Enzymatic mechanism during phytoextraction of heavy metals from fly ash amended soil

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Abstract: A pot culture experiment was carried out to study the phytoextraction potential of selected plant species, for remediation of fly ash contaminated soil. Plant species namely verbena speciosa, tagetes erecta, Cassia tora have been selected for the study. Fly ash samples were collected from coal based thermal power plants at gandhinagar from electrostatic precipitators. Soil samples were collected from Sabarmati river bed. Various physico-chemical parameters of fly ash and soil were conducted. Biomass and biochemical parameters of plants were studied for screening of plants for phytoextraction ability. Study of Photosynthetic pigment, protein content, antioxidative enzymes such as superoxide dismutase (SOD), Ascorbate peroxidase (APX), Catalase (CAT), Guaiacol peroxidase (GPX) was carried out in potential plant. Fly ash heavy metals may lead to oxidative stress which is a condition in which ROS or free radicals are generated extra- or intra-cellularly, which can exert their toxic effects to the cells. However, in cells antioxidant defense mechanisms is present to detoxify the harmful effects of ROS. The plant species were found in order verbena speciosa< tagetes erecta<Cassia tora for phytoremediation of fly ash heavy metals. Tanslocation factor and bioaccumulation factor was also determined. Results showed that cassia tora survived well in fly ash amendments and can be used well for phytoremediation and revegetation of fly ash effected sites.

Keywords: Antioxidative Enzymes, Phytoextraction, Fly Ash, Revegetation.

Introduction

In energy production coal serves to be a major fuel generates by-product named fly ash which is of great concern due to its fine particle size. This fly ash contains various toxic metals more in concentration than in respective coal, thus acts as environment pollutant. Various estimates indicate that in upcoming year's fly ash production will be increasing manyfolds. Due to this its management is of prime concerns in the country like India where availability of land is less for ash disposal. Also there is risk of other associated problems like metal leachability etc. FAUP, TIFAC estimates that annual ash generation is expected to reach about 225 million tonne by 2017 (Vimal Kumar, 2005). According to the international energy outlook 2013(Figure1) though renewable sources and natural gas are the fastest growing sources in electricity generation, still coal will be major fuel for electricity generation.

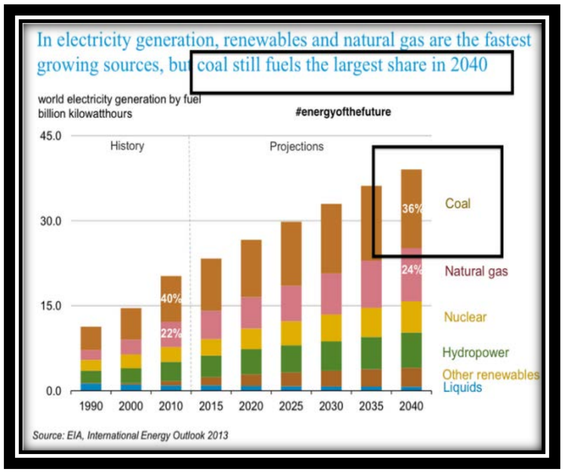

Figure1: Share of resources for electricity generation in coming years

Chemical nature of fly ash comprises of mainly SiO_2, Al_2O_3 and oxides of iron and other toxic metals. FA has a low bulk density, high surface area and light texture (Asokan et al., 2005; Jala and Goyal, 2006). Generally FA contains potentially toxic elements such as As, Cu, Zn, Cd, Pb, Ni, Cr, Se, etc. (Rautaray et al., 2003; Lee et al., 2006; Tiwari et al., 2008; Adriano et al., 2002). Depending upon the concentrations heavy metals may either inhibit or stimulate antioxidative enzymes activity. Various free radicals are generated due to heavy metals toxicity these may lead to oxidative stress. To overcome environmental issues arising out of fly ash phytoremediation technology is used to monitor dispersal of fly ash into environment and to develop bioaesthetic environment for local inhabitants. In this suitable plant species is used to revegetate fly ash dumpsites to accumulate fly ash heavy metals. Plants develops an antioxidative stress which comprises of various enzymes such as superoxide dismutase (SOD), Catalase (CAT), Guaiacol peroxidase (GPX), Ascorbate peroxidase (APX) etc. to overcome this stress.

Present study aim at finding suitable plant species which can easily colonize in fly ash, so that fly ash pollutant can be checked. Phytoextraction appears an encouraging science but it is necessary to transform this science into working system. For determination of real potential of this technology its land implication is important. Though a lot of work is done on phytoremediation yet in countries like India this technology is still in its starting stage, and search for new plant species still remains continue.

Material method

Sample collection

Samples of fly ash were collected from Thermal power station Gandhinagar Gujarat located at 23◦14' 57″ N latitude and 72◦ 40' 15″E longitude. Samples of soil were collected from a depth of about 30 cm along the banks of Sabarmati River, Gandhinagar. Stones and plant tissues were carefully removed from the soil prior to drying in the laboratory. The soil was screened through 2 mm stainless steel sieve and stored in a plastic bag at room temperature until use. Seeds of three ornamental plants namely *Cassia tora*, *Verbena speciosa*, *Tagetes erecta* were chosen for present study. Seeds of these plants were collected form nursery.

Experimental set up

Pot culture experiments were conducted using soil and soil amended with different concentration of fly ash. Fly ash was uniformly mixed with air dried soil sieved to <2 mm and placed in pots. Various amendments made were 0%, 25%, 50%, 75%, 100% respectively. Twenty seed were sown in soil to germinate. Experiment was conducted in triplicates under natural light and ambient temperature. No artificial fertilizers or soil amendments were added to soil during course of the experiment. Plants were water regularly with water obtained from Millipore water purification system.

Physicochemical analysis

The physicochemical parameters were measured by standard methods. The physico-chemical parameters analyzed were: pH, P, Total nitrogen, Ammonia, Nitrite, Nitrate, Alkalinity, Moisture content, Bulk density, Porosity, Sulphate, Conductivity, water holding capacity, Cation exchange capacity was determined after extraction with ammonium acetate at pH 7.0 and organic carbon was determined using the Walkley Black method. Concentrations of Pb and Cd were determined by atomic absorption spectrophotometer (APHA, 1998).

Photosynthetic parameters

Arnon (1949) formulated formula was used for calculating chlorophyll content in plants under study and Kirk and Allen (1965) for carotenoid contents.

Estimation of protein

The protein content was estimated by following the method of Lowry *et al*., (1951).

Enzymes assay

Superoxide Dismutase was estimated by method of Poonam et al., 1984. Catalase was estimated in the plant parts following the method of Aebi (1984). The ascorbate peroxidase activity was determined in plant by the method of Nakano and Asada (1981). Guaiacol peroxidase activity was determined following the formation of tetraguaiacol as described by Singh et al., (2006).

Statistical Analysis

Physiological, biochemical (photosynthetic pigments, protein content) parameters of plant, heavy metal concentrations of soil

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under different treatments were analyzed using one-way analysis of variance (ANOVA) to compare the means of different treatments.

Results and discussion

Fly ash, a by-product of coal combustion, is generated in large quantities in coal- based thermal power plants. Disposal of huge amount of fly ash is of prime concern because of its toxic nature. Phytoremediation is projected as a cost-efficient and ecologically benign process that uses plants to remove heavy metals from the environment by accumulation or transformation of these metals in vegetative biomass. Soil tests tell us condition of soil. Physico-chemical characteristics of soil is essential for the successful growth of plant for remediation of polluted soil.

Physico-chemical properties of the soil fly ash, and its various amendments

Physico-chemical parameter of fly ash amended in soil (Table 1) showed the increasing trend with the increasing concentration of fly ash in soil parameters such as pH, EC, TDS, Alkalinity,

water holding capacity, porosity of different amendments were increasing as compared to pure soil. While some parameters value decreased includes organic carbon, moisture content, Cation exchange capacity, bulk density. Nutrient content also found decreases with increasing concentration of fly ash in soil, nitrogen and phosphorous low concentration coupled with lack of organic content in fly ash mark it as a nutritive deficient substrate. Gupta et al., (2007) reported that with an increase in fly ash amendment ratio pH increased significantly this might be due to the precipitation of soluble cations in the fly ash amended soil. Fly ash amendment to soil significantly increased the electrical conductivity of the soil mixture by increasing the levels of soluble major and minor inorganic constituents (Eary *et al.*, 1990). Fly ash itself is not effective in retaining water but it significantly increases water holding capacity of the soil mixture (Chang *et al.*, 1977). Fly ash+soil medium has been reported for better soil porosity and thus increased water holding capacity (Gupta *et al.*, 2002) and decreased bulk density (Page *et al.*, 1979). While value for some parameters has decreased like organic carbon, moisture content, Cation exchange capacity, bulk density. The reduced CEC value indicates that fly ash treatment caused change in the mineralogy of treated soil. The result of the present study was similar to earlier finding showing decrease in CEC with increase of fly ash incorporation

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(Amrhein et al., 1996; Nalbantoglu, 2004; Sinha and Gupta, 2005; Gupta et al., 2007). Decrease in ammonium nitrogen may be due to the change in pH. Nutrient content also decreases with increasing concentration of fly ash, low levels of nitrogen and phosphorous coupled with lack of organic content in fly ash mark it as a nutritive deficient substrate.

Table 1 Physico-chemical properties of soil, fly ash, and its various amendments

Concentration	0%	25%	50%	75%	100%
Parameters					
Colour	Brown	Light brown	Greyish brown	Light gray	Gray
Ph	7.08±.01	7.8±.01	7.84±.02	7.96±.01	9.03±.03
EC	186.05±.01	192.675±.01	234.5±.12	354±.01	429.25±.02
TDS	80.4±.01	95.675±.01	110.3±.02	124.25±.01	178±.01
Organic carbon	0.84±1.4	0.7±.01	0.63±.01	0.59±.02	0.54±.01
Alkalinity	9.5±.05	16±.01	36±.01	50±.01	61.5±.04
Moisture content	1.1238±3.5	0.4396±.01	0.3568±.02	0.2986±.01	0.175±.01
WHC	35.87±.19	36.027±.13	36.427±.11	37.79±.12	39.27±.15
CEC	3.304±.20	2.38±.53	2.254±.07	0.504±.01	0.266±.01
BD	1.2938±.01	1.23581±.01	1.1428±.04	0.89952±.03	0.7404±.02
Porosity	46.5±.01	48.71±.02	49.83±.01	53.98±.02	58.13±.02
Sulphate	22.955±.13	16.42±.16	15.92±.12	12.37±.13	6.56±.16
Ammonia	8.9±.23	0.504±.22	0.347±.28	0.261±.21	0.219±.22
Nitrate	19.9±.95	19.12±.93	17.7±.93	14.45±.95	12.00±.90
Nitrite	3.26±.007	1.26±.05	1.08±.07	0.21±.05	0.148±.002
Phosphate	14.06±.005	11.05±.031	9.12±.059	8.54±.066	1.14±.055

All values are mean of triplicates ±S.D. (EC- electrical conductivity, TDS- total dissolved solids, WHC- water holding capacity, CEC cation exchange capacity, BD- bulk density).

Fly ash composition

Fly ash emissions from a variety of coal combustion show a wide range of composition. Variability is directly related to the source of the coal from which it is obtained, its pretreatment processes and the operation opted in the plant for burning the coal. Chemical composition of fly ash showed the dominance of silica followed by alumina and iron oxides. Decreasing order of chemical compound found in fly ash is as follows: SiO_2, > Al_2O_3, > FeO_3+Fe_3O_4, > MgO, > KO_2, > CaO, > SO_4,> NaO_2,> SO_3, > P_2O_5. > TiO_2 (Table 2).

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Table 2 Chemical composition of fly ash

S no.	Name	Symbols	Percentage
1.	Silica	SiO_2	63.2
2.	Alumina	Al_2O_3	26.3
3.	Iron oxides	$FeO_3+Fe_3O_4$	5.5
4.	Titanium di oxide	TiO_2	Nill
5.	Calcium oxide	CaO	0.95
6.	Magnesium oxide	MgO	1.8
7.	Sulphate	SO_4	0.5
8.	Sulphite	SO_3	0.22
9.	Sodium di oxide	NaO_2	0.26
10.	Potassium dioxide	KO_2	1.26
11.	Phosphate pento-oxide	P_2O_5	0.41
12.	Loss of ignition		1.8
13.	Specific gravity		2.6
14.	Finess		563

Source: Gandhinagar Thermal Power Plant

Biomass and biochemical parameters

Results demonstrate that with the increasing concentration of fly ash in soil physiological traits like fresh and dry biomass, showed significant retardation (Figure 2). Dose dependent growth responses, both positive and negative in relation to fly ash application have been earlier observed by several workers (Singh and Siddiqui, 2003; Mittra et al., 2005).

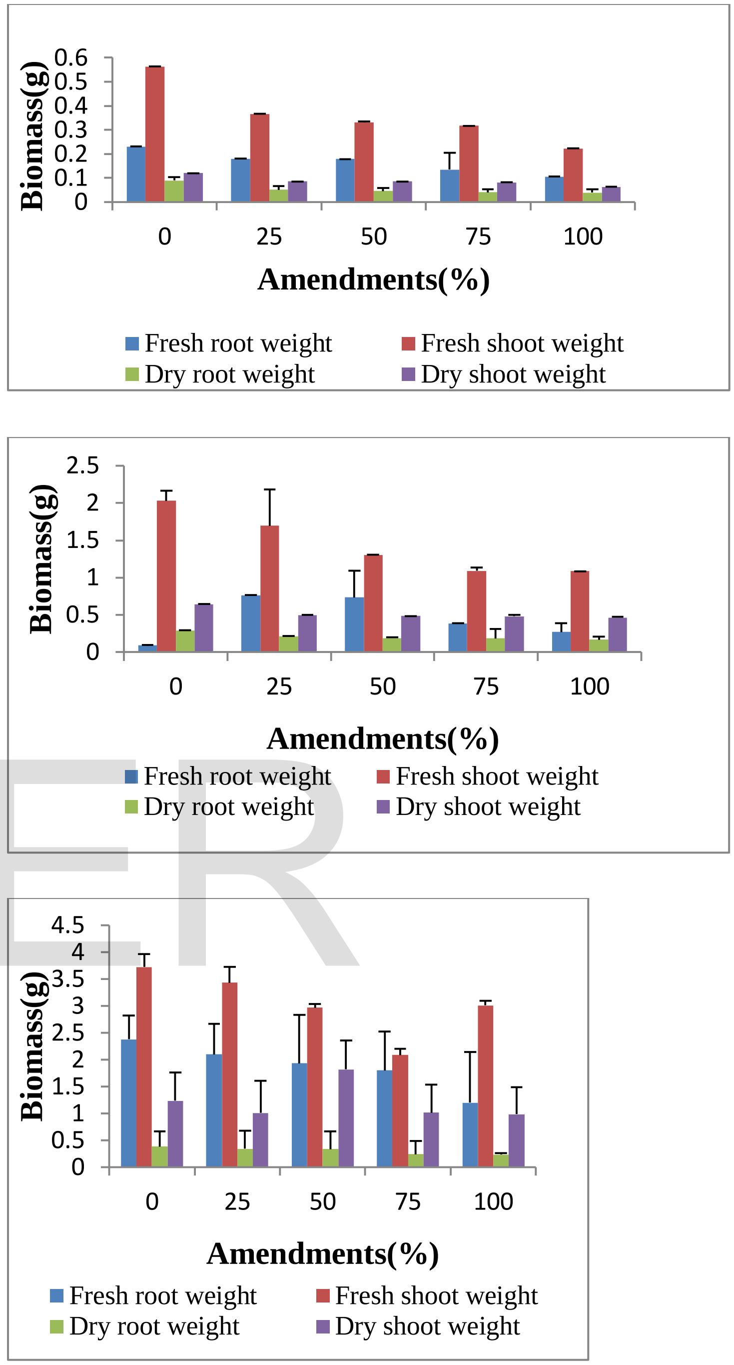

Figure 2: Biomass of selected plants after one month in fly ash amended soil

a. Biomass of *verbena speciosa* b. Biomass of *Tegetes erecta* c. Biomass of *Cassia tora*

The levels of chlorophyll in the plant species also decreased when the concentration of fly ash increases in soil. Results of Chlorophyll a content of *Verbena* species, revels that with the increased concentration of fly ash initially chlorophyll a concentration increases up to 25 % later on it starts to decreases. While for chlorophyll b and total chlorophyll content value decreased with increased concentration of fly ash. Means were not found significantly different (P value< 0.5080) as determined by one way ANOVA and there was inverse correlation among data. *Tagetes*, revealed similar trend as verbena. In *Cassia tora* Chlorophyll b showed increased concentration at 25% then value decreased while chlorophyll a and total chlorophyll showed decreasing trend with increased value (Table3). Decrease in chlorophyll content in fly ash grown plants may be either due to reduction synthesis of chlorophyll or its accelerated degradation. Toxic effect of heavy metals on photosynthesis is also reported by (Morita et al., 2006; Wang et al., 2004). Heavy metals are known to interfere with chlorophyll synthesis either through direct inhibition of an enzymatic step or by inducing deficiency of an essential nutrient (van Assche and Clíjsters 1990). Further d amino-levulinic acid dehydratase(ALAD) is a metal sensitive enzyme of chlorophyll biosynthesis which might have been affected by the higher amount of heavy metals in the fly ash thus reducing the chlorophyll content in the plant leaves. Under metal stress reduced ALAD activity (Pb, Cd, Cr, Hg) has been reported earlier in different plant (Vajpayee et al.,2000).

Carotenoid content of all plants was found to be increased with the increasing concentration of fly ash and exposure time. Increased carotenoid content has been considered as defence strategy of the plant to combat metal stress (Kenneth et al., 2000). Here it may be strategy adopted by plant to counteract the toxic effect of free radicals generated under fly ash stress conditions. Carotenoids (non-enzymatic antioxidant) play a significant role in the protection of chlorophyll pigment under stress conditions by quenching the photodynamic reactions, replacing peroxidation and collapsing of membrane in chloroplasts (Kenneth et al., 2000).

According to Tomar et al., (2000), the protein content (Table 3) under heavy metal stress may be affected due to (i) Enhanced protein hydrolysis resulting in decreased concentration of soluble proteins (Melinchuk et al., 1982). (ii) Catalytic activity of heavy metals (Bhattacharya and

Choudhari, 1997) and (iii) Protein Synthesis becoming reduced under all stress conditions. All the amendments of fly ash also showed inhibitory effect on protein content in all selected three plants species.

Table 3 Biochemical parameters of selected plant species

Biochemical parameters		Chlorophyll	Chlorophyll	Total	Caratenoid	Protein
Plants	concentration	a	b	Chlorophyll	content	content
Verbena speciosa	0%	1.22	0.76	1.99	0.28	0.60
	25%	1.24	0.55	1.79	0.56	0.41
	50%	1.08	0.52	1.60	0.64	0.37
	75%	0.98	0.40	1.39	0.73	0.32
	100%	0.70	0.24	0.94	0.92	0.26
Tagetes erecta	0%	0.82	0.49	1.32	0.25	1.04
	25%	0.92	0.41	1.33	0.40	0.31
	50%	0.82	0.39	1.21	0.50	0.27
	75%	0.75	0.35	1.11	0.83	0.25
	100%	0.61	0.31	0.93	1.51	0.24
Cassia tora	0%	1.59	0.63	2.23	0.61	1.91
	25%	1.29	0.68	1.97	0.74	1.00
	50%	1.24	0.62	1.86	0.81	0.91
	75%	1.13	0.60	1.73	1.01	0.86
	100%	0.90	0.57	1.47	1.53	0.70

Enzymatic mechanism

The antioxidative enzymes, guaicol peroxidase, catalase, ascorbate peroxidase, superoxide dismutase, exhibited an increasing trend in response to fly ash heavy metals. Heavy metals caused a significant increase in the activities of enzymes studied. Enzymatic activity in roots was found higher as compared to shoot (Figure 3). Heavy metals are known to generate toxic reactive oxygen species (ROS) like O^{2-}, OH^{-}, H_2O_2, etc. which degrade important cellular components by inducing oxidative stress (Panda 2003). The level of reactive oxygen species (ROS) in plant tissues is controlled by an antioxidant enzymes (guaicol peroxidase, catalase, ascorbate peroxidase, superoxide dismutase) and non enzymatic low molecular weight antioxidants (glutathione,proline,carotenoid,tocopherols etc.)(Schutzendubel and Polle, 2002). The results of the present study showed all the tested concentrations of fly ash caused oxidative stress. As fly ash concentrations increased, SOD, CAT and POD, guaicol peroxidase activity enhanced progressively.

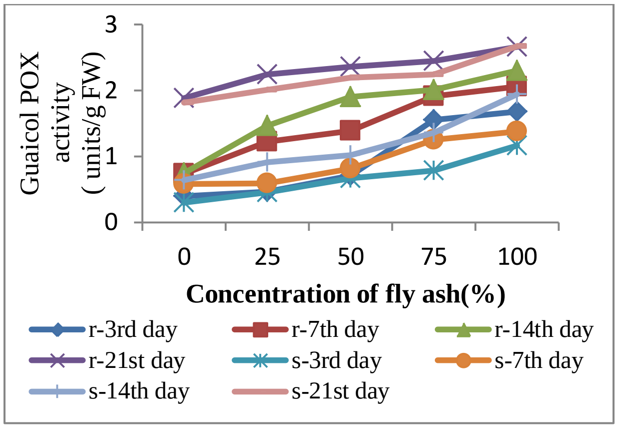

Figure: 3(a) Guaicol peroxidise activity in plant shoot(s) and root(r)

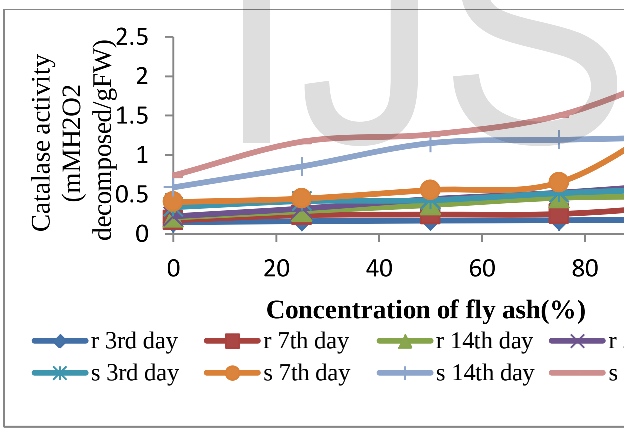

Figure: 3(b) Catalase activity in plant root and shoot

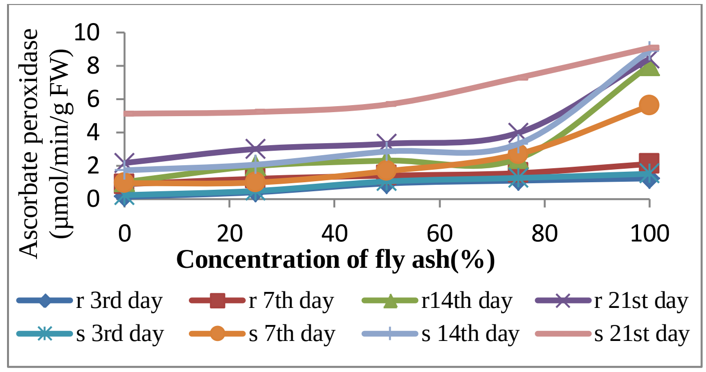

Figure: 3(c) Ascorbate peroxidise activity in plant roots and shoot

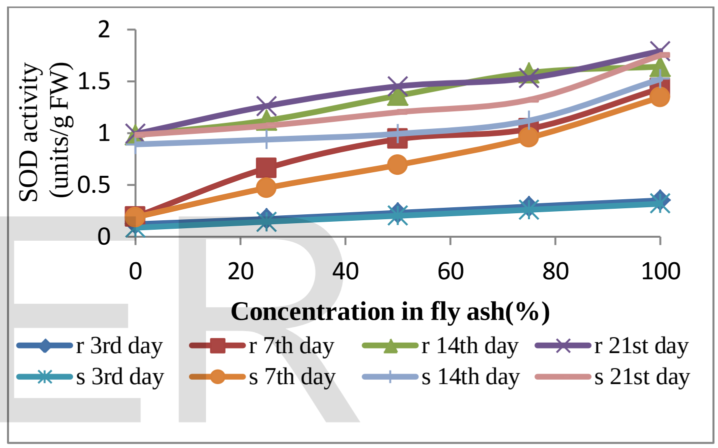

Figure: 3(d) SOD activity in plant shoot and root.

Heavy metal uptake by selected plants

With the increasing concentration of fly ash heavy metal concentration was found increased in roots and shoots. Concentration of metal was high in roots as compared to shoots (Figure 4). Among selected three plant species i.e *Verbena*, *Tagetes*, *Cassia*. *Cassia* was found to have maximum heavy metal concentration. Cadmium uptake was more compared to lead uptake in *cassia tora* specie, similar trends were seen in *verbena* and *tagetes* roots at 75% and 100% concentration. This can be explained by increased metal availability. While at concentration 0%, 25%, 50% lead uptake was more in *tagetes* roots. In *verbena* roots at 0% Cd uptake was more compared to Pb while at 25%, 50% Pb>Cd. In *tagetes* shoot Pb uptake was higher than Cd accumulation with the increasing concentration. This can be explained by more translocation of Pb from roots to shoots of plants. In verbena shoots at 0% concentration Cd and Pb uptake was almost same at 25% 50% concentration Cd uptake was high compared to Pb. But75% and 100% concentration Pb > Cd. Metal accumulation in the plants is depended on its mobility and availability in the soils and plant species growing on these soils (Sinha and Gupta, 2005).

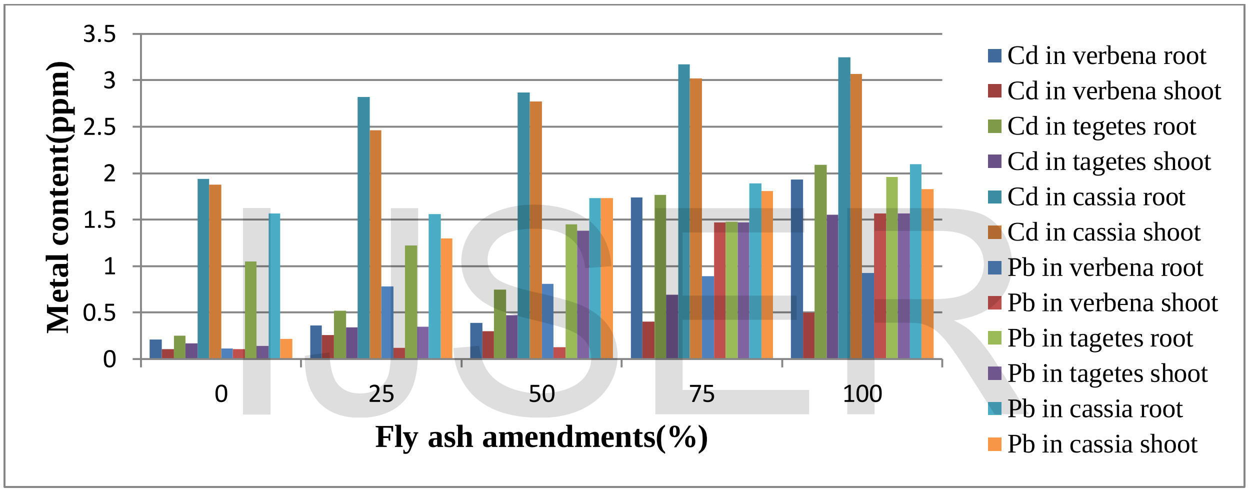

Figure 4 Heavy metal uptake by selected plants

Translocation factor

The rate of TF value ranged from 0.231 to 0.972 (Table 4) which represents translocation of Cd was effectively made from root to shoot. Result showed a TF<1 suggesting that Pb could not be effectively translocated from the roots to the shoots except in *verbena* plant in 75% and 100% concentration with values 1.64 and 1.68.

Table: 4 Translocation factor

Concentration ⇩	Cadmium			Lead		
	Verbena	*Tagetes*	*Cassia*	*Verbena*	*Tagetes*	*Cassia*
0%	.522	.691	.972	.967	.135	.140
25%	.723	.659	.875	.156	.286	.829
50%	.776	.635	.968	.157	.954	.997

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75%	.231	.390	.953	1.64	.997	.957
100%	.258	.744	.945	1.68	.798	.872

Bioaccumulation factor

Results show that bioaccumulation factor of cassia was greater than tagetes and verbena. In present research BAF of Cd and Pb had a range between .139- 1.678 and .054-.712 respectively (Table 5). This suggests a good performance of C. tora for phytoremediation of Cd, Pb-contaminated soil.

Table: 5 Bioaccumulation factor

Concentration ⇩	Cadmium			Lead		
	Verbena	*Tagetes*	*Cassia*	*Verbena*	*Tagetes*	*Cassia*
0%	.139	.182	1.678	.054	.288	.433
25%	.179	.250	1.533	.183	.321	.584
50%	.192	.342	1.569	.191	.573	.699
75%	.577	.661	1.665	.457	.569	.712
100%	.638	.957	1.659	.430	.609	.678

Difference in uptake and translocation of heavy metals is important in defining their potential utilization in the different phytoremediation procedures. All selected plant species were tolerant to heavy metal contamination of fly ash. Among them, the selected species cassia had good biomass production and could be considered more efficient for phytoextraction purposes.

Conclusion

Combustion of coal in thermal power plant continues to be the main source of energy production all over the world since past few decades. Due to which huge amount of fly ash is generated. This fly ash is harmful as it contains various toxic heavy metals and some radioactive substances. Thus disposal and utilization of fly ash is of prime concern. Complete utilization of fly ash could solve the problem associated with overflow of fly ash and its exposure to surrounding environment and its impact on human health and environment and also minimize the burden on land requirement for disposal.

The outcomes of the study will be helpful to control the human health hazards and effective conversion of wasteland, which is of paramount importance to an overpopulated country like India as well as open an avenue in solving coal ash disposal problem to avoid the environment degradation.

The present study was undertaken to find out the potential plant for remediation of soil contaminated with fly ash and to study various enzymatic activity in potential plant. It was observed that *Cassia tora* (Caesalpinioideae) has potential to remove contaminant in fly ash amended soil polluted by various heavy metals resent in fly ash. Several important aspects of metal tolerance in selected plant i.e *Cassia tora* has been identified.

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