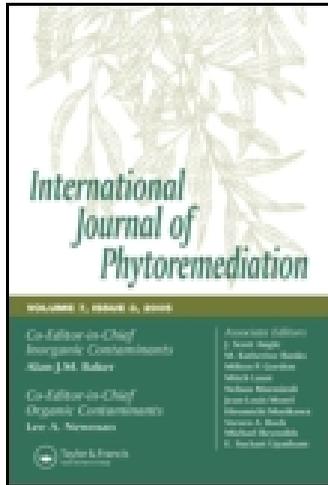


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TECHNICAL NOTE: VETIVER CAN GROW ON COAL FLY ASH WITHOUT DNA DAMAGE

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*Fly ash is a by-product of coal-fired electricity generation plants. The prevalent practice of disposal is as slurry of ash and water to open lands or ash ponds located near power plants and this has led to waste thousands of hectares all over the world. Wind and leaching are often the causes of off-site contamination from fly ash dumpsites. Vetiver (*Vetiveria zizanioides*) grown on fly ash for three months showed massive, mesh-like growth of roots which could have a phytostabilizing effect. The plant achieved this without any damage to its nuclear DNA as shown by comet assay done on the root nuclei, which implies the long-term survival of the plant on the remediation site. Also, when Vetiver is used for phytoremediation of coal fly ash, its shoots can be safely grazed by animals as very little of heavy metals in fly ash were found to be translocated to the shoots. These features make planting of Vetiver a practical and environmentally compatible method for restoration of fly ash dumpsites. Lack of DNA damage in Vetiver has been compared to that in a sensitive plant i.e. *Allium cepa*. Our results suggested that apart from traditional end-points viz. growth parameters like root length, shoot length and dry weight, comet assay could also be included in a battery of tests for initial, rapid and effective selection of plants for restoration and phytoremediation of polluted sites.*

KEY WORDS: comet assay, heavy metals, phytostabilization, *Vetiveria zizanioides*

INTRODUCTION

Thermal power generation through coal combustion produces minute particles of ash, commonly known as fly ash. Fly ash is a serious source of air pollution since it remains air-borne for a long period and causes hazard to the lungs through oxidative stress and inflammation (Donaldson *et al.*, 2005). To prevent the fly ash from being air-borne, the dumping grounds need to be kept wet all the time, for either which sprinklers are used or agencies are hired to water the grounds. This practice also leads to another problem: since these sites are not lined, seepage contaminates groundwater and soil. Fly ash itself and extracts of fly ash has been tested positive for mutagenity and genotoxicity (Kleinjans *et al.*, 1989), and involvement of heavy metals (Shifrine *et al.*, 1984) and aromatic hydrocarbons (Griest *et al.*, 1982) has been implicated. Thus, without compromising on health and environmental safety, fly ash should be handled judiciously and phytoremediation is an appropriate technique for this purpose.

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Phytoremediation is the direct use of living green plants to degrade, contain, or render harmless various environmental contaminants, including recalcitrant organic compounds or heavy metals (Macek *et al.*, 2004). It is important to select an appropriate pioneer plant species for successful site reclamation and in phytoremediation efforts to ensure a self-sustainable vegetative cover. We selected Vetiver for our study on phytoremediation of coal fly ash because it is a well-known phytoremediant with many useful properties. Vetiver grass or *Vetiveria zizanioides* (Linn.) Nash of the family Poaceae is a dense, bunch-type grass with stiff stem, and an extremely strong root system (up to 4.6 m deep), and grows to the height of over 2 m (The Wealth of India, 1976). It grows on all continents in tropical and subtropical regions, tolerates a wide range of soil pH and low fertility. It is both a xerophyte and a hydrophyte, and once established it can withstand drought, flood, and long periods of water logging. The plant is highly tolerant to elevated levels of heavy metals such as arsenic, cadmium, copper, chromium, lead, mercury, nickel, selenium and zinc (Truong and Baker, 1998; Truong and Hart, 2001). It does not compete with other plants and it has associated nitrogen-fixing mycorrhiza, which would explain its green growth throughout the year and tolerance to metal toxicity (Wong *et al.*, 2007).

The aim of the present work was two-fold. One was to establish whether Vetiver could be used as a phytoremediant for coal fly ash dumpsites. The second was to find the efficacy of comet assay as a molecular technique for initial rapid screening of plants for an appropriate phytoremediant in a particular polluted site. This was done by comparing the results of comet assay on root nuclei in a known phytoremediant (Vetiver) and in a sensitive plant (*Allium cepa*) grown on the same material (coal fly ash).

MATERIALS AND METHODS

Chemicals

Normal melting point agarose (NMPA), low melting point agarose (LMPA), ethylenediaminetetraacetic acid (EDTA) disodium salt, Tris buffer and ethidium bromide (EtBr, CAS no. 1239-45-8) were purchased from Sigma Chemical Co., St Louis, USA. Phosphate buffered saline (Ca^{++} , Mg^{++} free PBS) was purchased from Hi-Media Ltd., India. All other chemicals like sodium hydroxide (NaOH), sodium chloride (NaCl), *etc* were purchased locally and were of analytical grade.

Sample Collection

Fly ash was collected from fly ash dumpsite around the Kolaghat Thermal Power Station, in West Bengal, India. Sample was collected in plastic drum, dried under sunlight and kept at room temperature ($28 \pm 1^\circ\text{C}$). Authenticated specimen of *Vetiveria zizanioides* (Linn.) Nash was collected from the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India and grown in the experimental garden of Department of Botany, University of Calcutta for acclimatization and multiplication.

Treatment Conditions

Healthy Vetiver plants were planted and grown in pots containing fly ash or garden soil (3 pots / sample) under normal environmental conditions. The experiment was carried

for three months during the months of April to June. Equal-sized bulbs were chosen from a population of a local market variety of the common onion *Allium cepa* L. ($2n = 16$). The onions were positioned for germination and growth for 5 days on fly ash and garden soil—a method that mimics natural condition (Panda *et al.*, 1990).

Quantification of Heavy Metals

Fly ash sample: One gram of fly ash sample was digested in HNO_3 (70%) in a microwave digestion system (Ethos D Microwave Labstation, Milestone Inc, USA) and analyzed for Pb, Cd, Ni, Zn, and Cu by flame atomic absorption spectrometer (Avanta, GBC Scientific Equipments, Australia). Arsenic content in the fly ash was estimated by AAS after attaching the hydride generation system (S119—HG 3000 Hydride Generator, GBC Scientific Equipments, Australia).

Plant material: At the end of the experimental period, the plants were taken out of the pots, washed thoroughly in running tap water and demineralized water. Plant tissue was cut into small pieces, dried for 2 days at 80°C , and ashed in a muffle furnace at 500°C for 6 h. One gram of ash was dissolved in 5 mL of HNO_3 (70%). The solution was boiled until the emission of brown fumes stopped and filtered. The filtrate was diluted to 100 mL with double distilled water and analyzed for Zn, Pb, Cu, Ni, Cd and As.

Metal estimation was done following the methods of APHA (1998) and expressed as $\mu\text{g/g}$.

Comet Assay

The nuclei from *A. cepa* and *V. zizanioides* were processed for comet assay. The root meristem of *Allium* is considered highly sensitive to DNA damage (Navarrete *et al.*, 1997). It was the preferred tissue for analyzing DNA damage in Vetiver to keep the same sampling tissue in the two plant species. After respective treatment roots were removed, washed thoroughly in running tap water and then with demineralized water. The roots were placed for 2 min on ice to keep them turgid (Navarrete *et al.*, 1997). For isolation of nuclei, root tissues, treated or untreated as appropriate, were processed immediately according to the method described by Chakraborty *et al.* (2008). Briefly, roots were gently sliced with a fine razor blade in 250 μL of Tris buffer (400 mM), at pH 7.5 in a 60-mm Petri dish kept on ice.

Conventional microscope slides with frosted end were dipped into a solution of 1% NMPA prepared in water at 50°C , dried overnight at room temperature and kept in slide boxes until use. Nuclear suspension (40 μL) and 1% LMPA (40 μL) in phosphate-buffered saline were gently mixed at 40°C by repeated pipeting using a cut micropipette tip and added onto each slide. A cover slip was placed on the mixture to obtain a uniform layer. The gel was allowed to solidify on ice. The cover slip was removed and a final layer of 0.5% LMPA (80 μL) in phosphate-buffered saline was placed on the slide. A cover slip was placed on it and the slide was kept on ice for 5min. The cover slip was removed and the slides were placed in a horizontal gel electrophoresis tank (Biotech, Mumbai, India), side by side avoiding space, containing fresh and chilled electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, $\text{pH} > 13$). The slides were left in the solution for 15 min to allow DNA unwinding and expression of alkali labile sites as DNA breaks. Using power supply (Techno Lab, Kolkata, India) electrophoresis was conducted for 20 min at 4°C , at 20 V (0.7 V/cm) and 300 mA. All these steps were performed under dim light

and the electrophoresis tank was covered with a black paper to avoid additional DNA damage due to stray light. Lysis prior to DNA-unwinding step was avoided since it was confirmed previously that the lysis step in various comet experiments with plant material is unnecessary and it does not influence the effects of mutagenic treatment (Gichner and Plewa, 1998). After electrophoresis, the slides were rinsed 3 times with Tris buffer (400 mM Tris, pH 7.5) to neutralize excess alkali and air-dried.

Each slide was stained with 80 μ L ethidium bromide (20 μ g/mL) for 5 min, dipped in cold water to remove the excess ethidium bromide and covered with a cover slip (Gichner *et al.*, 2006). For each slide, 25 randomly chosen nuclei were analyzed using a fluorescence microscope. A computerized image analysis system (Komet version 5.5, Kinetic Imaging Ltd., Andor technology, Nottingham, UK) was employed. Three slides were evaluated per treatment and the median value of the comet parameter of percentage of tail DNA was calculated. Each treatment was repeated twice. From the repeated experiment, the mean of the calculated medians and standard deviation of percentage of tail DNA (% of DNA in comet tail) was calculated for each treatment group. The parameter of percentage of tail DNA was particularly selected because it was considered the most meaningful and easy to conceptualize among the parameters available for study (Kumaravel and Jha, 2006).

Statistical Analysis

Statistical analyses of the data were performed using the statistical programme-SigmaStat 3.0 (SPSS Inc., Chicago, IL, USA). Student's 't' test was done for the comet parameter- percentage of tail DNA between the *A. cepa* grown in garden soil and that grown in fly ash; between *V. zizanioides* grown in garden soil and that grown in fly ash sample.

RESULTS AND DISCUSSION

Phytoremediation is considered a practical, economical, and environmentally compatible solution for remediating some of heavy metal contaminated sites on a large scale. Categories of phytoremediation include phytoextraction, phytostabilization, phytovolatilization and rhizofiltration (Chaney *et al.*, 1997).

Phytoextraction is the use of plants to remove metals from soil (Kumar *et al.*, 1995). Data in Table 1 shows that the concentration of Zn in the roots of Vetiver plants grown on fly ash (100.66 ± 5.96) was higher than those grown on garden soil (59.54 ± 2.41). The other metals namely Pb, Cu, Ni, Cd, and As, did not show any increased values in the plant body grown on fly ash as compared to that grown in garden soil. The important implications of these findings are that when Vetiver is used for phytoremediation of fly ash dumpsites, animals can safely graze the shoots as very little of these metals are translocated to the shoots.

There was dense mesh like growth of root that was found entangling the fly ash in all the pots used for study showing the characters of a good phytostabilizer (Figure 1). This is particularly important in the case of fly ash since due to its lightweight it is easily carried away by wind and cause air pollution. Dense root system and vegetative cover may also help to retard the formation of hazardous leachate from fly ash. Phytostabilization in general depends on the roots ability to limit contaminant mobility and bioavailability in the soil

Table 1 Concentration of heavy metals ($\mu\text{g/g}$) (Mean of 3 samples \pm standard deviation)

Heavy metal	Garden Soil Day 1	Fly ash sample Day 1	<i>V. zizanioides</i>			
			Leaves		Roots	
			Control ^a	Treated ^b	Control ^a	Treated ^b
Zn	109.72 \pm 6.18	28.90 \pm 3.04	18.08 \pm 2.69	11.19 \pm 1.92	59.54 \pm 2.41	100.66 \pm 5.96
Pb	15.70 \pm 3.62	23.39 \pm 2.32	<3.18	<3.18	<3.18	<3.18
Cu	34.98 \pm 4.15	16.29 \pm 2.13	2.46 \pm 0.87	1.65 \pm 0.53	18.38 \pm 2.2	13.79 \pm 1.84
Ni	13.50 \pm 1.61	14.06 \pm 0.93	<2.4	<2.4	<2.4	<2.4
Cd	0.84 \pm 0.10	0.91 \pm 0.24	<0.44	<0.44	<0.44	<0.44
As	0.86 \pm 0.13	0.51 \pm 0.14	<0.14	<0.14	<0.14	<0.14

^a*V. zizanioides* grown for 3 months in garden soil.

^b*V. zizanioides* grown for 3 months in fly ash sample.

where the plants primary purpose is to decrease the amount of water percolating through the soil matrix, which may result in the formation of hazardous leachate and prevent soil erosion and distribution of the toxic metal to other areas. It is very effective when rapid immobilization is needed to preserve ground and surface water and disposal of biomass is not required (Ghosh and Singh, 2005). Result of the metal contents in plant tissues and root growth indicated that Vetiver was more suitable for phytostabilization than phytoextraction in case of coal fly ash.

The major elemental constituents of fly ash are Si, Al, Ca, C, Mg, K, Na, S, Ti, P, and Mn and nearly all naturally occurring elements like As, Mo, Se, Cd, and Zn can be found in trace quantities (el-Mogazi *et al.*, 1998). The presence of heavy metals in fly ash sample is given in Table 1. The concentration of Zn was highest followed by Pb, Cu, Ni, Cd, and

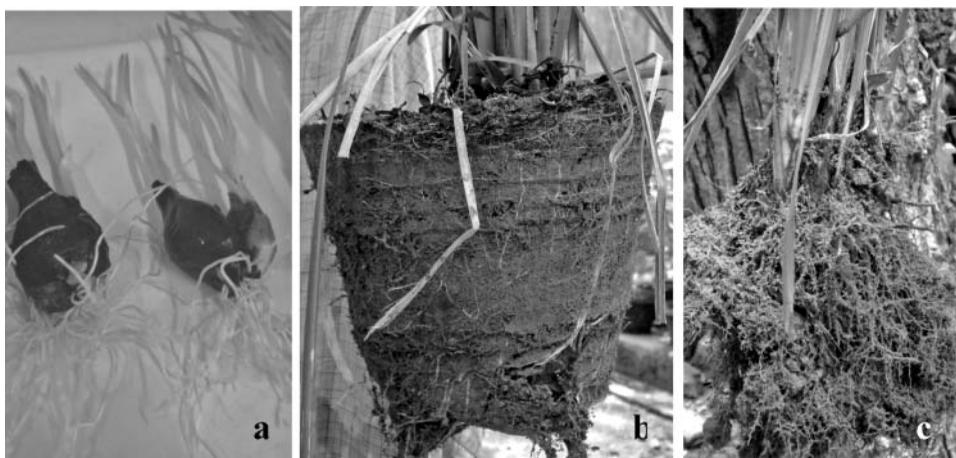


Figure 1 Condition of *Allium* after growth in coal fly ash for 5 days (a) Phytostabilizing effect of Vetiver on coal fly ash; (b) root system of Vetiver entangling compactly the fly ash on which the plant was grown for 3 months; and (c) huge, dense, mesh like growth of root of Vetiver grown on fly ash for 3 months (shown after removal of fly ash).

As. The concentrations of total Zn, Cu, and As were found higher in garden soil than in fly ash. Generally the bioavailability of metals is less in natural soil compared to coal fly ash due to chelation of metals with the organic matter present in soil that is absent in fly ash (Maiti and Jaiswal, 2008). Doygaliuk *et al.* (2001) reported that salts of metals—like Cd, Pb, Ni, Al, Cu, and Zn—could induce both clastogenic and aneugenic effects in plants. Again, the total amount of Polycyclic Aromatic Hydrocarbons (PAHs) in the fly ash was found much higher than in the raw coal by Liu *et al.*, 2000. Therefore, the adverse effect of coal fly ash on plant systems can be due to the presence of the inorganic and organic chemicals as well as due to the physical factors. Thus, it was important to assess any DNA damage in Vetiver grown on fly ash and for this purpose comet assay on root nuclei was performed.

The alkaline version of Comet assay is considered a rapid, sensitive, and relatively simple method for detecting DNA damage at the level of individual cells (Ostling and Johanson, 1984). The interpretation of the results is based on the hypothesis that, the nuclear DNA damage caused by a non-cross linking genotoxic agent produces low molecular weight DNA strands, either directly through DNA breaks or indirectly by affecting excision repair of damage or formation of alkali labile sites. These broken DNA strands are released in course of unwinding stage of the comet process and they produce comet tail upon electrophoresis, while undamaged high molecular weight DNA does not migrate and forms comet head. The more DNA is damaged by a non-crosslinking genotoxic agent and broken into low molecular weight pieces, the bigger the comet tail and larger the comet parameters like percentage of tail DNA (% of DNA in the Tail). In the present study, the results of comet assay show that *Allium* grown in fly ash gave significantly ($P \leq 0.001$) higher degree of DNA damage. Apparently, all the plants were equally healthy (Figure 1) and the percentage of tail DNA was 5-fold high in plants grown on fly ash than that of plants grown in garden soil. On the other hand, the DNA damage in Vetiver grown in fly ash for three months (8.31 ± 1.13) was not significantly different from that of the plants grown in garden soil (8.36 ± 1.16 , respectively). The data are represented in Table 2 and Figure 2. Absence of damage in nuclear DNA of Vetiver implies its long-term survival on the coal fly ash dumpsite. It also demonstrated that comet assay can suitably help us to segregate between a tolerant plant having the potential of a successful phytoremediant and a non-tolerant sensitive plant. Hence, apart from traditional end-points *viz.* growth parameters like root length, shoot length and dry weight, comet assay could also be included in a battery of tests for initial, rapid and effective selection of plants for restoration and phytoremediation of polluted sites.

Table 2 Percentage of tail DNA (% of DNA in comet tail) in root nuclei (Mean of 6 samples \pm standard deviation)

Plant material	Control ^a	Treated ^b
<i>A. cepa</i>	15.77 ± 1.63	$82.88 \pm 2.98^*$
<i>V. zizanioides</i>	8.36 ± 1.16	8.31 ± 1.13^{ns}

^a*A. cepa* grown for 5 days and *V. zizanioides* grown for 3 months in garden soil.

^b*A. cepa* grown for 5 days and *V. zizanioides* grown for 3 months in fly ash sample.

*Significant at $P \leq 0.001$; ns- non-significant at $P \leq 0.001$.

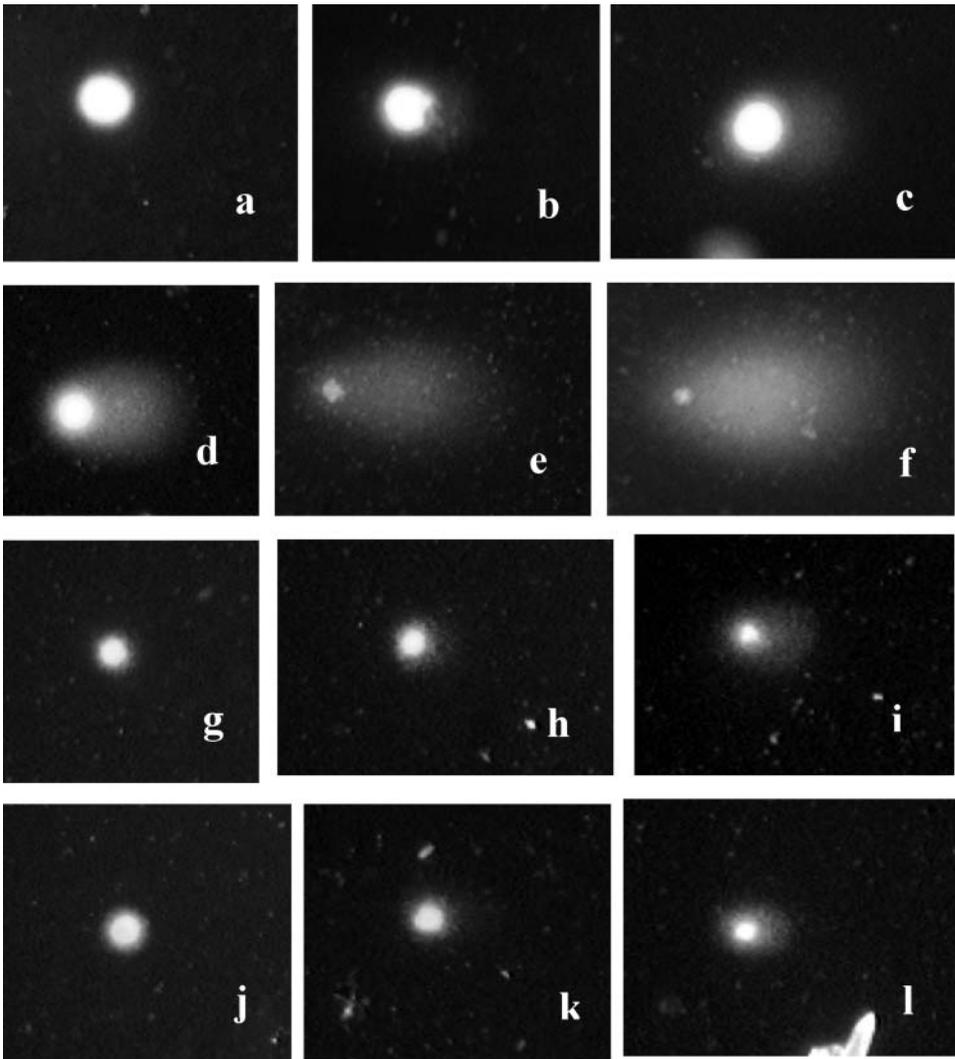


Figure 2 Comet character in nuclei isolated from root meristem: of *A. cepa* grown for 5 days in (a–c) garden soil and (d–f) fly ash sample; of *V. zizanioides* grown for 3 months in (g–i) garden soil and (j–l) fly ash sample.

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