Bull. Environ. Contam. Toxicol. (2002) 68:675–683

DOI: 10.1007/s00128-001-0307-4

Bulletin of Environmental Contamination and Toxicology

Biochemical Responses of *Cassia siamea* Lamk. Grown on Coal Combustion Residue (Fly-ash)

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Received: 7 August 2001/Accepted: 18 December 2001

Power generation from coal results in a variety of pollutants including fly-ash which is combustion by-product of coal. In India, about 79% of the electricity is generated by coal-based thermal power plants and annually 65 million tonnes of fly-ash is generated as a waste product (Sahu 1998). The fly-ash (coal combustion residue) is usually dumped in huge landfills, that has many environmental implications. Vegetating these landfills is a viable option for preventing many harmful affects attributable to fly-ash. However, the growth of plants of fly-ash is hindered due to some adverse physical and chemical properties of fly-ash (Mulhurn et al 1989). The possible limitations include high pH, negligible N, P and toxic concentrations of many heavy metals (Cu, Zn, Mn, Pb, Hg, Cd etc.). The presence of heavy metals in fly-ash was found to be most important factor detrimental to plant growth and caused stress to the plants. Many crop plants and vegetables have been grown on fly-ash and varying degree of success has been reported (Wong and Wong 1990; Singh et al 1997; Khan and Khan 1996). However, such studies are rare for tree species (Scalon and Duggan 1979; McMinn et al 1982; Vajpayee et al 2000). The accumulation of metals in various parts of higher plants is often accompanied by variety of cellular changes, some of which directly contribute to metal tolerance of the plants (Van Assche and Clijsters 1990). Primary metal induced toxic effects in plants include generation of free radicals and reactive oxygen species (superoxide : O_2, hydroxyradicals : $^{\cdot}OH$ and hydrogen peroxide : H_2O_2) (Halliwall and Gutteridge 1984). The inactivation of enzymes, degradation of membranes, proteins, nucleic acids, chlorophylls and increased peroxidation of lipids have been reported due to toxicity caused by free radicals generated under heavy metal stress (Dietz et al 1999). However, plants have internal defence mechanism to combat metal toxicity. Many antioxidants and antioxidative enzymes are present in plants to prevent them from damage caused by oxidative stress (Weckx and Clijeters 1996). Besides, it has been reported that plants synthesize phytochelatins (PC) in presence of Cu, Cd, Zn, Ni etc. which protect them from injuries caused by heavy metals (Zenk 1996). Plants having capability of synthesizing PCs exhibit high non-protein thiol and cysteine content. No report is available on biochemical changes induced in plants by fly-ash which is a complex substrate containing an array of heavy metals.

Cassia siamea L. is a non nodulated member of family leguminosae. It grows well in degraded soils. But it is not clear how the species could establish itself in degraded lands. Further, it has also been observed that *C. siamea* could grow in raw fly-ash and fly-ash ameliorated by different ameliorants (Cow dung manure, press mud, garden soil) in 1:1 ratio and accumulated significant amount of Cu, Zn, Fe, and Ni etc. in different plant parts (Tripathi et al 2000). Therefore, it seems necessary to study the biochemical responses of

N.B.R.I. Publication No. (510)
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C. siamea grown in different concentrations of fly-ash to understand the impact of heavy metal accumulation on different cellular processes. Hence, to study the effect of heavy metal accumulation on cysteine, non protein thiol contents, lipid peroxidation, superoxide dismutase (antioxidant enzyme) nitrate reductase (a key-SH containing enzyme of nitrate assimilation) activities, experiments were conducted on *C. siamea* plants grown on fly-ash and fly-ash +Garden soil, fly-ash + cow dung manure and fly-ash + press mud in 1:1 ratio and results are being presented in this paper.

MATERIALS AND METHODS

Fly-ash (FA) was collected from Feroze Gandhi Unchahar Thermal Power Project, Unchahar, Rae Bareli (U.P., India). Various physico-chemical parameters and metal contents of raw fly-ash, garden soil (GS), cowdung manure (CM) and pressmud (PM) were analysed (Piper 1967; APHA 1985). Thirty days old seedlings of *Cassia siamea* L. were obtained from Banthra Nursery, National Botanical Research Institute, Lucknow. Earthen pots (12") were filled by garden soil (GS), raw fly-ash (FA), garden soil + raw fly-ash (GS+FA, 1:1, w/w) and fly-ash + cowdung (FA+CM, 1:1, w/w) in triplicates. Oneseedling of *C. siamea* was planted in each earthen pot. Plants were harvested after 20, 40 and 60 d of plantation. Plants grown in garden soil served as control.

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content by the thiobarbutaric acid (TBA) reaction method (Heath and Packer 1966). Leaves (0.5 g) were homogenised in 4 ml 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 g for 10 min. To one ml of the supernatant, 4 ml 20% TCA containing 0.5% TBA was added. The mixture was heated at 95°C for 30 min and the absorbance of the cooled reaction mixture was read at 532 nm. The concentration of MDA was calculated using the extinction co-efficient of 155 mM^{-1} cm^{-1} and correcting for the specific absorbance at 600 nm (532-600 nm).

The activity of SOD (leaves) was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) (Beauchamp and Fridovich 1971) The reaction mixture lacking enzyme developed the maximum colour and this decreased with increasing volume of added enzyme extract. The volume of enzyme extract corresponding to 50% inhibition of the reaction has been calculated by plotting a graph between enzyme concentration in reaction mixture and abrobance of it at 560 nm and was considered as one unit enzyme. SOD activity was expressed as unit g^{-1} FW.

Cysteine content was measured following the method of Gaitonde (1967). Fresh leaves (0.5 g) were homogenized in 4 ml of 5% perchloric acid and centrifuged at 10,000 g for 10 min. To 1.0 ml aliquot, 1 ml acetic acid and 1.0 ml of freshly prepared acid ninhydrin reagent (250 mg ninhydrin + 6 ml acetic acid + 4 ml conc. HCl) were added and heated on water bath (90 °C) covered with aluminum foil for 10 min. The absorbance of reaction mixture was read at 560 nm. For estimation of non protein thiol content (NP-SH), frozen plant tissue (ca 700 mg fresh leaves) was extracted in 6.67% sulfosalicylic acid and centrifuged at 13000 g for 10 min. Supernatant was reacted with Ellman's reagent and absorbance was recorded at 412 nm (Ellman 1959).

In vivo nitrate reductase activity (NRA) was estimated according to the method of Srivastava (1974). The excised leaf tissue (0.5 g) was incubated in 10 ml reaction mixture containing 50 mM phosphate buffer (pH 7.5, 10 mM of KNO_3 and 0.5% n propanol v/v). The samples were vacuum infiltrated in a dessicator and incubated in dark at 30°C for 30

min. The mixture was boiled in water bath and the concentration of NO_2^- formed was estimated by adding 1.0 ml of 1% sulphanilamide in 1 N HCl (w/v) and 1.0 ml of 0.02% napthylethylene diamine dihydrochloride (NED) to 1 ml aliquot of above mixture. Absorbance was recorded at 540 nm and nitrate reductase activity was expressed as n mol NO_2^- $h^{-1}g^{-1}$ FW.

A two way analysis of variance (ANOVA) in complete randomised block design involving five treatments and three durations was performed to confirm the validity of data. Comparison from control and between the means of treatment was done by Duncan's multiple range test (Gomez and Gomez 1984).

RESULTS AND DISCUSSION

The physico- chemical properties as presented in Table 1 indicated that the fly-ash used in experiment was alkaline (pH: 8.8). The electrical conductivity of the fly-ash was very high indicating high ionic concentration. Total nitrogen (0.01%) and phosphorus (0.14%) contents were very low in fly-ash. Levels of almost all the elements were very high. However, Al, Si, Mo, Fe and Ni need special mention.

Table 1. Physico-chemical properties of fly-ash, garden soil, cowdung and press mud used in experiment

S.N.	Parameter	Fly-ash	Soil	Cow-dung	Press-mud
1.	pH	8.8 ± 0.42	7.7 ± 0.32	7.4 ± 0.030	7.2 ± 0.32
2.	Electrical conductivity(dSm^{-1})	7.61 ± 0.38	1.6 ± 0.08	2.2 ± 0.10	2.7 ± 0.12
3.	Total nitrogen (%)	0.02 ± 0.001	0.09 ± 0.002	1.13 ± 0.046	1.8 ± 0.80
4.	Total phosphorus (%)	0.14 ± 0.006	0.78 ± 0.032	1.20 ± 0.060	1.8 ± 0.70
5.	Organic carbon (%)	1.172± 0.059	1.483 ± 0.072	1.186 ± 0.049	2.653 ± 0.125
6.	Metals (μg/g)				
	Zn	82 ± 3.1	22.6 ± 1.10	24.7 ± 1.20	42.8 ± 2.10
	Fe	4150 ± 207	2850 ± 142	3040 ± 152	2960 ± 148
	Ni	204 ± 10.2	23.8 ± 1.10	39.4 ± 1.90	14.2 ± 0.71
	Mn	70 ± 3.4	45.8 ± 2.20	53.1 ± 2.60	38.2 ± 1.80
	Cu	58.6 ± 2.83	24.4 ± 1.16	24.1 ± 1.06	26.4 ± 1.18
	Cd	42.3 ± 2.12	ND	ND	ND
	Pb	40.1 ± 2.00	ND	ND	ND
	B	29.0 ± 1.26	ND	ND	ND
	Al	4615 ± 230	ND	ND	ND
	Si	5600 ± 280	ND	ND	ND

Values are mean (n =3)± SD; ND = not done; DW= dry weight.

Press mud had highest nitrogen and phosphorus contents, lowest pH followed by cowdung manure and garden soil. In general, there was significant difference in plants grown in raw fly-ash and raw fly-ash amended by soil, cowdung manure and press mud. Heavy metal uptake by *C. siamea* plants induced lipid peroxidation in leaves after 20 d exposure to raw fly-ash and fly-ash amended by soil,cowdung manure,press mud in 1: 1 ratio. Maximum MDA content (40.64 μ mol g^{-1} FW) was observed in plants grown in raw fly-ash followed by FA+GS, FA+ CM and FA+ PM in descending order. No significant difference (Duncan's multiple range test, $P>0.05$) between MDA content of plants grown in FA+PM and garden soil was observed during entire period (60 d) of study (Table 2).

Table 2. Malondialdehyde (MDA) content and Superoxide dismutase (SOD) activity of *C. siamea* grown on raw fly-ash (FA), fly-ash amended by garden soil (GS), press mud (PM), cowdung manure (CM) in 1:1 ratio and garden soil (control)

Treatments	MDA (μ mol g^{-1} FW)		
	20 d	40 d	60 d
GS	12.11^{d}±0.59	14.48^{d}±0.63	15.12^{d}±0.72
FA	28.13^{a}±1.06	33.75^{a}±1.29	40.64^{a}±1.53
FA+GS	21.73^{b}±0.84	23.76^{b}±1.02	26.58^{b}±1.21
FA + PM	13.12^{d}±0.63	14.78^{d}±0.71	15.78^{d}±0.73
FA + CM	17.12^{c}±0.72	19.58^{c}±0.83	21.58^{c}±1.03
	SOD (unit g^{-1} FW)		
GS	20.30^{e}±0.85	21.56^{e}±1.05	23.47^{e}±1.35
FA	25.15^{d}±1.05	28.00^{d}±1.35	30.57^{d}±1.06
FA+GS	36.27^{c}±1.02	39.50^{c}±1.12	41.73^{c}±1.05
FA + PM	41.44^{a}±1.22	45.24^{a}±1.92	49.20^{a}±1.61
FA + CM	39.07^{b}±1.90	42.44^{b}±1.45	44.79^{b}±1.32

Values are Mean ± SD (n=3); ANOVA $P<0.05$; Identical superscripts denote no significant difference ($P > 0.05$) between means in a column (MDA/SOD) according to Duncan's multiple range test; FW = fresh weight.

The stimulation of superoxide dismutase activity was observed in plants grown in raw fly-ash and all the three types of amendments (GS+FA, FA+PM, FA+CM). However, this antioxidant enzyme was most active in plants grown in FA+PM amendment followed by FA+CM, FA+GS and raw fly-ash (Table 2).

An increase in cysteine content of *C. siamea* (leaves) plants grown in raw fly–ash, Fly-ash +garden soil, fly-ash + cowdung manure and fly-ash + pressmud was observed (Fig. 1A). However, it was maximum in leaves of plants grown on fly–ash + pressmud (1:1) for 20d while its minimum concentration was recorded when plants were grown in garden soil (control) for same duration. A comparison of the data shown in figure 1A revealed that cysteine contents in leaves of *C. siamea* grown on raw fly-ash and fly-ash ameliorated by pressmud, cowdung manureand garden soil were high than control (garden soil) at all the three treatment duration. However, a treatment duration dependent toxicity to cysteine content in all the four treatments (FA, FA+GS, FA+CM, FA+PM) was recorded.

The presence of toxic metals in fly-ash increased the non-protein thiol contents (index of phytochelatins) in plants grown in raw fly-ash and fly-ash amended by soil, press mud, cowdung manure in 1:1 ratio (Fig. 1B). Maximum increase in non-protein thiol content was recorded when plants were grown in fly-ash amended by press mud for 20 days while minimum non-protein thiol content was observed when plants were grown in raw fly-ash for 60d . The toxicity to non protein thiol content of *C. siamea* exposed to different treatments (Raw fly-ash; fly-ash + garden soil, fly-ash + press mud, fly-ash + cowdung manure in 1:1 ratio) was observed in duration dependent manner. However, NP–SH content of plants (leaves) grown in garden soil remained unaffected during entire period of study (i.e. upto 60d).

Nitrate reductase activity (NRA) was also found affected by toxicity of fly-ash. Maximum inhibition in NRA was recorded in plants grown for 60 d in raw fly-ash. However, it was minimum when plants were growing in fly-ash amended garden soil in 1:1 ratio (Fig. 1C).

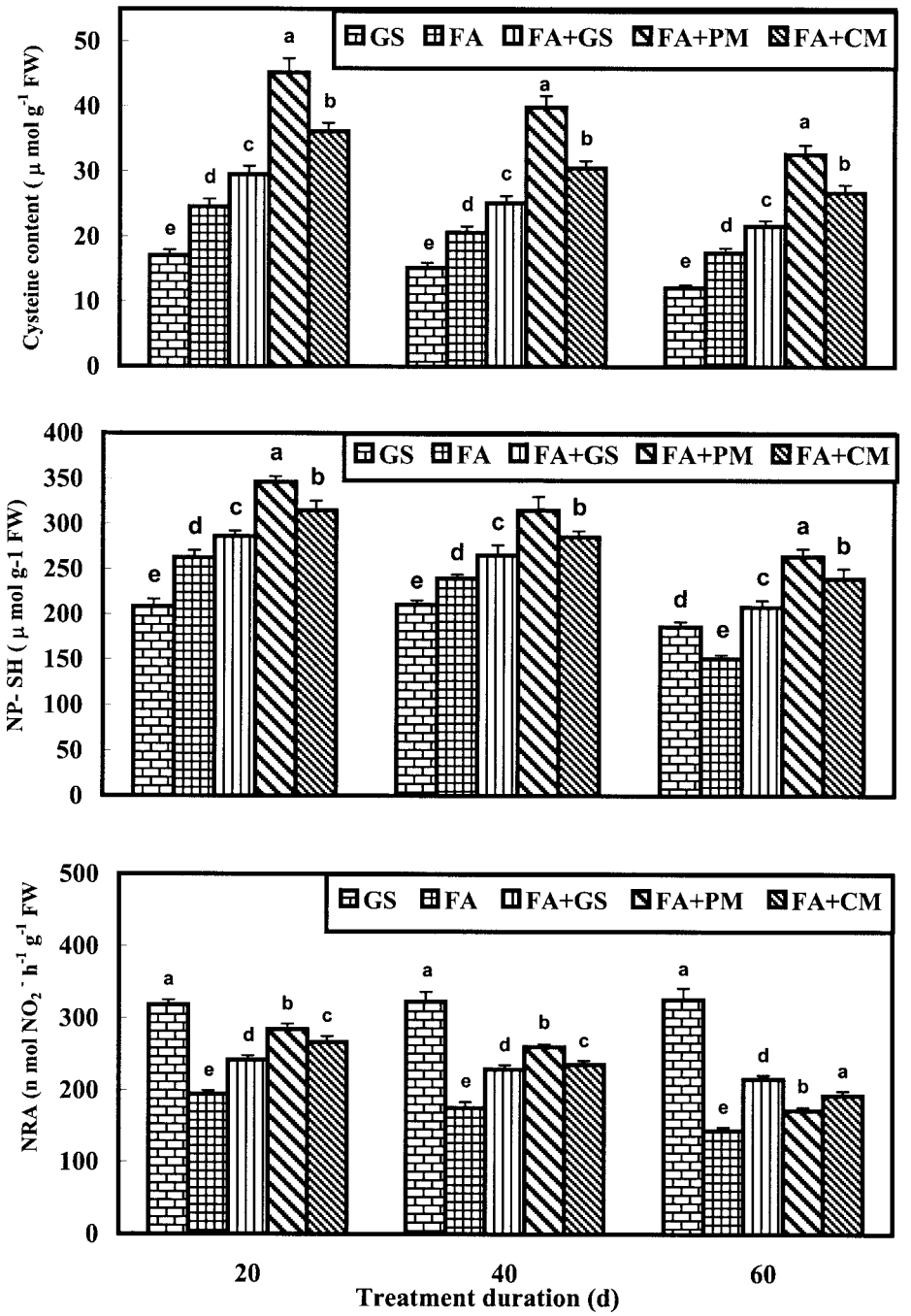

Figure 1: Effect of fly-ash on cysteine (A), NP-SH content (B) and NRA (C) of *C. siamea*. Different superscripts denote significant ($p < 0.05$) differences between bars at each treatment duration according to DMRT. FA = fly–ash; GS = garden soil; PM = pressmud, CM = cowdung manure.

Metal analysis in plant tissues indicates that *C. siamea* is good accumulator of toxic metals (Tripathi et al 2000). Although the concentration of metals was much higher in raw fly-ash than in fly-ash ameliorated by the cowdung manure, pressmud and garden soil, the subsequent uptake of metals by the plants grown in raw fly-ash was prevented due to high pH of fly-ash. Hence, The highest concentration of each metal (Cu, Zn, Fe and Ni) was recorded in the plants grown in fly-ash amended with press mud followed by fly-ash + cowdung manure, raw fly-ash and fly-ash + garden soil respectively. Probably, this was due to maximum decrease in pH of fly-ash after addition of press mud (Tripathi et al 2000).

All the metals accumulated by *C. siamea* have been reported to induce reactive oxygen species (Mazhoudi et al 1997; Chaoui et al 1997; Deitz et al 1999) The protective mechanisms adapted by plants to scavenge free radicals and peroxides include several antioxidant enzymes and antioxidant substances (Allen 1995). The antioxidant enzymes are important components in preventing oxidative stress. This is based on the fact that the activity of one or more of these enzymes is generally increased in plants under stress conditions and these elevated enzyme activities have been correlated to increased tolerance of the plants (Mazhoudi et al 1997). During present study, an increase in MDA content was recorded in plants grown in raw fly-ash and fly-ash amended by garden soil (1:1) press mud (1:1) and cowdung manure (1:1). However, the change in MDA content of plants grown in fly-ash and press mud mixture (1:1) was insignificant (Duncan's multiple range test $P>0.05$) when compared to control plants (garden soil). This might be due to the enhanced activity of superoxide dismutase and other antioxidant enzymes. Maximum SOD activity in leaves of *C. siamea* was recorded in plants grown in fly-ash amended by press mud. Quenching of active oxygen species by antioxidant enzymes like SOD under metal stress condition has been reported by Devi and Prasad (1998) in *Ceratophyllum* when plants were subjected to enhanced copper level. Similarly, when *Brassica juncea* was exposed to high concentrations of zinc, it showed an increase in the activities of SOD, catalase, and guaiacol peroxidase (Prasad et al 1999). Further, iron induced oxidative stress and quenching of active oxygen species by enhanced SOD activity in *Hydrilla verticillata* has been reported by Sinha et al (1997). However, the responses of antioxidant enzymes varied with plant species and metal involved (Mazhoudi et al 1997).

Plants like other organisms, have many mechanisms to overcome various stresses. One response of plants to heavy metal stress is the induction of phytochelatins (PCs), a family of related peptides that have the structure (γ-Glu-Cys) n-Gly (where n = 2-11) and are clearly derived from the tripeptide glutathione (Mehra and Tripathi 1999). Further, non protein thiol content is considered to be an indicator of PC synthesis in plants. Apart from antioxidant enzymes, some antioxidants like GSH thiols, carotenoids and ascorbate may also play a role in inducing resistance to metals by protecting labile macromolecules against attack by free radicals which are formed during various metabolic reactions leading to oxidative stress (Galli et al 1996; Rauser 1987). In the present study, the levels of non protein thiols and cysteine exhibited different response to the raw fly-ash and amended fly-ash. Increased non-protein thiol and cysteine contents correspond to the level of tolerance exhibited by metal treated plants (Gupta and Chandra 1996; Sinha et al 1997). Though level of PCs was not measured in plants grown on raw fly-ash/amended fly-ash, an increase in non-protein thiol and cysteine contents indicate their induction. Increased activities of the sulphate reduction enzymes, ATP sulphurylase and adenosine 5' phosphosulphate sulphotransferase under heavy metal stress leading to more cysteine content, have been reported (Nussabaum et al 1988). The decrease in cysteine content

after 60 d exposure to fly-ash was probably due to toxic effects of heavy metals.

Nitrate reductase is a key enzyme of nitrogen metabolism. Nitrate reductase catalyses the first step in the assimilatory reduction of nitrate to ammonia, which reduces nitrate to nitrite using NADH as an electron donor (Lillo 1994). NR was found to be severely affected by metals, salinity and drought stresses (Botella et al 1993; Rai et al 1998; Vajpayee et al 1999a). Since, fly-ash has low water retention capacity and high pH, inhibition in NRA of raw fly-ash grown plants might be due to following reasons (a) low substrate availability (a) salinity caused by presence of salts (b) uptake of toxic levels of metals (Cu, Zn, Ni, etc) which may exchange the active metal site of enzyme (VanAssche and Clijesters 1990) or generate active oxygen species which cause oxidation and cross linking of SH groups (Luna et al 1997).

A few reports (Scanlon and Duggan 1979; Mulhurn et al 1989; Cheung et al 2000; Vajpayee et al 2000) have indicated that certain trees including leguminous trees having high metal accumulation potential can withstand in fly-ash stress. During present study, it has been observed that *C. siamea* having adequate metal tolerance mechanisms (hyperactive antioxidant enzymes, accumulation of non protein thiols (index of metal detoxifying peptides - the phytochelations) and accumulation of cellular antioxidants) could provide good tool for the management of fly-ash dykes. As such trees could provide a good green cover on fly-ash disposal sites to minimise the pollution.

It could be concluded from present study that *C. siamea* is highly suitable species for plantation on fly-ash land fills as it thrives well and has metal detoxification potential. Among the different fly-ash amendments, the press mud was found to be most suitable amendment for amelioration of fly-ash to achieve desired vegetation cover.

Acknowledgements:

We thank the Director, National Botanical Research Institute, Lucknow for facilities and encouragement. Dr. P. Vajpayee and Mr. A. Kumar are grateful to Directorate of Environment, U.P. (Ministry of Environment & Forests) Lucknow for financial assistance.

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