DISCUSSION

Soil pollution by heavy metal results from both natural causes and also due to activities of humans. Activities such as mining and smelting as well as agriculture have contaminated extensive areas of world mostly by heavy metal such as Cd, Cu, Zn, Pb, Cr and Ni (Smith *et al.* 1996; Zantopolus *et al.* 1999; Herawati *et al.* 2000). Inorganic and organic fertilizers are most important sources of heavy metals to agricultural soils which include liming, sewage/sludge, irrigation water and pesticides (Sharma and Agrawal, 2005). While many of the heavy metal ions are essential micronutrients higher concentrations of these have adverse effects on plants. These are known to effect growth, biomass and yield as well as several physiological and biochemical processes.

The present study was undertaken to determine the effects of four heavy metals i.e. Cd, Cu, Hg and Pb on okra [*Abelmoschus esculentus* (L.) Moench] plants. Heavy metals were applied as the salts of cadmium nitrate 4-hydrate [$Cd(NO_3)_24H_20$], copper(II) sulphate-5-hydrate [$CuSO_45H_2O$], mercury(II) chloride [$HgCl_2$] and lead(II) nitrate [$Pb(NO_3)_2$] respectively at two concentrations i.e. 100 and 1000 $\mu g\ ml^{-1}$ which are higher than the amounts present in the normal soil.

Since seed germination is the first step in the establishment of plants in soil, initially the effects of these heavy metals on six cultivars of okra (cv. Arka Anamika, Deepti, Najuka F1, Paras Soumya, Parbhani Kranti and PB-57) were determined. Among the four heavy metals Hg inhibited germination to the greatest degree though all the other salts also inhibited germination. Peralta *et al.* (2000) reported that a reduction in germination of alfalfa was observed with increasing concentration of heavy metals Cd, Cr, Cu, Ni and Zn. Neelima and Reddy (2003) reported that Hg inhibited germination percentage in *Solanum melongena* while Cd induced seed germination even upto 20,000 ppm. However, both inhibited growth of seedlings. Bhattacharjee and Mukherjee (2004) reported that germination declined with increasing concentration of both $PbCl_2$ and $CdCl_2$. Maximum inhibitory effect was observed for seeds treated with 10^{-3} M $PbCl_2$ and $CdCl_2$. It was also reported by Parmar and Chanda (2005) that both Hg and Cr inhibited root and hypocotyls length of *Phaseolus vulgaris* seedlings by about 50-80%. Germination was also found to be gradually delayed with increasing concentration of heavy metals. They also reported

that Cd was found to be more inhibitory than Pb under the same stress. In the present study also it was observed that heavy metals delayed germination and after 72 h a comparatively higher germination percentage was recorded.

Influence of heavy metals on growth and yield of okra was measured on the basis of several parameters which included relative growth index (RGI), leaf area (LA), absolute growth rate (AGR), relative growth rate (RGR), specific leaf area (SLA) and specific leaf weight (SLW). All the heavy metal exerted a negative influence on growth of okra plants. Differences were observed in response of different cultivars to the different heavy metals. Tolerance index (TI) studies revealed that plants were more susceptible to Hg followed by Cd. Among the cultivars Deepti was the most tolerant. Among root and shoot, roots were more susceptible with lower TI values. Previous studies on heavy metal tolerance in plants also indicate that root growth is particularly sensitive to heavy metals (Punz and Sieghardt, 1993). Oncel *et al.* (2000) reported reduction in root growth due to heavy metals in wheat seedlings. Inhibition of growth of cucumber and *Brassica junceae* have also been reported (Moreno Caselis *et al.* 2000; Singh and Tewari, 2003) All the tested growth parameters revealed that Hg was the most inhibitory and reductions were obtained in LA, total biomass as well as yield. A decrease in leaf area of mungbean seedlings with increasing concentration of $CdSO_4$ was reported by Bindu and Bera (2001). They suggested that reduction in LA at higher concentration may be due to decreased turgor as well as cell division and expansion. Cd at a concentration of 1-5 μM decreased leaf area and biomass whereas at 1 μM increased the same in barley (Wu *et al.* 2003). Dewan and Dhingra (2004) reported that three varieties of pea differed in the effects of Cd on root and above ground biomass production. Cd treatment did not affect dry matter accumulation in the roots of above ground part of cv. Arkel except at higher concentration which was also inhibitory to accumulation of the above ground parts. However, in the cv. HFP4 low Cd enhanced the dry matter accumulation in roots as well as above ground parts. Zengin and Munzuroglu (2004) reported that Pb and Cu applied in the form of chloride inhibited the growth of root and shoot in bean seedlings. They also reported that roots were more sensitive followed by shoot and leaf. Decrease in total plant biomass in

Spinaceae oleraceae subjected to Cd and Ni were also reported by Mishra and Agrawal (2006). Gianazza *et al.* (2007) showed that in *Lepidium sativum* seedlings growth was inhibited by higher concentration of Cd. Thus, results of present study and those of previous authors indicate that different heavy metals inhibit growth of the plants at higher concentration though in lower concentration it may be stimulatory in some cases. Reduction in biomass accumulation is often a reliable indication of the plant's sensitivity to various stresses as it represents the cumulative effects of damaged or inhibited physiological functions.

Since all the heavy metals at higher concentration inhibited germination and growth of okra plants to some extent experiments were further conducted to determine their effects on metabolic processes in the plant. At the onset the influence of all heavy metals on major biochemical components including carbohydrates, pigments and proteins were determined. Total soluble sugar, reducing sugar and starch contents of leaves and roots were determined at different stages of growth. Though there was an overall reduction in the carbohydrate contents the responses of the different cultivars to various heavy metals varied. While Hg and Pb decreased total soluble sugar to the greatest degree, Cu was not very inhibitory. Accumulation of starch was inhibited in leaves, stems and roots following heavy metal treatments but inhibition was maximum in the stem. It seems possible that at times of stress maximum mobilisation of starch occurred in the stems. Though total soluble sugar and starch contents decreased following heavy metal stress, reducing sugar increased to some extent. This could be due to the breakdown of starch or other soluble sugars. Shukla *et al.* (2003) reported that Cd altered the levels of several biochemical constituents including starch and soluble sugars in wheat seedlings. It was also reported that higher concentration of arsenate induced decrease in total soluble sugars in *Vigna radiata* seedlings (Debnath and Srivastava, 2003).

Pigments were adversely affected by all the heavy metals with Hg being the most inhibitory. Chlorophyll as well as carotenoids showed similar response. Increased chlorophyll a/b in treated plants indicates that the influence was greater on chlorophyll b than chlorophyll a. In previous studies heavy metals such as Cd, Ni and Cu have been reported to affect the photosynthetic function of higher plants

either directly or indirectly (Baszynski, 1980; Hou *et al.* 2007). Similar reports are also available for radish (Krupa *et al.* 1987), *Cajanas cajan* (Sheoran *et al.* 1990), *Beta vulgaris* (Gregor and Orwin, 1991) and *Silene* (Ouzounidou, 1993). Chlorophyll biosynthesis was inhibited by Cu stress in *Thalaspi ocrolucum* (Ouzounidou, 1992) and *Phaseolus vulgaris* (Gadallah, 1995). It has also been reported that chlorophyll accumulation is highly sensitive to heavy metal toxicity (Gupta and Chandra, 1996). There are also reports that Pb inhibits chlorophyll synthesis and consequently leads to decrease in chlorophyll content (Miranda and Ilagowan, 1996; Mohan and Hosetti, 1997). Similar results were also reported by Saygideger and Dogan (2005) in *Nasturtium officinalis*. Chlorophyll a/b was found to either decrease (Sheoran *et al.* 1990) or increase (Singh and Tewari, 2003). Bhattacharjee and Mukherjee (2004) reported that increasing concentration of both $CdCl_2$ and $PbCl_2$ decreased chlorophyll a, chlorophyll b and carotenoid contents in primary leaves of *Amaranthus lividus* seedlings. Decreasing trends of all the pigments were correlated with increasing concentrations. In their study Cd was more detrimental than Pb. However, in the present study Pb was more inhibitory than Cd but less than Hg. Impaired chlorophyll biosynthesis due to heavy metal may be due to interference of structural components of chloroplast or inhibition of biosynthetic enzymes. Other heavy metals like Mn and Zn also inhibited chlorophyll and carotenoid pigments (Sinha *et al*, 2002; Singh *et al*, 2005).

Proline accumulation in general increased following heavy metal stresses though maximum accumulation was observed at 100 μgml^{-1} concentration and slightly lower at 1000 μgml^{-1}. In this case also the increment of proline accumulation varied to some extent with the treatment and cultivar. Proline is one of the common stress metabolites and is known to accumulate under different stress conditions, mostly abiotic stresses. Heavy metal stress could also create an osmotic stress in the cell and hence could have induced accumulation of proline. Schat *et al.* (1997) reported that massive accumulation of proline occurs in leaves of *Silene vulgaris* in response to Cu, Cd and Zn. Basak *et al.* (2001) also obtained significant accumulation of proline in leaves of tea plants subjected to elevated levels of Hg, Cu and Ni. Singh and Tewari (2003) reported significant accumulation of proline in

Brassica junceae plants subjected to Cd stress. They suggested that the increased accumulation of proline might be caused by increase in water saturation deficit in plants exposed to higher doses of Cd.

Protein being major biochemical components of plants, influence of the different heavy metals on protein contents of roots and leaves of okra were determined. Significant variations were not generally obtained following various treatments though a general decline in root proteins and slight increase in leaf proteins were obtained. Singh *et al.* (1987) reported that soluble protein content of pea leaves decreased at higher concentration of applied Cd which was also confirmed in pigeon pea by Sheoran *et al.* (1990). Dewan and Dhingra (2004) reported that Cd treatment in general did not affect the seed protein appreciably in the two parent cultivars of pea and their hybrid.However, high doses of Cd (7.5 mM) decreased protein content in one of the cultivar HFP 4. Reports of induction of phytochelatin synthesis by Cd are numerous (Ranieri *et al.* 2005; Mishra *et al.* 2006). Gianazza *et al* (2007) reported inhibition of storage protein catabolism and plant protein anabolism in *Lipidium sativum* plantlets exposed to Cd stress. Besides they also reported the appearance of two proteins which may be related to cellular stress and another two which may be involved in embryogenesis. SDS PAGE analysis of the proteins in the present investigation revealed only few new proteins in okra seeds subjected to heavy metal stress. New induced proteins were generally of low molecular weight or intermediate molecular weight.

In plants, generation of reactive oxygen species (ROS) is one of the common responses to a wide range of biotic and abiotic stresses. Heavy metals have been known to induce oxidative stress because they are involved in several types of ROS generating mechanisms (Stohs and Bagchi, 1995; Dietz *et al.* 1999). Considering the importance of heavy metals in inducing oxidative stress, in the present study oxidative responses of okra cultivars exposed to the different heavy metals were determined. Activities of antioxidative enzymes catalase (CAT), peroxidase (POX), ascorbate peroxidase (APOX), glutathione reductase (GR) and superoxide dismutase (SOD) as well as lipid peroxidation of membranes were determined. A decline in activity of CAT and increase in activity of all other antioxidative enzymes were

obtained. However, the degree of antioxidative responses varied with the heavy metals and cultivars. Significant decline in CAT activity is in confirmity with the works of some previous researchers while not in agreement with those of others. CAT is an important heme-containing enzyme that catalyses the dismutation of H_2O_2 to H_2O and is localized in the peroxisomes. Milone *et al.* (2003) reported that CAT as well as SOD and POX are inhibited in the roots of the most sensitive cultivar subjected to Cd stress in wheat. Cd induced inhibition of APOX and CAT was shown to be associated with H_2O_2 accumulation in kopler roots and *Arabidopsis* (Schutzendubel and Polle, 2002; Cho and Seu, 2004). Cd induced decrease in CAT activity was also reported by previous worker (Skorzynska-Polit *et al.* 2003; Panda and Choudhuri, 2005; Mishra *et al.* 2006). There are also several reports of enhanced activity of CAT following heavy metal stresses (Metwally *et al.* 2005; Singh and Tewari, 2003; Ruley *et al.* 2004). Since CAT is involved in the breakdown of H_2O_2 and H_2O_2 itself plays a dual role as a signaling molecule as well as toxic metabolite, the activity of CAT may vary. H_2O_2 production being an ongoing process in plants inhibition of CAT activity – one of the main routes of H_2O_2 degradation would result in H_2O_2 accumulation which would than activate defence related genes by acting as a second messenger (Keshamma *et al.* 2004).

All the other tested antioxidative enzymes showed increase in activities. Pb and Cd were found to be most effective in inducing the activities. Several previous reports also confirm these findings (Prasad *et al.* 2001; Verma and Dubey, 2003; Ruley *et al.* 2004; Mishra *et al.* 2006). One of the common reaction patterns during exposure to heavy metals has been a severe depletion of GSH. This may be due to increased consumption of glutathione for phytochelatin production (Schutzendubel and Polle, 2002). Since glutathione is also an important component for redox balance of the cell as it is involved in regulation of cell cycle, detoxification of oxidant and acts as a transport form of reduced sulphur it is quite probable that a short term lack of GSH may favour the accumulation of reactive oxygen and disturb developmental processes. The idea that Cd and perhaps also other toxic metals act in cells through a depletion of antioxidative defences is further supported by the observation that GR, APOX and CAT activities were inhibited at time scales similar to those found for

depletion of GSH (Schutzendubel and Polle, 2002). However, with prolonged heavy metal exposure antioxidative defence enzymes increase indicating the cell's recovery from stress. In the present study as the enzymes were analysed after 48 h of treatment increased activities of the antioxidant enzymes may indicate a recovery from the early stresses.

Thus results of the present study and those of previous workers revealed that though heavy metals induced oxidative stress, increased activities of antioxidant enzymes led to the ability of plants to withstand stress. Activation or inhibition of antioxidative enzyme not only depends on stress intensity and duration but also on tissue type and age of plants (Sgherri *et al.* 2002; Schutzendubel and Polle, 2002).

Lipid peroxidation which is considered an indication of oxidative stress in plants can be induced by free radicals and ROS that are generated as a result of heavy metal toxicity in plants. Lipid peroxidation can degrade biological membranes making them susceptible to oxidative damage (Panda, 2002; Panda and Choudhuri, 2005). In the present study increase in lipid peroxidation was obtained in all heavy metal stresses. Several previous workers have reported increased lipid peroxidation following heavy metal stresses, e.g. under Cd stress in *Oryza sativa* (Shah *et al.* 2000), *Holcus lanatus* (Hendry *et al.* 1992) and *Phaseolus vulgaris* (Chaoui *et al.* 1997). Verma and Dubey (2003) reported that in rice plants Pb stress induced increased lipid peroxidation. Singh and Tewari (2003) also reported increased lipid peroxidation in *Brassica juncea* plants subjected to Cd stress. Metwally *et al.* (2005) observed that while treatment with Cd stimulated accumulation of lipid peroxide in roots of all tested pea genotypes, the Cd induced increase of MDA level was lower in the less sensitive genotype 3429 and 1658 compared with the more sensitive genotype 4788 and 188. However, they also observed the maximum value of lipid peroxide in the 8456 which showed relatively high tolerance to Cd as deduced from growth parameters. Thus they suggested that the resistant genotype 8456 had other abilities to counteract or cope with oxidative stress. In the present study also the degree of lipid peroxidation was varied in the different cultivars. Deepti which showed high tolerance as indicated from TI values also showed lower MDA content following the different stresses.

Bhattacharjee and Mukherjee (2004) also reported that content of MDA increased in $PbCl_2$ and $CdCl_2$ treated *Amaranthus* seedlings. They reported that there was almost two fold increase in the extent of membrane lipid peroxidation in 10^{-3} M $CdCl_2$ treated seedlings. Cd stress was also reported to induce increased lipid peroxidation and electrical conductivity in *Bacopa monieri* (Mishra *et al.* 2006). Results of all the studies taken together indicate that increased lipid peroxidation is one of the major effects of oxidative stress induced by heavy metals. Increased level of lipid peroxidation causes modifications of membrane properties such as fluidity and permeability and modulate activities of membrane bound ATPase (Sharma and Agrawal, 2005).

Besides the well studied antioxidant system consisting of low molecular weight antioxidant and specific enzymes recent works are now highlighting the potential role of flavonoids, phenyl propanoids and phenolic acids as effective antioxidants. The ultra-violet absorbing characteristic of flavonoids have long been considered to be evidence for role of flavonoids in UV protection (Baiza and Lyos, 2001; Winkel-Shirley, 2002). There is also evidence that flavonoids play a role in resistance to aluminium toxicity in maize (Kidd *et al.* 2001). In the present study flavonoid accumulation in fruits of three cultivars of okra subjected to various heavy metals were analysed by high performance liquid chromatography. Results revealed that differences in flavonoid accumulation were heavy metal dependent and also varied with the cutivars. Pb and Hg were most inhibitory to flavonoid accumulation. It has been reported that tocopherol, carotenoids, flavonoid aglycones and other hydrophobic antioxidant protect cellular membrane from Cu^{2+} induced lipid peroxidation by breaking the autocatalytic cycle of lipid hydroperoxide formation. Such water soluble compounds as proline, simple phenolic acid and their glycocides, flavonoid glycoside may also protect plant membrane by directly quenching ROS or chelating excess Cu (Faure *et al.* 1990; Hanasaki *et al.* 1994; Cao *et al.* 1997). Caldwel (2001) also reported that Cu(II) altered the levels of flavonoids, increasing the levels of some compounds at low concentration and decreasing the levels at higher concentration. In their work with cucumber phenolics the magnitude of Cu induced changes of the phenolics including flavonoids was shown to be dependent

on tissue type, light condition, pH and treatment duration. Michalak (2006) also opined that during heavy metal stress phenolic compounds can act as metal chelators and on the other hand phenolics can directly scavenge molecular species of active oxygen. Phenolics, specially flavonoids and phenylpropanoids are oxidized by peroxidase and act in H_2O_2 scavenging phenolics/ ASC/ POX system (Michalak, 2006). Thus phenolics play important role in protecting plants against various stresses and enhancement of the metabolism is one of the responses to heavy metal stress (Gorecka *et al.* 2007). In particular their carboxyl or hydroxyl groups can strongly bind Cu^{2+} and Fe^{2+} and thus decrease heavy metal toxicity in cells (Fernandez *et al.*1991).

The accumulation of various heavy metals in different tissues and organs of plants are important considerations for humans. Dietary exposure to heavy metals mainly Cd, Pb, Zn and Cu has been identified as a risk to human health through consumption of vegetable crops (Kechenko and Singh, 2006). The absorption, mobilisation and accumulation of heavy metals vary from plant to plant and are also dependent on the heavy metal. In the present study the actual content of heavy metal in roots and fruits of okra were determined after application of high concentration (1000 $\mu g\ ml^{-1}$) of the salt in the soil. In all cases it was observed that contents varied with the plant parts and the heavy metal. Maximum accumulation was observed in roots and minimum in fruit (Moreno-Casalis *et al.* 2000; Aery and Rana, 2003). Pb was found to accumulate more in leaves of *Silene vulgaris* while in *Thalaspi alpestra* and *Armeria meritima* maximum accumulation was found in roots (Baker, 1981). Maximum accumulation was also reported in *Mentha aquatica* and *Nasturtium officinale* by Saygideger and Dogan (2005). In the present study when compared to Cd and Cu mobilisation of Pb was found to be greater. However Bibi *et al.*(2006) reported that both Cu^{2+} and Pb^{2+} ions were predominantly sequestered in roots of blackgram cultivars rather in leaves or seeds but with increase in external concentration of heavy metal their uptake with respective treated plants also increased.

Cd content in roots and seeds of pea were found to be dependent upon the varieties subjected to the same dose of external application (Dewan and Dhingra,

2004). They observed that Cd content in root and seed was maximum in HFP-4 and least in the hybrid and Arkel respectively. Increase in Cd concentration in roots, xylem, fruiting branches, petioles and boll shell of cotton with increasing Cd concentration in the nutrient solution was reported by Wu *et al.* (2004). In the vegetative organs maximum Cd concentration was reported in root and minimum in leaf. Significant differences in Cd concentration among genotype were also observed. Similar results have also been obtained in the present study where differences were evident among the different cultivars.

The uptake of Cu^{2+} is still a controversial issue for plant scientist (Hall, 2002). Inspite of its being a plant micronutrient it does not show high translocation from the roots to the different plant organs, though, some studies have indicated its considerable accumulation and translocation to different plant organ (Liu *et al.* 2003). Cook *et al.* (1997) observed a positive correlation between concentration of external application and those accumulated by roots, leaves and stems.

Microscopic observation of roots of seedlings and both roots and fruits of older plants subjected to heavy metal stress revealed darkening of intercellular spaces in the cortical region and accumulation of granular substances in both cases, after staining for starch and alkaloids respectively. Benaroya *et al.* (2004) reported that microscopic structural studies of Pb treated *Azolla* plants revealed the accumulation of dark deposited aggregate in the cell wall and vacuoles of leaf cells. In previous studies Cd accumulation in *Azolla* was characterised by the appearance of small dark grains in plant epidermal cells (Sela *et al.*1990). Han *et al.* (2004) in studies related to Cr toxicity in *Brassica* carried out both light microscopic and electron microscopic studies of various plant parts. In light microscopic studies they observed thickly stained areas surrounding the vascular bundles in stems. Such thickly stained areas were also obtained in the present study. Starch accumulation was localized in stroma of *Citrus volkameriana* plants under increased Mn concentration as revealed by microscopic studies (Papadakis *et al.* 2007).

Results of previous experiments revealed that the four tested heavy metals imposed stresses on the different okra cultivars which were partly overcome by the plant by its own defence mechanism. As a result it was observed that even the high

concentration of the metals were not lethal to plants as they could withstand the stress to some extent. In order to investigate whether treatment with some signalling molecules like Ca^{2+} and K^{2+} could further enhance the plant's capacity to withstand heavy metal stress, few experiments were conducted using $CaCl_2$ and KNO_3 as amendments or ameliorating chemicals. Experiments on the influence of Ca and K treatment in combination with heavy metal treatment on germination of okra seeds revealed that both the compounds increased germination percentage which had declined following heavy metal treatment. Following this, the protein content and activities of antioxidative enzymes were determined. It was observed that both $CaCl_2$ and KNO_3 increased the antioxidant responses in the treated seedlings. Kochhar *et al.*(2004) reported that inhibition of germination by Cd in mungbean was ameliorated by Ca. They also observed that Ca increased the activities of antioxidant enzymes in comparison to control. However, the role of Ca in heavy metal toxicity is not well documented. There are previous reports on the amelioration of heavy metal induced oxidative damage by signalling molecules like salicylic acid (SA) and polyamines (Choudhuri and Panda, 2004; Hsu and Kao, 2007).

In conclusion, results of all the experiments in the present study bring out the various metabolic changes in okra plants subjected to different heavy metal stresses. However, the changes were not identical in all the cultivars neither did all the heavy metal induce similar quantum of changes. On the other hand the general trend of responses was that, while stress induces changes in various components and processes, recovery was also evident as indicated by enhanced antioxidative responses. Though some cultivars were more tolerant than the others in growth and yield studies definite correlations were not possible in all the cases. On the whole cultivar Deepti seems to be the most tolerant as evidenced by high tolerance index (TI) values and certain biochemical markers including lesser lipid peroxidation of membrane.