 1), micronuclei were formed in significant quantity ($p \leq 0.05$). The spectrophotometric analysis of the extracted DNA from roots subjected to different treatments showed an absorbance ratio ($A_{260/280}$) of 1.80-2.00 (Table 1).

Arsenic induced cell death significantly ($p \leq 0.05$) at concentrations 500 and 1000 µg $L^{-1}$ (Table 1). Interestingly, arsenic at ≤50 and ≤100 µg $L^{-1}$, failed to induce significant cell death, which was similar to or less ($p \leq 0.05$) than that of control.

The antioxidant enzymes responded differently to the different concentrations of arsenic (Table 1). In our experiments, the mechanism of protection by CAT (catalase) action is rather inefficient in root meristem cells of *Allium cepa* because, except the bulbs treated with ≤50 µg $L^{-1}$ arsenic which shows a significant increase in compare to that in control, the others treated groups showed a more or less marked decline comparatively with control.

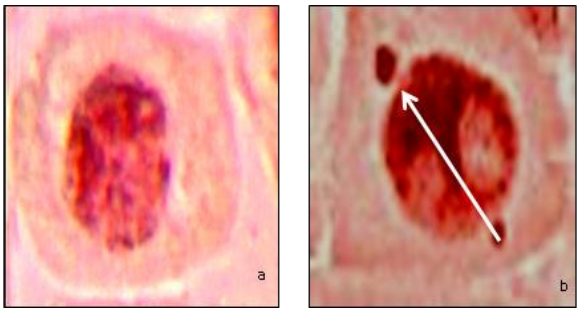


**Fig. 1:** (a) Normal cell in interphase and (b) Microneulceus in interphase cell (arsenic treated)

The responses of SOD to arsenic treatments were different. Concerning SOD activity under arsenic stress, the treatment resulted in a considerable rise of enzyme activity in all treated groups, exposed to arsenic, fact proving activation of root meristem cell detoxification mechanisms.

**Table 1:** Frequency distribution of cytogenetic endpoints, antioxidant enzymes and ROS after 4 day treatment of root meristematic tissue cells with arsenic

| Treatment | Mitotic index (MI) | MN % | DNA absorbance ratio ($A_{260/280}$) | Cell death | Antioxidant Enz. | | ROS | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | CAT | SOD | $O_2^{-}$* | $H_2O_2$** | $OH^{-}$*** |
| Control ($dH_2O$) | 19.93± 0.68 | 0.2± 0.01 | 1.82± 0.2 | 0.38±0.3 | 20.98±1.9 | 105± 1.5 | 0.7± 0.3 | 209± 2.0 | 21± 1.32 |
| Treated group I | $24.2± 0.53 | $0.11± 0.01 | #1.85± 0.2 | $0.19± 0.07 | $24.59± 0.97 | $111± 1.97 | $0.46± 0.04 | $201± 1.8 | #22.04± 0.49 |
| Treated group II | #21.16± 0.24 | #0.21± 0.02 | #1.89± 0.5 | #0.33± 0.04 | $18.1± 1.09 | $93.01± 1.12 | $0.42± 0.01 | $283± 2.33 | $18.66± 0.82 |
| Treated group III | $18.03± 0.1 | $0.38± 0.02 | #1.87±0.23 | $0.55± 0.03 | #19.01± 1.01 | $87.62± 1.5 | $1.0± 0.05 | $323± 1.74 | $37.31± 1.0 |
| Treated group IV | $16.29± 0.3 | $0.6± 0.2 | #1.92± 0.4 | $0.98± 0.2 | $12.80± 1.15 | $83.27± 1.09 | $1.24± 0.8 | $568± 1.3 | $47.81± 1.3 |

*nmoles $g^{-1}$ FW, **µmoles NBT reduced $g^{-1}$ FW, ***nmoles MDA generated $g^{-1}$ FW; $- significant difference in comparison with control; #- insignificant difference in comparison with control

The generation of ROS was determined in all experimental sets and in control (Table 1). In comparison with control, Treated groups I and II resulted in a significant concentration dependent decrease in generation of $O_2^-$ ($p \leq 0.05$). But Treated groups III, and IV resulted in a significant concentration dependent increase in generation of $O_2^-$ ($p \leq 0.05$). The level of $H_2O_2$ was decreased in Treated group I but showed significant increase in Treated groups II, III, and IV ($p \leq 0.05$) that showed significant correlation with the concentration of arsenic in experimental solution. Treated group I showed no significant change in $OH^-$ level and a significant decrease in treated groups II was also observed. Concentration-dependent increase was only noticed in Treated groups III and IV in comparison with control ($p \leq 0.05$). Generation of ROS was also significantly correlated with inhibition of mitosis ($p \leq 0.05$).

## 4. Discussion

Arsenic treatments suppressed mitosis in root cells of *A. cepa* significantly from 100 µg $L^{-1}$ onwards but, remarkably, stimulated mitotic division at the low concentration of 50 µg $L^{-1}$. A hormetic dose–response indicating low dose stimulation and high-dose inhibition of mitosis in *A. cepa* was thus evident (Calabrese and Blain, 2009; Belz *et al*., 2011). Such a biphasic (hormetic) dose-response might be dependent on the concentration and exposure duration together. High concentration and short treatment-time resulted in stimulation of mitosis to the same degree (data not shown) comparable with that induced by low concentration and long treatment-time.

Toxicity has been defined as a function of exposure (cumulative dose), and exposure is a function of concentration and exposure duration (Rozman *et al*., 2010). Arsenic depending on the magnitude of the dose, either inhibited or stimulated mitosis indicating toxicity or hormesis. The aforesaid findings provided evidence that stimulation of root growth, like inhibition, too was a function of both concentration and treatment-time of arsenic.

Arsenic toxicity was evident by the dose-dependent increase in generation of ROS and cell death. Among ROS, $OH^-$ showed the best correlation with cell death. In the 4-day regimen, arsenic a higher doses down regulated CAT, and SOD contributing to the generation of ROS leading to oxidative stress and genotoxicity.

The protective mechanisms adapted by plants to scavenge free radicals and peroxides involve two major antioxidative enzymes: SOD and catalase. The antioxidative enzymes are important in preventing the oxidative stress in plants. The activity of one or more of these enzymes is generally increased in plants when exposed to stressful conditions (Allen, 1995; Stevens *et al*., 1997; Schutzendubel *et al*., 2001). SOD has 3 major isoforms in higher plants, the Cu, Zn-SOD in thylakoid membranes as well as in the cytosol, Mn-SOD in the mitochondria and the Fe-SOD in the chloroplast. SOD is induced by its own substrate, the superoxide radical (Colepicolo *et al*., 1992; Allen and Tresini, 2000), and thus activation of cellular SOD may be an indication that the cell is experiencing pollutant induced superoxide radical stress.

The changes in SOD presented in this study reflect the changes in total SOD and not a particular isoform. Increase in SOD and catalase has been reported with environmental stresses (Tsang *et al*., 1991; Chen *et al*., 2000). Arsenic contaminated groundwater treatment of the onion bulbs in this study resulted in significant increase in the activities of SOD and catalase at lower arsenic concentrations while higher concentrations led to enzyme inhibition. These findings are consistent with earlier reports with heavy metal treatment in the same plant (Fatima and Ahmad, 2005).

The findings in this study clearly show that variations in the antioxidant enzymes of *A. cepa* can also serve as useful biomarkers for the detection of pollutants in the aqueous medium. Catalase and the peroxidases are the major enzymes involved in $H_2O_2$ detoxification; CAT did not exhibit consistent change upon arsenic exposure and it is known that glutathione peroxidase (GPX) has higher affinity for $H_2O_2$ than CAT; thus it is more effective in decomposing $H_2O_2$ (Halliwell and Gutteridge, 1984).

Micronuclei were the consequences of misrepaired DNA damage (Achary and Panda, 2010). Increasing number of MN with dose escalation from 100-1000 µg $L^{-1}$ clearly indicated genotoxic effect of arsenic at higher doses. But the DNA purity was maintained in the present study (1.82-1.92) based on the $A_{260/280}$ absorbance was an indication that DNA-protein complex in *Allium cepa* root meristematic cells was not affected by arsenic.

In conclusion, the findings suggested that arsenic depending on the magnitude of the dose (concentration *x* time) could either inhibit mitosis and induce genotoxicity at high doses, or stimulate mitosis at low doses apparently causing no genotoxic damage to the root cells of *A. cepa* that pointed to the dual role of ROS in the underlying mechanisms. Work is on-going to determine activities of ascorbate peroxidase (APX), glutathione peroxidase (GPX) in *A. cepa* root tips exposed to arsenic contaminated groundwater. The findings underscore that environmental toxicants such as metallic pollutants, in low doses induce hormesis—a beneficial stress response, which may have practical implications on agriculture, post harvest technology and even human health, thus warranting further exploration.

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