Studies on heavy metal stresses in okra [*Abelmoschus esculentus* (L.) Moench]

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TO WHOM IT MAY CONCERN

This is to certify that Ms. Belinda Lahon has carried out her research work under my supervision. Her thesis entitled **"Studies on heavy metal stresses in okra [*Abelmoschus esculentus* (L.) Moench]"**, is based on her original research work and is being submitted to the University of North Bengal for the award of Doctor of Philosophy (Science) degree in Botany, in accordance with rules and regulations of the University of North Bengal.

Usha Chakraborty

(Usha Chakraborty)

Acknowledgement

In reporting a scientific research, perhaps the acknowledgements are the only major deviation from the temper of science. But it is customary and in line with the cultural norms that one should be thankful to those who have helped in the research work. There are many who have helped me, in one way or other, while carrying out this work and to whom thanks are due.

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Contents

INTRODUCTION

Vegetables are an integral part of human diet and a meal without vegetable is supposed to be incomplete in any part of the world. The daily minimum requirement of vegetable according to a dietician is 284 g per capita, i.e. about 20 per cent of the daily requirement of the total food of an adult, but at present the per capita intake is only 210g per day (Singh and Kalloo, 1998). Lady's Finger/Okra/Bhindi [*Abelmoschus esculentus* (L.) Moench] is a fruit vegetable commonly grown on the plains of India and consumed in all the states (Plate I). Tender fruits are cooked as vegetables. It is a rich source of vitamin A, B and C with little iron and nitrogen. Matured fruits and stem containing crude fibre are used in paper industry. In India APEDA has identified okra as one of the vegetables with good export potential. Keeping in view the large demand of vegetables for domestic consumption and enormous scope for export, the yield can be increased manifold by using advanced technology.

Crop productivity is governed by the interaction of many factors like variety, environment and agronomic practices. Soil contamination with heavy metals has become a worldwide problem, leading to the loss of crop yield and health hazard as they enter the food chain (Salt *et al.* 1995; Schlickler and Caspi, 1999). Heavy metals are defined as metals with a density higher than 5 g cm^{-3}. Fifty-three of the ninety naturally occurring elements are heavy metals (Weast, 1984). Though some of these are essential for life processes, all of these are toxic to organisms at higher concentrations. Heavy metals are natural components of the earth's crust. They cannot be degraded or destroyed. To a small extent they enter our bodies via food, drinking water and air. As trace elements, some heavy metals (e.g. copper, selenium, zinc) are essential to maintain the metabolism of the human body. However, at higher concentrations they can lead to poisoning. Heavy metal poisoning could result, for instance, from drinking-water contamination (e.g. lead pipes), high ambient air concentrations near emission sources, or intake via the food chain. Heavy metals are dangerous because they tend to bioaccumulate.

Cadmium is a byproduct of the mining and smelting of lead and zinc. It is used in nickel-cadmium batteries, PVC plastics, and paint pigments. It can be found in soils because insecticides, fungicides, sludge, and commercial fertilizers that use

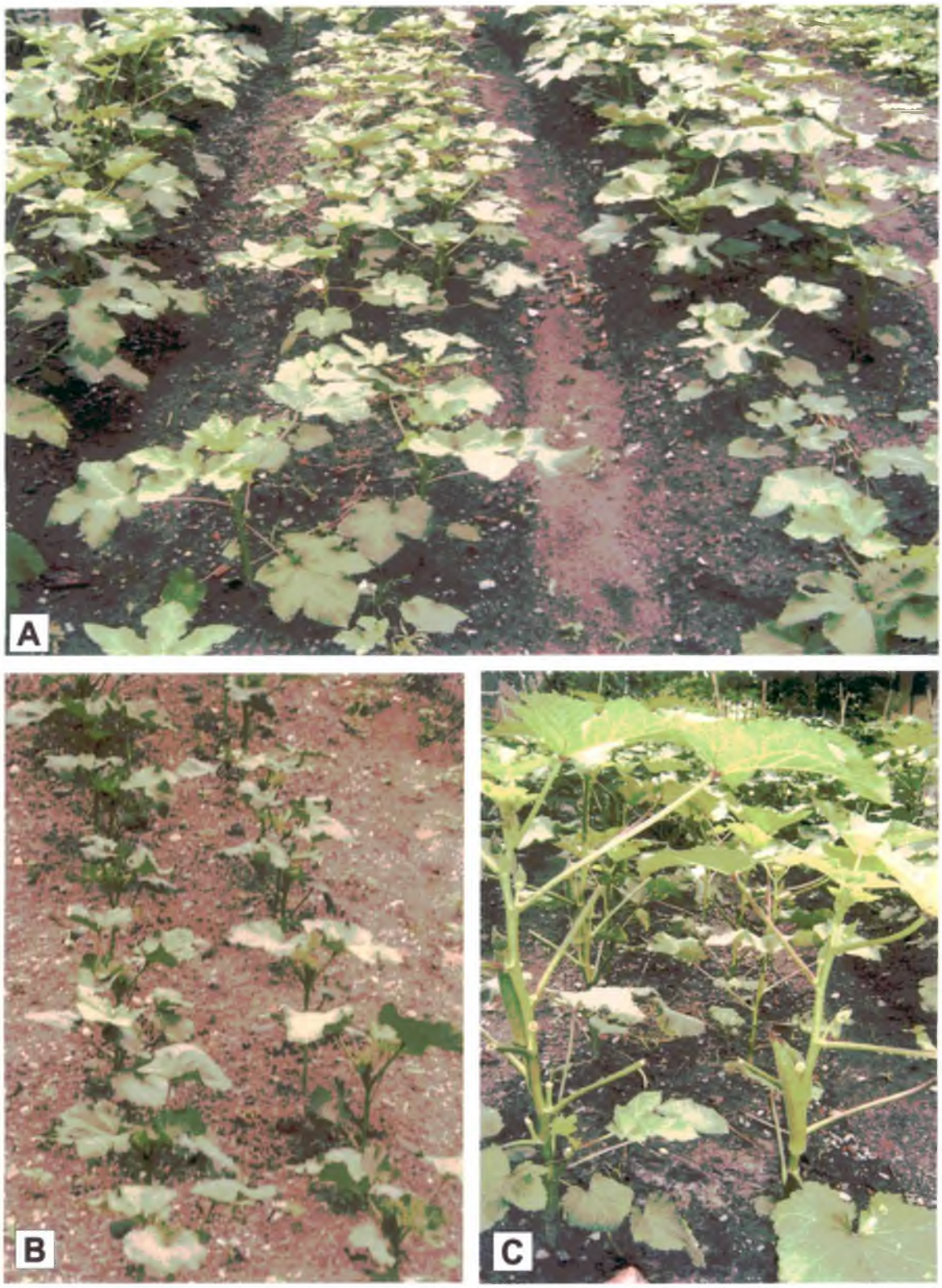


Plate I: Okra plants growing in the field. A: Flowering stage B: Seedling stage and C: Fruiting stage

cadmium are used in agriculture. Among various toxic metals, Cd is recognized as one of the most hazardous elements that is not essential for plant growth but is easily taken up by plants (Nigam *et al.* 2002).

Copper is a micronutrient and is essential for the plants in small amounts. Sources of copper in soil are copper containing fertilizers, fungicides and insecticides.

Mercury is generated naturally in the environment from the degassing of the earth's crust, from volcanic emissions. It exists in three forms: elemental mercury and organic and inorganic mercury. Mining operations, chloralkali plants, and paper industries are significant producers of mercury. Mercury continues to be used in thermometers, thermostats, and dental amalgam.

Lead is a very soft metal and was used in pipes, drains, and soldering materials for many years. Every year, industry produces about 2.5 million tons of lead throughout the world. Most of this lead is used for batteries. The remainder is used for cable coverings, plumbing, ammunition, and fuel additives. Other uses are as paint pigments and in PVC plastics, x-ray shielding, crystal glass production, pencils, and pesticides.

The development of industry, intensive agriculture with modern agricultural techniques, has been so rapid and extensive in the last few decades that it has led to soil and environmental pollution. Among most heavy metals cadmium and arsenic are extremely poisonous, mercury, lead, nickel and fluoride are moderately poisonous whereas boron, copper, zinc, iron, manganese, molybdenum etc. are less poisonous (Das, 2000). The heavy metals are generally present in areas with high anthropogenic pressure. The main sources of contamination in agricultural soil are fertilizer impurity (Cd^{2+}), pesticide composition (Cu^{2+} and Hg^{2+}), and use of refuge derived compost and sewage sludge (Cd^{2+}, Ni^{2+}, Pb^{2+} etc.) and to a lesser extent weathering of rocks. Soils influenced by human activities show a wide range of heavy metal contamination. Toxicity from metallic elements having high atomic weights, such as cadmium, cobalt, iron, lead, mercury, nickel and zinc, may result in cellular injury, chromosomal damage, tumours, birth defects as well as specified

poisonings following ingestion or breathing of particles of such elements alone or in compound form.

Heavy metals can directly or indirectly interfere with the various physiological processes of the plant. The contamination of agricultural land with heavy metal is a widely recognized problem. In the soil, these metals operate as stress factors that cause physiological constraints and impair metabolism after their uptake. This then results in decreased vigour and the stunted growth of the plants. Characteristic features of heavy metal toxicity are inhibition of seed germination, seedling growth, chlorosis, reduction in net photosynthetic rate leading to decreased growth and productivity (Burton *et al.* 1986; Bhattacharjee and Mukherjee, 2004; Neelima and Reddy, 2003). Metal toxicity is counteracted by the alteration in the biochemical parameters. Plant species respond differently to the same level of stress. Stress is a change in the physiology that occurs when plants are exposed to extremely unfavourable conditions that need not be fatal to the organism but will induce an "alarm response". It has been reported that oxidative stress induced by heavy metal toxicity leads to the production of reactive oxygen species (Aravind and Prasad, 2005). The reactive oxygen species (ROS) cause a variety of harmful effects in plant cells, such as inhibition of germination and photosynthetic activity, lipid peroxidation and DNA damage (MacFarlane, 2003; Malecka *et al.* 2001 ; Shaw *et al.* 2004). The ROS are scavenged in plants by antioxidant enzymes like superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), and glutathione reductase (GR). Measurement of activities of antioxidant enzymes is helpful in indicating the oxidative stress in plants (Geebelen *et al.* 2002). When the oxidative stress induced metabolic changes exceed the scavenging capacity by antioxidants, cell death occurs. At the whole plant level, stunted growth as a result of decreased root and shoot elongation is the most common symptom of oxidative stress (Metwally *et al.* 2005; Zengin and Munzuroglu, 2004).

Okra [*Abelmoschus esculentus* (L.) Moench] is a common vegetable crop cultivated in all parts of India. It is an annual and grows in different soil conditions. As it is also generally grown in soil adjacent to tea gardens where soil contaminants from tea garden pesticides/fungicides run off are expected to be present, the present

work was undertaken to determine how different cultivars of this plant responds to heavy metal induced stress. The objectives of the present work were:

(i) To screen Lady's Finger/ okra/ bhindi [*Abelmoschus esculentus* (L.) Moench] cultivars for their relative resistance/ tolerance to heavy metal stress.

(ii) To study the effect of heavy metal stress on the growth parameters.

(iii) To study the effect of heavy metal stress in terms of yield.

(iv) To study the biochemical parameters associated with heavy metal stress in terms of changes in the cellular constituents of shoots and roots including carbohydrates, carotenoids, chlorophylls, proline and protein.

(v) To study metabolite partitioning during stress.

(vi) To study the effect of heavy metal stress on enzyme activities like catalase, peroxidase, ascorbate peroxidase, glutathione reductase and superoxide dismutase.

(vii) To study the specific expression of new protein(s) during heavy metal stress.

(viii) To study the accumulation of heavy metals in different plant tissues.

(ix) To ameliorate the effect of heavy metals by $CaCl_2$ and KNO_3.

In order to achieve the above-mentioned objectives, standard methods have been used which are described in the following pages. Besides, a brief review of literature in the line of work is also being presented.

LITERATURE REVIEW

Heavy metals are a heterogenous group of elements which contaminate the soil following anthropogenic as well as natural activities. They are persistent and tend to bioaccumulate in nature. Elevated concentration of these metals in the soil lead to toxicity, which may be due to a range of interactions at the cellular/molecular level. Some plant species have evolved tolerant races that can survive and thrive on such metalliferous soils presumably by adapting mechanisms that may also be involve in the general homeostasis and constitutive tolerance to essential metal ions as found in all plants (Hall, 2002). With increasing soil pollution worldwide, more and more efforts are on to determine the toxicity effects, mechanisms of tolerance and means of detoxification. Hence, several reviews have been published on heavy metal toxicity in plants (Hall, 2002; Schutzendubel and Polle, 2002; Sharma and Agrawal, 2005). A brief review of literature pertaining to the heavy metal stresses of plants in line with the present work have been presented in the following pages.

2.1. Growth response of plants to heavy metal stress

Wang and Yang (1990) studied the effect of copper pollution on wheat and rice in calcareous soil fertilized with sludge containing copper. Their results demonstrated that high concentration of copper in soil affected on the growth of the crops and their yields. Rice was more susceptible than wheat, and reduced the yield by about 10% when the soil had been treated with copper by 100 ppm. The order of absorption and accumulation of copper within the organs was as follows: root > stem > leaf > grain. The copper content in grains of wheat and rice both were not higher than 20 ppm. In the soil fertilized with sludge, the variation of available copper, its cation speciation and soil capacity were also studied. They suggested that 130 ppm of copper as critical value and 800 ppm as a maximum permissible limit in sludge when it is fertilized to calcareous soil.

Distribution of cadmium and induced Cd-binding proteins in roots, stems and leaves of *Phaseolus vulgaris* was studied by Leita *et al.* (1991). Roots, stems and leaves of *Phaseolus vulgaris* L. (cv.Rubino PF 1H) grown in Hoagland's solution supplemented with 1, 2 and 2.5 mM $Cd(NO_3)_2$ were analyzed. In comparison with control plants grown in a nutrient solution containing equivalent amounts of NO_3

added as KNO_3, plants grown in the presence of 1mM $Cd(NO_3)_2$ showed a significant decrease in fresh weight and per cent dry matter in roots, whereas stems were slightly affected and leaves not at all. At 2 and 2.5 mM Cd concentrations the fresh weight of roots was unaffected, but their dry matter content was strongly reduced: stems showed significant, even limited decrease of both fresh weight and % dry matter. In comparison, the fresh weight and foliar area of leaves were strongly reduced, the dry matter content being unaffected.

The metal content of *Juncus acutus* (Juncaceae) seedling using $Pb(NO)_3$; $CuSO_4$ and $CdCl_2$ were determined and it was observed that the germination was not affected by any of these tested metals, whereas the initial growth was strongly inhibited by $Pb(NO_3)_2$, concentration from 0.12×10^{-5} M. Results showed that root was affected more than the shoot and failed to develop at all the tested concentration of $CuSO_4$. Moreover the accumulation of Cd in the seedlings was higher than that of Pb and Cu (Stefani *et al.* 1991).

Seven-day old seedlings of *Vigna catjang* Endl. were treated with distilled water or 10^{-5} M $PbCl_2$ or 10^{-5} M $CdCl_2$ or (10^{-4} M $PbCl_2$ + 5 times 10^{-5} M reduced glutathione (GSH)) or [10^{-5} M $PbCl_2$ + 5 times 10^{-5} M buthionine sulfoximine (BSO)] or (10^{-5} M CdI_2 + 5 times 10^{-5} M GSH) or (10^{-5} M $CdCl_2$ + 5 times 10^{-5} M BSO) for 6 days under open air conditions in a net house. Heavy metal treated plants showed significant decline in biomass, leaf area, root length, relative water content, root metabolic activity, pigment and protein content on one hand and a significant rise in MDA, alpha-NH^2, proline content and electrical conductivity of leaf leachate on the other. In all the cases Cd^{2+} was more effective than Pb^{2+}. Treatment with GSH showed different degrees of recovery of stress-induced damages whereas BSO treatment augmented the stress-induced damages. These results of Bhattacharyya and Choudhuri (1995) suggested the possible involvement of phytochelatin-like substances in the mitigation of metal-induced damages.

Gigliotti *et al.* (1996) performed a 6-year field study to evaluate heavy metal accumulation in the top 20 cm of a clay-loam calcareous soil (Fluventic Xerochrept) amended with urban waste compost and to determine heavy metal uptake and distribution in corn plants grown in this soil. Compared with untreated

soils, amended soils showed a significant increase only in Cu, Zn, Pb and, in the last 2 years, Cr concentrations. They concluded that corn plants grown on the amended soil showed a general increase in metal uptake, which was about three times greater for Pb and two times greater for the other heavy metals than in plants grown on untreated soil. At times, the diluting effect resulting from enhanced growth rates of the plants with compost application resulted in lower concentrations in the plants grown on treated plots. Cr and Pb were less mobile in the corn plant and were accumulated only in root tissues. In the last 3 years of the experiment traces of Pb were found in the stalks. The limited mobility of Pb was confirmed in a contemporary hydroponic greenhouse experiment. The values of the plant/soil transfer coefficients were within the lower range reported in the literature, indicating that in the soil studied (which contained 14% $CaCO_3$) there was limited transfer of heavy metal ions from the soil to the corn plants. It is concluded that the long-term application of large amounts of urban waste compost to $CaCO_3$-containing soils does not necessarily cause medium-term problems to plant, animal or human health.

Seeds of rice (*Oryza sativa* L. cv. IR-36 and Ratna) subjected to heavy metal (Pb^{2+} and Hg^{2+}) stress showed inhibition in germination percentage, shoot and root length and in their fresh and dry mass after 7 days (Mishra and Chowdhury, 1997). Both Pb^{2+} and Hg^{2+} inhibited starch hydrolysis due to inhibition of L-amylase. Hg^{2+} was more effective than Pb^{2+} in inhibiting germination, and IR-36 was more tolerant than Ratna to these heavy metals. When metal treated embryos were grown in vitro, 2% sucrose in the medium could overcome the inhibitory effect of Pb^{2+} on embryo while the same, could not erase the inhibitory effect of Hg^{2+} on embryo growth significantly. Thus, Pb^{2+} inhibited germination and seedling growth by impairing the hydrolysis of endosperm starch without significantly affecting the embryo, while Hg^{2+} inhibited the same by damaging the embryo itself. Thus, Hg^{2+} was shown to be potentially more lethal than Pb^{2+} in inhibiting germination of rice seeds.

Ouariti *et al.*(1997) investigated cadmium and copper uptake and distribution, as well as their effects on growth and lipid composition in 17 day old tomato seedlings (*Lycopersicon esculentum* Mill. cv. 63/5 F1) grown in culture

solution supplied with two concentrations of Cd or Cu (0, 5 and 50 mu-M). The accumulation of Cd and Cu increased with external metal concentrations, and was considerably higher in roots than in primary leaves. They concluded that biomass production of the growing roots and primary leaves was strongly depressed at high metal levels.

The relationship between the stage of leaf maturity and the response of the photosynthetic apparatus to Cd in rye plants was determined. Because of growth characteristics, leaves of monocotyledonous plants represent all stages of leaf maturity in this organ – from the youngest cells at the base to the oldest at the tip of the leaf. Since some recent studies carried on dicotyledonous plants proved the level of heavy metals toxicity to be related to the leaf age and its stage of growth. Krupa and Moniak (1998) decided to test this hypothesis on monocotyledonous plants where they could observe all stages of maturity within different sections of one leaf. Basic growth analysis (total leaf length, FW content of leaf sections), determination of Cd content and chloroplast pigment levels in individual leaf sections, measurements of the photosynthetic apparatus efficiency using chlorophyll fluorescence techniques, were carried out. They have shown a close relation between the stage of leaf maturity and the efficiency of the photosynthetic apparatus in rye plants with the Calvin cycle being the most sensitive to Cd toxicity.

Wierzbicka and Obidzinska (1998) conducted a study to determine the extent to which seed coats are a barrier to lead and to what degree germination is affected by this heavy metal. The study was carried out on 25 species of plants from 12 families, represented by different varieties and populations. In all, 34 types of seeds were tested. Comparative observations were conducted on the effect of barium nitrate on seed imbibition and germination. Seeds were treated with solutions of the following salts during imbibition: 100, 1000, and 10000 mg dm^{-3} Pb^{2+} from $Pb(NO_3)_2$ and 1000 mg dm^{-3} Ba^{2+} from $Ba(NO_3)_2$. The histochemical rhodizonate method was used to determine the distribution of Pb and Ba in the seeds. Water uptake and germination dynamics were also studied. It was found that 28% of the studied plant species had seed coats that were permeable to lead ions, 39% were permeable to Ba ions. In both cases these species belonged to three families:

Papilionaceae, Crucifereae and Graminae. Pb delayed germination and lowered the ability of seeds to germinate in a dose-dependent manner in the species with highly Pb-permeable seed coats. In some other species, germination was delayed only a few hours. In all, a significant effect of Pb on germination was found in over half of the studied species. It was shown that Pb did not act by inhibiting water uptake during imbibition. It was also shown that seed coat permeability varied during imbibition of seeds with coats highly permeable to Pb. Seed coats were impermeable to lead in the first period of imbibition when water uptake is intense. In the final stages of imbibition, when water uptake is reduced, seed coats became more permeable to lead. The Pb that penetrated into the embryos in the final stage of imbibition delayed germination. This shows that seed coats are selectively permeable to lead ions.

Mishra and Choudhury (1999) monitored the phytotoxic effect of lead and mercury of two rice (*Oryza sativa* L.) cultivars (Ratna and IR 36). They observed a decrease in germination percentage, germination index (GI), shoot and root length, tolerance index (TI), vigour index (VI) and dry mass of shoot and root but increased percentage difference from control (% DFC) of germination and percentage phytoxicity in both the cultivars. From these indices they concluded that the phytotoxic effect of mercury was greater than lead at identical concentrations and that IR36 appeared more tolerant than Ratna to these metals. Among the monitoring indices examined, TI, VI and % phytotoxicity seemed to serve as good biological monitoring methods for evaluating the relative toxicity of lead and mercury to rice cultivars.

The distribution and excretion of Cu, Pb and Zn in the root and leaf tissue of the grey mangrove, *Avicennia marina* was studied using scanning electron microscope (SEM) X-ray microanalysis and atomic absorption spectroscopy. SEM X-ray microanalysis of nutritive root tissue in seedlings dosed with 4 g L^{-1} Cu, Pb and Zn revealed accumulation of all metals predominantly in cell walls. The root epidermis provided a major barrier to the transport of Pb only. The endodermal casparian strip was shown to provide a barrier to movement of all three metals into the stele. Washings from mature leaves contained significantly higher amounts of Zn and Cu than control plants after 1 month, suggesting excretion of both metals from

the glandular trichomes. In addition, salt crystals exuded from the glands on the adaxial surface of mature leaves were composed of alkaline metals: Zn in Zn-treated plants, and Cu in Cu-treated plants. Leaf tissue in seedlings dosed with 4 g L^{-1} Zn showed a decreasing gradient of the metal from xylem tissue, through photosynthetic mesophyll, to hypodermal (water) tissue, with a subsequent increase in concentration in the glandular tissue. A similar gradient was observed across leaf tissue in seedlings dosed with 4 g L^{-1} Cu, however, there was no subsequent increase in Cu concentration in glandular tissue. For both metals leaf cell wall metal concentrations were consistently higher than intracellular concentrations (MacFarlane and Burchett 2000). In a further study MacFarlane and Burchett (2001) observed that mangroves possess a tolerance to high levels of heavy metals. Six month-old seedlings of the grey mangrove, *Avicennia marina* (Forsk.) Vierh, were exposed to a range of Cu (0–800 μg g^{-1}), Pb (0–800 μg g^{-1}) and Zn (0–1000 μg g^{-1}) concentrations in sediments under laboratory conditions, to determine leaf tissue metal accumulation patterns. Limited Cu uptake to leaves was observed at low sediment Cu levels, with saturation and visible toxicity to Cu at sediment levels greater than 400 μg g^{-1}. Leaf Pb concentrations remained low over a range of Pb sediment concentrations, up to 400 μg g^{-1} Pb, above which it appeared that unrestricted transport of Pb occurred, although no visible signs of Pb toxicity were observed. Zn was accumulated linearly with sediment zinc concentration, and visible toxicity occurring at the highest concentration, 1000 μg g^{-1} Zn.

Peralta *et al.* (2000) reported that preliminary studies have shown that alfalfa plants (*Medicago sativa*) can grow in some heavy metal contaminated soils. Based on it they studied the individual effects of several doses of Cd(II), Cr(VI), Cu(II), Ni(II), and Zn(II) on the growth of live alfalfa plants using solid media. The doses used in this study were 0, 5, 10, 20, and 40 ppm. The seed germination and plant growth was significantly affected by Cd(II) and Cr(VI) at 10 ppm, as well as by Cu(II) and Ni(II) at 20 ppm and higher concentrations ($P<1\%$). Zn(II) did not affect seed germination. The roots of the plants exposed to 5 ppm-dose of Cd(II), and 5 and 10 ppm-dose of Cr(VI), Cu(II), Ni(II), and Zn(II), grew more than the roots of the control treatment by more than 30%. Exposures of 5 ppm of Cd(II) reduced the shoot

size by 16% as compared to the control. While Cr(VI), Cu(II), Ni(II), and Zn(II) increased the shoot size by 14.0%, 60.0%, 36.0%, and 7.7%, respectively; only Zn(II) promoted the shoot growth at the doses of 20 and 40 ppm.

The capability of common reed and cattail (*Phragmites australis* (Cav.) Trin. ex Steud. and *Typha latifolia* L.) to accumulate and translocate Cd^{2+} from roots to shoots and their defense mechanisms induced by Cd^{2+} at the levels of thiol metabolism and antioxidant enzyme activity were studied by Fediuc and Erdei (2002). They carried out the experiment by using young, small sized plants grown hydroponically and originating either from regenerating tissue culture (reed) or aseptically germinated seeds (cattail). Cadmium treatment was applied as a concentration series between 0.1 and 100 μmol L^{-1} for 40 and 100 days for reed and cattail, respectively. For assays and analysis, plant samples were taken in 2-4 weeks intervals. They reported that most of the Cd^{2+} taken up was retained in roots in both species, however, *Typha* accumulated more cadmium in the shoot compared to *Phragmites*. In *Typha*, increasing accumulation of cadmium was in positive correlation with the increase of free thiol content while in *Phragmites* increased glutathione reductase, catalase and peroxidase activities were found. They concluded that under Cd^{2+} stress different defense strategies operate in *Typha* and *Phragmites*. In *Typha*, this strategy relies more on thiol induction and metal binding leading to heavy metal avoidance, while in *Phragmites* it is based on increased antioxidant enzyme activities and thus scavenging of active oxygen species.

Because plant wilting has been described as a consequence of cadmium (Cd^{2+}) toxicity, Perfus-Barbeoch *et al.* (2002) investigated the Cd^{2+} effects on plant water losses, gas exchanges and stomatal behaviour in *Arabidopsis thaliana* L. Effects of 1 week Cd^{2+} application in hydroponic condition ($CdCl_2$ 10–100 μM) were analyzed. A 10 μM Cd^{2+} concentration had no significant effect on the plant–water relationship and carbon assimilation. At higher Cd^{2+} concentrations, a Cd^{2+} - dependent decrease in leaf conductance and CO_2 uptake was observed despite the photosynthetic apparatus appeared not to be affected as probed by fluorescence measurements. In epidermal strip bioassays, nanomolar Cd^{2+} concentrations reduced stomatal opening under light in *A. thaliana*, *Vicia faba* and *Commelina communis*.

Application of 5 mM ABA limited the root-to-shoot translocation of cadmium. However, the Cd^{2+}-induced stomatal closure was likely ABA-independent, since a 5-day treatment with 50 μM Cd^{2+} did not affect the plant relative water content. Additionally, a similar Cd^{2+} induced stomatal closure was observed in the ABA insensitive mutant abi1-1. Interestingly, this mutant displayed a higher transpiration rate than the wild type but did not accumulate more Cd^{2+}, arguing that Cd^{2+} uptake is not dependent only on the transpiration flow. Application of putative calcium channels inhibitors suppressed the inhibitory effect of Cd^{2+} in epidermal strip experiments, suggesting that Cd^{2+} could enter the guard cell through calcium channels. Patch-clamp studies with *V. faba* guard cell protoplasts showed that plasma membrane K^+ channels were insensitive to external Cd^{2+} application whereas Ca^{2+} channels were found permeable to Cd^{2+}. In conclusion, they proposed that Cd^{2+} affects guard cell regulation in an ABA-independent manner by entering the cytosol via Ca^{2+}channels.

Shu *et al.* (2002) designed an experiment to investigate both Fankou and Lechang lead/zinc (Pb/Zn) mine tailings located at Guangdong Province contained high levels of total and DTPA-extractable Pb, Zn and Cu. *Paspalum distichum* and *Cynodon dactylon* were different populations of the two grasses growing on the tailings. Tillers of these populations including those from an uncontaminated area were subjected to the following concentrations: 5, 10, 20, 30 and 40 mg l^{-1} Pb, 2.5, 5, 10, 20 and 30 mg L^{-1} Zn, or 0.25, 0.50, 1 and 2 mg L^{-1} Cu for 14 days, respectively. The dominant species colonized naturally on the tailings. Pb, Zn and Cu accumulation and tolerance of tolerance index (TI) and EC_{50} (the concentrations of metals in solutions which reduce 50% of normal root growth) were calculated. The results indicated that both Lechang and Fankou populations of the two grasses showed a greater tolerance to the three metals than those growing on the uncontaminated area, which suggested that co-tolerant ecotypes have evolved in the two grasses. *P. distichum* collected from Fankou tailings had the highest tolerance to Cu while Lechang population had the highest tolerance to Pb and Zn among the tested populations, and tolerance levels in *P. distichum* were related to metal concentrations in the plants. *P. distichum* had a better growth performance than *C.*

dactylon when both of them were grown on the tailings sites. Tolerant populations of these species would serve as potential candidates for re-vegetation of wastelands contaminated with Pb, Zn and Cu.

The repeated use of copper (Cu) fungicides to control vine downy mildew, caused by *Plasmopara viticola*, has been responsible for the heavy increase of Cu concentration in the upper layers of vineyard soils (Brun *et al.* 2003). To determine the effects of elevated soil Cu on plant development, they created an artificial soil gradient with Cu enrichments ranging from 0 to 400 mg kg^{-1}. On this gradient, and for five ruderal plant species commonly found in vineyards in southern France (*Poa annua* L., *Dactylis glomerata* L., *Senecio vulgaris* L., *Hypochoeris radicata* L., and *Andryala integriflolia* L.), they quantified survival, growth, and reproduction throughout one flowering season. High concentrations of Cu in the soil resulted in low survival, low total plant biomass, delay in flowering and fruiting, and low seed set. They concluded that the effects differed among species. Furthermore, high soil Cu concentrations had contrasting effects on patterns of resource allocation depending on the plant species.

The toxicity of sodium arsenate on germination physiology of *Vigna radiata* (L.) Wilczek was studied. Soaking treatment of different doses (1, 5 and 10 μM) of $NaHAsO_4$ was given to the seeds for 8 h in petri dishes. The percentage of seed germination was recorded at an interval of 24, 48 and 72 h. Seedling growth (shoot and root length) and fresh and dry weight of different parts of seedlings were determined 96 h after soaking. They concluded that $NaHAsO_4$ caused inhibition of germination and other growth parameters (Debnath and Srivastava, 2003).

Liu *et al.* (2003) conducted a pot experiment on 20 rice cultivars of different genotypes and origins by adding 100 mg kg^{-1} of cadmium (Cd) to soil. The aim was to investigate the effects of Cd on the dry matter accumulation and grain yield of different rice cultivars, the differences among rice cultivars and genotypes in Cd uptake and translocation, the interactions between Cd and five mineral nutrients Fe, Zn, Mn, Cu and Mg in response of the uptake and translocation in rice plant. The results showed that the effects of Cd on rice growth and development were variety dependent; some cultivars were strongly tolerant to soil Cd stress, while others were

very sensitive. Differences existed among the cultivars for Cd uptake and distribution in rice plants, but the differences were not necessarily related to rice genotypes. Cd concentrations fell rapidly from roots to brown rice along rice plants, so the concentrations of Cd were very low in brown rice compared with other parts of rice plants. The effects of Cd on the concentrations of the mineral nutrients in the rice roots and leaves were mostly significant, however, the results varied with metal elements, rice plant organs and growing stages. Under soil Cd stress, the variations of the grain and straw yield of the cultivars were not correlated with the changes of any mineral nutrient in the rice plant. The regression analysis showed that, for their concentrations in roots and leaves, significant positive correlations between Cd and Fe, Cd and Zn, Cd and Cu existed, but no significant correlation between Cd and Mg, and the relationship between Cd and Mn varied with the organs of rice plant. These results suggested that rice cultivars differed greatly in growth and development responses to Cd and in absorption and translocation of Cd, Fe, Zn, Cu, Mn and Mg. The effects of Cd on the five mineral nutrients were not the main causes of the inhibition of Cd on rice growth and development. The interactions of Cd and Fe, Zn, Cu are synergetic in uptake and translocation from root to shoot by rice plant.

Growth parameters of six fast growing trees showed that the roots responded to Cd treatment more sensitively than shoots. Cd-treatment suppressed rooting and root growth (length and biomass production) as well as its development in all tested species. Root systems of *Salix cinerea*, *Salix alba*, and *Populus* cv. Robusta were more tolerant to Cd stress than the root system of the other studied species. Shoot growth parameters of *Salix* species were significantly reduced unlike *Populus* species, which were not affected by Cd treatment (Sottnikova *et al.*, 2003).

Parida *et al.* (2003) conducted a screenhouse experiment using an alkaline sandy loam soil equilibrated with graded levels of Ni (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 and 300 mg kg^{-1} soil) to assess the Ni accumulation pattern and its influence on growth and micronutrient distribution in fenugreek plants. Green as well as the dry matter yields of fenugreek increased slightly up to 20 g Ni kg^{-1} soil but decreased significantly with the application $\geq$40 mg Ni kg^{-1} soil. They observed that crops showed characteristic toxicity symptoms of interveinal

chlorosis in pots receiving ≥40 mg Ni kg^{-1} soil. While the total content of Ni in the plant tissues increased consistently with increasing rates of applied Ni, the roots accumulated much higher amount of this element compared to the shoot. The content of Fe in plants showed an increase whereas that of Cu and Zn experienced a decrease with the rise in the applied Ni.

The influence of cadmium (Cd^{2+}) on the wheat (*Triticum aestivum* L.) plant was analyzed by Shukla *et al.* (2003). Cd^{2+} accumulation and distribution in 3 week old seedlings grown in nutrient medium containing varying concentrations of Cd^{2+} (control, 0.25, 0.50, 1.0, 2.5, and 5.0 mg L^{-1}). The effect of varying Cd^{2+} concentrations up to 21 days on biomass productivity, plant growth, photosynthetic pigments, protein, amino acids, starch, soluble sugars, and essential nutrients uptake was studied in detail to explore the level up to which the plant can withstand the stress of heavy metal. Plants treated with 0.5, 1.0, 2.5, and 5.0 mg L^{-1} Cd^{2+} showed symptoms of heavy-metal toxicity as observed by various morphological parameters which were recorded with the growth of plants. The root shoot-leaf length and the root, shoot-leaf biomass progressively decreased with increasing Cd^{2+} concentration in the nutrient medium. Cd^{2+} uptake and accumulation was found to be maximum during the initial growth period. Cd^{2+} also interfered with the nutrients uptake, especially calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^{+}), iron (Fe^{2+}), zinc (Zn^{2+}), and manganese (Mn^{2+}) from the growth medium.

Soltan and Rashed (2003) studied the survival and behaviour of water hyacinth [*Eichhornia crassipes* (Mart.) Solms] under varying conditions of heavy metal concentrations, groups of the plants were grown in different media (distilled water, Nile water, wastewater and different concentrations of heavy metals). They observed visual changes in the plants during each experiment. The heavy metal (Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn) concentrations, pH and conductivity of the media were measured before, during and at the termination of experiments. In addition, analyses for heavy metals were carried out on the plant samples after termination of the experiments to determine the effect of different media on metal accumulation by the plants. Their results showed that water hyacinth can survive in a mixture of heavy metal concentrations up to 3 mg L^{-1} and in 100 mg Pb L^{-1} solution, whereas

higher concentrations of metals as mixtures and 100 mg Cd L^{-1} led to rapid fading of the plants. Water hyacinth exhibited a deprotonation reaction during the uptake of metal ions, which was detected as a result of a decrease in pH of the growth media. Their results indicated that water hyacinth plays an outstanding role as a heavy metal decontaminator; in addition, its role as a pollutant by releasing metal ions into the aquatic environment was also noted. They suggested that elevated pH and ammonia concentrations, along with the low dissolved oxygen content in the microenvironment around the root hairs, are the main factors for the rapid wilting of plants growing in Kima drain wastewater.

Stolt *et al.* (2003) studied PC accumulation in 12 day old seedlings of two cultivars of spring bread wheat (*Triticum aestivum*), and two spring durum wheat cultivars (*Triticum turgidum* var. durum) with different degrees of Cd accumulation in the grains. Shoots and roots were analysed for dry weight, Cd and PC accumulation. There were no significant differences between the species or the varieties in the growth response to Cd, nor in the distributions of PC chain lengths or PC isoforms. At 1 μM external Cd, durum wheat had a higher total Cd uptake than bread wheat, however, the shoot-to-root Cd concentration ratio was higher in bread wheat. When comparing varieties within a species, the high grain Cd accumulators exhibited lower rates of root Cd accumulation, shoot Cd accumulation, and root PC accumulation, but higher shoot-to-root Cd concentration ratios. Intraspecific variation in grain Cd accumulation is apparently not only explained by differential Cd accumulation as such, but rather by a differential plant-internal Cd allocation pattern. However, the higher average grain Cd accumulation in the durum wheats, as compared to the bread wheats, is associated with a higher total Cd accumulation in the plant, rather than with differential plant-internal Cd allocation. The root-internal PC chain length distributions and PC–thiol-to-Cd molar ratios did not significantly differ between species or varieties, suggesting that differential grain Cd accumulation is not due to differential PC-based Cd sequestration in the roots.

In a study by Ajasa *et al.* 2004 the concentration levels (ppm) of selected toxic trace metals (Fe, Mn, Cu, Pb and Zn) and macronutrients (Na, K, Mg and Ca), along with P, were estimated in some of the important herbal plants of the southwest

part of Nigeria. The atomic absorption spectrophotometer was employed for the estimation conducted on 10 plant species collected from different locations within Ogbomoso. The plants were *Anacardium occidentale, Azadirachta indica, Butyrospermum paradoxum, Mangifera indica, Morinda lucida, Ocimum canum, Solanum erianthum, Solanum torvum, Zingiber officinale* and *Hyptis suaveolens*. The metal contents in the samples were found at different levels. The highest mean levels (ppm) of Zn (35.1±0.01) and Cu (24.4±0.01) were found in *Hyptis suaveolens* while those of Mn (685±0.02) and Ca (51 340±21) were found in *Morinda lucida*. Their result also showed that *Ocimum canum* had the highest amounts of K (36 600±350), P (3700±35) and Fe (241±0.05). *Anacardium occidentale* had the highest concentration of Na (613±0.60) while *Azadirachta indica* had the highest mean concentrations of Pb (0.49±0.03) and Mg (5630±12).

Flax, hemp and cotton, grown in industrially polluted region, were studied by Angelova *et al.* 2004. The experimental plots were situated at different distances (0.5 and 15 km) from the source of pollution—the Non-Ferrous-Metal Works (MFMW) near Plovdiv. They investigated the level of pollution and the way heavy metals enter the fibre crops, by taking soil and plant samples. The contents of heavy metals in plant materials (roots, stems, leaves, seeds, flowers) were determined after the method of the dry mineralization. The quantitative measurements were carried out with inductively-coupled plasma (ICP). They reported that a clearly distinguished species peculiarity exists in the accumulation of heavy metals in the vegetative and reproductive organs of flax, hemp and cotton. Flax is the crop that most strongly absorbs and accumulates heavy metals from the soil, followed by hemp and cotton. The distribution of the heavy metals along the plant axis of the studied crops seemed to be selective, therefore their contents in flax and hemp are decreased in the following order: roots>stems>leaves>seeds, while in cotton: leaves>seeds> roots> stems. A strongly exhibited tendency towards decrease of the contents of heavy metals in the fibre crops was observed as the distance from the NFMW increases. They concluded that flax and hemp are cultures, suitable for growing in industrially polluted regions—they remove considerable quantities of heavy metals

from the soil with their root system and can be used as potential crops for cleaning the soil from heavy metals.

Barazani *et al.* (2004) conducted an experiment in which the ability of *Allium schoenoprasum* L. (chives) to accumulate and tolerate cadmium in aqueous Hoagland medium at 50 μM and 250 μM were tested under continuous growth or several successive harvests of shoots. After 28 days of continuous growth, chives accumulated the metal up to 0.2% and 0.5% of its dry weight, when grown in 50 μM and 250 μM, respectively. In the experiments the leaves were successively harvested every 16 days, there were no obvious stress symptoms after six harvests during a period of 96 days at 50 μM Cd. At 250 μM, after 64 days and four harvests, inhibition of growth occurred. In each treatment, a total of 1.2 g kg^{-1} DW and 2.4 g kg^{-1} DW were accumulated in the leaves, respectively. Total SH compounds concentration in leaf was found significantly higher by 3 and 7.4 times in plants treated with Cd at 50 μM and 250 μM in comparison to the control, respectively, while no difference in the concentration of glutathione (GSH + GSSG) were found. Thus, it is assumed that sulphur-containing compounds, yet unknown, are involved in defensive mechanisms against heavy metals in chives. The results pointed to chives phytoremediation potential, but also on the potential risk in accumulation of heavy metals in a commonly edible plant.

The concentrations of lead, zinc, copper and cadmium accumulated by 12 emergent-rooted wetland plant species including different populations of *Leersia hexandra*, *Juncus effusus* and *Equisetum ramosisti* were investigated in field conditions of China by Deng *et al.* 2004. Their results showed that metal accumulation by wetland plants differed among species, populations and tissues. Populations grown in substrata with elevated metals contained significantly higher metals in plants. Metals accumulated by wetland plants were mostly distributed in root tissues, suggesting that an exclusion strategy for metal tolerance widely exists in them. They observed that some species/populations could accumulate relatively high metal concentrations (far above the toxic concentration to plants) in their shoots, which indicated that internal detoxification metal tolerance mechanism(s) were also included. The factors affecting metal accumulation by wetland plants included metal

concentrations, pH, and nutrient status in substrata. Mostly concentrations of Pb and Cu in both aboveground and underground tissues of the plants were significantly positively related to their total and/or DTPA-extractable fractions in substrata while negatively to soil N and P, respectively.

To assess the contamination induced by traffic at the vicinity of a highway (A31, France), several complementary studies were carried out by Viard *et al.* 2004 on two sites, with different profiles and traffic intensity. Concentrations of zinc, lead and cadmium were measured by atomic absorption spectrophotometry in deposits, roadside soil and autochthonous plants (Graminaceae) gathered at the vicinity of the highway (1–320 m). According to the results obtained for different compartments, the highway induces a contamination on the surrounding environment, up to 320 m, but with the maximum contamination observed between 5 and 20 m: the concentrations measured in plants at the vicinity of the highway were 2.1 mg Pb kg^{-1} DW, 0.06 mg Cd kg^{-1} DW, 62 mg Zn kg^{-1} DW and the concentrations measured in snails were 21.3 mg Pb kg^{-1} DW, 5.7 mg Cd kg^{-1} DW, 510.8 mg Zn kg^{-1} DW. The levels measured decreased with increasing distance from the highway. The results of the three metals studied indicated that lead seems to be the best metal to evaluate road transport contamination.

An investigation of alfalfa plants grown in soil at different growth stages exposed to separate batches of Cr(VI) at 100 mgl^{-1}, and Cd(II), Cu(II), Ni(II), or Zn(II) at 500 mg L^{-1} was carried out by Videa *et al.* (2004). Four days after germination, all metals, except Zn(II), had lethal effects on the alfalfa seedlings. Furthermore, when applied 16 days after germination, Cr(VI) and Ni(II) still had lethal effects on the seedlings and Cd(II) and Cu(II) destroyed more than 50% of the plant population. While approximately 90% of the plants exposed to Cd(II), Cu(II) and Zn(II) were able to grow without apparent negative effects 20 days after germination, Cr(VI) and Ni(II) still showed lethal effects. The concentration of heavy metals in shoot dry tissues was 1209 mg kg^{-1} for Cd, 887 mg kg^{-1} for Cu and 645 mg kg^{-1} for Zn. These results demonstrated that the tolerance of alfalfa plants to Cd, Cu and Zn was positively correlated with the age of the plants. Also, these results opened the possibility of using alfalfa plants, via transplant, to clean up soils

where the concentration of Cd, Cu or Zn is high enough to avoid alfalfa seed germination.

Zengin and Munzuroglu (2004) studied the effects of copper and lead applied in form of chloride salts on root, shoot and leaf growth of the bean seedlings. They reported that both heavy metals significantly prevented the growth of root, shoot and leaves of seedlings. A parallel relation was observed between an increase in the concentration of the heavy metal salt and rate of inhibition of root, shoot and leaf growth. Extend of the exposing time to heavy metals of seedling lead to more decreasing of root, shoot and leaf growth. They concluded that copper and lead stress is more sensitive to root growths, followed by shoot and leaf growth, respectively.

Drazic and Mihailovic (2005) reported that the treatment of soybean seedlings with 6 mg kg^{-1} Cd during 72 h induces a slight growth inhibition in roots, stems and leaves and a significant desiccation of cotyledons and leaves with a decrease of chlorophyll content in leaves. Salicylic acid (SA) applied simultaneously at the concentrations of 10^{-6}, 10^{-5} and 10^{-4} M significantly alleviates the effect of Cd. Cd and SA act synergistically on K content inducing its significant decrease in roots. Under the influence of Cd, Fe content is decreased in roots and increased in leaves, while SA removes this effect. Magnesium content is substantially decreased in root and stem under the influence of Cd, and SA attenuates the effect of Cd only in roots, while in leaves it induces a significant increase of the content of this element. SA does not decrease Cd uptake, but changes its distribution in plant organs depending on the concentration of added Cd. The obtained results indicate that the influence of SA on the alleviation of toxic effects of Cd is probably indirect, through a development of general antistress response of the seedlings which includes also the regulation of K and Mg distribution.

Kovalchuk *et al.* (2005) analyzed the influence of salts of two heavy metals - lead and cadmium (Pb^{2+} and Cd^{2+}) on plants, including plant and root size, plant genome stability as well as global genome expression. Measurement of the metal uptake showed that there was a significantly higher incorporation of Cd than of Pb, 0.6 and 0.15 uM g^{-1} of dry weight, respectively. The analysis of the root length and plant size showed a dose dependent decrease in plants exposed to Cd. In contrast

there was little difference in the size of plants exposed to Pb, although there was nearly four-fold increase of the root length. Analysis of the genome stability revealed that Cd led to a dose dependent increase of homologous recombination whereas Pb had no effect. Analysis of the global genome expression of plants chronically exposed to 50 uM of Cd and Pb revealed 65 and 338 up and down regulated genes by Cd and 19 and 76 by Pb, respectively. Interestingly, half of the genes that changed their expression in Pb-treated plants also changed their expression in Cd-treated ones. The greater number of genes regulated by Cd reflects generally higher genome instability of plants as well as higher uptake as compared to Pb.

Growth responses were analyzed in *Prunus cerasifera*, a peach rootstock after exposure to various copper concentrations. *P. cerasifera* plantlets tolerated Cu concentrations up to 50 μM and unexpectedly showed improved iron uptake under low to moderate concentrations (from 0.1 to 50 μM). At 100 μM of Cu, plantlets reduced relative growth rate for both fresh and dry weight and developed severe browning which progressed to necrosis. (Lombardi and Sebastiani, 2005).

Papazoglou *et al.* (2005) grew the giant reed (*Arundo donax* L.) on surface soil and irrigated with mixed heavy metal solutions of Cd(II) and Ni(II) to study the impact of these heavy metals on its growth and photosynthesis. The tested concentrations were 5, 50, and 100 ppm for each heavy metal against the control and resulted in high cadmium and nickel (DTPA extractable) concentrations in the top zone of the pot soil. The examined parameters, namely, stem height and diameter, number of nodes, fresh and dry weight of leaves, and net photosynthesis (Pn) were not affected, indicating that plants tolerate the high concentrations of Cd and Ni. They suggested that as giant reed plants are very promising energy plants, they can be cultivated in contaminated soils to provide biomass for energy production purposes.

The effect of different concentration of Cd on *Phyllanthus amarus* Schum. and Thonn. was investigated, because *P. amarus* is mostly grown as weed in agricultural and waste lands. It is a reputed plant used in Indian indigenous systems of medicine with hepatoprotective, diuretic, stomachic properties and is recently being used for the treatment of hepatitis B. It was observed that Cd causes significant

decrease in fresh and dry weight, length of root and shoot. Moreover, ultramorphological changes were also observed in stomatal opening and wax deposition on both the surfaces of leaves (Rai *et al.* 2005).

Vitoria *et al.* (2005) reported that mesophyll cells from the leaf of radish seedlings exposed to 0.25 and 1.0 mM of $CdCl_2$ during 24 h exhibited structural changes of chloroplasts, mitochondria and nuclei when compared to non-treated control plants. They observed that chloroplasts from Cd^{2+}-exposed samples exhibited changes in the organelle shape, an increase in the stroma volume and a deposition of electron-dense material in the double membrane. The changes in the chloroplast membranes were not very drastic; however a reorganization of the thylakoids and stroma could be detected. In contrast, the breakdown of the nuclear envelope of the plant cells treated with Cd^{2+} was very clear. The accumulation of electron-dense granules was also observed in mitochondria. But no alterations were observed in the vacuoles of radish seedlings grown at different Cd^{2+} concentrations for the periods tested.

Youssef *et al.* (2005) reported that treatment of rape seedlings with increasing $CdCl_2$ concentrations in the culture medium resulted in a cadmium accumulation within plant tissues, which increased with external metal dose; such accumulation was more important in roots than in leaves. Biomass production was severely inhibited, even at low cadmium concentration. They also reported that metallic ion seemed to affect selectively chloroplastic membranes due to an inhibition of polyunsaturated fatty acid biosynthesis. Moreover, a lipid peroxidation occurred because of the spectacular increase of malondialdehyde (MDA) content observed in cadmium treated leaves.

Zheljazkov *et al.*(2005) tested the hypothesis that some essential oil crops could be to grown as alternatives to edible crops in heavy metal enriched soils. Experiments were conducted to evaluate the effect of Cd, Pb, and Cu on yields and essential oils of peppermint, basil, and dill. The accumulation of Cd, Pb, and Cu in plant parts, in plant material and water after distillation, and in the essential oils, were also determined. Metal treatments of peppermint and basil consisted of Cd, Pb, Cu, Cd + Pb, Cd + Cu, Pb + Cu, Cd + Pb + Cu, and unamended control. Metal

treatments of dill consisted of (in mg L^{-1}): Cd at 2, 6, and 10; Pb at 50, 100, and 500; Cu at 20, 60, and 150 and an unamended control.They reported that peppermint and basil yields were not affected by the treatments. Copper at 60 and Cu 150 mg L^{-1} reduced both yields and height of dill, Cu 150 mg L^{-1} resulted in Cu phytotoxicity symptoms and retarded growth. High Pb and Cu reduced Cd uptake by peppermint and basil. At elevated Cd concentrations in the growth medium, Cd transport from roots to shoots of the three species was impaired. The tested treatments slightly altered chemical composition of the essential oils of basil and dill, and reduced the menthol content in the peppermint oil. Oil content in basil from the Cd, Pb, Cu treatment was lower that in the control. Copper application at 150 mg L^{-1} reduced oil content in dill relative to the control. No detectable amount of Cd, Cu, or Pb in the oils of any of the three species were found. They concluded that peppermint, basil, and dill can be grown in soils enriched with Cd, Pb, and Cu medium without risk for metal transfer into the oils, and without significant alteration of essential oil composition that may impair marketability.

In a study by Aina *et al.* (2006) rice seedlings were exposed to a range of Cd concentrations (0.1 μM, 1 μM, 10 μM, 100 μM and 1 mM) for 15 days and a combination of different molecular approaches were used to evidence Cd effects and to assess the plants ability to counteract metal toxicity. At a macroscopical level, only the highest Cd concentration (1 mM) caused a complete plant growth inhibition, whereas the lowest concentrations seemed to stimulate growth.

Giachetti and Sebastiani (2006) evaluated the effects of different levels of industrial wastes on growth traits and metal accumulation in aerial portions for *Populus* × *euramericana* clone I-214. The experiment started in April 2003. Scions of *Populus* × *euramericana* clone I-214, were grown outdoor near Pisa (Italy), in lysimeters filled with soil naturally present in the land around the experimental site. Four increasing treatments were applied: soil non-amended, soil amended with 4.8 kg m^{-2}, with 9.6 kg m^{-2} and with 19.2 kg m^{-2} of fresh tannery waste. The climatic parameters were daily recorded throughout the whole experiment and growth relieves were performed during the growing season. After six months since the plantation of the scions, aerial portions of every plant were harvested for biomass

and metal content analyses. Data demonstrated that the waste exerted beneficial effects on poplars mainly through a general increase of growth traits and that the nutrients relocation is the mechanisms involved in modulating growth rate. The concentration and the amount of the mineral elements analysed (N, P, K, Na, Ca, Mg, S, B, Fe, Mn, Cu, Zn, Cr) changed determinately among treatments, organs and position. They concluded that phytoremediation strategies of tannery wastes might be possible and sustainable for polar plantations in soil amended with non-hazardous levels of industrial waste, which maintain total heavy metals concentration close to background values.

Makino *et al.* (2006) tested the efficiencies of neutral salts, strong acids, and chelates for extracting cadmium (Cd) from three paddy soils. Their test revealed that higher the selectivity of the cations of the added neutral salts toward soil adsorption sites, the lower the pH in the extracts and the more soil Cd could be extracted. In addition, soil carbon and nitrogen contents and mineral composition were closely associated with the amount of Cd extracted. Calcium chloride and iron(III) chloride were selected as wash chemicals to restore Cd-contaminated paddy soils in situ. They concluded that washing with calcium chloride led to the formation of Cd chloride complexes, enhancing Cd extraction from the soils. The washing also substantially decreased soil levels of exchangeable and acid-soluble Cd, which are the major forms of bioavailable Cd for rice (*Oryza sativa* L.).

The effects of low levels of heavy metals on plant growth, biomass turnover and reproduction for *Hieracium pilosella* was evaluated by Ryser and Sauder (2006). Plants were grown for 12 weeks on substrates with different concentrations of heavy metals obtained by diluting contaminated soils with silica sand. It was found that the more metal-contaminated soil the substrate contained, the lower the leaf production rate and the plant mass. The phenological development was also delayed. Flowering phenology was very sensitive to metals. Leaf life span was reduced at the highest and the lowest metal levels, the latter being a result of advanced seed ripening. Even if the effect of low metal levels on plant growth may be small, the delayed and reduced reproduction may have large effects at population, community and ecosystem level, and contribute to rapid evolution of metal tolerance.

Sharma *et al.* (2006) reported that the heavy metal contamination of soil resulting from wastewater irrigation is a cause of serious concern due to the potential health impacts of consuming contaminated produce. They assessed the impact of wastewater irrigation on heavy metal contamination of *Beta vulgaris,* which is a highly nutritious leafy vegetable that is widely cultivated and consumed in urban India, particularly by the poor. A field study was conducted at three major sites that were irrigated by either treated or untreated wastewater in the suburban areas of Varanasi, India according to normal practice. Samples of irrigation water, soil, and the edible portion of the palak (*Beta vulgaris* L. var All green H1) were collected monthly during the summer and winter seasons and were analyzed for Cd, Cu, Zn, Pb, Cr, Mn, and Ni. Their results showed that heavy metals in irrigation water were below the internationally recommended (WHO) maximum permissible limits set for agricultural use for all heavy metals except Cd at all the sites. Similarly, the mean heavy metal concentrations in soil were below the Indian standards for all heavy metals, but the maximum value of Cd recorded during January was higher than the standard. However, in the edible portion of *B. vulgaris*, the Cd concentration was higher than the permissible limits of the Indian standard during summer, whereas Pb and Ni concentrations were higher in both summer and winter seasons. The results of linear regression analysis computed to assess the relationship between individual heavy metal concentration in the vegetable samples and in soil showed that Zn in soil had a positive significant relationship with vegetable contamination during winter. Concentrations of Cd, Cu, and Mn in soil and plant showed significant positive relationships only during summer. Concentration of Cr and Pb during winter season and Zn and Ni during summer season showed significant negative relationships between soil and plant contamination. They concluded that the use of treated and untreated wastewater for irrigation increased the contamination of Cd, Pb, and Ni in edible portion of vegetables causing potential health risk in the long term from this practice. They also pointed to the fact that adherence to standards for heavy metal contamination of soil and irrigation water does not ensure safe food.

A study was conducted to evaluate the use of reclaimed lake sediment as a growth media for vegetable production and to estimate whether accumulation of

micronutrients and heavy metals in the vegetables would impact human nutrition or health, respectively (Stephen *et al.* 2006). Five plant species, bean (*Phaseolus vulgaris* L.), broccoli (*Brassica oleracea* L.), carrot (*Daucus carota* L.), pepper (*Capsicum annum* L.), and tomato (*Lycopersicon esculentum* L.), were grown in pots containing either reclaimed sediment from the Illinois River or a reference soil. Edible and vegetative tissues from the plants were analyzed for 19 elements, including As, Cd, Cr, Cu, Hg, Mo, Ni, Pb, Se, and Zn. Tomato and pepper grown in sediment showed significantly greater biomass and yield as compared to plants from the reference soil. Elemental analysis of the tissues revealed that Zn and Mo were the only elements that were significantly greater in sediment-grown plants on a consistent basis. While significant, Zn concentrations were no more than 3-fold higher than those in plants from the reference soil. The same trend was observed for Mo, except for bean tissues, which showed a 10-fold greater concentration in sediment-grown plants. The results from this study suggest that this reclaimed sediment can be utilized for the production of vegetables intended for human consumption. The results from this case study also suggest that sediment material with similar physicochemical characteristics and elemental concentrations that fall within the pertinent regulatory guidelines should also be a suitable and safe medium for vegetable production.

Xiong *et al.* (2006) reported that copper (Cu) from various anthropogenic and natural sources is one of the major heavy metal contaminants in the environment. To study Cu-induced nitrogen (N) metabolism damage in the popular vegetable Chinese cabbage (*Brassica pekinensis* Rupr.), aquatic culture experiments with this plant were performed. Two Cu levels [0.3 μmol L^{-1} (control) and 10.3 μmol L^{-1}] and two N levels (0.05- and 1-fold Hoagland's solution) were used for the aquatic culture experiments. The results demonstrated adverse effects of Cu on N metabolism and plant growth. Cu exposure elevated Cu concentration in the roots and shoots. It also shortened root length and produced fewer leaves and lower plant biomass. The results also demonstrated effects of N deficiency on N metabolism and plant growth. N deficiency increased the root/shoot ratio of biomass. In addition, there were interactive effects between Cu exposure and N level on plant biomass and

root/shoot ratio. The results suggested that Cu toxicity to the plant was at least partly due to an influence on N metabolism.

Yoon *et al.* (2006) investigated whether phytoremediation can be potentially used to remediate metal-contaminated sites. They evaluated the potential of 36 plants (17 species) growing on a contaminated site in North Florida. Plants and the associated soil samples were collected and analyzed for total metal concentrations. While total soil Pb, Cu, and Zn concentrations varied from 90 to 4100, 20 to 990, and 195 to 2200 mg kg^{-1}, those in the plants ranged from 2.0 to 1183, 6.0 to 460, and 17 to 598 mg kg^{-1}, respectively. None of the plants were suitable for phytoextraction because no hyperaccumulator was identified. However, plants with a high bioconcentration factor (BCF, metal concentration ratio of plant roots to soil) and low translocation factor (TF, metal concentration ratio of plant shoots to roots) have the potential for phytostabilization. Among the plants, *Phyla nodiflora* was the most efficient in accumulating Cu and Zn in its shoots (TF = 12 and 6.3) while *Gentiana pennelliana* was most suitable for phytostabilization of sites contaminated with Pb, Cu and Zn (BCF = 11, 22 and 2.6). Plant uptake of the three metals was highly correlated, whereas translocation of Pb was negatively correlated with Cu and Zn though translocation of Cu and Zn were correlated. They concluded that native plant species growing on contaminated sites may have the potential for phytoremediation.

2.2. Biochemical response of plants to heavy metal stresses

2.2.1.Pigments and other metabolites

The single and combined effects of metals lead, cadmium, nickel and UV and γ-radiation on protein content and protein synthesis in leaves of barley seedlings was investigated by Bhattacharya (1991). He observed that the treatment produced an increase in soluble protein content and rates of protein synthesis. Combined treatments produced changes depending on the stage of irradiation, nature of irradiation, metal content of the substratum and storage period of seeds after irradiation.

Distribution of cadmium and induced Cd-binding proteins in roots, stems and leaves of *Phaseolus vulgaris* was studied by Leita *et al.* (1991). Roots, stems and leaves of *Phaseolus vulgaris* L. (cv.Rubino PF 1H) grown in Hoagland's solution supplemented with 1, 2 and 2.5 mM $Cd(NO_3)_2$ were analyzed. The distribution of Cd in plant tissues showed that total Cd in roots exceeded by about one and two orders of magnitude total Cd of stems and leaves, respectively. Results showed that most of Cd in the apoplast of root, stem and leaf tissues was extractable by complexing it with EDTA. Water extractable Cd in intercellular spaces was present in ionic form as Cd^{2+}. Gel filtration of tissue extracts showed that 83.4% of total Cd was present as free metal ion in extracts of leaves, whereas 56.6% and 48.7% was found in stem and roots extracts, respectively. The remaining part of the total Cd was associated with protein fractions. One type of Cd-protein fraction of about 10 KDa molecular weight (K_{av} 0.54) was present in roots, stems and leaves, binding 24.1%, 43.4% and 16.6% of total Cd, respectively. A second protein fraction with apparent molecular weight > 30 KDa was present only in roots, binding 27.2% of total root Cd. This result was confirmed by SDS-PAGE electrophoresis, showing a Cd-induced protein band common to leaves, stems and roots with an apparent molecular weight 9.2 KDa, which can be interpreted as phytochelatins, and an intensively stained Cd-induced band, present only on root extracts of about 42 kDa apparent molecular mass.

The influence of Cd-stress on the structure and functional activity of the photosynthetic apparatus of 15-day-old barley plants cvs. Obzor and Hemus was investigated by Vassilev *et al.* (2005). Two concentrations, toxic for the growth of plants, namely 3 and 6mg Cd L^{-1} 1/2 Knops' solution were used. The contact of plants with heavy metal lasted 12 days. They observed that in the Cd-treated plants there was a tendency to decrease the photosynthetic rate. At the same time the changes in PS2 functional activity were insignificant, although a part of the chloroplasts were characterized by disturbed ultrastructural organization. Their data evaluating growth reaction, structural and functional changes in the photosynthetic apparatus suggest that at the initial stages of plant development, cv. Hemus is more susceptible to Cd-stress than cv. Obzor.

Baruah and Bharatnath (1996) observed the changes in growth, ion uptake and metabolism of rice (*Oryza sativa* L.) seedlings at excess level of iron. Six rice genotypes *viz.*, Mahsuri, Pankaj, IET 66, TTB 101-14, Biraj and Khoram were grown in sand in a green house with different levels of Fe in nutrient solutions *viz.*, control (2 ppm), 100 ppm and 200 ppm. Leaf yellowing was observed in the seedlings when grown at higher Fe concentration in the medium. Pankaj and TTB 101-14 maintained higher leaf chlorophyll alongwith higher total soluble protein and nitrate reductase activity in the leaves at 100 and 200 ppm Fe. Higher Fe concentration in the medium exerted an inhibitory effect on the contents of macro and micro nutrients in different genotypes. However, Pankaj and TTB 101-114 had relatively higher N, K, Mn and Zn content in the when grown in higher Fe level. They concluded that Pankaj and TTB 101-114 are suitable for growing under higher toxic concentration of iron.

The results of Blinda *et al.* (1997) suggest that the leaf apoplast is a site of preferential accumulation of heavy metals in the shoot when barley seedlings are grown in the presence of cadmium, nickel or zinc in hydroponic medium. They demonstrated that apoplastic proteins increase to an even greater extent in Ni-treated plants, and that the response is intermediate for Cd-treated plants. The possible causes for the increase in apoplastic proteins was focused by the authors.

The effect of cadmium and copper on lipid composition in 17 days old tomato seedlings (*Lycopersicon esculentum* Mill. cv. 63/5 F1) grown in culture solution supplied with two concentrations of Cd or Cu (0, 5 and 50 mu-M) was determined by Ouariti *et al.* (1997). Significant decreases in the content of lipid classes and changes of fatty acid composition were recorded in heavy metal-stressed plants in comparison with controls. Glycolipid contents were decreased more in leaves than in roots by Cd-treatment, but Cu decreased both to similar extents in both organs. Likewise, both metals reduced the phospholipid and neutral lipid contents more in roots than in leaves. In almost all lipid classes the proportion of palmitic acid (16:0) increased, and that of linoleic (18:2) or linolenic (18:3) acid decreased, suggesting that heavy metal treatment induced an alteration in the fatty acid desaturation processes. Furthermore, the accumulation of palmitate (16:0) rather than

stearate (18:0) indicated an alteration in the ratio of products from the fatty acid synthase. Cu was found to be the most unfavourable for plant growth and lipid metabolism.

Massive accumulation of proline in the leaves of *Silene vulgaris* in responses to copper, cadmium and zinc was reported by Schat *et al.* (1997). On the basis of their results they suggested that metal induced proline accumulation depends on the development of the metal induced H_2O_2 deficit in the leaves.

Extractable proteins, free amino acids and the activities of the enzymes protease, leucine amino-peptidase and carboxypeptidase were determined in seedlings of two rice cultivars, Roma and Jaya, raised in $Cd(NO_3)_2$ containing medium. With 500 μM $Cd(NO_3)_2$ the protein level increased by 1.7 to 3.0 times in roots and 0.23 to 1.8 times in shoots of 20 days old seedlings. Also 15 days old plants contained 0.2 to 0.4 times higher amino acid level in roots and 0.4 to 0.8 times higher in shoots compared to non-stressed seedlings. Cd^{2+} treatments significantly reduced protease activity in the shoots. *In vitro* activity of protease was inhibited markedly at concentration in excess of 10 μM Cd^{2+} and leucine amoino-peptidase and carboxypeptidase activities were inhibited by 48-68% respectively in roots, whereas in shoots the activity increased by 36-47% with 500 μM Cd^{2+} concentration (Shah and Dubey, 1997).

Jemal *et al.* (1998) reported that pepper plants (*Capsicum annuum*), like many other plant species, respond when stressed with cadmium chloride by the synthesis of phytochelatins [(γGlu-Cys)$_n$Gly] (PCs) and desglycyl phytochelatins [(γGlu-Cys)$_n$], where n=2–4. Higher molecular weight PCs with a chain length longer than four have also been detected; their synthesis was shown to be dependent upon the duration of the experiments and the concentrations of Cd used in the culture medium. The synthesis of PCs and related peptides in Cd-stressed pepper plants was also strongly suggested by the use of buthionine sulfoximine a specific inhibitor of the γ-glutamyl-cysteine synthetase (enzyme involved in the synthesis of glutathione, the precursor of PCs). Indeed no thiol-containing compounds were detected in crude extracts of Cd-treated pepper plants, when they were grown in the presence of BSO. In addition to the synthesis of PCs and PC derivatives, Cd treatment of pepper plants

also leads to the synthesis of two 10-kDa proteins, which differ in their amino acid composition and are absent in untreated plants. The function and role of these two proteins is still unknown, but they might also be involved in defense mechanisms against heavy metals.

In vivo substitution of magnesium, the central atom of chlorophyll, by heavy metals (mercury, copper, cadmium, nickel, zinc, lead) leads to a breakdown in photosynthesis and is an important damage mechanism in heavy metal-stressed plants. Kuepper *et al.* (1998) presented a number of methods for the efficient *in situ* detection of this substitution (i.e. in whole plants or in chloroplasts). While macroscopic observations pointed to the formation of heavy metal chlorophylls at higher concentrations, fluorescence microscopy enabled the detection of this reaction at very low substitution rates. Furthermore, absorbance spectroscopy of whole cells or isolated chloroplasts also enabled the *in situ* detection of heavy metal chlorophylls. These methods provide practicable approaches in detecting the formation of these compounds *in situ*, avoiding artefacts that might occur using extraction methods based on polar solvents. In addition to the new methods for in situ detection, an extreme heterogeneity in the reaction of cells in the same tissue upon heavy metal stress was observed: while some cells are already disintegrating, others still show normal fluorescence and photosynthetic activity. Measurements of fluorescence kinetics gave a further hint that in high light intensity a substitution of Mg by heavy metals might take place specifically in PS II reaction centres.

Accumulation of proline in response to toxic heavy metal exposure was reported by Sharma *et al.* (1998). To elucidate the role for proline in plant responses to heavy metal stress, they studied the effect of proline on Cd-induced and Zn-induced inhibition of glucose-6-phosphate dehydrogenase (G-6-PDH; EC 1.1.1.49) and nitrate reductase (NR; EC 1.6.6.2) *in vitro*. Proline appeared to protect both enzymes against Zn and, though less effectively, against Cd. Measurements with a Cd^{2+}-specific electrode strongly suggested that this protection was based on a reduction of the free metal ion activity in the assay buffer, due to the formation of metal-proline complexes. There were no indications of any significant role for proline-water or proline-protein interactions.

The production of stress ethylene in carrot plants was reported to be highly stimulated by 1 mM Cd. A pre-treatment with buthionine sulfoximine (BSO) did not further increase ethylene production. After being treated with Cd, both plants and cell suspensions produced phytochelatins, and no lipid peroxidation was detected. In cell cultures, the in vitro activity of phytochelatin synthase was assayed in the presence of Cd and glutathione: the first product (PC_2) was detected in less than 30 min. Absence of ethylene (after treatment with aminoethoxyvinylglycine (AVG), an inhibitor of ethylene-biosynthesis, or use of ethylene traps) caused both a decrease in the phytochelatin synthase activity of cell suspensions and a strong lowering in the Cd-induced SH groups in plants. However 1-aminocyclopropane-1-carboxylic acid (ACC) supply did not increase either phytochelatin synthase activity or total SH level (Toppi *et al.* 1999).

An experiment was conducted by Chugh and Sawhney (1999) to determine the effect of cadmium in one-month-old pea seedlings (*Pisum sativum* L. cv. Bonneville). Seedlings were raised in sand culture and provided with a nutrient solution containing 0, 2.5, 5, 7.5 and 10 mM Cd and the effect on various aspects of photosynthesis were investigated after 6 and 12 days of the treatment. The rate of photosynthesis, chlorophyll content, activities of photosystem I (PS I) and II (PS II) and a few selected photosynthetic enzymes *viz.* ribulose-1, 5-bisphosphate carboxylase (EC 4.1.3.9), NADP-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13), fructose-1,6-bisphosphatase (EC 3.1.3.11) and NADP-malate dehydrogenase (EC 1.1.1.82) declined progressively with increasing concentration of applied Cd. As compared to the other parameters such as dm^{-2} leaf area, mg^{-1} fresh weight (FW) and mg^{-1} chlorophyll (chl), the rate of photosynthesis showed maximum decline on per plant basis. On extending the period of exposure to 12 days, the rate of photosynthesis and activities of enzymes showed a further decline. Cd had a more pronounced effect on the activity of PS II during the initial stages; however, on prolongation of the exposure for 12 days to the heavy metal, the functioning of PS I was also equally affected. Addition of Cd to the chloroplasts isolated from untreated plants impaired the functioning of PS II without any discernible effect on that of PS I. In presence of 0.1 mM Cd in the reaction mixture,

activity of PS II was inhibited by 46 % whereas that of PS I remained unaffected. Compared to the effect on the rate of photosynthesis (mg^{-1} chl basis) and on photosystems, activities of photosynthetic enzymes showed far greater inhibition 12 days after the treatment.

An experiment on reed plants fed with heavy metal concentrations of 100 μM Cu^{2+} and 10 mM Fe^{2+} under hypoxia was carried out. Authors found 1 mg g^{-1} dry weight (DW) Cu^{2+} and 8 mg g^{-1} DW Fe^{2+} in roots of reed plants when fed with heavy metal concentrations of 100 μM Cu^{2+} and 10 mM Fe^{2+} under hypoxia. Roots seemed to act as a kind of filter since the amounts in rhizomes were only 0.06 mg Cu^{2+} g^{-1} DW and 2 mg Fe^{2+} g^{-1} DW. Increased contents of both ions reduced post hypoxic respiration capacity by 40–50% and also the sum of adenylates (ATP, ADP, AMP) by the same order of magnitude, although energy charge values remained above 0.85 in Cu^{2+} and 0.79 in Fe^{2+} treatments. Although the energy metabolism of rhizomes was not affected. Cu and Fe contents of roots as well as of rhizomes were high enough to induce oxidative stress when roots were fed with 40 μM Cu^{2+} and 1 mM Fe^{2+}, respectively. Furtig *et al.* (1999) concluded that increased, but environmentally attainable, amounts of copper and reduced iron ions disturb root energy metabolism, and therefore root functioning and development. Latent injuries, based on oxidative stress, may be harmful for roots and rhizomes under long term exposure.

The induction and heavy metal binding properties of phytochelatins in heavy metal tolerant (*Silene vulgaris*) and sensitive (tomato) cell cultures, in water cultures of these plants and in *Silene vulgaris* grown on a medieval copper mining dump were investigated by Leopold *et al.* (1999). Application of heavy metals to cell suspension cultures and whole plants of *Silene vulgaris* and tomato induced the formation of heavy metal–phytochelatin-complexes with Cu and Cd and the binding of Zn and Pb to lower molecular weight substances. The binding of heavy metal ions to phytochelatins seemed to play only a transient role in the heavy metal detoxification, because the Cd- and Cu-complexes disappeared in the roots of water cultures of *Silene vulgaris* between 7 and 14 days after heavy metal exposition. Free heavy metal ions were not detectable in the extracts of all investigated plants and cell

cultures. *Silene vulgaris* plants grown under natural conditions on a mining dump synthesized low molecular weight heavy metal binding compounds only and showed no complexation of heavy metal ions to phytochelatins. Authors suggested that the induction of phytochelatins is a general answer of higher plants to heavy metal exposition, but only some of the heavy metal ions are able to form stable complexes with phytochelatins. The investigation of tolerant plants from the copper mining dump showed that phytochelatins are not responsible for the development of the heavy metal tolerant phenotypes.

The relationship between the toxicity of Cr(III) ions and oxidative reactions in plant cells was studied by Panda and Patra (2000). Leaves from 7 and 9 days old wheat seedlings were incubated in various concentrations of Cr(III) ion containing solutions for 24 h in the light. Breakdown of chlorophyll, carotenoid and increase in membrane permeability and lipid peroxidation was noticed at higher concentrations of Cr(III) ions. Free radical scavengers such as mannitol and sodium benzoate prevented the increase in the senescence parameters. Both mannitol and sodium benzoate were effective for the leaves of both ages. Cr(III) ions increased the catalase activity in younger leaves while the activity decreased in older ones. Peroxidase activity decreased with increasing Cr(III) ion concentration. A slight increase in superoxide dismutase activity was seen in both the leaves at lower Cr(III) level while it decreased at higher concentrations. Free radical scavengers protected these enzymes against inactivation. Their results indicate an excess Cr(III) mediated oxidative reactions in the light, which accelerated leaf senescence.

In a study it was observed that mangroves possess a tolerance to high levels of heavy metals, yet accumulated metals may induce subcellular biochemical changes, which can impact on processes at the organism level. Six month-old seedlings of the grey mangrove, *Avicennia marina* (Forsk.) Vierh, were exposed to a range of Cu (0–800 $\mu g\ g^{-1}$), Pb (0–800 $\mu g\ g^{-1}$) and Zn (0–1000 $\mu g\ g^{-1}$) concentrations in sediments under laboratory conditions, to determine effects on photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and carotenoids), and the activity of the antioxidant enzyme peroxidase. Significant increases in peroxidase activity and decreases in photopigments were found with Cu and Zn at concentrations lower than

those inducing visible toxicity. Significant increases in peroxidase activity only, were found when plants were exposed to Pb. Positive linear relationships between peroxidase activity and leaf tissue metal concentrations were found for all metals. Significant linear decreases in photosynthetic pigments with increasing leaf tissue metal concentrations were observed with Cu and Zn only. Photosynthetic pigments and peroxidase activity may be applicable as sensitive biological indicators of Cu and Zn stress, and peroxidase activity for Pb stress in *A. marina* (MacFarlane and Burchett, 2001).

Chlorophyll, organic (citric and malic acids) and abscisic acid (ABA) contents and stem water potential was measured by Monni *et al.* (2001) to indicate possible physiological effects of heavy metal deposition on *Empetrum nigrum* L. (crowberry). The leaves and stems of *E. nigrum* were collected at distances of 0.5 and 8 km from the Cu–Ni smelter at Harjavalta, south-west Finland. All the investigated parameters were clearly affected by heavy metal emissions. Chlorophyll contents in the leaves and organic acid contents in the leaves and stems were lower close to the emission source. In contrast, ABA contents in stems and leaves in general, were higher in plants growing 0.5 km from the pollution source. Close to the smelter the stem water potential of *E. nigrum* was less negative during the day but more negative during the night. These results suggest that smelter emissions have a negative effect on the ecophysiology of *E. nigrum* even though it is considered to be a tolerant species to heavy metals.

An experiment was conducted by Prasad *et al.* (2001) to find out the effect of heavy metals in aquatic plants known to accumulate and bioconcentrate heavy metals. Several physiological responses of aquatic vascular plant *Lemna trisulca* L. to elevated concentrations of cadmium (up to 10 mM) and copper (up to 50 μM) were investigated. They found that *Lemna* fronds were able to accumulate both Cd and Cu, but Cu-treated material showed pronounced toxic symptoms at concentrations 1000-fold lower in comparison to Cd. *Lemna trisulca* could tolerate elevated levels of Cd, i.e. up to 10 mM, without significant changes in photosynthetic pigments concentration. On the contrary, Cu in concentrations 25 and 50 μM promoted significant pigment degradation. The main processes affected by

Cd in *Lemna* fronds were total gas exchange and net photosynthesis. On the contrary, the inhibition of total gas exchange and net photosynthesis caused by Cu (2–50 μM) correlated with Chl *a* and carotenoid concentrations decrease as well as with the decay of fluorescence from PS II. Also, an increasing impact of respiration in total oxygen exchange was observed after treatment of *Lemna* with increasing Cd concentrations (up to 5 mM) and with Cu in concentration range between 2 and 50 μM. In Cd-treated fronds, a dose-dependent accumulation of two polypeptides with apparent molecular weights 18 and 10 kDa, respectively as well as the appearance of two smaller polypeptides (apparent molecular weights 8 and 7 kDa) was observed in SDS-PAGE. On the contrary, in Cu-treated fronds neither accumulation of existing proteins nor appearance of any extra protein was observed.

Kim *et al.* (2003) determined the effect of cadmium (II), lead (II), copper (II) and zinc (II) in *Polygonum thunbergii* and soil from the Mankyung River watershed, Korea. Soil samples contained detectable lead (<17.5 μg g^{-1}), copper (<8.4 μg g^{-1}) and zinc (<24.5 μg g^{-1}), whereas Cd was undetectable. Whole plants of *P. thunbergii* contained detectable Pb (<320.8 μg g^{-1}), Cu (<863.2 μg g^{-1}) and Zn (<2427.3 μg g^{-1}), whereas Cd was detectable only in the stem (<7.4 μg g^{-1}) and root (<10.1 μg g^{-1}). Whole plant concentrations were very different for each metal, particularly in the case of Zn. The mean content of heavy metal in the whole plants increased in the order of Cd (8.5 μg g^{-1}) < Pb (183.3 μg g^{-1}) < Cu (548.1 μg g^{-1}) < Zn (1506.7 μg g^{-1}). Soil Pb, Cu and Zn were correlated with each metal's accumulation in the plants (Pb, r=0.841, P<0.005; Cu, r=0.874, P<0.001; Zn, r=0.770, P<0.005). Pb content in roots and leaves was highly correlated (r=0.5529, P<0.001), as was Pb content in roots and stems (r=0.5425, P<0.001). Mean bioconcentration factors for the aboveground tissues were 4.2 (Pb), 14.8 (Cu) and 27.7 (Zn), and for the underground tissues, were 22.2 (Pb), 92.9 (Cu) and 62.7 (Zn). After hydroponic growth, bioaccumulation coefficients were 2.0 (Cd), 3.2 (Pb), 17.2 (Cu) and 13.1 (Zn) for whole plants.

It was observed that higher concentrations of Zn and Cr decreased the pigment content in senescing rice leaves with the increasing duration of excision. Accumulation of osmolyte proline was noticed with increasing metal concentration.

Lipid peroxidation measured in terms of thiobarbituric acid reactive substance content increased in senescing leaves concurrently in total peroxide content. Ascorbate content showed an increase under heavy metal treatment whereas the activities of catalase, guaiacol peroxidase and superoxide dismutase decreased with the increase in the period of excision and metal concentration. Their investigation showed acceleration senescence in rice under heavy metal toxicity stress (Panda and Khan, 2003).

Nielsen *et al.* (2003) studied the effects of elevated Cu^{2+} on developmental processes in embryos of the brown algae *Fucus serratus* (Phaeophyceae). Elevated Cu^{2+} was shown to inhibit fixation of the zygotic polar axis but not its formation. Actin localization was unaffected by elevated Cu^{2+} but polarized secretion, which occurs downstream, was inhibited. Significant differences in tolerance to Cu^{2+} were observed for polarization and rhizoid elongation of embryos derived from adults from Cu^{2+} contaminated and uncontaminated locations. Moderate Cu^{2+} exposure inhibited the generation of cytosolic Ca^{2+} signals in response to hypo-osmotic shocks. In contrast, cytosolic Ca^{2+} was elevated by treatments with high (Cu^{2+}) and this coincided with production of reactive oxygen species. Their results indicate that direct effects on signalling processes involved in polarization and growth may in part explain complex, concentration-dependent effects of Cu^{2+} on early development.

The response of cultured spruce cells to heavy metals in aqueous solution, and at characterizing these basic cellular responses as potential biomarkers was investigated by Schroeder *et al.* (2003). In order to characterize cell reactions toward heavy metals, spruce cell cultures were incubated with $CdSO_4$ (50 to 500 muM), Na_2HAsO_4 (1.5 to 80 muM) or $PbCl_2$ (10 to 150 muM). Alternatively, the cells were incubated with a standard heavy metal mixture containing 80 muM Na_2HAsO_4, 150 muM $CdSO_4$ and 150 muM $PbCl_2$ in medium and with aqueous original soil eluates. Measurement of oxidative stress, antioxidants and basic detoxification enzymes involved in plant defence reactions were performed with the treated cells. After 5 h of incubation, the onset of a strong oxidative burst was observed. H_2O_2 concentrations exceeded 40 μM in the culture media after 20 h. Concomitantly, glutathione levels showed drastic changes indicating the influence of the metals

and/or the H_2O_2 on antioxidative systems. Following Cd treatment, GSH and GSSG were elevated by 50 and 200% above controls. Whereas As doubled GSSG levels, treatment with Pb did not cause significant changes. However, a mixture of the metals decreased both metabolites by 50%. The effect of the metals was concentration-dependent and disappeared at high concentrations. Furthermore, strong induction of glutathione S-transferase (GST) subunits was observed and, although no novel subunit was expressed, the rise of a new GST isoform occurred. Authors concluded that the most potent inducer of plant defense reactions is Cd, followed by As and Pb in descending order of effectiveness. Counter ions seem to play an important role, e.g. lead chloride influenced the investigated parameters much more than lead acetate. The investigated metals activate gene expression through signal transduction pathways previously not associated with these metals, which points to new end points for resistance and toxicity testing. Especially a monitoring of GST subunit behaviour together with quantifying the oxidative burst seem to be promising for a biomonitoring concept. The close regulation of plant observed may facilitate the setup of an integrated biotest for heavy metal pollution that could be based on enzymological as well as proteome data. They further concluded that heavy metals cause stress to plant cells and elicit a whole range of answers, although specific for individual metal species. The differences observed in plant answers are suitable to distinguish between metals bioavailable in soil eluates and water samples, however only at concentrations in the muM range. It will be necessary to evaluate the effects on the RNA and transcript level. They recommended that similar plant metabolic end points and enzyme reactions be screened for their suitability as biotest systems.

The effect of varying Cd^{2+} concentrations up to 21 days on biomass productivity, plant growth, photosynthetic pigments, protein, amino acids, starch, soluble sugars, and essential nutrients uptake was studied in detail by Shukla *et al.* (2003) to explore the level up to which the wheat plant can withstand the stress of heavy metal. Wheat plants treated with 0.5, 1.0, 2.5, and 5.0 mg L^{-1} Cd^{2+} showed symptoms of heavy-metal toxicity as observed by various altered levels of major biochemical constituents such as chlorophyll, protein, free amino acids, starch, and

soluble sugars. All these constituents play a major role in plant metabolism in response to varying concentrations of Cd^{2+} in the nutrient medium.

An *et al.* (2004) tested *Cucumis sativus* (cucumber) to assess an ecotoxicity in soils contaminated by the heavy metals copper, cadmium and lead separately and in combinations. The toxicity endpoint was plant growth, which was measured as shoot and root lengths after 5 days exposure. Sum of toxic unit (TU) at 50% inhibition for the mixture ($EC50_{mix}$) was calculated from the dose (TU-based)–response relationships by the Trimmed Spearman–Karber method. Binary metal combinations of Cu+Cd, Cu+Pb, and Cd+Pb produced all three types of interactions; concentration additive ($EC50_{mix}$=1TU), synergistic ($EC50_{mix}$<1TU), and antagonistic ($EC50_{mix}$>1TU) responses. Ternary combination of Cu+Cd+Pb produced an antagonistic response for the growth of *Cucumis sativus*. Bioaccumulations of Cu, Cd, and Pb were observed in *Cucumis sativus* and the bioaccumulation of one metal was influenced by the presence of other metals in metal mixtures. In general, antagonistic and/or synergistic responses reflected bioaccumulation patterns in some binary combinations, but the patterns in mixtures were not always consistent with toxicity data. The study indicated that TU approach appears to be a good model to estimate the combined effect of metals in plant systems, and mixture toxicity may be closely-related to the bioaccumulation pattern within plants.

Backor *et al.* (2004) determined copper uptake, potassium efflux and free proline accumulation in copper-enriched liquid cultures of wild-type *Trebouxia erici* as well as in copper-tolerant strain. By using inductively coupled plasma atomic emission spectrometry they found that the highest intracellular copper uptake from 2 mM Cu media occurred within 4 h in both strains, but significantly less was accumulated by the tolerant strain. The copper-tolerant strain exhibited significantly more intracellular proline and significantly less potassium efflux than the wild strain. By 24 h differences between strains in intracellular Cu diminished, as concentrations in both strains reached their highest levels. At the same time proline accumulations decreased significantly. Growth, pigment content, chlorophyll *a* degradation and chlorophyll *a* fluorescence were decreased by high copper concentration in the agar media after 2 weeks of cultivation, more pronounced in wild-type of *T. erici*. Adding

proline alleviated the toxic effects of Cu in both strains, but markedly so in the case of the tolerant strain.

The changes in fresh weight, total protein amounts, cadmium concentration and glutathione content in maize kernels cultivated for 5 days at three different Cd concentrations (0, 10 and 100 μmol L^{-1} $CdCl_2$) were determined in a study. Maize kernels exposed to the highest cadmium concentration (100 μmol L^{-1}) germinated formerly and much better. A rapid increase of the fresh weight probably relates with more intensive uptake of water in order to decrease cadmium concentration. An intensive preservation of homeostasis of Cd^{2+} ions in the germinating plants by defending mechanisms might explain differences of uptake rate of cadmium. The linear increase of GSH content with the exposure time at all studied concentration suggests the defending mechanisms might be triggered by concentrations of a heavy metal (Klejdus *et al.* 2004).

Mazen (2004) reported that *Corchorus olitorius* plants treated by 5 μg cm^{-3} of Cd, Pb, Al or Cu in hydroponic culture accumulated in leaves 190, 150, 350 and 325 μg g^{-1}(dm) of these metals, respectively, after 6 days of exposure. Exposure of *Corchorus* plants to tested metals resulted in a sharp rise in content of amino acids in leaf tissues, however the magnitude of accumulation was different from one metal to another. Presence of sulphur in the growth medium significantly increased uptake of Cd and Pb; and cysteine (cyst) was more effective than K_2SO_4. Similarly, addition of salicylic acid (SA) in the growth medium significantly enhanced the ability of *Corchorus* plants to accumulate all these metals. Growth of *Corchorus* plants was significantly reduced by treatment with any of the four metals except Cu and added cys, K_2SO_4 or SA alleviated the growth retarding effect of metals.

The potential accumulation of Cd(II), Cr(VI), and and Cu(II) in *Convolvulus arvensis* L. using an agar-based medium was determined by Torresday *et al.* (2004). They found that shoots of *C. arvensis* plants exposed to 20 mg L^{-1} of these heavy metals, demonstrated capability to accumulate more than 3800 mg of Cr, 1500 mg of Cd, and 560 mg of Cu kg^{-1} of dry tissue. The outcome of this study and the field data previously reported corroborate that *C. arvensis* is a suitable candidate for the phytoremediation of Cd(II), Cr(VI), and Cu(II) contaminated soils.

Furthermore, the concentration of Cr determined in the dry leaf tissue (2100 mg kg^{-1}) indicates that *C. arvensis* could be considered as a potential Cr-hyper accumulator plant species.

The effects of sewage sludge application to barley (*Hordeum vulgare* L.) var. Sunrise were investigated. Treatments were: (1) fertilization with a conventional inorganic fertilizer (M); (2) 15 t ha^{-1} of sludge in 1998 only (RS); (3) cumulative sewage sludge application, i.e., repeated applications of 15 t ha^{-1} every year (CS); and (4) unammended soil as control (C). Cumulative application of sewage sludge to barley crop increased grain yield significantly, which might be associated with improved early establishment of seedlings. The plants had higher dry matter yields and leaf protein concentrations from the beginning of development to ear emergence. These CS plots had lower pH, and increased total organic C (TOC), cation exchange capacity (CEC) and DTPA-extractable heavy metals. The treatment also improved soil microbiological properties, such as basal respiration, microbial biomass and some soil enzyme activities (urease, BAA-protease, phosphatase and β-glucosidase), which promote the recycling of nutrients for crop. Sewage sludge had a positive but short residual effect after only 1-year application. Results of this study conducted by Antolin *et al.* (2005) indicate that relatively low application rates of sewage sludge could be used for several years to maintain crop production in Mediterranean-type climates. However, there was a significant increase of grain heavy metal concentrations that must be taken into consideration under long-term applications of sludge.

In an investigation by Chaoui and El Ferjani (2005) 12 days old seedlings of pea were treated for 4 days by 20 and 100 μM of $Cd(NO_3)_2$ or $CuSO_4$. They observed that in leaves, all treatments caused an increase in the lipoperoxidation product rate but 20 μM of Cu did not affect the growth. Moreover, except for 20 μM of Cu, the activity of unspecific peroxidases, used as stress marker, was enhanced in cell walls of metal-stressed plants. However, no change in the antioxidant capacities was observed in plants treated with 20 μM of metal. At this dose, the Cd-reduced growth could be associated to an elevation in the activities of IAA oxidase and of lignifying peroxidases. Increase of these latter, in concert with loss in antioxidant

capacities, would be responsible for the growth diminution after exposure to 100 μM of metal.

An investigation was conducted by Marcano *et al.* (2005) on the effects that the extracts of a non-carbonaceous meteorite could have on the nutrient source for young plants of [(*Lycopersicon esculentum* and *Daucus carota*) and one monocotyledon (*Zea mays*)] edible types. Solution cultures were developed using seeds, seedlings and seed-embryos. Phaeophytinization index and chlorophyll a/b ratio, suggesting a negative effect of the heavy metals or acidic ions over the photosynthetic activity when extracts having high meteoritic concentrations were utilized. However, they found a higher chlorophyll a production in comparison to that of chlorophyll b in extracts (Type-A and -B) with low concentrations of meteoritic matter. On the other hand, *Z. mays* seed-embryos growing in extracts (Type-D) having 3.53×104 mg L^{-1} of meteoritic matter showed a protein production (9.81×10^{-2} mg protein mg^{-1} wet wt) higher than that observed in seed-embryos coming from extracts having lower concentrations. However, in Murashige medium, the seed-embryos exhibited a higher protein production (10.3×10^{-2} mg protein mg^{-1} wet wt). Further, chlorophyll (a+b) synthesis was higher in Murashige medium than in meteoritic extracts but chlorophyll a/b ratio was <1 in all extracts and controls. Their results suggest the usefulness of the non-carbonaceous meteoritic resource as a complementary soil component or fertilizers for culture of edible plants in space settlements and mainly for the production of young plants due to the positive metabolic effects on the chlorophyll synthesis, mitochondrial metabolism and cellular division caused by PO_4^{3-}, Fe^{2+}, Cu^{2+} and Ca^{2+} ions.

Rai *et al.* (2005) investigated the effect of different concentration of Cadmium (Cd) on *Phyllanthus amarus* Schum. and Thonn. The study revealed that Cd causes significant decrease in protein, chlorophyll, carotenoids and sugar and increase in starch content. They noted that the therapeutically active compounds—phyllanthin and hypophyllanthin, enhanced at certain levels of Cd due to abiotic stress.

In an experiment conducted by Aina *et al.* (2006) rice seedlings were exposed to a range of Cd concentrations (0.1 μM, 1 μM, 10 μM, 100 μM and 1 mM)

for 15 days and a combination of different molecular approaches were used to evidence Cd effects and to assess the plants ability to counteract metal toxicity. At genome level, the amplified fragment length polymorphism (AFLP) technique was applied to detect DNA sequence changes in root cells, showing that all the Cd concentrations induced significant DNA polymorphisms in a dose-dependent manner. Data also evidenced the absence of preferential mutation sites. Plant responses were analysed by measuring the levels of gluthatione (GSH) and phytochelatins (PCs), the thiol-peptides involved in heavy metal tolerance mechanisms. Results showed a progressive increase of GSH up to 10 μM of Cd treatment, whereas a significant induction only of PC_3 was detected in roots of plants exposed to 100 μM of Cd. As suggested by the proteome analysis of root tissues, this last concentration strongly induced the expression of regulatory proteins and some metabolic enzymes. Furthermore, the treatment with 10 μM of Cd induced changes in metabolic enzymes, but it mainly activated defense mechanisms by the induction of transporters and proteins involved in the degradation of oxidatively modified proteins.

An investigation was undertaken by Labra *et al.* (2006) to examine the influence of different concentrations of potassium dichromate on the *Zea mays* L. plantlets. A clear effect of chromium on maize plantlets growth and seed germination was observed starting from 100-300 ppm up to 1500 ppm. Chromium uptake was dependent on the concentration in the medium. They reported that the metallothioneins, involved in heavy metal binding, were measured by capillary electrophoresis (CE), and showed a dose-response induction. Protein profile analyzed by two-dimensional gel electrophoresis showed differential expression of several proteins. Their results showed that proteins induced by heavy metal exposure are principally involved in oxidative stress tolerance or in other stress pathways. Induction of proteins implicated in sugar metabolism was also observed. They inferred that the identification of factors involved in plant response may lead to a better understanding of the mechanisms involved in cell protection and tolerance.

In an investigation by Li-an *et al.* (2006) the growth responses of *Poa pratensis* to the stresses of Cu^{2+}, Zn^{2+},Cd^{2+} and Pb^{2+} at different concentrations were

studied by sand culture. The results revealed that with Cu^{2+},Zn^{2+},Cd^{2+} concentrations reaching 100 mg L^{-1},both the seed germination rates and young-seedling heights of *P. pratensis* declined to some extent and their decrements increased as the concentrations increased. Pb^{2+} did not show significant effect on these two indexes. Cu^{2+} inhibited the root and aboveground biomasses and the inhibitory effect on the root growth was particularly significant; with Cu^{2+} concentration coming to 600 mg L^{-1}, the root lengths decreased by as high as 96. 67%, compared with those in the control. With Zn^{2+}, Cd^{2+} and Pb^{2+} concentrations going above 200 mg L^{-1}, the root and aboveground biomasses of *P. pratensis* appeared to be inhibited, and the inhibitory effects became intensified as the concentrations increased. The four heavy metals appeared undifferentiated in their action patterns on chlorophyll, i. e. they enhanced chlorophyll synthesis with their concentrations standing below 200 mg L^{-1} and the chlorophyll contents declined as their concentrations continued to increase after reaching 200 mg L^{-1}.

Michalak (2006) reported that one of the adverse effects of heavy metals on plants is the generation of harmful active oxygen species, leading to oxidative stress. The results showed 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. In the study it was concluded that phenolics, especially flavonoids and phenylopropanoids, are oxidized by peroxidase, and act in H_2O_2-scavenging, phenolic/ASC/POX system

Page *et al.* (2006) conducted an experiment on seedlings of wheat (*Triticum aestivum* L.) and white lupin (*Lupinus albus* L.) radiolabelled for 24 h with 65Zn, 109Cd, 54Mn and 57Co via one seminal root (wheat) or via the main root (lupin). Plants were afterwards grown on rhizoboxes containing soil. Samples were collected throughout the experiment and analysed afterwards for their radionuclide contents. A strong retention in the labelled part of the root was observed for 57Co in wheat and lupin and for 109Cd in lupin, while 65Zn and 54Mn were transported to the shoot in both plants. While 65Zn was redistributed via the phloem from older to younger leaves, 54Mn accumulated in the first leaves and no major redistribution within the shoot was observed. 109Cd was present in the shoot of wheat but not in the shoot of

lupin. The redistribution of 65Zn, 109Cd, 54Mn and 57Co in the phloem differed between wheat and lupin. The 65Zn content in the wheat roots appearing after the labelling phase represented 34% of the total content in the plant at the end of the experiment and less than 3% remained in the labelled root, while a high percentage of 65Zn was retained in the originally labelled part of the main root of lupin. Smaller quantities of 109Cd, 54Mn and 57Co accumulated in all parts of the root system of wheat and lupin. Nevertheless, heavy metals were found in rhizosphere soil (1–2 mm soil around the roots) and bulk soil (no contact with roots) from both plants. Higher quantities of heavy metals were found in the rhizosphere soil close to the labelled part of the root. 65Zn was present in large quantities in the rhizosphere soil close to all parts of the root system of wheat. For both plants, 65Zn, 109Cd, 54Mn and 57Co were found in the bulk soil indicating that the plant itself might play a role in the redistribution of heavy metals in the soil around its own roots. Phloem-mobile elements may be transported to growing parts of the root system and may reach deeper soil layers. The redistribution of heavy metals in the soil may be in vertical and horizontal directions; at least as far as the root system grows.

Pendergrass and Butcher (2006) carried out an experiment in the Barber Orchard, Haywood County, NC site which has been designated a U.S. EPA Superfund site, primarily because of elevated levels of lead and arsenic. In this experiment carrots, lettuce, and tomatoes were cultivated in a greenhouse in control soil and soil obtained from Barber Orchard. The resulting samples were then analyzed for Pb and As using inductively coupled plasma optical emission spectrometry (ICP–OES). Except for carrot roots grown in the contaminated soil, the concentrations of Pb and As in the plants were below the ICP-OES detection limit. The concentration of Pb in carrot roots was $20 \pm 11 \mu g\ g^{-1}$, which represents a bioconcentration factor (BCF) of 0.03.

Cu-induced nitrogen metabolism damage in the popular vegetable Chinese cabbage (*Brassica pekinensis* Rupr.), were observed by Xiong *et al.* (2006) in aquatic culture experiments. Two Cu levels [0.3 μmol L^{-1} (control) and 10.3 μmol L^{-1}] and two N levels (0.05- and 1-fold Hoagland's solution) were used for the purpose. Cu exposure decreased nitrate reductase (NR) activity in the roots and

shoots and reduced total chlorophyll content. Treatments increased total free amino acid content in the leaves and decreased the nitrate contents and NR activity in roots and leaves. In addition, there were interactive effects between Cu exposure and N level on chlorophyll and nitrate content in leaves.

Exposure of seedlings of *Lepidium sativum* (L.) to increasing concentrations of Cd resulted in the growth inhibition and in the accumulation of proteins in the 10–25 kDa range in cotyledons and hypocotyls of the plantlets. Most of these proteins were also found in extracts of *L. sativum* seeds. Analysis by ESI-MS after two-dimensional electrophoresis showed that these proteins exhibit sequences similar to those of storage proteins from various Cruciferae species. According to Gianazza *et al.* (2007) the response to metal exposure during germination and initial plantlet elongation thus involves inhibition of both storage protein catabolism and plant protein anabolism. In addition, two of the proteins present in higher amounts in plantlets exposed to Cd heat-shock, in agreement with literature data, and jasmonate-like inducible protein are related to cellular stress and another two (LEAs or late embryogenesis abundant) are involved in embryogenesis. Changes in protein expression can be detected by two-dimensional electrophoresis after exposure to heavy metal concentrations lower than those at which morphometric changes become evident. Proteomics of germinating *L. sativum* thus constitutes a very sensitive tool for evaluating environmental pollution.

2.2.2. Antioxidative enzymes

The toxicological effect of Cr(VI) on some biochemical parameters in pepper were studied both in soil culture and nutrient culture experiments by Zhou *et al.* (1990). They reported that treatments of heavy metals decreased fresh weight and promoted senescence of the pepper plant by decreasing chlorophyll and activities of superoxide dismutase and catalase as well as increasing iron content and peroxidase activities over control values.

In a later study, it was reported that pepper (*Capsicum annuum* L.) plants growing in a nutrient solution with excess copper, showed an increase in shikimate dehydrogenase (SKDH, EC 1.1.1.25) and peroxidase (EC 1.11.1.7) activities in the

hypocotyl. In the roots, peroxidase was also induced, but SKDH activity per organ was depleted rather than enhanced. Cu stress caused stunting in the plants, reflected by a decrease in the fresh weight of all the organs. In the hypocotyl, the induction of both enzymatic activities was associated with the accumulation of soluble phenolics and lignin. The two SKDH isozymes present in the control hypocotyls (SKDH-3 and SKDH-4) increased in a similar proportion after Cu stress. In the case of peroxidases, two new isozymes (PRX-A2 and PRX-A4) were detected in cu-stressed hypocotyls, and the other two isoperoxidases, PRX-B and PRX-A3, were enhanced ten and three times, respectively, with respect to the control. The application of the chelator EDTA was able to counteract all the stress effects of the metal cited above. (Díaz *et al.* 2001).

The influence of Cd was also studied on pepper by Leon *et al.* (2002). They investigated the effect of growing five different cultivars of pepper plants (*Capsicum annum* L.) with $CdCl_2$ concentrations ranging from 0.125 to 0.5 mM on different physiological parameters, and antioxidative enzyme activities of leaves was investigated by They found that on the basis of growth parameters, pepper plants were relatively tolerant to Cd, although metal concentrations higher than 0.125 mM produced a significant inhibition of growth and net photosynthesis, and water use efficiency. Different sensivities to Cd^{++} ions were observed among cultivars, Abdera being the most resistant to cadmium stress, while Mondo and Herminio were the most sensitive cultivars. Cadmiun concentrations of 0.5 mM produced an increase in the activity of glutathione reductase, and guaiacol peroxidase in most cultivars, while catalase and superoxide dismutase (SOD) were slightly depressed. The analysis of the SOD activity pattern by native-PAGE showed the presence in most cultivars of four SODs which were identified as Mn-SOD, Fe-SOD, CuZn-SOD I and CuZn-SOD II. However, the CuZn-SODs were absent in the Cd-sensitive cv. Herminio. The growth of pepper plants with 0.5 mM Cd inhibited the activity of CuZn-SODs in all cultivars, while the activity of Mn-and Fe-SOD was enhanced. The activity of NADPH - dehydrogenase (glucose - 6 - P- dehydrogenase, 6 - phosphogluconate dehydrogenase, NADP - isocitrate dehydrogenase and malic enzyme) showed a Cd-dependent enhancement in most cultivars, the highest increase being in the tolerant

cv. Abdera. Their results suggest that in pepper plants the tolerance to Cd toxicity is more dependent on the availability of NADPH than on its antioxidant capacity.

Hegedus *et al.* (2001) evaluated the effects of cadmium stress induced alterations in the activities of several representatives of the enzymatic antioxidant defense system such as guiacol peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) in green and greening barley seedlings that represent two different stages of plant development. Although roots were the main site of Cd accumulation, 1.5–3% of Cd was translocated into leaves and it induced oxidative damage which was indicated by the reduced chlorophyll and increased malondialdehyde content of the leaves. In roots of both types of seedlings exposed to various Cd concentrations, the APX activity was enhanced without any increase in the activity of POD. In leaves, however, elevated activities of both POD and APX could be observed. In roots of green seedlings at high concentration of Cd, the APX activity was reduced on the fourth day of culture but no inhibition was found in the POD activity. Leaf CAT which mainly represented the peroxisomal enzyme activity did not display any changes under Cd stress. Their results show that at both developmental stages barley seedlings exhibit a well-defined activity of the enzymatic antioxidant system, which operates differentially in roots and shoots subjected to Cd stress.

In a study by Landberg and Greger (2002) different clones of *Salix viminalis* with different resistances to Cd, Cu, and Zn were cultivated hydroponically in the presence of 7 μmol L^{-1} Cd, 3 μmol L^{-1} Cu or 70 μmol L^{-1} Zn for 20 days. The clones were then compared with regard to the concentrations of free radicals, estimated by measuring thiobarbituric acid-reactive material (TBA-rm) and glutathione (GSH). The activities of the enzymes, aspartate peroxidase (APX), guaiacol peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT) were also analysed. Salicylic acid was also measured since it is known to be involved in antioxidative activities. The results showed that some differences could be observed between resistant and sensitive clones. The SOD activity was higher in untreated resistant clones compared with the sensitive ones. Under metal treatment, however, the SOD activity was similar. Furthermore, TBA-rm was higher in shoots of resistant clones compared to sensitive ones, while the opposite was found in roots.

Pandey and Sharma (2002) reported that exposure of cabbage plants to excess (500 μM) of Co^{2+}, Ni^{2+} and Cd^{2+} in sand culture led to increased accumulation of the metals, inhibition of growth and induction of visible symptoms of metal toxicity. In addition to chlorosis, Co^{2+} treated plants exhibited reddish purple coloration along leaf margins, Ni^{2+} treated plants exhibited black spots near leaf margins, and Cd^{2+} treated plants developed purple coloration along leaf margins. At equimolar concentration, inhibition of growth was most severe with excess Cd^{2+} and induction of visible symptoms was most severe with excess Ni^{2+}. Exposure to excess concentration of the heavy metals decreased the uptake of Fe and its translocation to leaves. Exposure to each Co^{2+}, Ni^{2+} and Cd^{2+} decreased chlorophyll content (Ni^{2+}>Cd^{2+}>Co^{2+}), concomitant with decrease in the activities of the Fe enzymes - catalase and peroxidase, suggesting reduced availability of Fe for chlorophyll - heme biosynthesis. Each Co^{2+}, Ni^{2+} and Cd^{2+} decreased water potential and transpiration rate, associated with increase in diffusive resistance showing development of water stress. This was further substantiated by enhanced accumulation of proline in the leaves of plants exposed to Co^{2+}, Ni^{2+} and Cd^{2+}.

Changes in the content of reactive oxygen species (ROS) and the activity of the antioxidant system were measured in leaves of *Arabidopsis thaliana* (L.) Heynh exposed to Cd^{2+}. Mature plants growing in the nutrient solution were treated with Cd^{2+} at different concentrations (0, 5, 25, 50, 100 muM). An increase of O^{2-} content in leaves was observed at 5, 25 and 50 muM Cd^{2+}. A strong accumulation of H_2O_2 was found only at the lowest Cd^{2+} concentration. The content of OH was high at 50 and 100 muM Cd^{2+}. Superoxide dismutase (SOD) activity was always higher in Cd^{2+} treated plants than in control. Catalase (CAT) activity decreased with increasing Cd^{2+} concentration in the nutrient solution. Guaiacol peroxidase (POX) activity was particularly high at the lowest and highest Cd^{2+} concentrations and ascorbate peroxidase (APX) activity additionally at 50 muM Cd^{2+}. Enhanced activity of monodehydraye ascorbate reductase (MDHAR) and strong reduction in ascorbate (AA) content were observed at 25 muM Cd^{2+}. Glutathione reductase (GR) activity was always higher than in the control but decreased as Cd^{2+} concentration increased.

However it was accompanied by gradual content increase of SH-groups. (Skorzynska-Polit *et al.* 2003).

Changes in nitrate reductase (NR) activity and subsequent induction of oxidative stress in *Polytrichum* under chromium toxicity were investigated by Panda and Choudhury (2004). It was observed that exposure of Cr at different concentrations for 24 h and 48 h reduced the NR activity in moss cells with significant inhibition after 48 h at 100 mM of Cr. Reduction of total chlorophyll occurred in moss cells after Cr treatment. High accumulation of Cr was seen after 24 h and 48 h. Cr prompted malondialdehyde (MDA) production. Increase in MDA content suggested oxidative stress in moss cells. The increase in MDA content was followed by activation of antioxidant enzymes like catalase(CAT), guaiacol peroxidase(GPx), glutathione reductase(GR) and superoxide dismutase(SOD). Increasing trend for all the enzymes was seen after 24 h and 48 h of Cr treatment. Increase in CAT, GR or SOD was highly significant with increase in concentration and duration of treatment of metal. In case of GPx, the enzyme activity decreased after 48 h of Cr exposure.

Qadir *et al.* (2004) studied ten *B. juncea* cultivars (V_1–V_{10}) commonly grown in India to determine their Cd extraction potential and degree of resistance to Cd stress. Ten-day-old seedlings of *B. juncea* cultivars were exposed to various levels of cadmium chloride (0.0–2.0 mM) for 72 h in hydroponics culture and leaf samples were analyzed at 24, 48 and 72 h after treatment (HAT) for the changes in the rate of lipid peroxidation, plant length, biomass accumulation, cadmium accumulation and activities of catalase (CAT, 1.11.1.6), superoxide dismutase (SOD, 1.15.1.1), ascorbate peroxidase (APX, 1.11.1.11) and glutathione reductase (GR, 1.6.4.2) along with ascorbate (Asc) and glutathione contents. A reduction in the plant length, biomass accumulation, CAT activity and ascorbate content was noted in all the cultivars, however, a significant increase in lipid peroxidation rate, Cd accumulation, activities of APX, GR, SOD and glutathione content was observed. *B. juncea* cv. Pusa Jai Kisan (V_5) showed the least increase in the rate of lipid peroxidation but accumulated highest levels of biomass, Cd and glutathione contents among the cultivars studied. These results indicate that cv. Pusa Jai Kisan possesses

a better Cd-sequestering and antioxidant system. They suggested that high increase in the levels of glutathione suggests its possible incorporation in synthesis of the phytochelatins and metallothioneins to sequester Cd and combat Cd-stress.

Twelve-day-old seedlings of pea treated for four days by 20 and 100 μM of $Cd(NO_3)_2$ or $CuSO_4$ were investigated by Chaoui and Ferjani (2005). In leaves, all treatments caused an increase in the lipoperoxidation product rate. However, 20 μM of Cu did not affect the growth. Moreover, except for 20 μM of Cu, the activity of unspecific peroxidases, used as stress marker, was enhanced in cell walls of metal-stressed plants. No change in the antioxidant capacities was observed in plants treated with 20 μM of metal. At this dose, the Cd-reduced growth could be associated to an elevation in the activities of IAA oxidase and of lignifying peroxidases. Increase of these latter, in concert with loss in antioxidant capacities, would be responsible for the growth diminution after exposure to 100 μM of metal. However, the activity of lignifying enzymes was not affected by 100 μM of Cu.

CAT and SOD activity levels and the modulation of transcription of catalase and superoxide dismutase genes were analyzed in *Prunus cerasifera* after exposure to various copper concentrations. Stress due to Cu toxicity resulted in an increase in total catalase and superoxide dismutase activity and a simultaneous induction of *Sod* and *Cat* gene expression. The study demonstrated that *P. cerasifera* is quite tolerant to Cu and mobilizes catalase and superoxide dismutase in order to mitigate Cu-stress damages (Lombardi and Sebastiani, 2005).

A kinetic model of GSH and phytochelatin synthesis in plants was constructed by Mendoza-Cózatl and Sánchez (2006) using the software *GEPASI* and the kinetic data available in the literature. The main conclusions drawn by the model concerning metabolic control analysis are (1) γ-ECS is indeed a rate-limiting step in GSH synthesis, but only if GSH-consuming enzymes are not taken into account. (2) At low demand, GSH-consuming enzymes exert significant flux-control on GSH synthesis whereas at high demand, supply and demand blocks share the control of flux. (3) In unstressed conditions, flux to GSH is controlled mainly by demand, so that γ-ECS determines the degree of homeostasis of the GSH concentration. Under cadmium exposure, the GSH demand increases and flux-control is re-distributed

almost equally between the supply and demand blocks. (4) To enhance phytochelatins synthesis without depleting the GSH pool, at least two enzymes (γ-ECS and PCS) should be increased and/or, alternatively, a branching flux (GSH-S-transferases) could be partially diminished.

MATERIALS AND METHODS

This chapter deals with the approach and procedure that have been followed to accomplish the objectives of the study. The study was carried out in four stages, i.e., germination stage, seedling stage, vegetative stage and reproductive stage. The experiments were conducted in the experimental garden of Department of Botany, University of North Bengal, India (26.42° N Latitude and 88.25° E Longitude) between February 2004 and March 2007.

3.1. Growth and maintenance of plant material

Okra [*Abelmoschus esculentus*(L.)Moench] seedlings of different cultivars (Arka Anamika, Deepti, Najuka F1, Paras Soumya, PB-57 and Parbhani Kranti) were raised in the experimental garden of Botany Department, North Bengal University. Seeds were surface sterilised in 0.01% $HgCl_2$. The seeds were then thoroughly washed with distilled water and set for germination in Petri plates lined with moistened filter paper. Germinated seeds were then sown in 10″ inches diameter earthenware pots lined with plastic sheets and filled with sandy–loam soil during the summer and kharif season. The pots were filled with garden soil and farmyard manure. Recommended package of practices were followed for growing the crop. Two weeks after germination the seedlings not showing the optimum growth and development were eliminated/thinned out. Plants of the different cultivars were maintained until fruiting stage (Plates II and III).

3.2. Heavy metal treatment

Heavy metal solutions were applied to the seeds and plants at different intervals of time to determine their effect at the different stages of the plant growth and development.

3.2.1. Selection of compounds

The heavy metal compounds selected for the study were cadmium nitrate 4-hydrate [$Cd(NO_3)_2$ $4H_20$], copper(II) sulphate-5-hydrate [$CuSO_45H_2O$], mercury(II) chloride [$HgCl_2$] and lead(II) nitrate [$Pb(NO_3)_2$].

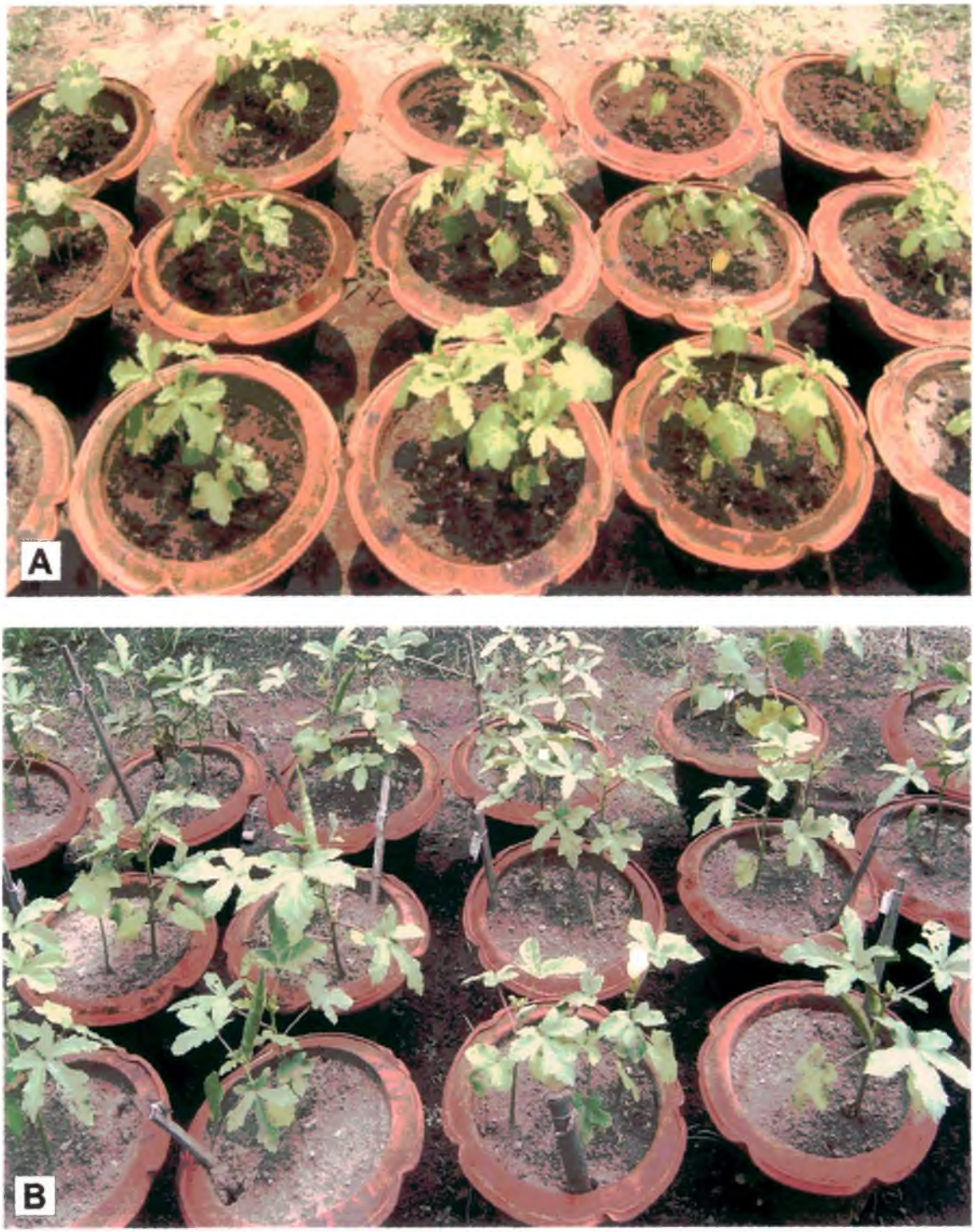


Plate II: Okra plants grown in pots. A: Seedling stage (cv. Arka Anamika) B: Fruiting stage (cv. Parbhani Kranti)

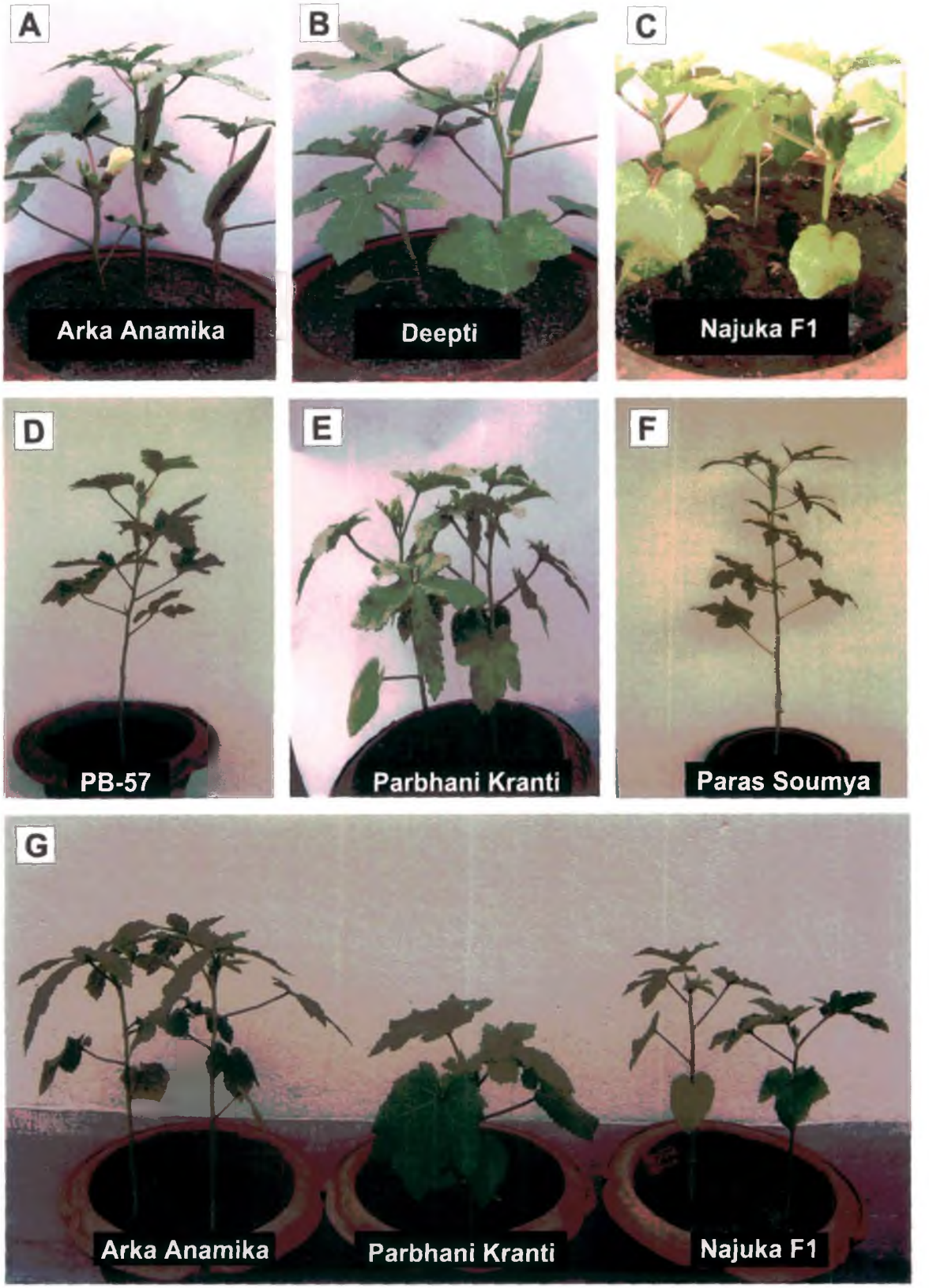


Plate III (A - G): Different cultivars of okra grown in pots.

3.2.2. Application of chemicals

Solution of $Cd(NO_3)_2$ $4H_20$, $CuSO_45H_2O$, $HgCl_2$ and $Pb(NO_3)$ were prepared at concentrations of 100 μg ml^{-1} and 1000 μg ml^{-1}. These solutions were then applied as respective treatments from time to time at different stages. For control only water was applied.

3.2.2.1 . Seed

The seeds of all the cultivars were surface sterilized in 0.01% mercury chloride ($HgCl_2$) solution for 1 min and repeatedly washed with distilled water. 10 seeds were put in each Petri plate lined with filter paper moistened with respective solutions in three replicates for germination test. The Petri plates were covered to prevent loss of moisture by evaporation. Whenever needed, the treatment solution was again applied to avoid drying. Germination percentage was recorded every 24 h for 3 days after putting the seeds in the Petri plates with respective solutions. Seeds were considered germinated when the emergent radicle reached 2 mm in length.

3.2.2.2. Soil

The seedlings were allowed to grow for 3 weeks. After 3rd week the underdeveloped/unhealthy seedlings were thinned out. 200 ml of the respective treatment solution were applied in each pot. For each treatment, 3 applications were done-once each in seedling stage, vegetative stage and reproductive stage. On control only water was applied.

3.2.2.3. Application of ameliorating compounds

Ameliorating compounds were also applied to amend the effects of heavy metals. The ameliorating compounds used were calcium chloride ($CaCl_2$) and potassium nitrate ($KNO_3.2H_2O$) at concentration of 1000 μg ml^{-1} each.

The treatments were done by soaking the seeds in a combination of the respective ameliorating compounds and heavy metals. For control the seeds were treated only with heavy metals.

3.3. Seed germination

The seeds of all the cultivars were surface sterilized in 0.01% mercury chloride ($HgCl_2$) solution for 1 min and repeatedly washed with distilled water. 10

seeds were put in each Petri plate lined with filter paper moistened with respective treatment solutions in three replicates for germination test. The Petri plates were covered to prevent loss of moisture by evaporation. Whenever needed, the treatment solution was again applied to avoid drying. Germination percentage was recorded every 24 h for 3 days after putting the seeds in the Petri plates in respective treatment solution. Seeds were considered germinated when the emergent radicle reached 2mm in length.

3.4. Determination of growth parameters and yield

The determination of growth parameters mainly depended on estimation of fresh weight, dry matter and leaf area over a period of time.

3.4.1. Fresh weight and dry weight

The plants were uprooted and washed with water. The excess water was soaked with blotting paper and the different parts were separated. The fresh weight of the different parts of representative sample on plant basis was recorded. The samples were than oven dried at 60°C to 70°C for 72-90 h till a constant dry weight was obtained.

3.4.2. Tolerance index

Heavy metal tolerance of the cultivars were calculated as the tolerance index (TI) which gives the percentage of shoot and root fresh biomass(g plant^{-1}) of heavy metal treated (FWt) over untreated control (FWc) plants according to the equation given by Metwally *et al.* 2005.

$$TI\ (\%) = FWt / FWc\ X\ 100 - 100$$

3.4.3. Relative growth index

Relative growth index (RGI) is the ratio of average dry matter of seedling in treatment to the average dry matter of seedling in control. It is expressed in per cent.

$$RGI = \frac{\text{Average dry matter of seedling in treatment}}{\text{Average dry matter of seedling in control}} X\ 100$$

3.4.4. Leaf area

Leaf area was estimated by graph paper method. Leaves were placed in the graph paper and the outline was drawn. The area covered by the outline was then calculated out.

3.4.5. Absolute growth rate

Absolute growth rate (AGR) is the increase in dry matter per unit time. The unit is mg day^{-1}.

$AGR = w_2 - w_1 / t_2 - t_1$

where, w_1 and w_2 are the total dry weights at time t_1 and t_2.

3.4.6. Relative growth rate

Relative growth rate (RGR) is the increase in dry matter over initial dry matter over a period of time. It is calculated by the formula given by Blackman (1919) and expressed as $g^{-1}g^{-1}day$.

$RGR = \log_e w_2 - \log_e w_1 / t_2 - t_1$

where, w_1 and w_2 are the total dry weights at time t_1 and t_2 respectively.

3.4.7. Specific leaf area and Specific leaf weight

Specific leaf area (SLA) is defined as leaf area per unit leaf biomass. It is expressed in $cm^2\ g^{-1}$. Specific leaf weight is defined as leaf area per unit leaf area. It also gives the measure of leaf thickness and is expressed in mg cm^{-2}.

3.4.8. Yield

Yield was calculated by recording the fresh weight of fruits per plant.

3.5. Biochemical analyses

Various biochemical analyses were performed from the treated plants at different stages of growth. Sampling at each stage was done after 48 h of application in the soil.

3.5.1. Extraction and quantification of carbohydrate

3.5.1.1.Extraction of total soluble and reducing sugar

Sugar was extracted following the method of Harbone (1973). Fresh tissues were crushed with 95% ethanol and centrifuged at 5000 rpm. Then the alcoholic

fractions of the filtrate were evaporated off on a boiling water bath. The volume was finally made up to 5 ml with distilled water and was used for estimation of both total soluble and reducing sugar.

3.5.1.2. Estimation total soluble and reducing sugar

Estimation of total soluble sugar was done following Anthrone's method as described by Plummer (1978). To 1ml of test solution 4 ml of Anthrone's reagent was added and mixed thoroughly. Mixtures were placed in a boiling water bath for 10 min. The reaction mixture was then cooled under running tap water. Absorbance was measured at 570 nm in a colorimeter. Total soluble sugar content was then calculated from the standard curve of dextrose solution.

Somogyi's method as described by Plummer (1978) was followed for the estimation of reducing sugar. 1 ml alkaline Cu-tartarate solution was added to 1 ml of test solution.Reaction mixture was then allowed to boil in a water bath for 15 min. The mixture was then cooled under running tap water and to each reaction mixture 1ml of Nelson's arseno molybdate reagent was added. Reaction mixtures were diluted to 5 ml by adding 2 ml more distilled water and absorbances were measured in a colorimeter at 540 nm. The concentration of reducing sugar was determined by plotting the values on the standard curve of dextrose solution.

3.5.1.3.Starch

Starch was extracted by homogenizing 0.5 g of the plant tissue in hot 80% ethanol to remove sugars (Thimmaiah, 1999). The extract was centrifuged and the residue was retained. The residue was repeatedly washed with hot 80% ethanol till washings did not give any colour with Anthrone reagent. The residue was then dried over a water bath. To the dried residue 5 ml of water and 6.5 ml of 52% perchloric acid was added and centrifuged at 0°C for 20 minutes. The supernatant was saved and the process was repeated using fresh perchloric acid each time. The supernatant was pooled and the volume was made up to 100 ml.

For estimation, to 1 ml of test solution 4 ml of Anthrone reagent was added and mixed thoroughly. Mixtures were placed in a boiling water bath for 8 min. The reaction mixture was then cooled under running tap water. Absorbance was

measured at 630 nm in a colorimeter. Starch content was then calculated from the standard curve of glucose solution.

3.5.2.Extraction and estimation of proline content of leaves

Proline was extracted from leaves in 3% sulfosalicylic acid. The leaf tissues were homogenized in 3% sulfosalycylic acid and filtered through Whatman No.1 filter paper. The filtrate were collected and used for the estimation of proline.

Proline was estimated following the method of Bates *et al.* (1973). To 1 ml of the test solution 1 ml of Ninhydrin solution was added [1 g Ninhydrin in 25 ml acetone:water (2:3)]. After this the solution was boiled in hot water bath for 30 min. The solution was cooled and separated with 5 ml toluene in a separating funnel. The lower layer was collected and the upper layer was discarded. The absorbance of the lower layer was measured in a colorimeter at 520 nm.

3.5.3.Extraction and quantification of pigments

3.5.3.1.Chlorophyll

Chlorophyll was extracted according to the method of Harbone (1973) by homogenizing 1 g of leaf sample in 80% acetone and filtering through Whatman No. 1 filter paper. 80% acetone was repeatedly added from the top till the residue became colourless. The filtrate was collected and the total volume was made up to 25 ml.The chlorophyll content was estimated by observing the absorbance values at 645 nm and 663 nm respectively in a UV-VIS spectrophotometer (DIGISPEC-200GL) and calculation was done following the method of Arnon (1949).

Total chlorophyll = $(20.2\ A_{645} + 8.02\ A_{663})$ mg g^{-1} fresh weight

Chlorophyll a = $(12.7\ A_{663} - 2.69\ A_{645})$ mg g^{-1} fresh weight

Chlorophyll b = $(22.9\ A_{645} - 4.68\ A_{663})$ mg g^{-1} fresh weight

3.5.3.2.Carotenoids

Carotenoids were extracted and estimated according to the method given by Lichtenthaler (1987). Extraction of carotenoids was done by homogenizing 1 g of the plant sample in methanol. The homogenate was filtered through Whatman No.1 filter paper and the volume was made up. Absorbance of filtrate was observed at 480 nm,

645 nm and 663 nm in a UV-VIS spectrophotometer (DIGISPEC-200GL) and the carotenoid content was calculated by using the formula

Carotenoids = A_{480} - (0.114 x A_{663}) - 0.638(A_{645}) μg g^{-1} fresh weight

3.6. Protein analysis

3.6.1. Extraction of soluble proteins from seeds and different plant parts

Soluble protein was extracted from seeds, leaves, roots of different cultivars following the method of Chakraborty *et al.* (1995). Plant tissues were homogenized using 0.05 M sodium phosphate buffer (pH 7.2) to which PVPP(Polyvinyl pyrollidone phosphate) was added and centrifuged at 4°C for 20 min at 10,000 rpm. The supernatant was used as crude protein extract for the estimation of protein content.

3.6.2. Estimation of protein content

Soluble proteins were estimated following the method as described by Lowry *et al.* (1951). To 1 ml of protein sample 5 ml of alkaline reagent (1 ml of 1% $CuSO_4$ and 1 ml of 2% sodium potassium tartarate, dissolved in 100 ml of 2% Na_2CO_3 in 0.1 N NaOH) was added. This was incubated for 15 min at room temperature and then 0.5 ml of 1 N Folin Ciocalteau reagent was added and again incubated for further 15 min following which absorbance was noted at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.6.3. SDS-PAGE analysis of total soluble protein

Total soluble proteins extracted in 0.05 M sodium phosphate buffer were used as crude protein extract for analysis of protein pattern. Analysis was carried out on 10% SDS-PAGE gels following the method of Sambrook *et al.* (1989). Protein samples were loaded on the gel and separated for 2 h at 200 V and 15-20 mA current. Following electrophoresis the gel was fixed, stained in Coomassie Brilliant Blue (R-250) solution and finally destained in a solution of methanol: glacial acetic acid: dH_2O(4.5: 4.5: 1).

3.6.3.1. Preparation of stock solution

For the preparation of gel the following stock solutions were prepared:

(A) Acrylamide and N' N'- methylene bis acrylamide

A stock solution containing 29% acrylamide and 1% bisacrylamide was prepared in warm water. As both of these amides are slowly deaminated to acrylic and bis acrylic acid by alkali and light the pH of the solution was kept below 7. The stock solution was then filtered through Whatman No. 1 filter paper, kept in brown bottle and stored at 4°C and used within one month.

(B) Sodium Dodecyl Sulphate (SDS)

A 10% stock solution of SDS was prepared in warm water and stored at room temperature.

(C) Tris Buffer

i) 1.5 M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with concentrated HCl and stored at 4°C for use.

ii) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was adjusted to 6.8 with conc. HCl and stored at 4°C for use.

(D) Ammonium Persulphate (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

(E) Tris –Glycine electrophoresis buffer

Tris running buffer consists of 25 mM Tris base, 250 mM Glycine (pH 8.3) and 0.1% SDS. The solution was made by dissolving 3.02 g Tris base, 18.8 g Glycine and 10 ml of 10% SDS in 1 L of distilled water.

(F) SDS gel loading buffer

This buffer contains 50 mM Tris-HCl (pH 6.8), 10 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. The solution was prepared by dissolving 0.5 ml of 1 M Tris buffer (pH 6.8), 0.5 ml of 14.4 M β-mercaptoethanol, 2 ml of 10% SDS, 10 mg bromophenol blue, 1 ml glycerol in 6.8 ml of distilled water.

3.6.3.2. Preparation of gel

Mini slab gel (plate size 8 cm x10 cm) was prepared for the analysis of protein patterns by SDS-PAGE. For gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm thick spacers were placed between the glass plates at three sides and sealed with high vacuum grease and clipped tightly to prevent any leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following order and poured by Pasture pipette leaving sufficient space for comb in the stacking gel (comb + 1cm).

Composition of solutions

10% resolving gel		**5% stacking gel**	
Name of the compond	**Amount (ml)**	**Name of the Coumpound**	**Amount (ml)**
Distilled water`	2.85	Distilled water	2.10
30% acrylamide	2.55	30% acrylamide	0.50
1.5 M Tris buffer (pH 8.8)	1.95	1 M Tris buffer (pH 6.8)	0.38
10% SDS	0.075	10% SDS	0.030
10% APS	0.075	10% APS	0.030
TEMED	0.003	TEMED	0.003

After pouring the resolving gel solution, it was immediately over layered with water and kept for polymerization for 2 h. After polymerization of the resolving gel was complete, overlay of water was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30-45 min. After polymerization of the stacking gel the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris-Glycine buffer was added

sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom of the gel, was removed carefully with a bent syringe.

3.6.3.3. Sample Preparation

Sample (50 µl) was prepared by mixing the sample protein (35 µl) with the SDS gel loading buffer (15 µl) in cyclomixer. All the samples were floated in boiling water bath for 3 min to denature the protein sample. The samples were immediately loaded in a pre-determined order into the bottom of the wells with a micro litre syringe. Along with the samples, protein markers consisting of a mixture of six proteins ranging from high to low molecular weight (Phosphorylase b- 97,4000; Bovine Serum Albumin- 68,000; Ovalbumin- 43,000; Carbolic Anhydrase- 29,000; Soyabean Trypsin inhibitor-20,000; Lysozyme- 14,300) was treated as the other sample and loaded in a separate well.

3.6.3.4. Electrophoresis

Electrophoresis was performed at a constant 18 mA current for a period of 2 h until the dye front reached the bottom of the gel.

3.6.3.5. Fixing and staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid: methanol: d H_2O(10:20:70). The staining solution was prepared by dissolving 250 mg of Coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. After the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No.1 filter paper.

The gel was removed from the fixer and stained in this stain solution for 4 h at 35°C with constant shaking at low speed. After staining the gel was finally distained with distaining solution containing methanol:dH_2O:acetic acid (4.5: 4.5: 1) at 35°C with constant shaking until the background became clear.

3.7. Extraction of enzymes

3.7.1. Catalase (CAT, EC 1.11.1.6), Peroxidase (POX, EC 1.11.1.7) and Ascorbate peroxidase (APOX, EC 1.11.11)

CAT, POX and APOX were extracted by homogenizing the plant tissues in 0.05 M sodium phosphate buffer (pH 7.2) containing 1% (w/v) insoluble polyvinyl pyrrolidone phosphate (PVPP) and 2 mM β mercaptoethanol under ice cold condition. The homogenate was centrifuged immediately at 10,000 rpm for 20 min at 4°C. After centrifugation the supernatant was collected and after recording its volume was used immediately for assay.

3.7.2. Glutathione reductase (GR, EC 1.6.4.2) and Superoxide dismutase (SOD, EC 1.15.1.1)

GR and SOD were extracted by homogenizing the plant tissue in 0.1 M potassium phosphate buffer (pH 7.8) containing 1% (w/v) insoluble polyvinyl pyrrolidone phosphate (PVPP) and 2 mM β mercaptoethanol. Insoluble material was removed by centrifugation at 12,000 rpm for 15 min at 4°C.

3.8. Assay of enzyme activities

3.8.1. Catalase

CAT activity was assayed according to Chance and Machly (1955). Enzyme extract(20 µl) was added to 3 ml of H_2O_2-phosphate buffer(0.16 ml of H_2O_2 to100 ml of phosphate buffer, pH 7.2) and the breakdown of H_2O_2 was measured at 240 nm in a spectrophotometer. An equivalent amount of buffer containing H_2O_2 was used as reference. The enzyme activity was expressed as enzyme units mg protein^{-1} where one enzyme unit was defined as a change of 0.01 absorbance min^{-1} caused by the enzyme aliquot.

3.8.2. Peroxidase

Peroxidase activity was assayed by adding 100 µl of freshly prepared crude enzyme extract to the reaction mixture containing 1 ml of 0.2 M sodium phosphate buffer (pH 7.2), 100 µl of 4mM H_2O_2, 100 µl of O-dianisidine (5 mg ml^{-1} methanol) and 1.7 ml of distilled water. Peroxidase activity was assayed spectrophotometrically

at 460 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2. Specific activity was expressed as the increase in absorbance at 460 nm g^{-1}tissue min^{-1} (Chakraborty *et al.* 1993).

3.8.3. Ascorbate peroxidase

APOX activity was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm according to the method of Asada (1994) with some modification. The reaction mixture consisted of 100 µl of enzyme extract, 100 µl of 0.5 mM ascorbic acid, 100 µl of H_2O_2 and 2.7 ml of sodium phosphate buffer (pH 7.2). Enzyme activity was finally expressed as change (decrease) in absorbance (ΔA_{290}) mg protein $^{-1}$ min $^{-1}$.

3.8.4. Glutathione reductase

GR activity was assayed by the oxidation of NADPH at 340 nm with extinction coefficient of 6.2 mM cm^{-1} as described by Lee and Lee (2000). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM glutathione (oxidized form, GSSG) with an appropriate volume of enzyme extract. The reaction was initiated by the addition of NADPH at 25°C. Enzyme activity was finally expressed as µM NADPH oxidized min^{-1} mg $protein^{-1}$.

3.8.5. Superoxide dismutase

SOD activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Dhindsa *et al.* (1981) with some modification. Each 3 ml of the assay mixture constituted of 0.1 ml enzyme extract, 1.5 ml phosphate buffer(0.1 M, pH 7.8), 0.1 ml Na_2CO_3 (1.5 M), 0.1 ml NBT (2.25 mM), 0.2 ml methionine (200 mM), 0.1 ml EDTA (3 mM), 0.1 ml riboflavin (60 µM) and 0.8 ml of distilled water.

The reaction tubes containing enzyme samples were illuminated with 15 W fluorescent lamp for 10 min. The other set of tubes lacking enzymes were also illuminated and served as control. A non- irradiated complete reaction mixture did not develop any colour and served as blank. The absorbance of samples was measured at 560 nm and 1 unit of activity was defined as the amount of enzyme

required to inhibit 50% of the NBT reduction rate in the controls containing no enzymes.

3.9. Isozymes analysis of peroxidase by PAGE

Extract for isozyme analysis was prepared by crushing 1 g of leaf tissue in a mortar and pestle in 0.1 M sodium phosphate buffer (pH 7.0) on ice and insoluble material was removed by centrifugation at 12,000 rpm for 15 min at 4°C. Polyacrylamide gel electrophoresis (PAGE) was performed for isozyme analysis of peroxidase as described by Davis (1964) and immediately used for the isozyme analysis.

3.9.1. Preparation of stock solution

(A) Acrylamide stock solution (Resolving gel)

Acrylamide stock solution for resolving gel was prepared by dissolving 28 g of acrylamide and 0.74 g of N' N' methelene bisacrylamide in distilled water and the volume was made up to 100 ml. The stock solution was filtered with Whatman No. 1 filter paper and stored at 4°C in dark bottle.

(B) Acrylamide stock solution (stacking gel)

Acrylamide stock solution for stacking gel was prepared by dissolving 10 g of acrylamide and 2.5 g of bis- acrylamide in distilled water and the volume was made up to 100 ml. The stock solution was filtered and stored at 4°C in dark bottle.

(C) Tris- HCl (Resolving gel)

Tris-HCl for resolving gel was prepared by dissolving 36.6 g of Tris base in distilled water and 0.25 ml of TEMED was added. The pH was adjusted to 8.9 with concentrated HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was then stored at 4°C for further use.

(D) Tris- HCl (Stacking gel)

Tris-HCl for stacking gel was prepared by dissolving 5.98 g of Tris base in distilled water and 0.46 ml of TEMED was added. The pH was adjusted to 6.7 with conc. HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was stored at 4°C for further use.

(E) Ammonium persulphate solution (APS)

Fresh solution of APS was prepared by dissolving 0.15 g of APS in 10 ml of distilled water.

(F) Riboflavin solution

Fresh solution of riboflavin was prepared by dissolving 0.4 mg of riboflavin in 10 ml distilled water. The solution was kept in dark bottle to protect from light.

(G) Electrode buffer

Electrode buffer was prepared freshly by dissolving 0.6 g of Tris base and 2.9 g glycine in 1 L of distilled water.

3.9.2. Preparation of gel

Mini slab gel for the polyacrylamide gel electrophoresis of peroxidase isozymes was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease and then dried. Spacers of 1.5 mm thickness were placed between the glass plates on three sides and these were sealed with high pressure vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. 7.5% resolving gel was prepared by mixing solution A: C: E: dH_2O (1: 1: 4: 1) leaving sufficient space for (comb + 1 cm) the stacking gel. This resolving gel was immediately over layered with water and kept for polymerization for 2 h. After polymerization of the resolving gel was complete, over layer was poured off and washed with water to remove any unpolymerized acrylamide.

The stacking gel solution was prepared by mixing solutions B: D: F: dH_2O (2:1:1:4). Stacking gel solution was poured over the resolving gel and comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30-45 min in strong sunlight. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was now finally mounted in the electrophoresis apparatus. Tris-Glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

3.9.3. Sample preparation

Sample (32 µl) was prepared by mixing the sample enzyme (20 µl) with gel loading dye (40% sucrose and 1% bromophenol blue in distilled water) in cyclomixer in ice. All the solutions for electrophoresis were cooled. The samples were immediately loaded in a predetermined order into the bottom of the wells with a microlitre syringe.

3.9.4. Electrophoresis

Electrophoresis was performed at constant 15 mA current for a period of around 3-4 h at 4°C until the dye font reached the bottom of the gel.

3.9.5. Fixing and Staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally stained. Staining of the gel was performed following the method of Reddy and Gasber (1973).

The gel was incubated in an aqueous (80 ml) solution of benzidine(2.08 g), acetic acid (18 ml), 3% H_2O_2 (100 ml) for 5 minutes. The reaction was stopped with 7% acetic acid after the appearance of clear blue coloured bands. Analysis of isozyme was done immediately.

3.10. Determination of peroxidation of cell membrane lipids

Lipid peroxidation was measured according to the method described by Heath and Packer (1968). Leaf tissue was homogenized in 2 ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000 rpm for 10 min. To 0.5 ml of the supernatant 2 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA was added. The reaction mixture was incubated at 95°C for 30 min. After cooling it was again centrifuged. The absorbance of the supernatant was recorded at 532 nm and corrected for 600 nm. Amount of malonedialdehyde produced was calculated by using the extinction coefficient of 155 mM cm^{-1}.

3.11. Extraction of flavonoids

Fruits samples (0.5 g dry weight) of okra were extracted in 25 ml of 1.2 M HCl in 50% aqueous methanol. For extraction hydrolysis was carried out for 2 h at 98°C reflux as per the method described by Vargas-Alvarez *et al.* (2004). After refluxing the pH was adjusted to 2.5 and 500 µl aliquots from the hydrolyzed mixture were filtered with 0.45 µm membrane filters.

3.12. Analysis of flavonoids

For analysis of flavonoids high performance liquid chromatography (HPLC) was carried out.

3.12.1. High performance liquid chromatography (HPLC)

HPLC analyses of the samples were carried out in the HPLC system (Shimadzu Advanced VP Binary Gradient) using C-18 hypersil column with linear gradient elution system as follows: mobile phase A 100% acetonitrile; mobile phase B 2% acetic acid in water. Elution: 88% B for 6 min then linear gradient to 75% B over 5 min. The elution was complete after a total of 25 min. Flow rate was fixed as 1 ml min^{-1} with sensivity of 0.5 aufs, injection volume 20 ml and monitored at 278 nm (Obanda and Owuor, 1994).

3.13. Determination of heavy metal contents in plant material

Heavy metal content in the various parts of the plants was estimated with the help of atomic absorption spectrophotometer (AAS). The plant materials were oven dried to a constant dry weight and digested in a ternary acid mixture of nitric acid: perchloric acid: sulphuric acid (10:4:1). One gram of the plant material was digested at a temperature of 180°C for 15 min. After the digestion was complete the volume was made up to 100 ml with distilled water and the AAS (Perkin Elmer model 1100) reading was recorded.

3.14. Microscopic studies

Microscopic studies were carried out to observe the accumulation of starch and alkaloids in the different parts of okra plants treated with the heavy metals at 1000 µg ml^{-1} concentration.

3.14.1. Starch

In case of starch the transverse sections of roots were cut and dipped in Melzers reagent(aqueous solution of 1% iodine and 1% potassium iodide)for 15 min. Mounting was done with glycerol and then the slides were observed under microscope in both low and high power.

3.14.2. Alkaloids

For observing the alkaloids the transverse sections of roots and fruits were cut and dipped in Wagners reagent (1.27 g iodine and 2 g potassium iodide dissolved in 100 ml of distilled water) for 10 min. The sections were then thoroughly washed in distilled water and mounting was done with lactophenol. The mounted slides were then observed under light microscope in both low and high power.

3.15. Treatment with chemicals for amendment of heavy metal stress

Ameliorating compounds were applied as treatments to amend the effects of heavy metals. The ameliorating compounds used were calcium chloride ($CaCl_2$) and potassium nitrate (KNO_3) at a concentration of 1000 $\mu g\ ml^{-1}$. For seed treatment, the seeds were sown in Petri dishes lined with filter paper moistened with the ameliorating compounds and with combination with the different heavy metals (cadmium nitrate 4-hydrate [$Cd(NO_3)_2\ 4H_20$], copper(II) sulphate-5-hydrate [$CuSO_45H_2O$], mercury(II) chloride [$HgCl_2$] and lead(II) nitrate [$Pb(NO_3)_2$] at concentrations of 1000 $\mu g\ ml^{-1}$). One set was soaked in combination with distilled water and the respective heavy metal solutions to serve as control.

3.16. Glass wares and chemicals used in the experiment

All the glass wares *viz.*, Petri plates, test tubes, conical flask, pipettes, beakers, funnels used in the experiment were either of Borosil and Riviera make. Chemicals used in the experiment were of analytical reagent grade.

3.17. Statistical Analysis

All the data were recorded in triplicate and analysed using the standard error mean and ANOVA was done with the application of randomised block design. The test of significance was calculated with the help of Students 't' test.

Results of all experiments conducted in the present study have been detailed in the following pages. All the experiments were conducted using standard procedures as given under materials and methods.

4.1. Effect of heavy metals on seed germination

Seeds of six cultivars of okra (Arka Anamika, Deepti, Najuka F-1, Paras Soumya, Parbhani Kranti and PB-57) were treated with $Cd(NO_3)_24H_20$, $CuSO_45H_2O$, $HgCl_2$ and $Pb(NO_3)_2$ at both 100 and 1000 μg ml^{-1} concentrations. Maximum inhibition of germination was observed by Hg at 1000 μgml^{-1} after 24 h of germination. In this case no germination was recorded for the cultivar PB-57. However, after 48 h 30% germination was recorded. In most of the cultivars 1000 μg ml^{-1} treatments with the other heavy metal salts also inhibited germination significantly (Table 1). Analysis of variance revealed that there was significant difference between control and all the treatments after 24 h of germination. There was also significant difference in germination per cent between 100 μg ml^{-1} concentrations of the different salts. After 48 h of germination significant difference was also obtained between control and all heavy metal salts at both concentrations except $CuSO_45H_2O$ at 100 μg ml^{-1}(Tables 1A and 1B; Plate IV). After 72 h different cultivars exhibited germination between 77% (Parbhani Kranti) and 100% (Arka Anamika) in control. Higher concentration of Pb and Hg inhibited germination in the range between 30 to 40% (Figs.1 and 2).

4.2. Analysis of the effect of heavy metals on growth and yield of okra

Effect of the different heavy metals on the growth and yield of okra cultivars was determined on the basis of different parameters.All growth parameters were determined at the vegetative stage. Treatments of heavy metal salts were at 1000 μg ml^{-1} concentrations. Morphological effects of the heavy metals on different cultivars of okra have been presented in Plates V-IX.

4.2.1. Tolerance Index

Tolerance of the different cultivars and different plant parts was determined by the tolerance index computed from the fresh weight biomass. Results revealed that among the different cultivars Deepti was most tolerant as evidenced by

relatively high tolerance index values. Plants were most susceptible to Hg followed by Cd. Among the plant parts roots were the most susceptible (Table 2).

Table 1: Effect of heavy metals on seed germination of okra cultivars

Cultivars	Time (h)	Germination per cent								
		Control	Cadmium		Copper		Mercury		Lead	
			100	1000	100	1000	100	1000	100	1000
Arka Anamika	24	33.33 ± 2.72	23.33 ±2.72	13.33 ±2.72	16.66 ±2.72	16.66 ±2.72	20.00 ± 0.0	3.33 ±2.72	20.00 ±4.72	10.00 ±4.72
	48	100 ± 0.00	66.67 ±2.72	63.33 ±2.72	83.33 ±2.72	66.67 ±2.72	63.33 ±2.72	33.33 ±2.72	46.67 ±2.72	36.67 ±5.45
Deepti	24	30.00 ± 4.72	10.00 ±4.72	3.33 ±2.72	16.67 ±2.72	6.67 ±2.72	10.00 ±4.72	10.00 ±4.72	20.00 ±4.72	13.33 ±7.21
	48	80.00 ± 4.72	66.67 ±2.72	36.67 ±2.72	76.67 ±2.72	63.33 ±2.72	56.67 ±2.72	40.00 ±4.72	56.67 ±5.45	53.33 ±2.72
Najuka-F1	24	26.67 ± 2.72	13.33 ±2.72	6.67 ±2.72	10.00 ±4.72	6.67 ±2.72	10.00 ±0.00	3.33 ±2.72	10.00 ±2.72	10.00 ±0.00
	48	66.67 ± 2.72	63.33 ±2.72	53.33 ±2.72	70.00 ±4.72	30.00 ±4.71	43.33 ±2.72	30.00 ±4.71	16.63 ±2.72	20.00 ±4.72
Paras Soumya	24	23.33 ± 2.72	10.00 ±4.72	6.67 ±2.72	10.00 ±4.72	6.67 ±2.72	10.00 ±4.72	3.33 ±2.72	6.67 ±2.72	3.33 ±2.72
	48	70.00 ± 2.72	63.33 ±2.72	53.33 ±2.72	53.33 ±2.72	53.33 ±2.72	40.00 ±4.72	23.33 ±2.72	26.67 ±2.72	26.67 ±2.72
Parbhani Kranti	24	23.33 ± 2.72	16.67 ±2.72	6.67 ±2.72	13.33 ±2.72	6.67 ±2.72	10.00 ±4.72	3.33 ±2.72	10.00 ±4.72	6.67 ±2.72
	48	46.67 ± 2.72	26.67 ±2.72	23.33 ±2.72	33.33 ±2.72	23.33 ±2.72	33.33 ±2.72	13.33 ±2.72	23.33 ±2.72	20.00 ±4.72
PB-57	24	23.33 ± 2.72	13.33 ±2.72	3.33 ±2.72	10.00 ±4.72	3.33 ±2.72	6.67 ±2.72	0.00 ±0.00	3.33 ±2.72	3.33 ±2.72
	48	53.33 ± 2.72	33.33 ±2.72	30.00 ±.00	33.33 ±2.72	30.00 ±0.00	30.00 ±4.72	30.00 ±4.72	23.33 ±2.72	30.00 ±4.72

100 & 1000 represent 100 & 1000 μg ml^{-1} concentration of the salts
Values are mean of 3 replicates; ± = SEm

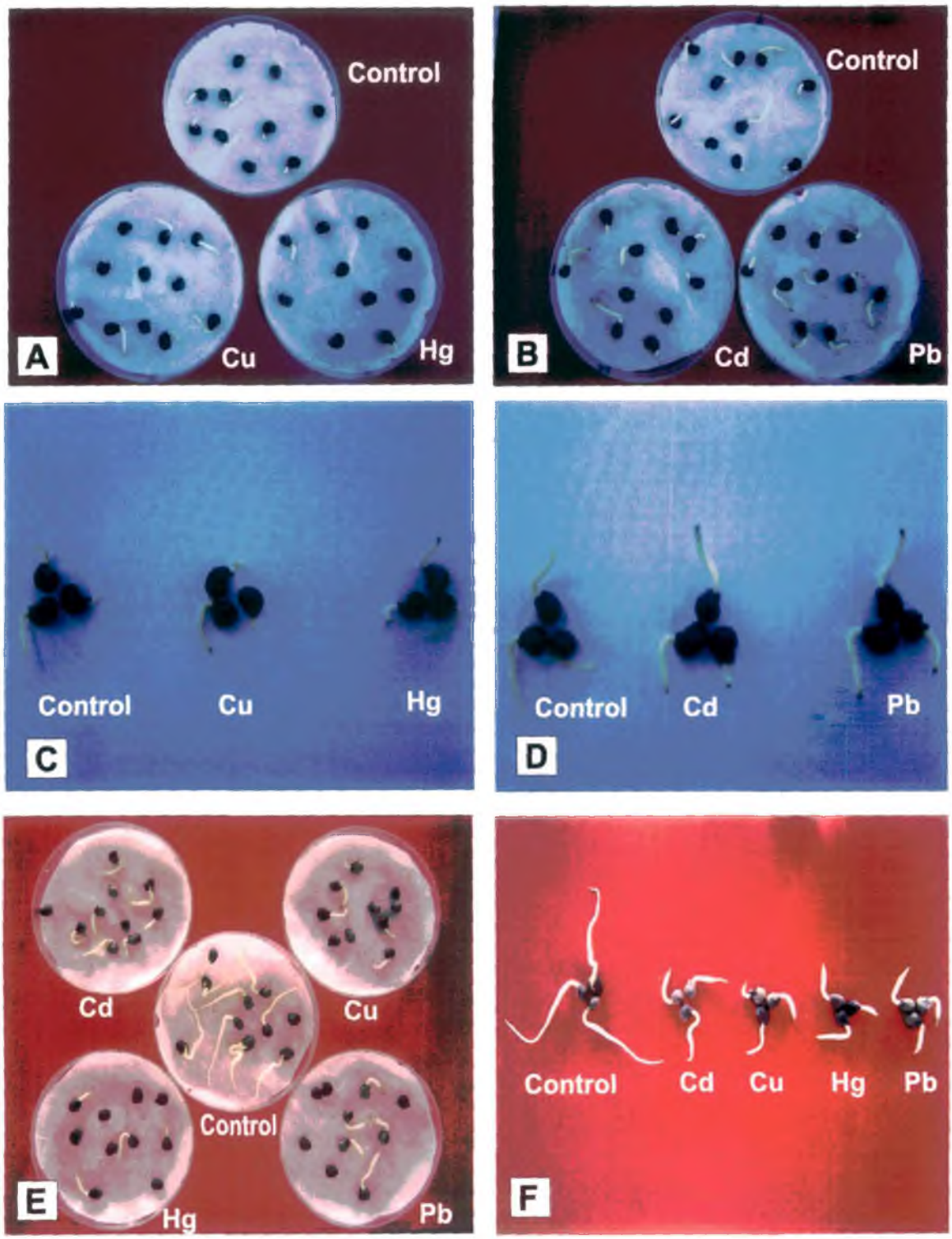


Plate IV: Effect of heavy metals on seed germination in okra cultivars A: Deepti; B: Najuka F1; C: Parbhani Kranti; D: PB-57; E-F: Arka Anamika.

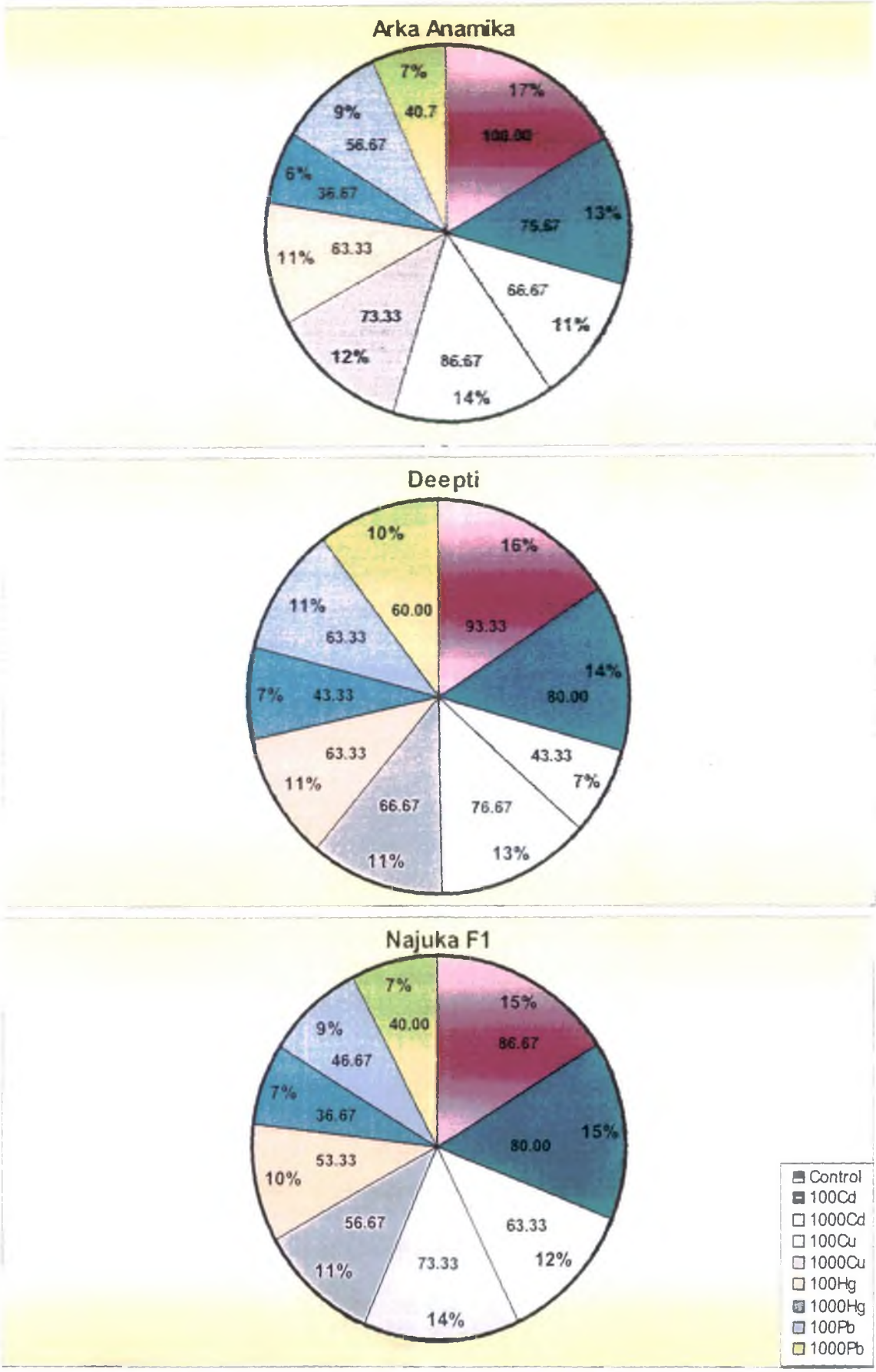


Fig.1:Effect of heavy metals at 100 and 1000 μg ml^{-1} concentration on germination of okra seeds (cv. Arka Anamika, Deepti, Najuka-F1) after 72 hours

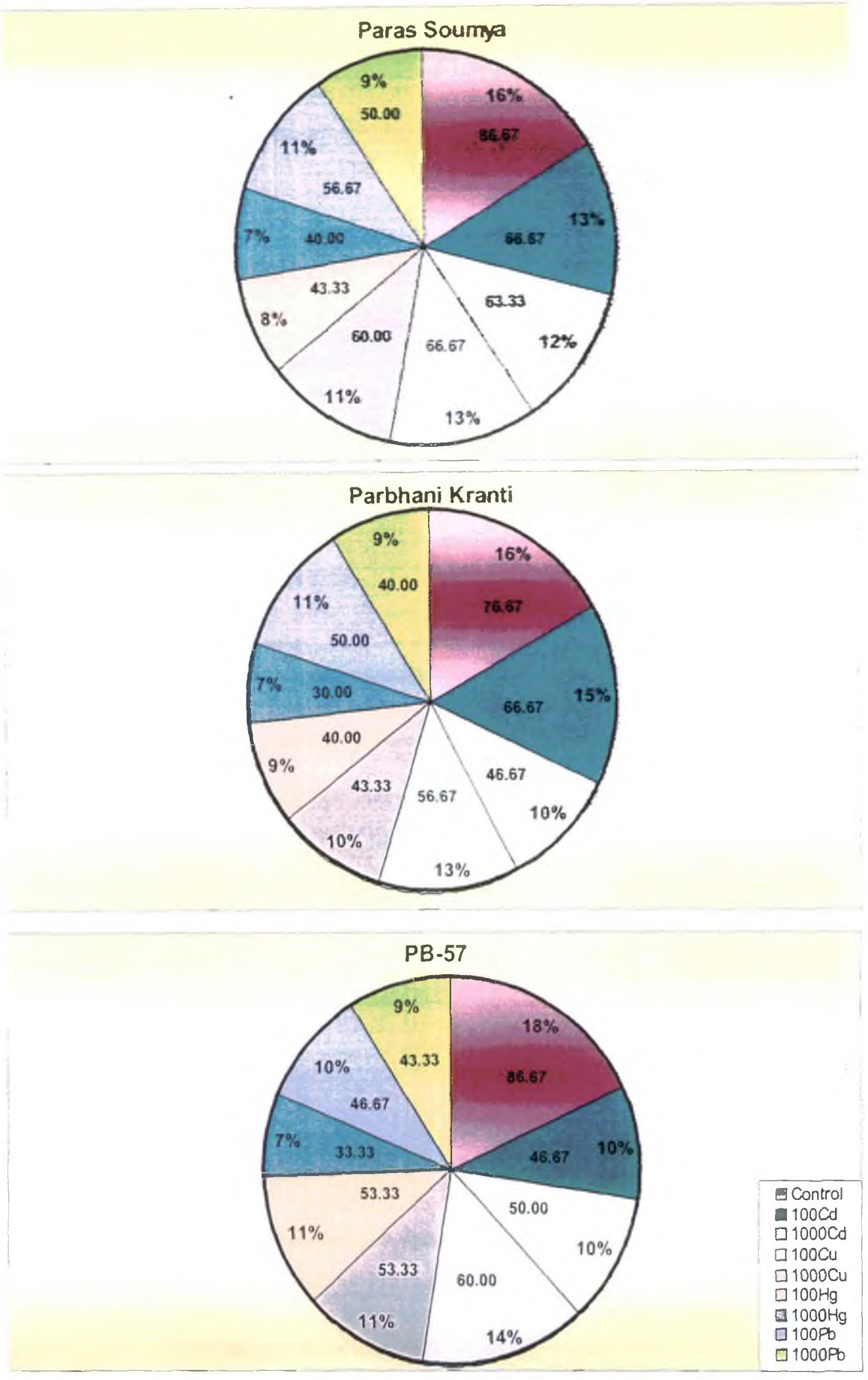


Fig.2: Effect of heavy metals at 100 and 1000 μg ml^{-1} concentration on germination of okra seeds (cv. Paras Soumya, Parbhani Kranti, PB-57) after 72 hours

Table 1A: Analysis of variance of data presented in Table 1 (24 h)

Source	D.F.	S.S.	M.S.	F	C.D.
Replication	2	134.57	67.29		
Variety	5	1699.38	339.88	6.21	3.82
Treatment	8	6290.13	786.27	14.37	4.68
Interaction	40	1050.62	26.26	0.48	11.47
Error	106	5798.76	54.71		
Total	161	14973.46			

Table 1B: Analysis of variance of data presented in Table 1 (48 h)

Source	D.F.	S.S.	M.S.	F	C.D.
Replication	2	103.71	51.86		
Variety	5	26405.56	5281.11	105.69	3.66
Treatment	8	27055.56	3381.95	67.68	4.48
Interaction	40	9966.66	249.17	4.99	10.97
Error	106	5296.29	49.97		
Total	161	68827.78			

4.2.2. Growth analysis

Influence of the different heavy metals on plant growth was determined on the basis of different parameters – relative growth index (RGI), leaf area (LA), absolute growth rate (AGR), relative growth rate (RGR), specific leaf area (SLA) and specific leaf weight (SLW).

4.2.2.1.Relative growth index

Relative growth index was computed from the dry weight biomass as per the formula given in materials and methods. RGI in control was taken to be 100. Response of different cultivars showed different trends with Hg being most inhibitory (lowest RGI values) to Arka Anamika, Deepti and PB-57. All the treatments differed significantly from control (Tables 3 and 3A). Minimum inhibition in Arka Anamika, Najuka-F1and Parbhani Kranti was by Pb and in Deepti, Paras Soumya and PB-57 by Cu.

Table 2: Tolerance index of different cultivars of okra to heavy metals stress

Cultivars	Parts	Tolerance index (Fresh weight basis) Control	Cadmium	Copper	Mercury	Lead
Arka Anamika	Whole plant	0.00	-34.02 ± 4.02	-21.89 ± 1.97	-27.70 ± 3.28	-13.86 ± 1.77
	Shoot	0.00	-31.89 ± 3.61	-20.77 ± 2.04	-27.53 ± 2.71	-11.73 ± 1.70
	Root	0.00	-43.83 ± 5.95	-27.47 ± 1.50	-31.60 ± 4.50	-22.66 ± 2.62
Deepti	Whole plant	0.00	-10.49 ± 0.84	-2.44 ± 0.24	-13.76 ± 0.66	-10.54 ± 0.68
	Shoot	0.00	-9.95 ± 1.05	-7.66 ± 0.43	-11.99 ± 1.03	-8.87 ± 1.54
	Root	0.00	-24.45 ± 2.28	-11.49 ± 0.91	-28.64 ± 1.57	-22.09 ± 2.62
Najuka-F1	Whole plant	0.00	-39.24 ± 3.64	-34.92 ± 5.17	-50.95 ± 1.60	-18.84 ± 2.62
	Shoot	0.00	-41.63 ± 2.65	-33.57 ± 2.32	-53.95 ± 0.23	-19.66 ± 1.96
	Root	0.00	-39.43 ± 3.10	-31.17 ± 3.02	-47.76 ± 2.94	-17.34 ± 4.2
Paras Soumya	Whole plant	0.00	-39.85 ± 2.49	-27.79 ± 4.25	-46.68 ± 1.55	-45.63 ± 5.37
	Shoot	0.00	-35.46 ± 0.08	-21.53 ± 2.95	-47.88 ± 3.39	-45.33 ± 5.43
	Root	0.00	-64.67 ± 7.60	-48.09 ± 5.35	-60.42 ± 10.99	-49.01 ± 6.51
Parbhani Kranti	Whole plant	0.00	-31.97 ± 1.30	-30.53 ± 1.72	-25.10 ± 6.22	-15.34 ± 1.76
	Shoot	0.00	-31.12 ± 0.50	-33.20 ± 1.74	-24.96 ± 3.20	-10.43 ± 0.13
	Root	0.00	-49.19 ± 5.30	-32.34 ± 1.53	-26.84 ± 2.78	-44.87 ± 5.14
PB 57	Whole plant	0.00	-30.48 ± 2.02	-22.35 ± 2.07	-31.47 ± 0.09	-26.76 ± 0.76
	Shoot	0.00	-32.14 ± 2.59	-18.25 ± 2.00	-27.25 ± 0.46	-25.09 ± 0.82
	Root	0.00	-22.15 ± 1.11	-38.48 ± 2.15	-48.43 ± 0.90	-40.28 ± 3.52

Treatments were applied at 1000 μg ml^{-1} concentration of the salts

Values are mean of 3 replicates; ± = SEm

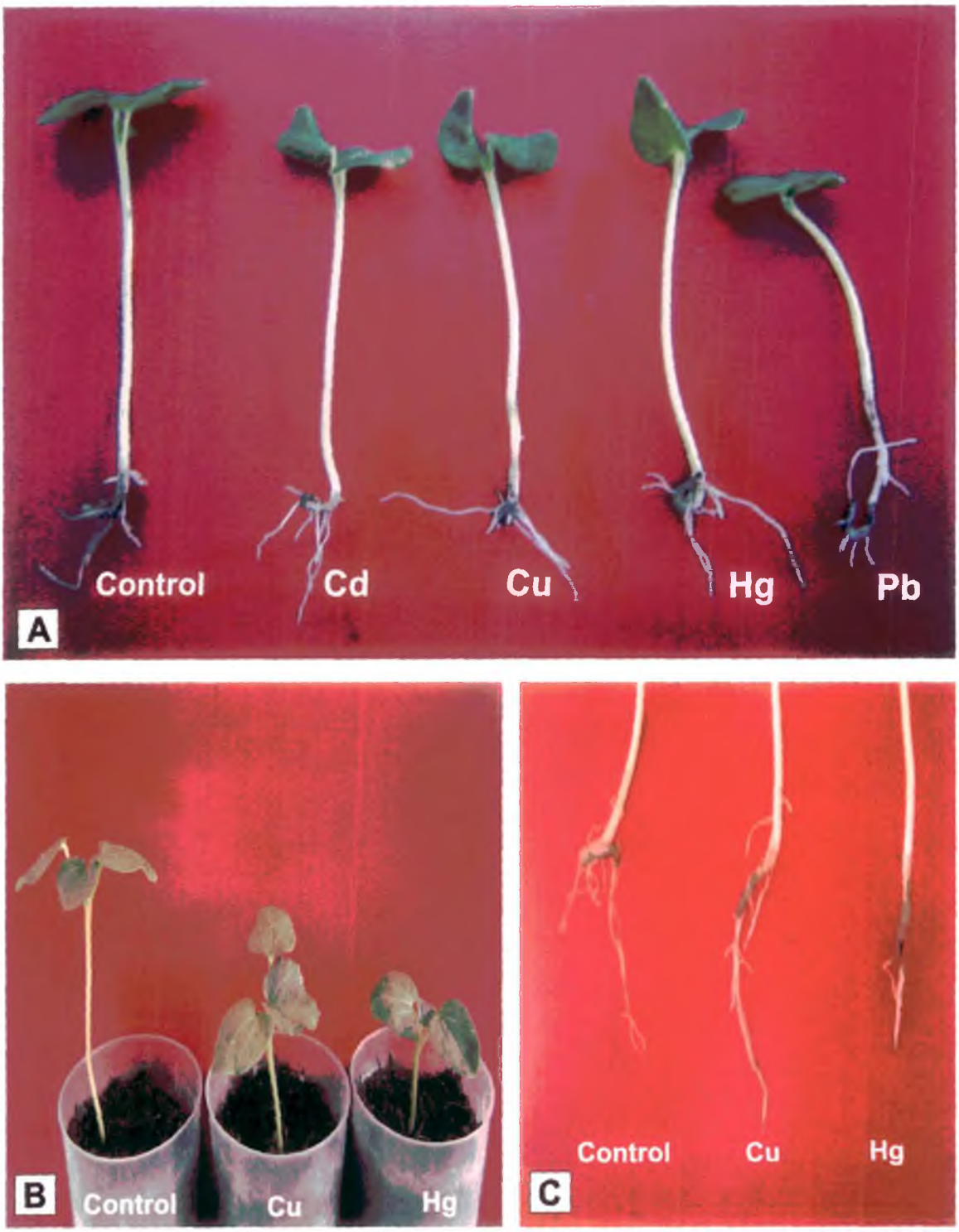


Plate V(A-C): Effect of heavy metal salts on seedling growth of different okra cultivars.

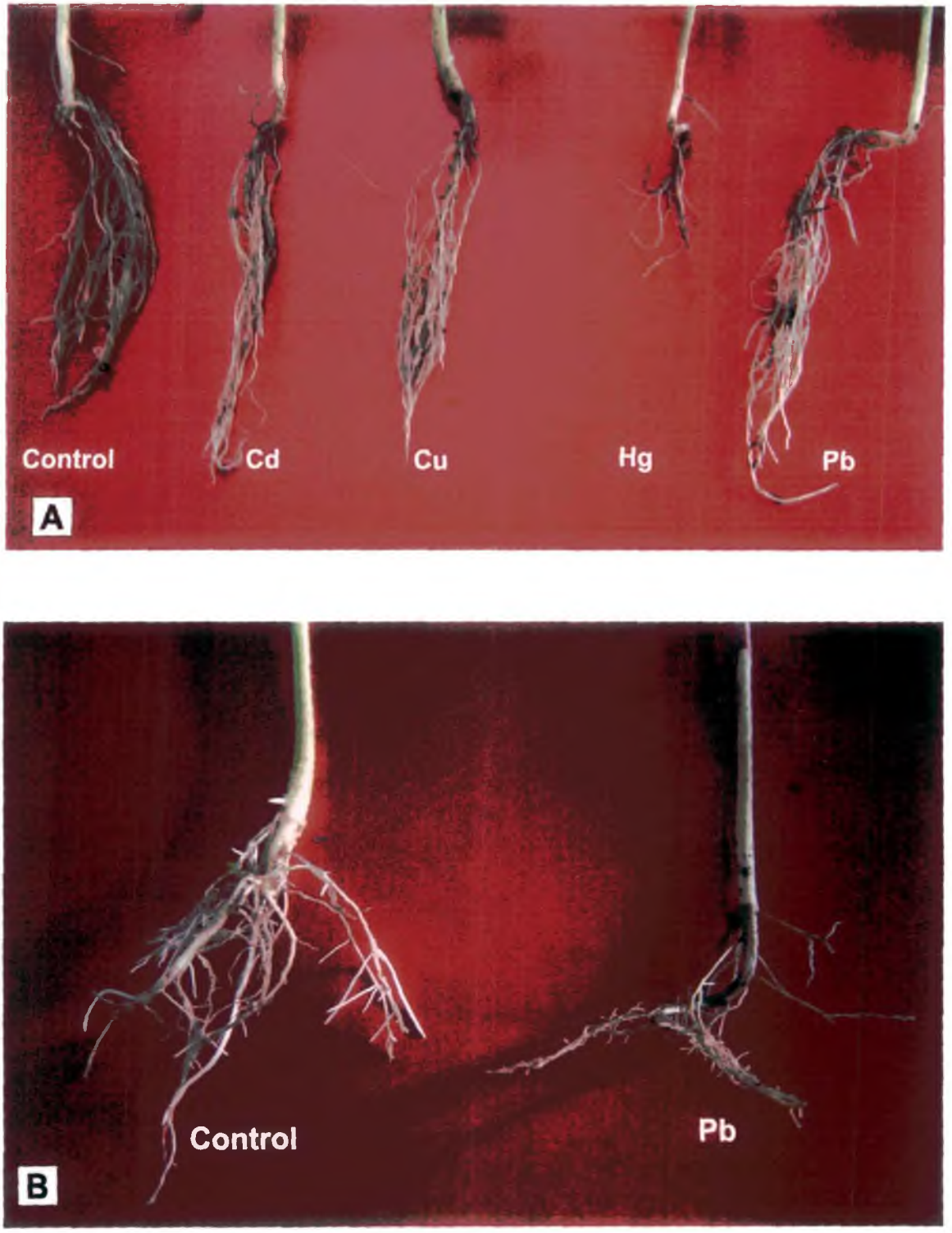


Plate VI (A-B): Root damage in okra plants caused by heavy metal treatments (cv. Arka Anamika) at vegetative stage.

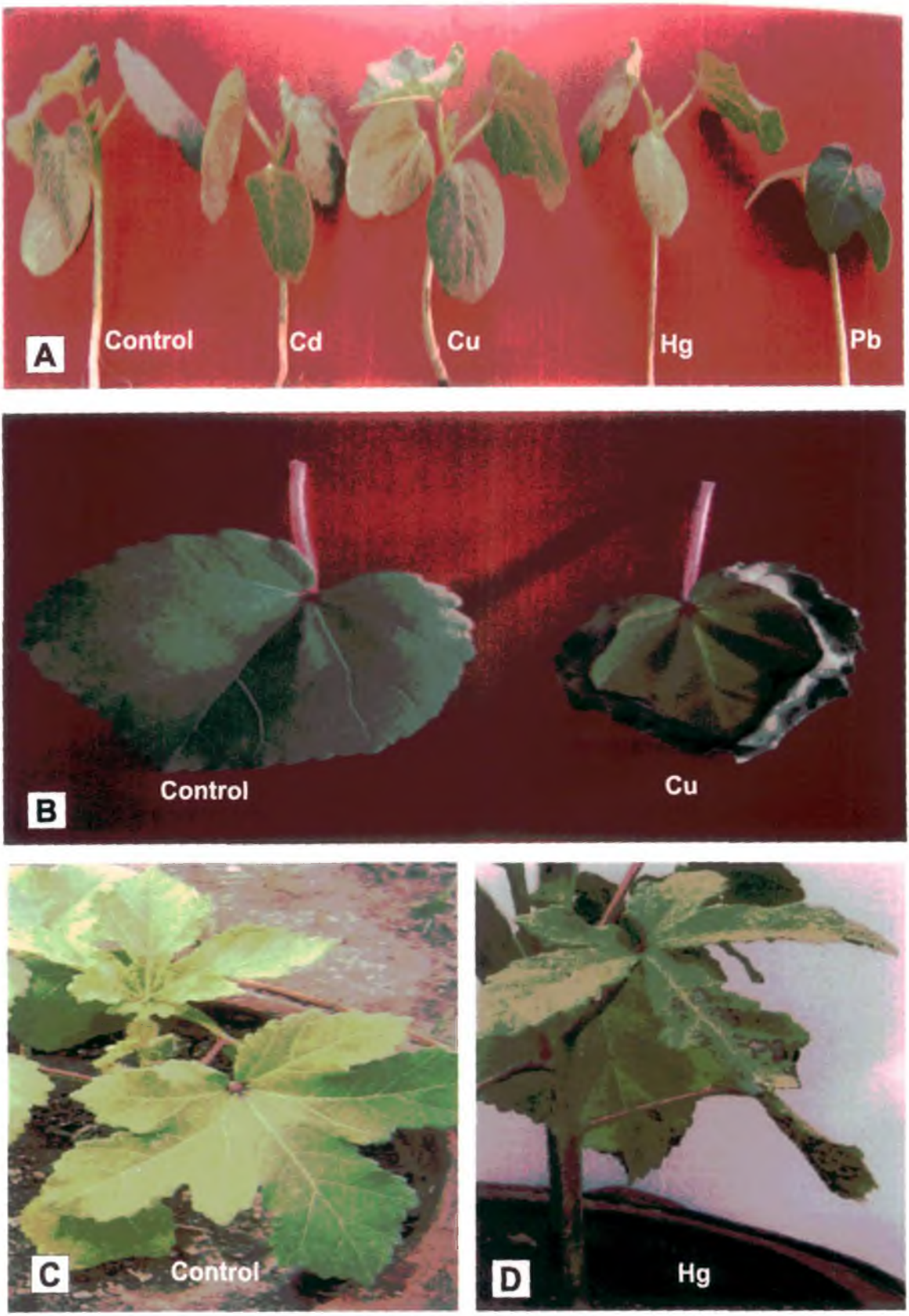


Plate VII : Leaf damage in okra plants (cv. Arka Anamika) A: Seedling stage; B: Vegetative stage; C-D: Reproductive stage.

Table 3: Effect of heavy metals treatments on growth of okra cultivars

Cultivars	Relative growth index (%)				
	Control	Cadmium	Copper	Mercury	Lead
Arka Anamika	100 ± 0.00	81.51 ±7.45	70.79 ± 4.08	67.00 ± 5.37	98.16 ±7.76
Deepti	100 ± 0.00	80.71 ± 5.39	86.31 ± 5.70	69.92 ± 4.17	83.75 ± 3.20
Najuka-F1	100 ± 0.00	67.32 ± 5.14	73.59 ± 5.72	80.29 ± 0.87	87.97 ± 1.47
Paras Soumya	100 ± 0.00	69.02 ± 2.24	87.87 ± 5.22	70.23 ± 0.66	66.07 ± 8.15
Parbhani Kranti	100 ± 0.00	67.02 ± 3.46	55.49 ± 1.61	63.73 ± 4.68	91.18 ± 1.33
PB-57	100 ± 0.00	84.52 ± 1.19	95.55 ± 2.15	75.28 ± 2.20	91.07 ± 3.17

Treatments were applied at 1000 μg ml^{-1} concentration of the salts
Values are mean of 3 replicates; ± = SEm

Table 3A: Analysis of variance of data presented in Table 3

Source	D.F.	S.S.	M.S.	F	C.D.
Replication	2	2600.85	1300.43		
Variety	5	1702.75	340.55	4.75	6.19
Treatment	4	9450.42	2362.61	32.96	5.66
Interaction	20	4768.74	238.44	3.33	13.85
Error	58	4158.30	71.69		
Total	89	22681.06			

4.2.2.2.Absolute growth rate

Absolute growth rate which indicates the increase in dry matter per unit time was found to be significantly lower in plants treated with the different heavy metals. As with all other growth indices AGR was minimum in Arka Anamika, Parbhani

Kranti and PB-57 treated with Hg. Pb and Cu inhibited growth to a lesser extent in comparison to Hg (Fig. 3A).

4.2.2.3.Relative growth rate

Relative growth rate decreased with heavy metal application in most of the cases. However, the decrease was not significant in some of the treatments (Fig. 3B).

Table 4: Changes in leaf area of okra cultivars following heavy metal treatments

Cultivars	Leaf area (cm^2)				
	Control	Cadmium	Copper	Mercury	Lead
Arka Anamika	14.40 ± 1.45	9.84 ± 0.17	9.60 ± 0.23	9.06 ± 0.29	10.54 ± 0.67
Deepti	45.00 ± 1.46	38.28 ± 4.61	33.95 ± 3.13	26.05 ± 2.24	27.73 ±1.39
Najuka-F1	19.36 ± 2.45	12.96 ± 1.72	11.16 ± 0.62	11.60 ± 1.36	12.18 ± 1.16
Paras	26.35 ± 0.49	21.43 ± 0.64	29.44 ± 0.62	20.87 ± 0.42	19.71 ± 0.25
Soumya					
Parbhani Kranti	36.46 ± 0.74	21.78 ± 0.35	19.80 ± 0.77	24.92 ± 0.68	30.55 ± 1.05
PB-57	11.42 ± 0.54	7.06 ± 0.35	8.48 ± 0.09	6.80 ± 0.28	8.13 ± 0.07

Treatments were applied at 1000 μg ml^{-1} concentration of the salts
Values are mean of 3 replicates; ± = SEm

Table 4A: Analysis of variance of data presented in Table 4

Source	D.F.	S.S.	M.S.	F	C.D.
Replication	2	211.73	105.87		
Variety	5	7838.79	1567.76	124.33	2.60
Treatment	4	863.26	215.82	17.11	2.37
Interaction	20	855.76	42.79	3.39	5.81
Error	58	731.38	12.61		
Total	89	10500.92			

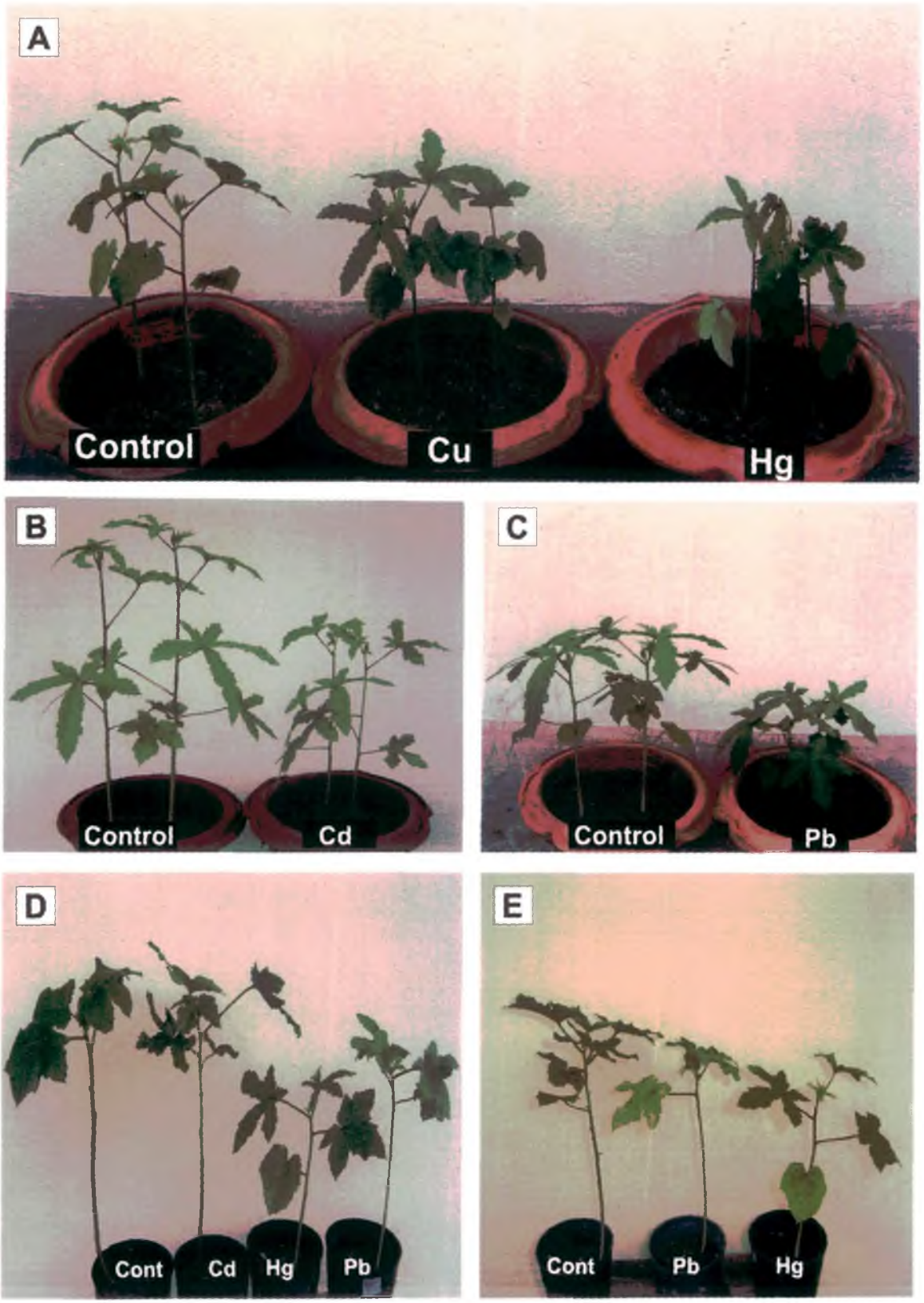


Plate VIII : Control and treated plants of different cultivars of okra. A: Arka Anamika; B: PB-57; C: Najuka -F1; D: Parbhani Kranti and E:PB-57.

4.2.2.4.Leaf area

Significant decrease in leaf area was observed in the treated plants as compared to the control. Among all the heavy metals Hg was most inhibitory to the cultivars Arka Anamika, Deepti and PB-57 (Tables 4 and 4A).

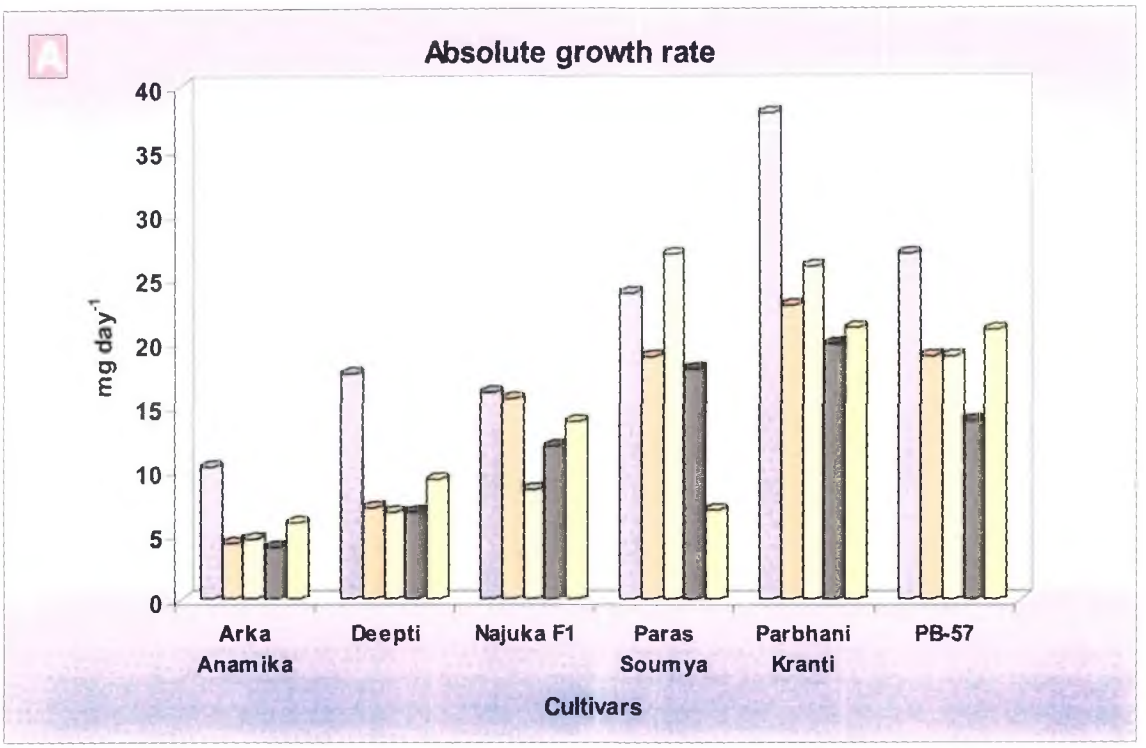
Table 5: Yield of different cultivars of okra plants following heavy metal treatments

Treatments		Yield (fresh wt. g plant^{-1})					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		83.19 ± 0.95	75.66 ± 0.67	71.35 ± 0.64	54.92 ± 0.86	76.26 ± 0.66	72.92 ± 0.99
Cadmium	100	51.55** ± 1.41	48.26** ± 0.71	40.17** ± 0.22	34.09** ± 0.76	48.22** ± 0.61	49.12** ± 0.65
	1000	42.84** ± 1.45	39.20** ± 0.86	37.59** ± 0.90	31.34** ± 0.55	44.96** ± 1.02	37.72** ± 0.57
Copper	100	52.03** ± 0.61	45.08** ±0.75	42.10** ± 0.58	36.49** ± 0.82	50.06** ± 0.83	44.15** ± 0.93
	1000	45.06** ± 0.15	36.70** ± 0.91	39.98** ± 0.37	33.30** ± 0.57	45.55** ± 0.85	38.82** ± 0.83
Mercury	100	38.05** ± 1.28	43.36** ± 0.17	38.54** ± 0.67	32.87** ± 0.32	39.46** ± 0.76	39.35** ± 0.91
	1000	35.96** ± 0.99	35.81** ± 1.48	36.55** ± 2.57	29.08** ± 0.89	35.62** ± 0.70	32.28** ± 0.79
Lead	100	48.55** ± 1.15	42.07** ± 0.99	42.03** ± 0.46	33.28** ± 0.87	44.18** ± 0.41	43.55** ± 0.79
	1000	40.98** ± 0.60	37.37** ± 0.30	39.77** ± 1.05	30.93** ± 0.74	38.29** ± 0.70	34.80** ± 0.38

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

4.2.2.5.Specific leaf area

The heavy metal treatments showed an inhibitory effect on the specific leaf area. The inhibition was maximum with Hg treatment in Deepti, Paras Soumya and Parbhani Kranti. In case of Arka Anamika and Najuka-F1 the inhibition was maximum due to Pb whereas, Cd was most inhibitory to PB-57 (Fig. 4A).



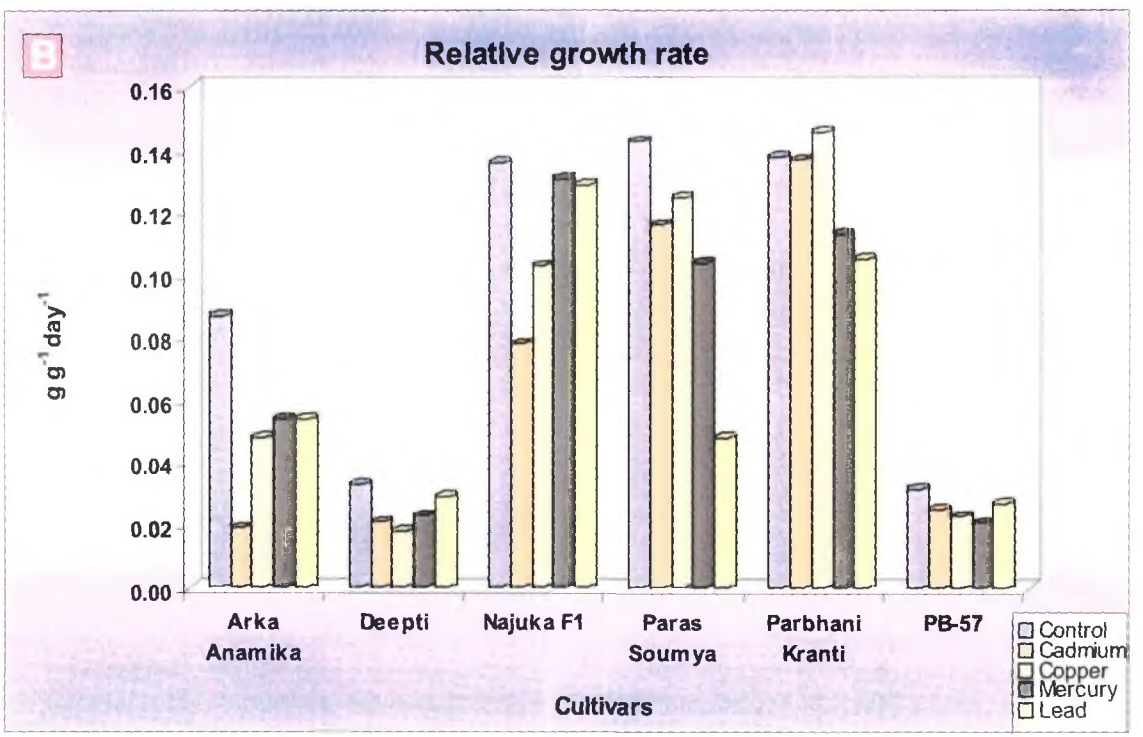


Fig.3 (A&B): Effect of heavy metals at 1000 μg ml^{-1} concentration on Absolute growth rate (A) and Relative growth rate (B) of okra plants

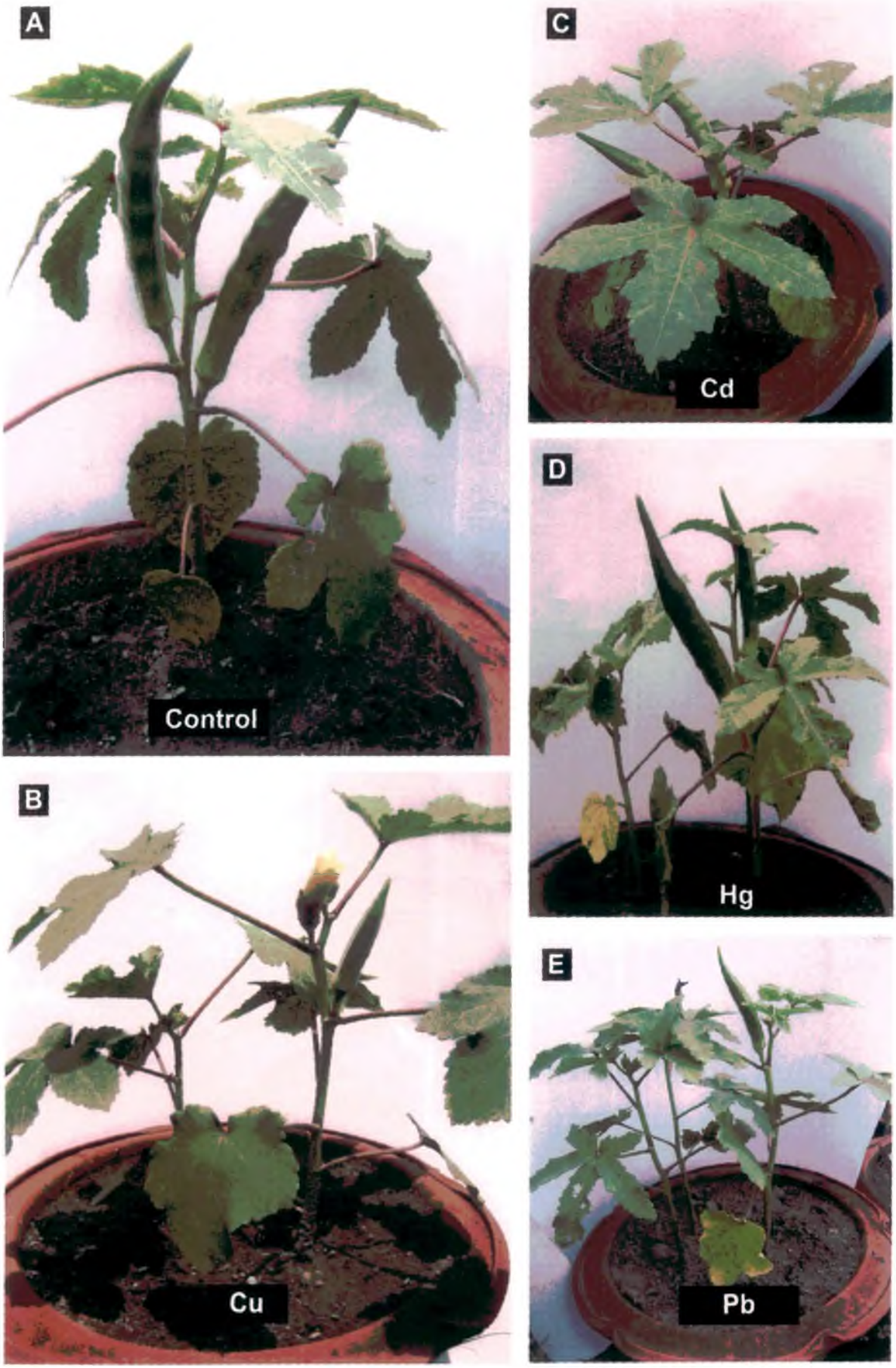


Plate IX(A-E): Fruiting plants of okra (cv. Arka Anamika) subjected to different heavy metal treatments.

4.2.2.6.Specific leaf weight

In case of specific leaf weight there was an increase due to the different treatments in all the varieties. Significant differences were however obtained only between control and Cd and control and Hg (Fig. 4B).

4.2.3. Yield

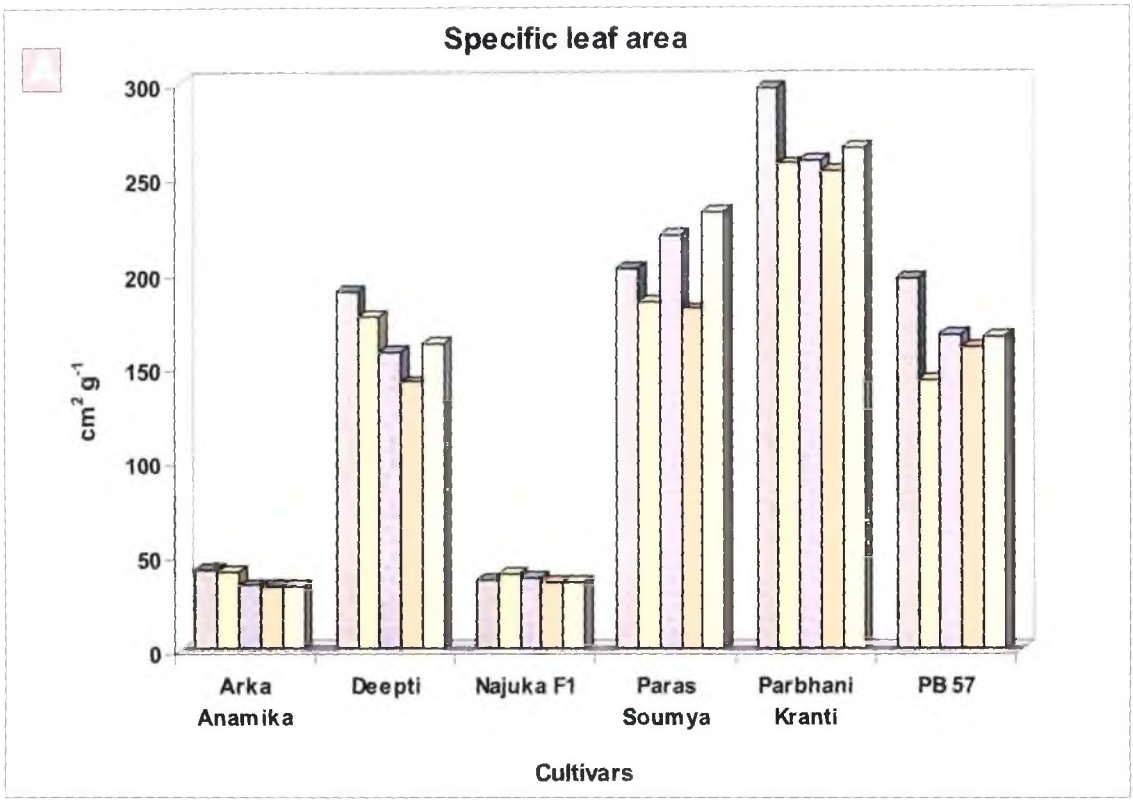
In order to determine the effect of different heavy metals on yield of okra cultivars the fresh weight of the fruits were taken and computed as yield per plant. Highly significant decrease in yield was observed in treatments of all cultivars. In 100 μg ml^{-1} of Hg treatment there was a decrease of yield by about 57% in Arka Anamika (Table 5).

4.3. Studies on carbohydrates in okra plants subjected to heavy metal treatments

Influence of the heavy metal on the carbohydrate metabolism was quantitatively analyzed by determining the total soluble sugar, reducing sugar and starch contents of the different plant parts of okra plants at seedling, vegetative and reproductive stages. An overall reduction in the carbohydrates as a whole was evident.

4.3.1. Total soluble sugar

In the seedling stage total soluble sugar of leaves was reduced but was statistically significant only in some cases. Among the different cultivars Hg at 1000 μg ml^{-1} decreased the total soluble sugar to the greatest degree in Najuka-F1, Parbhani Kranti and PB-57 while Pb at 1000 μg ml^{-1} was most effective in Arka Anamika and Paras Soumya (Table 6). In case of leaves at vegetative and reproductive stages also a similar trend was noticed with Hg and Pb being most effective in bringing about noticeable changes in total soluble sugar content. However, copper was not very effective in inducing changes in total soluble sugar content (Tables 7 and 8). Total soluble sugar of roots determined at seedling, vegetative and reproductive stages also showed a decrease in the contents which in most cases were significant at 5%. At vegetative stage inhibition was maximum by Cd at 1000 μg ml^{-1} while in case of reproductive stage Hg was found to inhibit total soluble sugar content in 4 cultivars (Tables 9-11).



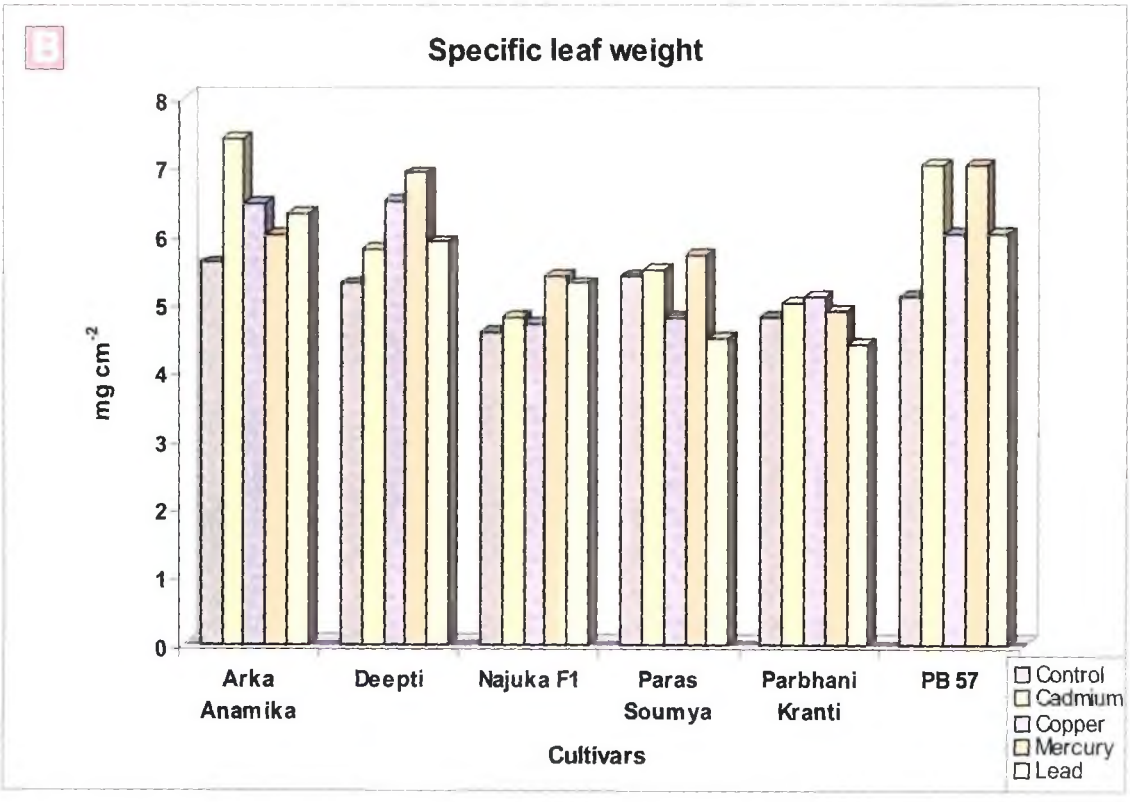


Fig.4 (A&B): Effect of heavy metals at 1000 µg ml^{-1} concentration on specific leaf area (A) and specific leaf weight (B) of okra plants

It was observed that overall among the different heavy metals Cu was least effective in inhibition of total soluble sugar of leaves and roots.

Table 6: Total soluble sugar content of leaves of okra at seedling stage following heavy metal treatments

Treatments		Total sugar content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		23.50 ± 1.18	12.71 ± 0.21	17.00 ± 0.47	16.67 ± 0.45	18.75 ± 0.34	18.86 ± 0.67
Cadmium	**100**	19.25 ± 0.35	7.36** ± 0.41	11.50** ± 0.24	14.79 ± 0.34	18.50 ± 0.24	13.70* ± 0.40
	1000	15.75* ± 0.83	5.58** ±0.16	10.00** ± 0.47	13.83* ± 0.47	18.00 ± 0.24	11.80* ± 0.94
Copper	**100**	18.86 ± 1.26	11.16 ± 0.51	13.00* ± 0.47	12.72 ± 0.84	18.25 ± 0.12	11.53* ± 0.60
	1000	14.76* ± 0.46	10.96* ± 0.12	11.50** ± 0.24	11.67* ± 0.95	17.50 ± 0.23	9.95** ± 0.38
Mercury	**100**	17.75 ± 0.83	11.12 ± 0.31	8.00** ± 0.47	13.83 ± 0.79	17.25 ± 0.35	9.90** ± 0.57
	1000	16.50* ± 0.47	8.31** ± 0.09	7.00** ± 0.47	13.50* ± 0.24	16.25* ± 0.12	8.40** ± 0.36
Lead	**100**	19.83 ± 0.95	10.72* ± 0.31	12.00* ± 0.47	16.11 ± 0.50	17.00 ± 0.24	11.04* ± 0.64
	1000	13.00* ± 1.18	10.48* ± 0.23	11.00** ± 0.27	9.78* ± 0.59	16.50* ± 0.23	10.25** ± 0.54

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 7: Total soluble sugar content of leaves of okra at vegetative stage following heavy metal treatments

Treatments		Total sugar content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		10.33 ± 0.36	13.30 ± 0.33	12.90 ± 0.14	14.00 ± 0.47	17.82 ± 0.25	16.20 ± 0.22
Cadmium	100	9.33 ± 0.60	9.00* ± 0.57	10.15** ± 0.17	10.00* ± 0.34	15.50* ± 0.23	12.00 ± 0.09
	1000	9.17 ± 0.36	8.50** ± 0.24	10.05** ± 0.21	9.40* ± 0.24	13.42* ± 0.57	8.00** ±0.15
Copper	100	9.50 ± 0.24	13.20 ± 0.57	9.65** ± 0.07	11.50* ± 0.24	16.00 ± 0.38	14.00* ± 0.34
	1000	10.00 ± 0.24	11.40* ± 0.28	10.20** ± 0.14	9.50* ± 0.24	15.63* ± 0.34	10.76* ± 0.51
Mercury	100	9.00 ± 0.47	6.65** ± 0.21	9.20** ± 0.09	12.00 ± 0.54	15.00* ± 0.47	10.00** ± 0.27
	1000	8.00* ± 0.24	7.15* ± 0.26	8.85** ± 0.07	11.00* ± 0.47	13.89* ± 0.49	8.03** ± 0.26
Lead	100	8.33 ± 0.60	12.35 ± 0.31	10.40** ± 0.28	11.00* ± 0.27	14.50* ± 0.24	12.60* ± 0.58
	1000	6.83* ± 0.49	11.40* ± 0.28	10.45** ± 0.31	10.50* ± 0.24	13.14* ± 0.46	7.60** ± 0.33

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 8: Total soluble sugar content of leaves of okra at reproductive stage following heavy metal treatments

Treatments		Total sugar content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		14.17 ± 0.49	14.75 ± 0.35	18.50 ± 0.23	19.50 ± 0.24	20.75 ± 0.12	13.00 ± 0.23
Cadmium	100	13.50 ± 0.23	13.75 ± 0.12	16.00* ± 0.24	15.50* ± 1.65	18.25** ± 0.12	11.75* ± 0.12
	1000	11.50* ± 0.24	12.25 ± 0.35	15.50* ± 0.23	15.00* ± 0.94	17.25** ± 0.15	11.25* ± 0.15
Copper	100	12.50 ± 0.47	13.00 ± 0.47	16.25* ± 0.12	16.50* ± 0.71	18.75** ± 0.11	11.50* ± 0.23
	1000	12.00 ± 0.24	12.75* ± 0.12	15.15** ± 0.12	16.00* ± 0.77	18.00** ± 0.23	11.25* ± 0.12
Mercury	100	10.50* ± 0.24	12.00* ± 0.23	15.75* ± 0.59	14.50* ± 0.71	17.50** ± 0.24	10.75* ± 0.12
	1000	9.50* ± 0.71	10.75* ± 0.35	15.50* ± 0.24	13.50** ± 0.71	17.00** ± 0.23	10.25** ± 0.12
Lead	100	13.00 ± 0.71	12.50 ± 0.47	16.50* ± 0.23	14.50* ± 0.94	17.75** ± 0.12	11.00* ± 0.34
	1000	11.00* ± 0.47	11.75* ± 0.35	14.75** ± 0.12	14.00* ± 0.70	16.75** ± 0.35	10.75* ± 0.35

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 9: Effect of heavy metals and total soluble sugars of okra roots at seedlings stage

Treatments		Total sugar content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		19.33 ± 0.27	11.50 ± 0.24	11.33 ± 0.72	18.67 ± 0.32	19.25 ± 0.35	16.74 ± 0.10
Cadmium	100	14.00* ± 0.47	9.50* ± 0.24	8.67 ± 0.27	14.00* ± 0.47	16.00* ± 0.47	12.93** ± 0.08
	1000	11.50** ± 0.24	8.50* ± 0.24	6.50* ± 0.24	12.50** ± 0.24	13.75** ± 0.35	9.82** ± 0.42
Copper	100	15.00* ± 0.47	8.00* ± 0.47	9.67 ± 0.72	15.67* ± 0.54	17.50 ± 0.24	13.03** ± 0.15
	1000	15.50* ± 0.47	6.50** ± 0.24	5.67* ± 0.27	14.00* ± 0.47	15.75* ± 0.12	7.18** ± 0.05
Mercury	100	16.50* ± 0.24	9.00* ± 0.34	7.00* ± 0.47	14.33* ± 0.72	16.25* ± 0.33	7.68** ± 0.02
	1000	13.33** ± 0.29	7.00** ± 0.27	6.67* ± 0.72	12.00** ± 0.47	14.00* ± 0.47	7.36** ± 0.09
Lead	100	16.00* ± 0.47	7.50** ± 0.24	6.67* ± 0.27	17.00 ± 0.47	16.00* ± 0.47	5.88** ± 0.30
	1000	14.33* ± 0.54	6.30** ± 0.31	5.33* ± 0.72	13.33* ± 0.72	15.25* ± 0.35	5.42** ± 0.23

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 10: Effect of heavy metals and total soluble sugars of okra roots at vegetative stage

Treatments		Total sugar content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		8.67 ± 0.31	12.00 ± 0.47	10.50 ± 0.71	12.17 ± 0.72	17.00 ± 0.47	10.78 ± 0.19
Cadmium	100	3.99** ± 0.62	10.50 ± 0.23	7.50* ± 0.24	8.83* ± 0.49	15.00 ± 0.47	9.00 ± 0.47
	1000	3.33** ± 0.31	8.50* ± 0.24	5.00** ± 0.47	8.33* ± 0.49	13.56* ± 0.25	7.83** ± 0.08
Copper	100	5.33* ± 0.62	11.40 ± 0.28	5.50** ± 0.24	9.83* ± 0.27	14.37* ± 0.88	10.00 ± 0.94
	1000	4.00* ± 0.62	9.00* ± 0.42	5.20** ± 0.47	10.17* ± 0.27	14.14* ± 0.38	8.24** ± 0.14
Mercury	100	5.33* ± 0.62	8.75* ± 0.27	9.50 ± 0.24	8.63* ± 0.36	14.50* ± 0.24	7.00** ± 0.17
	1000	4.67* ± 0.31	8.25** ± 0.26	7.50* ± 0.21	8.00* ± 0.62	14.04* ± 0.33	7.97** ± 0.09
Lead	100	4.67* ± 0.31	10.15* ± 0.17	8.50* ± 0.24	12.00 ± 0.71	15.50 ± 0.24	9.90 ± 0.42
	1000	4.66** ± 0.94	10.00** ± 0.19	7.50* ± 0.24	8.83* ± 0.36	13.00* ± 0.09	9.76 ± 0.24

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 11: Effect of heavy metals and total soluble sugars of okra roots at reproductive stage

Treatments		Total sugar content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		13.50 ± 0.24	10.25 ± 0.35	12.00 ± 0.47	15.00 ± 0.47	18.50 ± 0.23	11.25 ± 0.12
Cadmium	100	10.00* ± 0.34	9.75 ± 0.58	10.00 ± 0.47	11.00* ± 0.34	16.50* ± 0.24	10.25* ± 0.12
	1000	8.50* ± 0.71	8.25 ± 0.59	8.50* ± 0.24	9.50* ± 0.71	13.50** ± 0.23	9.00* ± 0.23
Copper	100	11.50* ± 0.23	9.50 ± 0.23	9.00* ± 0.47	11.00* ± 0.47	16.25* ± 0.35	10.50 ± 0.24
	1000	9.00* ± 0.47	8.50 ± 0.24	8.50* ± 0.24	8.50** ± 0.24	14.50** ± 0.23	9.00* ± 0.23
Mercury	100	9.50* ± 0.71	7.50* ± 0.23	8.00* ± 0.47	10.50* ± 0.24	15.25* ± 0.35	9.25* ± 0.35
	1000	8.00** ± 0.34	6.75* ± 0.35	7.60* ± 0.23	8.00** ± 0.47	12.50** ± 0.24	8.25** ± 0.11
Lead	100	11.00* ± 0.47	8.75 ± 0.35	8.50* ± 0.31	10.50* ± 0.71	13.75** ± 0.12	9.50* ± 0.23
	1000	8.50** ± 0.23	8.00 ± 0.47	7.50* ± 0.71	9.48* ± 0.24	13.25** ± 0.12	9.25* ± 0.11

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

4.3.2. Reducing sugar

The results of quantification of reducing sugar at different stages following heavy metal treatments revealed that in all stages there was an increase of reducing sugar. The quantum of increase was not the same in the different cultivars or the

same cultivars following different treatments. The results have been tabulated in tables 12 to 17. All the observed increases were however not statistically significant or in some cases significant at 5% level. Hg was most effective in the vegetative stage as compared to the reproductive stage.

Table 12: Changes in reducing sugar content of leaves of okra cultivars at seedling stage following heavy metal treatments

Treatments		Reducing sugar content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		1.66 ± 0.04	1.01 ± 0.05	1.97 ± 0.03	2.30 ± 0.09	1.07 ± 0.03	1.07 ±0.06
Cadmium	100	2.02 ± 0.09	1.47 ± 0.10	2.31 ± 0.08	2.83* ± 0.07	1.24 ± 0.03	1.11 ± 0.05
	1000	2.11** ± 0.02	1.52* ± 0.03	2.65** ± 0.02	3.43* ± 0.09	1.30* ± 0.03	2.33** ± 0.09
Copper	100	1.70 ± 0.04	1.41 ± 0.09	2.48* ± 0.05	3.10* ± 0.05	1.27* ± 0.01	1.89* ± 0.09
	1000	1.73 ± 0.02	1.75* ± 0.13	2.42 ± 0.13	3.13* ± 0.05	1.41* ± 0.02	2.72** ± 0.08
Mercury	100	2.00* ± 0.04	1.63* ± 0.08	2.53* ± 0.08	3.32* ± 0.08	1.32* ± 0.01	1.50* ± 0.05
	1000	2.06** ± 0.01	1.80** ± 0.05	2.82* ± 0.10	3.67** ± 0.07	1.47** ± 0.02	1.91* ± 0.07
Lead	100	1.94* ± 0.02	1.58* ± 0.05	2.98** ± 0.08	2.17 ± 0.03	1.38** ± 0.01	1.21 ± 0.06
	1000	2.09** ± 0.01	1.69* ± 0.10	2.99** ± 0.02	4.00* ± 0.05	1.44** ± 0.01	1.24 ± 0.09

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 13: Changes in reducing sugar content of leaves of okra cultivars at vegetative stage following heavy metal treatments

Treatments		Reducing sugar content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		0.82 ± 0.02	0.85 ± 0.03	1.23 ± 0.05	0.84 ± 0.02	0.79 ± 0.03	1.00 ± 0.04
Cadmium	100	0.93 ± 0.04	1.02 ± 0.05	1.35 ± 0.05	1.24* ± 0.05	0.86 ± 0.02	1.24 ± 0.05
	1000	1.03** ± 0.01	1.07* ± 0.03	1.63* ± 0.02	1.30** ± 0.02	0.96 ± 0.07	1.27* ±0.05
Copper	100	0.92 ± 0.05	1.15** ± 0.03	1.46 ± 0.05	1.58** ± 0.05	0.87 ± 0.01	1.30* ± 0.03
	1000	0.98* ± 0.01	1.17* ± 0.03	1.52* ± 0.02	1.75** ± 0.07	1.12 ± 0.08	1.55* ± 0.08
Mercury	100	0.98* ± 0.02	0.98 ± 0.02	1.57* ± 0.05	1.52** ± 0.03	1.02 ± 0.05	1.18 ± 0.03
	1000	1.02* ± 0.03	1.24* ± 0.06	2.24** ± 0.05	1.63** ± 0.02	1.08 ± 0.06	1.36* ± 0.05
Lead	100	0.98 ± 0.03	1.00* ± 0.01	1.23 ± 0.05	1.35** ± 0.05	0.90 ± 0.05	1.23 ± 0.04
	1000	1.08** ± 0.01	1.05 ± 0.04	1.29 ± 0.02	1.57** ± 0.05	0.95 ± 0.05	1.35* ± 0.02

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 14: Changes in reducing sugar content of leaves of okra cultivars at reproductive stage following heavy metal treatments

Treatments		Reducing sugar content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		1.13 ± 0.05	0.96 ± 0.02	1.72 ± 0.01	1.63 ± 0.03	1.07 ± 0.03	1.04 ± 0.01
Cadmium	100	1.30 ± 0.07	1.35** ± 0.02	1.89* ± 0.03	2.30 ± 0.03	1.16 ± 0.04	1.13 ± 0.08
	1000	1.35 ± 0.05	1.43** ± 0.01	1.92* ± 0.03	2.42 ± 0.08	1.30 ± 0.02	1.24 ± 0.05
Copper	100	1.18 ± 0.08	1.30** ± 0.02	1.83* ± 0.03	3.19 ± 0.08	1.19 ± 0.03	1.16 ± 0.01
	1000	1.58* ± 0.05	1.41** ± 0.03	1.86* ± 0.02	3.47 ± 0.05	1.27 ± 0.01	1.21 ± 0.02
Mercury	100	1.18 ± 0.08	1.61** ± 0.04	1.94* ± 0.02	2.22 ± 0.04	1.21 ± 0.04	1.15 ± 0.01
	1000	1.24 ± 0.11	1.44** ± 0.07	1.89 ± 0.04	2.25 ± 0.11	1.24 ± 0.02	1.19 ± 0.03
Lead	100	1.30 ± 0.03	1.55** ± 0.09	1.94* ± 0.01	1.85 ± 0.08	1.33 ± 0.04	1.18 ± 0.02
	1000	1.41 ± 0.08	1.64** ± 0.02	1.97** ± 0.02	2.02 ± 0.11	1.38 ± 0.04	1.19 ± 0.03

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 15: Changes in reducing sugar content of roots of okra cultivars at seedling stage following heavy metal treatments

Treatments		Reducing sugar content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka -F1	Paras Soumya	Parbhani Kranti	PB-57
Control		0.80 ± 0.05	0.68 ± 0.05	0.43 ± 0.02	1.70 ± 0.02	0.90 ± 0.05	1.15 ± 0.08
Cadmium	100	1.67 ± 0.05	1.08 ± 0.04	0.67 ± 0.09	1.79 ± 0.05	1.13 ± 0.05	1.23 ± 0.05
	1000	1.87 ± 0.07	0.90 ± 0.05	0.70 ± 0.05	1.92* ± 0.02	1.36 ± 0.11	1.55* ± 0.04
Copper	100	1.03 ± 0.03	0.83 ± 0.06	0.73 ± 0.05	1.72 ±0.06	1.24* ± 0.05	1.36 ± 0.04
	1000	1.20 ± 0.05	1.98 ± 0.09	1.40 ± 0.05	1.78 ± 0.05	1.41* ± 0.03	1.58* ± 0.04
Mercury	100	1.30 ± 0.05	0.74 ± 0.04	0.60 ± 0.05	1.75 ± 0.04	1.47* ± 0.11	1.37 ± 0.08
	1000	1.47 ± 0.05	0.92 ± 0.04	0.07 ± 0.05	1.83* ± 0.01	1.52* ± 0.08	1.34 ± 0.04
Lead	100	1.53 ± 0.03	0.86 ± 0.00	0.67 ± 0.02	1.91* ± 0.02	1.58* ± 0.05	1.48 ± 0.02
	1000	1.80 ± 0.05	0.93 ± 0.05	1.12 ± 0.09	1.98** ± 0.01	1.69** ± 0.05	1.53 ± 0.06

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 16: Changes in reducing sugar content of roots of okra cultivars at vegetative stage following heavy metal treatments

Treatments		Reducing sugar content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka -F1	Paras Soumya	Parbhani Kranti	PB-57
Control		0.75 ± 0.07	0.71 ± 0.04	1.02 ± 0.05	0.53 ± 0.02	0.73 ± 0.03	1.06 ± 0.02
Cadmium	100	1.79* ± 0.14	1.07* ± 0.03	1.30 ± 0.08	0.93* ± 0.07	0.99 ± 0.07	1.19 ± 0.03
	1000	1.72** ±0.04	1.17* ± 0.07	1.36 ± 0.11	0.98* ± 0.06	1.04* ± 0.03	1.27 ± 0.07
Copper	100	1.95* ± 0.14	1.01* ± 0.03	1.35* ± 0.05	0.65 ± 0.04	0.84 ± 0.03	1.30* ± 0.03
	1000	2.31** ± 0.11	1.15* ± 0.03	1.41* ± 0.06	0.70 ± 0.04	1.13* ± 0.08	1.51* ± 0.06
Mercury	100	0.90 ± 0.07	0.73 ± 0.07	1.47* ± 0.05	1.08** ± 0.04	0.96* ± 0.03	1.35* ± 0.05
	1000	1.65* ± 0.14	1.05* ± 0.03	1.52* ± 0.07	1.08** ± 0.05	1.08* ± 0.05	1.32 ± 0.09
Lead	100	1.73* ± 0.11	0.73 ± 0.00	1.19 ± 0.03	0.80* ± 0.05	0.88 ± 0.04	1.27 ± 0.06
	1000	1.87* ±0.10	0.88 ± 0.03	1.24 ± 0.05	1.03** ± 0.05	1.02* ± 0.05	1.46** ± 0.04

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 17: Changes in reducing sugar content of roots of okra cultivars at reproductive stage following heavy metal treatments

Treatments		Reducing sugar content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka -F1	Paras Soumya	Parbhani Kranti	PB-57
Control		1.06 ± 0.03	0.85 ± 0.02	0.73 ± 0.03	1.50 ± 0.04	0.85 ± 0.08	0.83 ± 0.02
Cadmium	100	1.36 ± 0.11	1.10* ± 0.04	0.90 ± 0.05	2.25* ± 0.12	1.02 ± 0.05	0.90 ± 0.03
	1000	1.91* ± 0.11	1.21* ± 0.03	0.96* ± 0.03	3.00** ± 0.12	1.30* ± 0.07	0.93 ± 0.04
Copper	100	1.16 ± 0.08	1.24** ± 0.02	1.01* ± 0.05	1.87 ± 0.08	1.19 ± 0.02	0.90 ± 0.05
	1000	2.13** ± 0.05	1.27** ± 0.01	1.07* ± 0.03	2.19* ± 0.10	1.24* ± 0.05	0.93* ± 0.01
Mercury	100	1.40* ± 0.03	1.32** ± 0.01	0.84 ± 0.03	2.32* ± 0.09	1.36* ± 0.07	0.87 ± 0.07
	1000	1.57* ± 0.11	1.38** ± 0.01	0.96 ± 0.08	2.60* ± 0.15	1.41* ± 0.08	0.99* ± 0.01
Lead	100	1.51* ± 0.08	1.30** ± 0.02	1.01* ± 0.05	1.97 ± 0.11	1.47* ± 0.05	1.02* ± 0.02
	1000	2.86** ± 0.03	1.44** ± 0.01	1.13* ± 0.05	2.62** ± 0.07	1.52* ± 0.08	1.07* ± 0.03

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Results of influence of heavy metal on both total soluble sugar and reducing sugar had shown that while the total soluble sugars in general decreased with treatment reducing sugar showed an increase (Tables 6-17 and Figs. 5 and 6).

4.3.3. Starch

Starch contents in roots, stems and leaves of okra plants following heavy metal treatments were quantified. Results revealed that there was a general significant reduction in starch contents in all treatments (Table 18 A). Hg was most inhibitory.

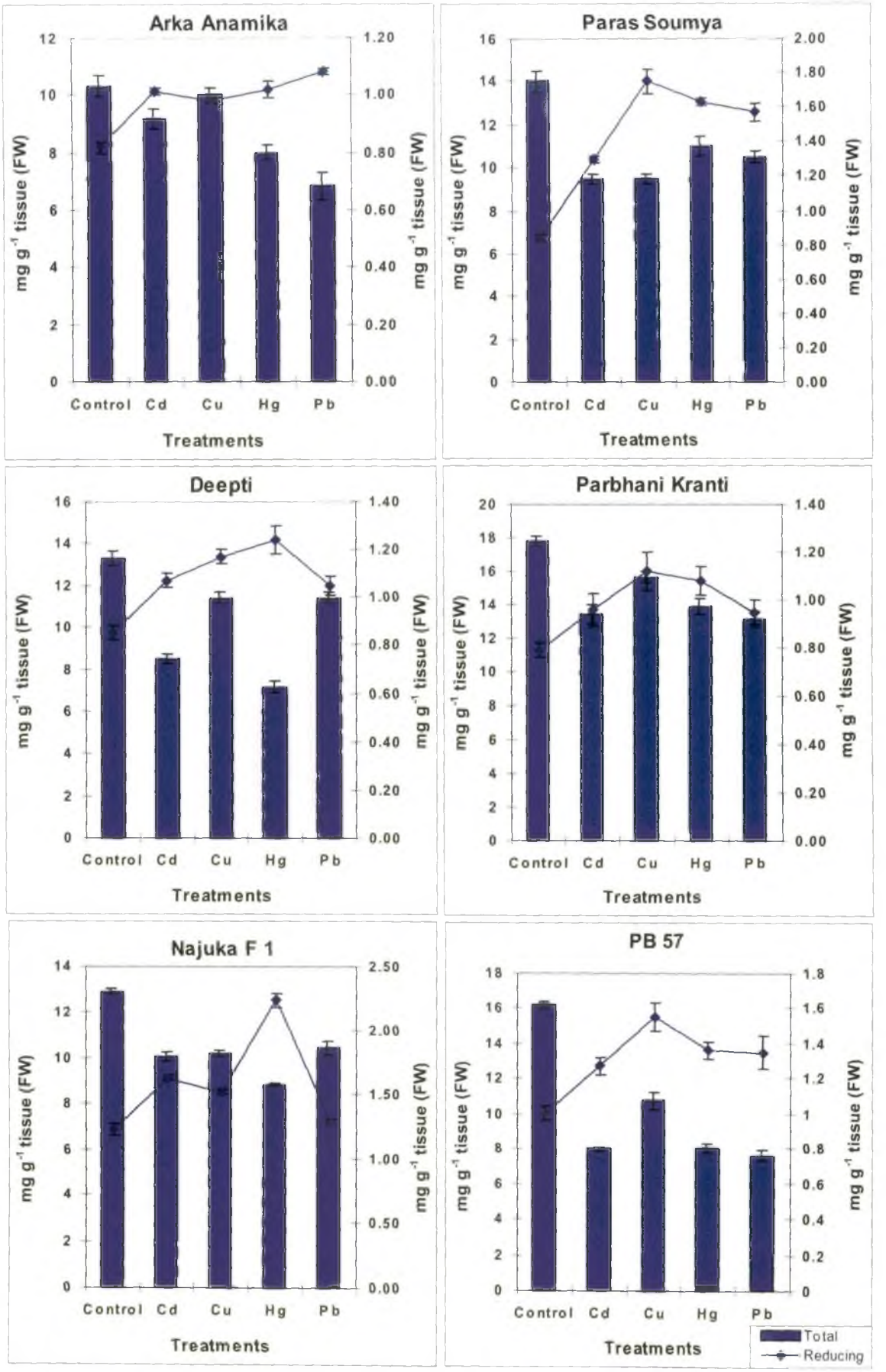


Fig. 5: Total and reducing sugar content of leaves of okra cultivars at vegetative stage after heavy metal treatment at 1000 μg ml^{-1} concentration.

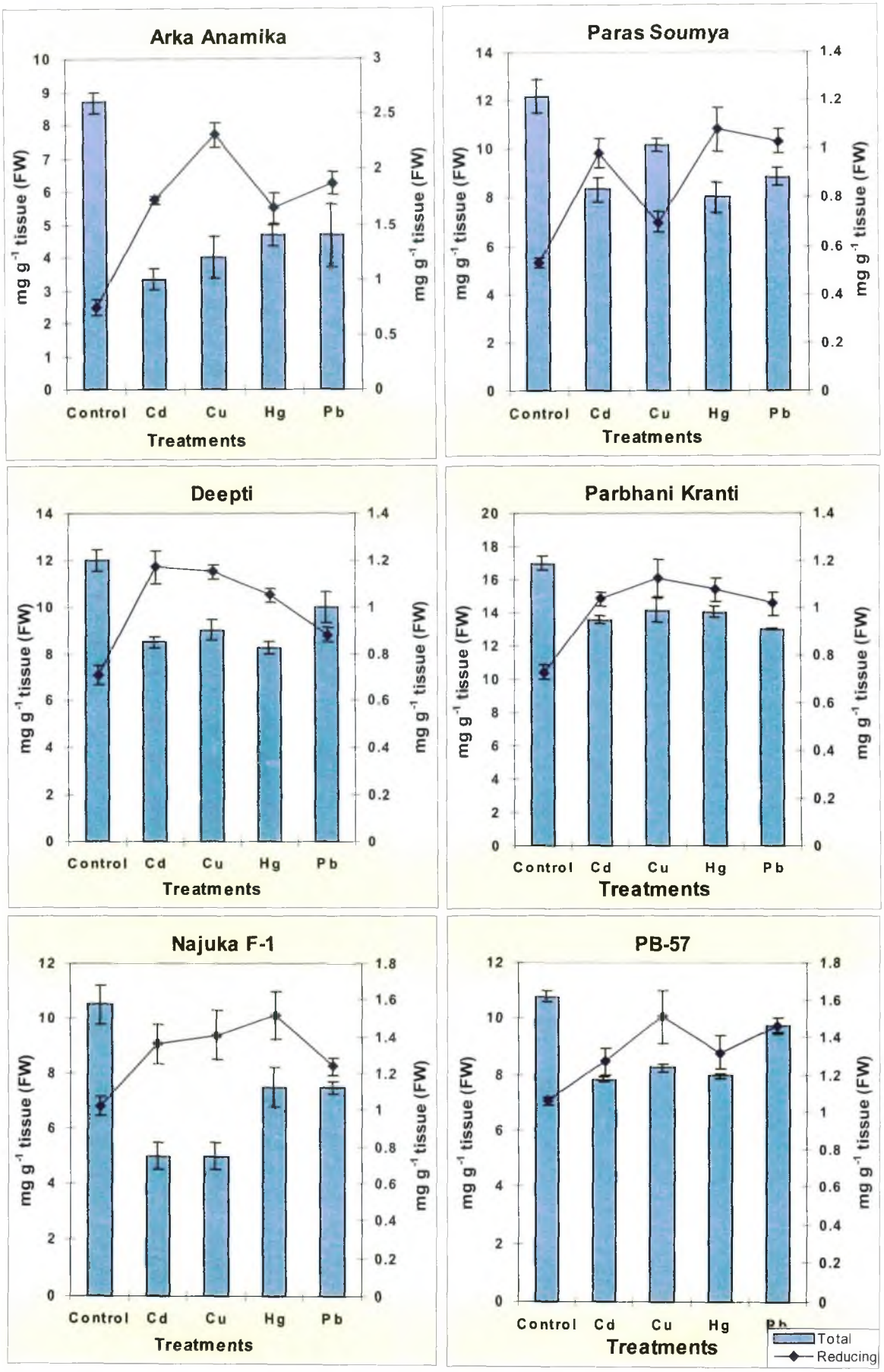


Fig. 6: Total and reducing sugar content of roots of okra cultivars at vegetative stage after heavy metal treatment at 1000 μg ml^{-1} concentration.

Table 18: Changes in starch contents of different plant parts of okra cultivars following heavy metal treatments

Cultivars	Starch content (mg g^{-1} tissue)					
	Parts	Control	Cadmium	Copper	Mercury	Lead
Arka Anamika	Leaf	248.00 ± 3.77	200.00 ± 3.77	192.00 ± 0.00	136.00 ± 3.77	101.00 ± 5.19
	Stem	300.00 ± 9.44	160.00 ± 18.87	133.00 ± 12.75	128.00 ± 30.21	160.00 ± 15.10
	Root	184.00 ± 3.77	120.00 ± 3.77	104.00 ± 3.77	100.00 ± 9.44	140.00 ± 9.44
Deepti	Leaf	268.80 ± 9.06	235.20 ± 6.80	197.60 ± 4.91	213.20 ± 7.36	230.40 ± 4.53
	Stem	403.20 ± 9.06	220.73 ± 13.60	163.20 ± 13.60	192.00 ± 9.06	86.20 ± 4.53
	Root	67.20 ± 4.53	52.50 ± 3.54	48.00 ± 4.53	44.00 ± 4.16	72.00 ± 2.27
Najuka-F1	Leaf	216.00 ± 0.00	165.50 ± 3.40	223.20 ± 10.20	151.20 ± 3.40	115.20 ± 0.12
	Stem	324.00 ± 8.49	243.00 ± 12.75	290.00 ± 9.55	270.00 ± 8.50	184.50 ± 6.40
	Root	65.45 ± 0.00	47.14 ± 3.71	65.46 ± 6.18	47.14 ± 3.71	47.79 ± 3.40
Paras Soumya	Leaf	189.79 ± 5.48	159.09 ± 4.64	166.01 ± 5.93	129.84 ± 1.23	143.13 ± 3.33
	Stem	335.84 ± 7.16	214.73 ± 6.27	189.66 ± 3.47	184.71 ± 5.99	192.77 ± 4.14
	Root	112.70 ± 3.46	93.41 ± 3.02	89.62 ± 3.50	80.71 ± 2.22	90.73 ± 0.60
Parbhani Kranti	Leaf	134.24 ± 8.16	93.71 ± 8.84	111.09 ± 8.74	88.42 ± 5.96	95.04 ± 4.08
	Stem	304.62 ± 5.75	188.57 ± 5.93	140.18 ± 6.01	152.30 ± 6.53	158.61 ± 4.40
	Root	121.30 ± 4.40	52.69 ± 4.97	85.91 ± 5.79	92.31 ± 8.71	82.08 ± 3.74
PB-57	Leaf	343.79 ± 7.73	278.26 ± 13.13	219.51 ± 11.51	185.19 ± 3.49	239.73 ± 3.23
	Stem	428.89 ± 4.13	280.00 ± 18.88	215.06 ± 20.30	227.42 ± 18.94	151.99 ± 6.52
	Root	45.67 ± 1.44	29.41 ± 2.78	26.93 ± 1.81	24.61 ± 1.94	25.06 ± 0.91

Treatments were applied at 1000 μg ml^{-1} concentration of the salts
Values are mean of 3 replicates; ± = SEm

Table 18A: Analysis of variance of data presented in Table 18 (Leaves)

Source	D.F.	S.S.	M.S.	F	C.D.
Replication	2	195.92	97.96		
Variety	5	208166.69	41533.34	35.73	24.98
Treatment	4	81446.79	20361.69	17.48	22.80
Interaction	20	20622.30	1031.12	0.89	55.85
Error	58	67572.31	1165.07		
Total	89	378004.01			

Table 18B: Analysis of variance of data presented in Table 18 (Shoot)

Source	D.F.	S.S.	M.S.	F	C.D.
Replication	2	15281.38	7640.69		
Variety	5	95830.14	19166.03	26.52	19.67
Treatment	4	407485.15	101871.29	140.97	17.96
Interaction	20	85306.08	4265.30	5.90	43.99
Error	58	41914.83	722.67		
Total	89	645817.58			

Table 18C: Analysis of variance of data presented in Table 18 (Root)

Source	D.F.	S.S.	M.S.	F	C.D.
Replication	2	101.66	50.83		
Variety	5	93086.60	18617.32	205.24	1.74
Treatment	4	14559.24	3639.81	40.13	6.36
Interaction	20	12302.02	615.10	6.78	15.58
Error	58	5261.46	90.71		
Total	89	125310.98			

Percentage reductions of starch content in the different organs were calculated in relation to control and it was observed that maximum reduction occurred in the stems of different cultivars. In case of leaves and roots percentages were lesser than that of stem. Cd and Cu caused minimum inhibition whereas Hg and Pb caused greater inhibitions in general (Fig. 7).

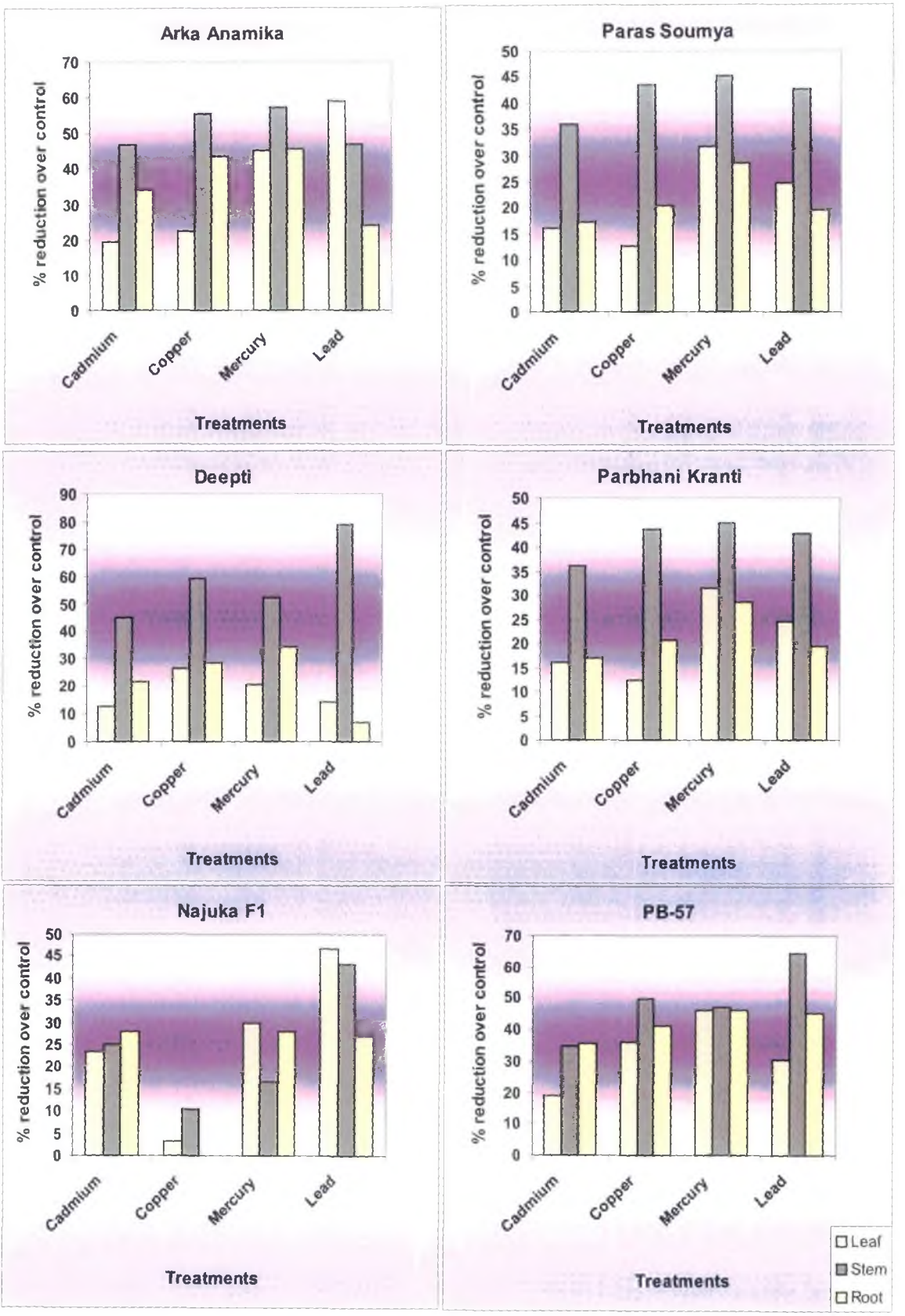


Fig. 7. Reduction in starch contents of different parts of okra plants subjected to heavy metal treatments (1000 μg ml^{-1}).

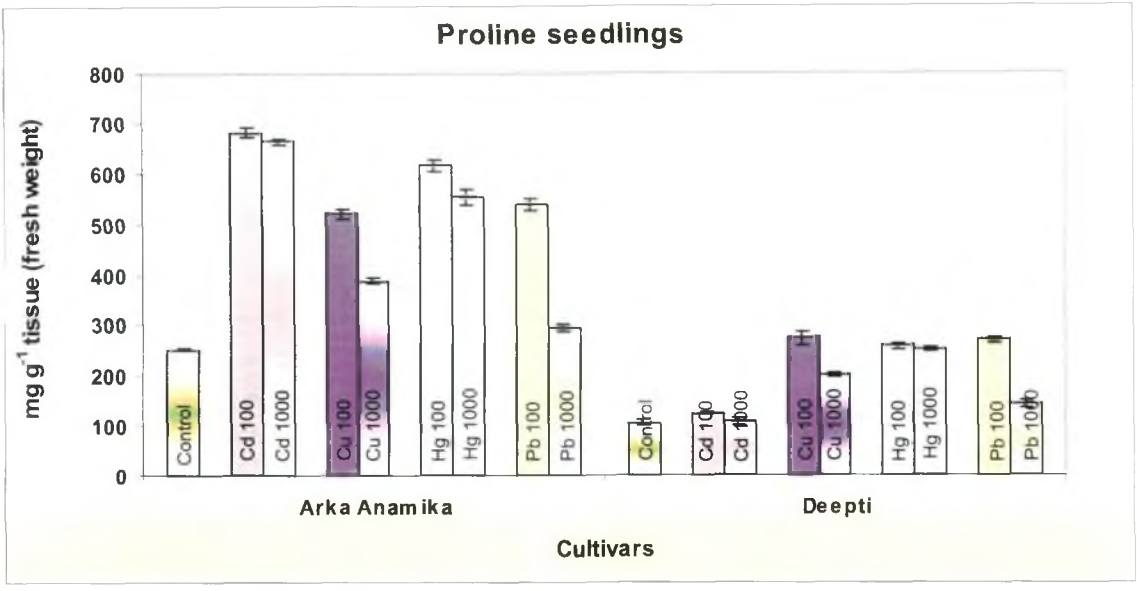
4.4. Effect of heavy metals on proline content of okra leaves

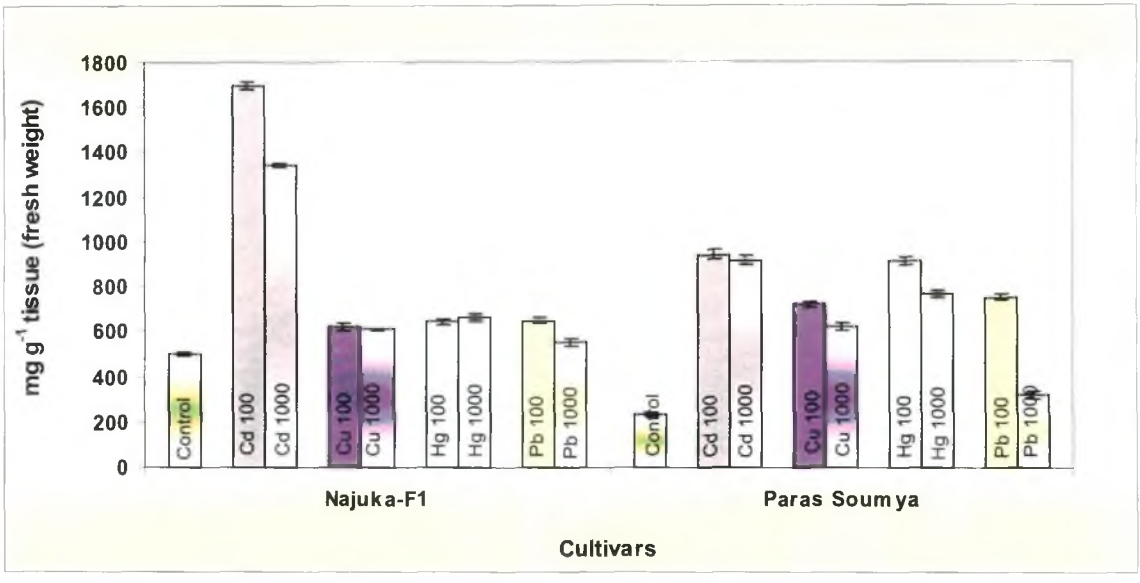
Proline content of leaves of all six cultivars was determined after heavy metal treatments at seedling, vegetative and reproductive stages. Constitutive proline was higher at the seedling stage as compared to vegetative and reproductive stages. In all treatments heavy metal induced accumulation of proline. However, 100 μg ml^{-1} treatments induced comparatively higher accumulation than 1000 μg ml^{-1} (Tables 19 and 20 and Fig. 8).

Table 19: Changes in proline content of leaves of okra cultivars at vegetative stage after heavy metal treatments

Treatments		Proline content (μg g^{-1} tissue)					
Heavy metal	Conc. (μg ml^{-1})	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		142.50 ± 6.13	185.80 ± 2.38	192.84 ± 15.17	130.00 ± 5.40	192.84 ± 7.58	176.77 ± 7.57
Cadmium	100	435.29** ± 7.14	273.19** ± 7.58	334.26** ± 12.14	473.53** ± 19.50	305.33** ± 7.58	321.40* ± 15.17
	1000	405.20** ± 8.93	233.02** ± 3.79	308.55** ± 12.13	424.25** ± 15.76	257.16* ± 7.57	208.91 ± 7.58
Copper	100	365.00** ± 14.74	260.34** ± 4.55	321.41** ± 10.11	327.5** ± 10.22	297.30** ± 3.79	241.05* ± 9.47
	1000	292.55** ± 12.76	219.30** ± 1.99	257.12* ± 8.03	283.33** ± 13.16	265.16* ± 3.79	224.98 ± 15.17
Mercury	100	362.13** ± 14.27	246.41** ± 5.06	353.54** ± 15.17	357.25** ± 12.45	337.47** ± 7.58	305.33** ± 7.58
	1000	312.50** ± 12.28	216.95* ± 6.83	336.23** ± 9.34	280.00* ± 14.31	289.26* ± 7.58	273.19* ± 7.58
Lead	100	292.5* ± 16.73	255.23** ± 13.38	295.69** ± 6.07	332.50** ± 8.91	345.51** ± 0.00	337.47** ± 7.58
	1000	225.00* ± 9.36	226.59** ± 2.27	276.89* ± 18.67	215.00* ± 10.22	323.40** ± 4.68	265.16* ± 11.38

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm





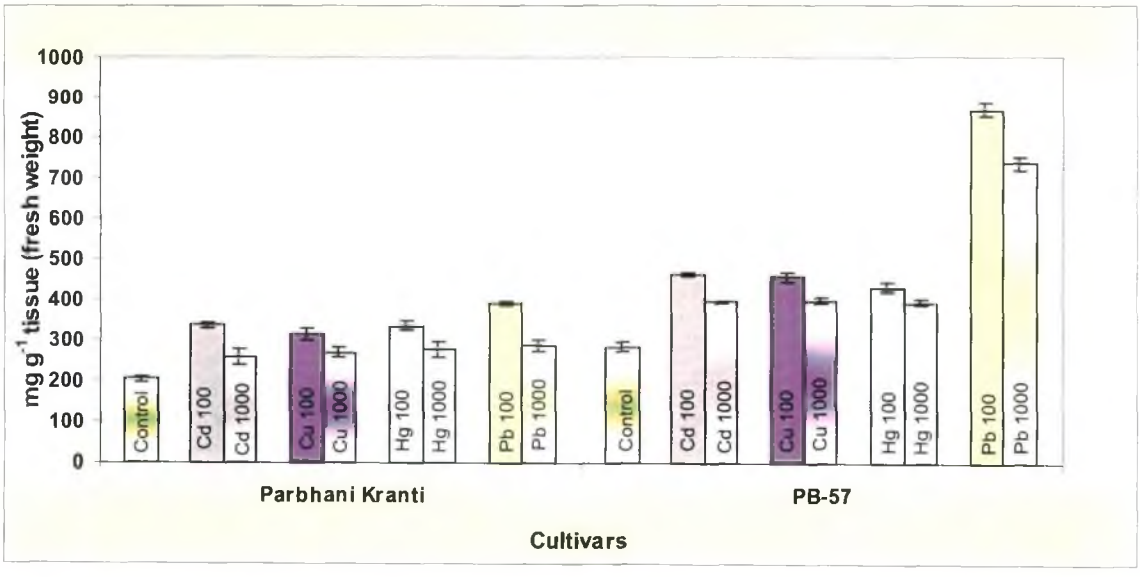


Fig.8: Changes in proline content of leaves of okra cultivars at seedling stage after heavy metal treatment.

Table 20: Changes in proline content of leaves of okra cultivars at reproductive stage after heavy metal treatments

Treatments		Proline content ($\mu g\ g^{-1}$ tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		208.00 ± 7.55	273.19 ± 7.66	224.98 ± 15.16	204.00 ± 7.55	289.26 ± 7.58	305.33 ± 15.17
Cadmium	100	562.50** ± 7.79	425.86** ± 3.79	385.68* ± 30.34	417.00** ± 14.63	435.89** ± 9.04	433.89* ± 7.58
	1000	450.00** ± 15.10	385.68** ± 7.58	289.26 ± 15.17	290.00* ± 16.05	377.65* ± 11.38	377.65 ± 11.38
Copper	100	403.00** ± 7.08	457.99** ± 3.79	353.54* ± 15.17	255.00 ± 15.57	409.79** ± 3.79	441.93* ± 3.79
	1000	272.00 ± 11.11	409.79** ± 14.44	257.12 ± 0.00	240.00 ± 7.55	369.61* ± 7.58	369.61 ± 7.59
Mercury	100	497.50** ± 8.02	449.96** ± 7.58	401.76* ± 22.75	411.00** ± 14.62	433.89** ± 7.58	466.03* ± 15.17
	1000	433.00** ± 7.08	369.61* ± 7.59	321.40* ± 0.00	321.00* ± 15.57	361.58* ± 3.79	385.68* ± 7.58
Lead	100	401.00** ± 8.02	482.10** ± 7.58	417.82* ± 15.17	385.00** ± 11.65	466.03** ± 7.58	514.24* ± 22.75
	1000	368.00** ± 7.55	361.57* ± 3.79	305.34 ± 22.75	304.00* ± 11.65	401.75** ± 7.58	393.72* ± 3.79

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Increment of proline due to heavy metal treatment varies to some extent with the cultivars. In PB-57 the difference of proline content with control at both vegetative and reproductive stages were either significant only at 5% level or non-significant (Cd and Cu 1000 $\mu g\ ml^{-1}$). In Arka Anamika most of the treatments were significant at 1%. Observations of all three stages in the different cultivars revealed that in Arka Anamika $Cd(NO_3)_24H_20$ at 1000 $\mu g\ ml^{-1}$ induced maximum accumulation of proline in all the three stages. However, $Pb(NO_3)_2$ at 100 $\mu g\ ml^{-1}$ induced maximum accumulation in Parbhani Kranti and PB-57 at all three stages. Response of other three varieties varied.

4.5. Changes in pigment content following heavy metal treatments

The effect of heavy metal treatment (1000 μg ml^{-1}) in pigment was determined on the basis of chlorophyll and carotenoid content.

4.5.1. Chlorophyll

Total chlorophyll content of the leaves showed a general decline following heavy metal treatments. Among all the heavy metals Hg reduced total chlorophyll content to the greatest degree in all the six cultivars. Decreases in almost all the treatments were statistically significant (Table 21). Similar trends were also observed in both chlorophyll a and chlorophyll b. Chlorophyll a/b showed an increase over control in most of the cultivars and treatments (Table 22).

4.5.2. Carotenoids

Carotenoid contents of the different cultivars showed great variations, ranging from 14 μg g^{-1} in Paras Soumya to 44 μg g^{-1} fresh weight leaf tissue in Arka Anamika. Heavy metal treatments significantly reduced the carotenoid contents in most of the cases (Table 21).

4.6. Effect of heavy metals on proteins

4.6.1. Protein content

Protein contents of the leaves and roots of okra cultivars at seedling, vegetative and reproductive stages were determined for all treatments. Protein content of leaves was much higher than those of roots. Leaves of both seedling and vegetative stage had slightly higher protein content than reproductive stage. In the leaves heavy metal treatments mostly increased the protein content though not very significantly (Table 23 - 25). In case of roots in most cases differences with control were either not significant or lesser (Table 26 – 28). No fixed pattern of protein change could be detected as the response was widely varied (Tables 23-28 and Fig. 9).

Table 21: Influence of heavy metals on total chlorophyll and carotenoid content in leaves of okra

Cultivars	Treatment	Total chlorophyll (mg g^{-1} tissue)	Carotenoid (μg g-1 tissue)
Arka Anamika	**Control**	2.49 ± 0.06	43.73 ± 0.50
	Cadmium	2.09 ± 0.07	37.09 ± 0.79
	Copper	1.98 ± 0.10	37.40 ± 0.71
	Mercury	1.69 ± 0.04	36.07 ± 0.30
	Lead	1.99 ± 0.08	38.75 ± 0.56
Deepti	**Control**	1.10 ± 0.08	18.00 ± 0.25
	Cadmium	0.76 ± 0.04	14.00 ± 0.60
	Copper	0.53 ± 0.02	15.70 ± 0.35
	Mercury	0.35 ± 0.03	15.09 ± 0.19
	Lead	0.73 ± 0.05	16.76 ± 0.30
Najuka-F1	**Control**	1.94 ± 0.06	27.50 ± 0.67
	Cadmium	1.46 ± 0.09	23.90 ± 0.17
	Copper	1.38 ± 0.07	13.20 ± 1.18
	Mercury	1.39 ± 0.07	14.20 ± 0.17
	Lead	1.37 ± 0.01	22.10 ± 0.53
Paras Soumya	**Control**	1.08 ± 0.05	14.25 ± 0.25
	Cadmium	0.78 ± 0.04	13.83 ± 0.44
	Copper	0.77 ± 0.06	13.29 ± 0.48
	Mercury	0.50 ± 0.01	7.20 ± 0.45
	Lead	0.62 ± 0.05	6.93 ± 0.32
Parbhani Kranti	**Control**	1.68 ± 0.24	24.39 ± 0.80
	Cadmium	1.22 ± 0.14	26.56 ± 0.66
	Copper	1.21 ± 0.12	19.88 ± 1.08
	Mercury	0.88 ± 0.35	7.86 ± 0.62
	Lead	1.16 ± 0.09	23.24 ± 0.62
PB-57	**Control**	1.31 ± 0.03	21.22 ± 0.25
	Cadmium	0.88 ± 0.02	16.99 ± 0.19
	Copper	0.87 ± 0.05	16.59 ± 0.23
	Mercury	0.70 ± 0.01	11.18 ± 0.15
	Lead	0.81 ± 0.01	12.71 ± 0.63

Treatments were applied at 1000 μg ml^{-1} concentration of the salts
Values are mean of 3 replicates; ± = SEm

Table 22: Chlorophyll a and b contents in leaves of okra cultivars following heavy metal treatments

Cultivars	Treatments	Chlorophyll a (mg g^{-1} tissue)	Chlorophyll b (mg g^{-1} tissue)	Chlorophyll a/b
Arka	**Control**	1.38 ± 0.06	1.11 ± 0.07	1.24 ± 0.02
Anamika	**Cadmium**	1.35 ± 0.04	0.74 ± 0.03	1.82 ± 0.02
	Copper	1.36 ± 0.07	0.62 ± 0.03	2.24 ± 0.02
	Mercury	1.21 ± 0.01	0.48 ± 0.02	2.52 ± 0.13
	Lead	1.06 ± 0.05	0.83 ± 0.03	1.27 ± 0.02
Deepti	**Control**	0.54 ± 0.05	0.56 ± 0.03	0.96 ± 0.04
	Cadmium	0.41 ± 0.04	0.35 ± 0.04	1.17 ± 0.01
	Copper	0.30 ± 0.04	0.22 ± 0.02	1.67 ± 0.44
	Mercury	0.14 ± 0.02	0.21 ± 0.01	1.56 ± 0.10
	Lead	0.49 ± 0.04	0.23 ± 0.01	2.03 ± 0.21
Najuka-F1	**Control**	1.02 ± 0.02	0.92 ± 0.03	1.12 ± 0.01
	Cadmium	0.85 ± 0.06	0.61 ± 0.04	1.39 ± 0.01
	Copper	0.62 ± 0.03	0.76 ± 0.04	0.81 ± 0.01
	Mercury	0.58 ± 0.04	0.81 ± 0.02	0.83 ± 0.05
	Lead	0.84 ± 0.03	0.53 ± 0.03	1.62 ± 0.14
Paras Soumya	**Control**	0.46 ± 0.01	0.62 ± 0.06	1.08 ± 0.02
	Cadmium	0.43 ± 0.02	0.35 ± 0.02	1.23 ± 0.09
	Copper	0.44 ± 0.02	0.33 ± 0.03	1.33 ± 0.10
	Mercury	0.22 ± 0.02	0.27 ± 0.02	0.81 ± 0.01
	Lead	0.28 ± 0.03	0.34 ± 0.02	0.82 ± 0.09
Parbhani	**Control**	0.83 ± 0.03	0.85 ± 0.03	0.98 ± 0.07
Kranti	**Cadmium**	0.75 ± 0.02	0.47 ± 0.02	1.60 ± 0.16
	Copper	0.65 ± 0.03	0.56 ± 0.02	1.16 ± 0.02
	Mercury	0.58 ± 0.02	0.30 ± 0.02	1.93 ± 0.04
	Lead	0.69 ± 0.04	0.47 ± 0.02	1.47 ± 0.01
PB-57	**Control**	0.64 ± 0.05	0.81 ± 0.04	0.70 ± 0.01
	Cadmium	0.42 ± 0.02	0.51 ± 0.02	0.87 ± 0.02
	Copper	0.37 ± 0.03	0.54 ± 0.03	0.75 ± 0.04
	Mercury	0.29 ± 0.01	0.40 ± 0.01	0.86 ± 0.03
	Lead	0.43 ± 0.03	0.53 ± 0.03	0.82 ± 0.03

Treatments were applied at 1000 μg ml^{-1} concentration of the salts
Values are mean of 3 replicates; ± = SEm

Table 23: Effect of heavy metal treatment on protein content of okra leaves at seedlings stage

Treatments		Protein content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka -F1	Paras Soumya	Parbhani Kranti	PB-57
Control		12.55 ± 0.22	18.50 ± 0.71	26.29 ±0.15	19.75 ± 0.35	24.31 ± 0.68	23.03 ± 0.22
Cadmium	100	14.90* ± 0.28	23.34* ± 0.31	30.97* ± 0.46	19.50 ± 0.71	21.88 ± 1.47	25.31* ± 0.15
	1000	15.87** ± 0.16	33.35** ± 0.64	36.67** ± 0.31	20.25 ± 0.35	24.20 ± 1.34	25.84* ± 0.49
Copper	100	13.83* ± 0.14	22.18* ± 0.36	32.01* ± 0.86	24.75** ± 0.59	22.00 ± 0.94	29.29* ± 0.67
	1000	14.00* ± 0.23	23.12* ± 0.18	32.67** ± 0.23	30.75** ± 0.59	26.29 ± 0.51	31.76** ± 0.37
Mercury	100	14.03* ± 0.22	19.42 ± 0.54	27.38* ± 0.08	17.00 ± 0.71	22.50 ± 1.18	26.59 ± 0.32
	1000	14.35* ± 0.17	20.33 ± 0.25	28.28* ± 0.48	23.50* ± 0.47	23.86 ± 0.66	28.83** ± 0.41
Lead	100	18.17** ± 0.36	24.28* ± 0.38	37.90** ± 0.85	16.00* ± 0.24	27.50 ± 1.18	25.84 ± 0.79
	1000	16.00** ± 0.24	19.55 ± 0.75	26.81 ± 0.20	21.00 ± 0.71	26.00 ± 0.94	31.36** ± 0.45

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 24: Effect of heavy metal treatment on protein content of okra leaves at vegetative stage

Treatments		Protein content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		12.35 ± 0.36	24.50 ± 0.55	26.17 ± 0.20	18.76 ± 0.56	25.50 ± 0.24	25.75 ± 0.35
Cadmium	100	13.53 ± 0.40	29.17 ± 1.10	29.59** ± 0.20	17.94 ± 0.22	29.75** ± 0.12	26.50 ± 0.47
	1000	14.50 ± 0.71	32.08* ± 1.38	32.00** ± 0.20	17.16 ± 0.20	30.75** ± 0.35	26.75 ± 0.35
Copper	100	11.58 ± 0.47	27.99* ± 0.55	30.00* ± 0.48	14.66 ± 0.36	30.25** ± 0.35	27.25 ± 0.35
	1000	12.42 ± 0.18	29.74* ± 0.28	34.80** ± 0.21	27.16* ± 0.75	32.25** ± 0.12	28.00* ± 0.24
Mercury	100	15.02* ± 0.36	30.00* ± 0.47	30.56* ± 0.65	17.70 ± 0.31	28.75** ± 0.12	27.75* ± 0.12
	1000	13.87 ± 0.47	32.00** ± 0.94	32.28** ± 0.39	16.60 ± 0.46	31.75** ± 0.35	27.50* ± 0.24
Lead	100	15.58* ± 0.38	26.67 ± 0.63	28.75 ± 0.59	19.10 ± 0.09	30.50** ± 0.24	28.25* ± 0.12
	1000	16.25* ± 0.31	31.33* ± 0.94	32.50* ± 0.59	19.16 ± 0.24	29.25* ± 0.35	29.75** ± 0.12

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 25: Effect of heavy metal treatment on protein content of okra leaves at reproductive stage

Treatments		Protein content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		11.50 ± 0.23	22.10 ± 0.14	15.50 ± 0.00	14.50 ± 0.23	20.25 ± 0.35	20.75 ± 0.23
Cadmium	100	13.25 ± 0.35	29.38* ± 0.88	19.50** ± 0.24	14.75 ± 0.35	22.25* ± 0.12	21.50 ± 0.24
	1000	12.25 ± 0.35	31.54** ± 0.27	20.65** ± 0.17	16.25 ± 0.59	21.50 ± 0.23	21.38 ± 0.18
Copper	100	13.75 ± 0.83	25.39* ± 0.46	18.25* ± 0.35	12.50* ± 0.08	23.25* ± 0.35	21.13 ± 0.17
	1000	15.00** ± 0.23	29.17** ± 0.39	20.05** ± 0.12	15.25 ± 0.35	22.50 ± 0.47	22.00* ± 0.12
Mercury	100	15.25** ± 0.15	32.43** ± 0.64	19.40** ± 0.28	15.00 ± 0.47	23.00* ± 0.47	21.75 ± 0.12
	1000	16.00** ± 0.23	37.50** ± 0.54	21.60** ± 0.42	15.50 ± 0.27	20.75 ± 0.12	22.13* ± 0.06
Lead	100	13.50 ± 0.47	26.57* ± 0.44	15.20 ± 0.38	18.25* ± 0.35	23.75* ± 0.12	22.38* ± 0.29
	1000	14.00* ± 0.23	32.50** ± 0.39	17.00** ± 0.00	15.50 ± 0.23	22.00 ± 0.24	23.75** ± 0.12

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 26: Changes in protein content of okra roots following heavy metal treatments at seedling stage

Treatments		Protein content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		3.63 ± 0.19	8.50 ± 0.12	8.74 ± 0.16	5.50 ± 0.24	4.68 ± 0.29	4.62 ± 0.14
Cadmium	100	5.20* ± 0.01	8.37 ± 0.06	7.80* ± 0.09	7.50 ± 0.71	4.50 ± 0.12	7.25* ± 0.26
	1000	4.32 ± 0.29	8.63 ± 0.18	7.39* ± 0.27	6.50 ± 0.49	4.25 ± 0.12	6.64* ± 0.28
Copper	100	6.25 ± 0.59	8.75 ± 0.12	8.00 ± 0.16	7.50* ± 0.24	4.75 ± 0.12	7.39** ± 0.23
	1000	3.91 ± 0.36	8.25 ± 0.12	5.21** ± 0.29	7.00 ± 0.48	5.55 ± 0.26	7.14** ± 0.00
Mercury	100	5.97** ± 0.00	8.13 ± 0.29	7.90 ± 0.25	9.00* ± 0.48	5.00 ± 0.35	8.12** ± 0.21
	1000	4.59 ± 0.43	8.44 ± 0.15	7.75* ± 0.08	8.00* ± 0.48	5.28 ± 0.26	7.03 ± 0.72
Lead	100	5.51* ± 0.37	7.97 ± 0.22	9.75* ± 0.12	7.00 ± 0.94	4.00 ± 0.24	8.51** ± 0.31
	1000	4.81 ± 0.25	8.88 ± 0.29	9.43 ± 0.12	6.50 ± 0.71	5.31 ± 0.30	5.42 ± 0.28

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 27: Changes in protein content of okra roots following heavy metal treatments at vegetatives stage

Treatments		Protein content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		5.75 ± 0.24	10.00 ± 0.24	8.75 ± 0.12	6.00 ± 0.12	3.75 ± 0.12	6.25 ± 0.06
Cadmium	100	6.07 ± 0.39	9.25 ± 0.12	8.00 ± 0.24	5.82 ± 0.19	3.50 ± 0.24	5.50 ±0.24
	1000	5.91 ± 0.35	8.00 ± 0.24	8.25 ± 0.12	5.60 ± 0.31	2.41 ± 0.24	5.25 ±0.12
Copper	100	4.42 ± 0.18	9.38 ± 0.18	8.12 ± 0.15	5.42 ± 0.24	3.25 ± 0.12	6.25 ± 0.44
	1000	3.50 ± 0.31	8.50 ± 0.24	7.22 ± 0.26	4.58 ± 0.24	2.19 ± 0.14	5.75 ± 0.06
Mercury	100	5.57 ± 0.36	9.75 ± 0.35	7.81 ± 0.30	6.60 ± 0.17	2.75 ± 0.12	5.00 ± 0.12
	1000	5.33 ± 0.42	7.88 ± 0.18	8.00 ± 0.12	6.44 ± 0.15	2.05 ± 0.10	6.56 ± 0.43
Lead	100	7.25 ± 0.43	8.76 ± 0.29	7.78 ± 0.13	6.17 ± 0.42	4.25 ± 0.12	5.50 ± 0.12
	1000	7.00 ± 0.12	8.13 ± 0.29	7.50 ± 0.12	5.67 ± 0.38	4.65 ± 0.17	6.30 ± 0.31

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

Table 28: Changes in protein content of okra roots following heavy metal treatments at reproductive stage

Treatments		Protein content (mg g^{-1} tissue)					
Heavy metal	Conc. ($\mu g\ ml^{-1}$)	Arka Anamika	Deepti	Najuka-F1	Paras Soumya	Parbhani Kranti	PB-57
Control		4.75 ± 0.35	8.93 ± 0.17	7.10 ± 0.30	6.00 ± 0.23	3.89 ± 0.06	6.00 ± 0.24
Cadmium	100	8.75** ± 0.12	9.75 ± 0.35	6.20 ± 0.09	5.75 ± 0.12	4.25 ± 0.35	7.25* ± 0.12
	1000	7.50* ± 0.23	11.96** ± 0.14	5.21* ± 0.27	5.50 ± 0.71	4.00 ± 0.23	6.50 ± 0.23
Copper	100	6.75* ± 0.12	8.25 ± 0.12	5.75 ± 0.35	6.00 ± 0.94	4.75 ± 0.37	7.63* ± 0.18
	1000	4.75 ± 0.12	7.50* ± 0.09	5.09* ± 0.34	6.25 ± 0.35	4.88** ± 0.06	6.75 ± 0.12
Mercury	100	7.25* ± 0.19	10.25* ± 0.12	4.83* ± 0.40	8.00* ± 0.24	4.50 ± 0.23	7.00 ± 0.24
	1000	7.00* ± 0.24	8.35 ± 0.22	3.34* ± 0.26	8.75* ± 0.35	3.75 ± 0.12	6.88 ± 0.29
Lead	100	7.50 ± 0.71	10.59 ± 0.51	6.31 ± 0.47	7.00 ± 0.24	4.13 ± 0.06	7.75* ± 0.12
	1000	8.25* ± 0.12	9.12 ± 0.14	4.36* ± 0.14	7.75 ± 0.59	3.63 ± 0.14	6.63 ± 0.18

Difference with control significant at p=0.05 (*) and at p = 0.01 (**) as tested by Students 't' test; Values are mean of 3 replicates; ± = SEm

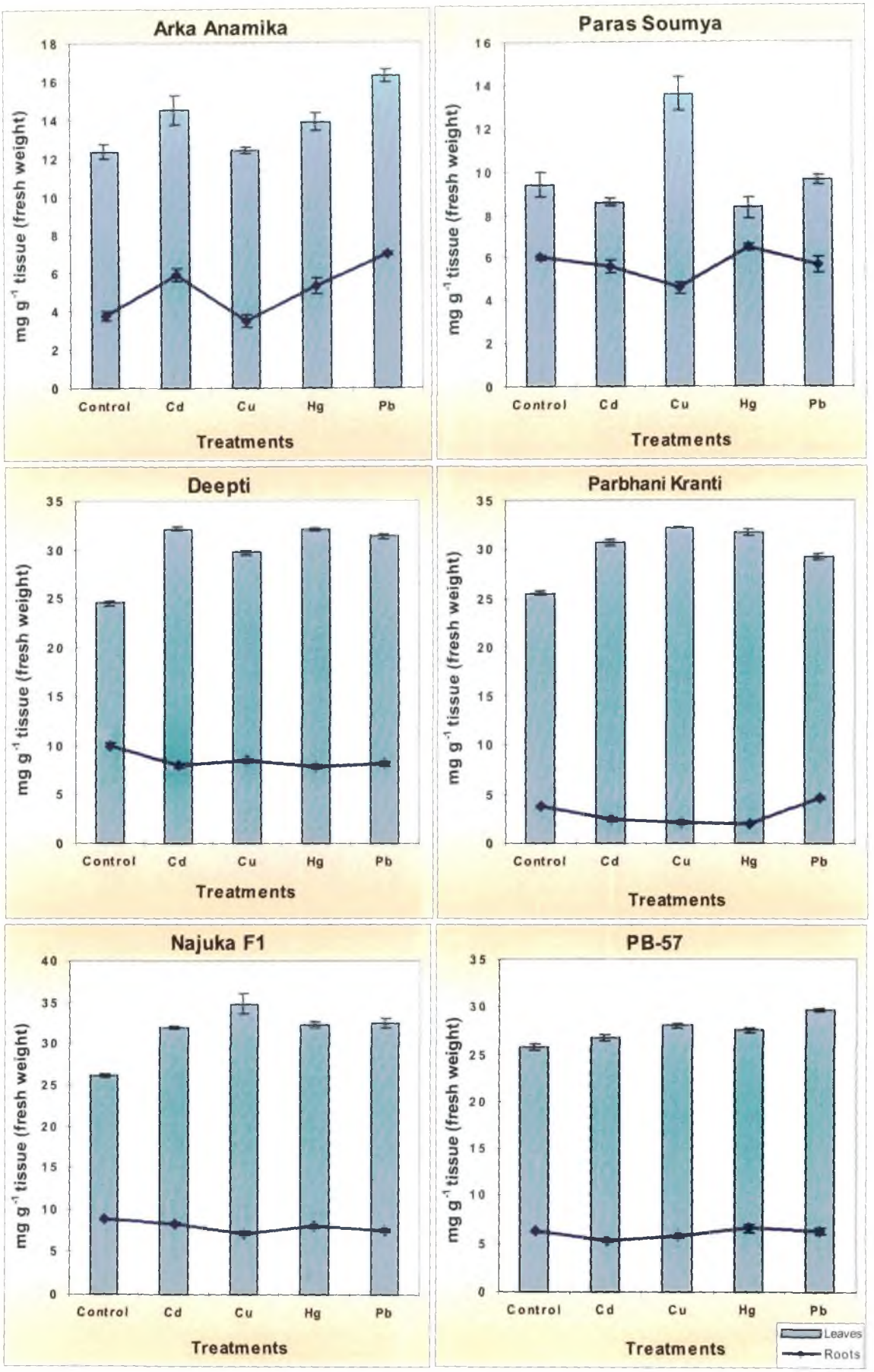


Fig.9: Protein contents of leaves and roots of okra plants following heavy metal treatments (1000 $\mu g\, ml^{-1}$).

4.6.2. Protein profile

In order to determine any changes in protein pattern following heavy metal stress treatments SDS-PAGE analysis of soluble protein was carried out. Proteins were extracted from the seeds of different varieties which were either untreated or treated with different heavy metals. Leaf proteins were also analysed. Comparison of the seed proteins of different cultivars revealed that the protein pattern of all six cultivars were similar. Three prominent bands of molecular mass 47.86, 22.39 and 14.79 KDa were present in all cases. Number of protein bands was in the range of 14 with the molecular mass ranging from approximately 14 to 83 KDa. Higher molecular weight proteins were not very prominent in Paras Soumya and Arka Anamika. In Paras Soumya 2 bands was found to be missing (Plate X A and Table 29). In case of leaves no differences in cultivars were observed (Plate X B and Table 30). SDS-PAGE analysis of the heavy metal treated seed proteins revealed accumulation of few new proteins which again varied with the cultivar. In Arka Anamika apparently two new proteins of approximately 47.90 and 15.8 KDa molecular masses were induced by all the heavy metals. In case of Deepti a high molecular weight protein is found to be induced by Cu (Plate XI A and XI B). No new bands could be distinctly observed in either Parbhani Kranti and PB-57 (Plate XII A and XII B). In Najuka F1 a band of approximately 83.10 KDa was found to be inhibited by all treatments while another of 72.5 KDa was inhibited by Pb and Hg. Similarly, no significant changes were observed in leaves of Arka Anamika (Plate XIII A and XIII B). Numbers of bands in leaf proteins were lesser than those observed for seeds. While seeds had 3 prominent bands with molecular masses of 47.86, 22.39 and 14.79 KDa, in leaves 2 protein bands of 50.12 and 17.12 KDa were apparent. However, the overall analysis of leaf protein did not yield sharp well resolved bands probably because of interference of mucilage like substances. Hence even under heavy metal stresses seed proteins showed some changes but no differences were obtained in the leaf protein.

Results of SDS-PAGE analysis revealed that on the whole no major difference could be detected after the heavy metal treatments.

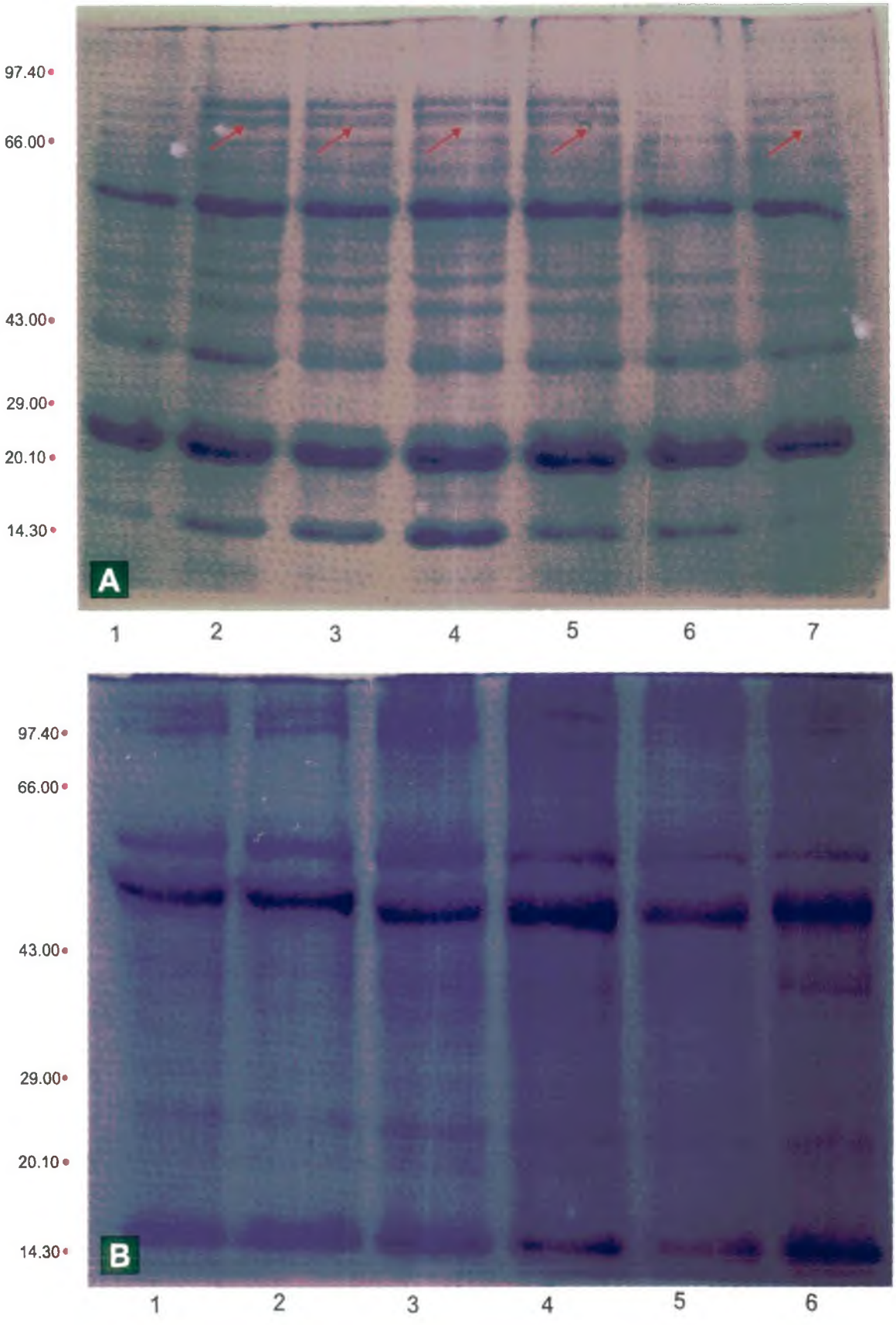


Plate X (A&B) : SDS PAGE analysis of different cultivars of okra A: Seeds (Lane 1 & 7: Arka Anamika; 2: Deepti; 3: Najuka F-1; 4: PB-57; 5: Parbhani Kranti and 6: Paras Soumya) B: Leaves (Lane 1 & 6: Arka Anamika; 2: Deepti; 3: Najuka F-1; 4: Parbhani Kranti; 5: PB-57).

Table 29: SDS-PAGE analysis of soluble proteins from seeds of different cultivars of okra

Cultivars	No. of bands	Molecular mass (kDa)
Arka Anamika	14	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Deepti	14	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Najuka F 1	13	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 23.99, 22.39, 17.38, 14.79, 14.13
PB-57	14	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Parbhani Kranti	14	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Paras Soumya	12	83.13, 79.43, 72.49, 57.54, 47.86, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Arka Anamika	14	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13

Table 30: SDS-PAGE analysis of soluble proteins from leaves of different cultivars of okra

Cultivars	No. of bands	Molecular mass (kDa)
Arka Anamika	9	95.50, 87.10, 58.88, 50.12, 38.90, 34.67, 27.54, 24.55, 17.35
Deepti	9	95.50, 87.10, 58.88, 50.12, 38.90, 34.67, 27.54, 24.55, 17.35
Najuka-F1	9	95.50, 87.10, 58.88, 50.12, 38.90, 34.67, 27.54, 24.55, 17.35
Parbhani Kranti	9	95.50, 87.10, 58.88, 50.12, 38.90, 34.67, 27.54, 24.55, 17.35
PB-57	9	95.50, 87.10, 58.88, 50.12, 38.90, 34.67, 27.54, 24.55, 17.35
Arka Anamika	9	95.50, 87.10, 58.88, 50.12, 38.90, 34.67, 27.54, 24.55, 17.35

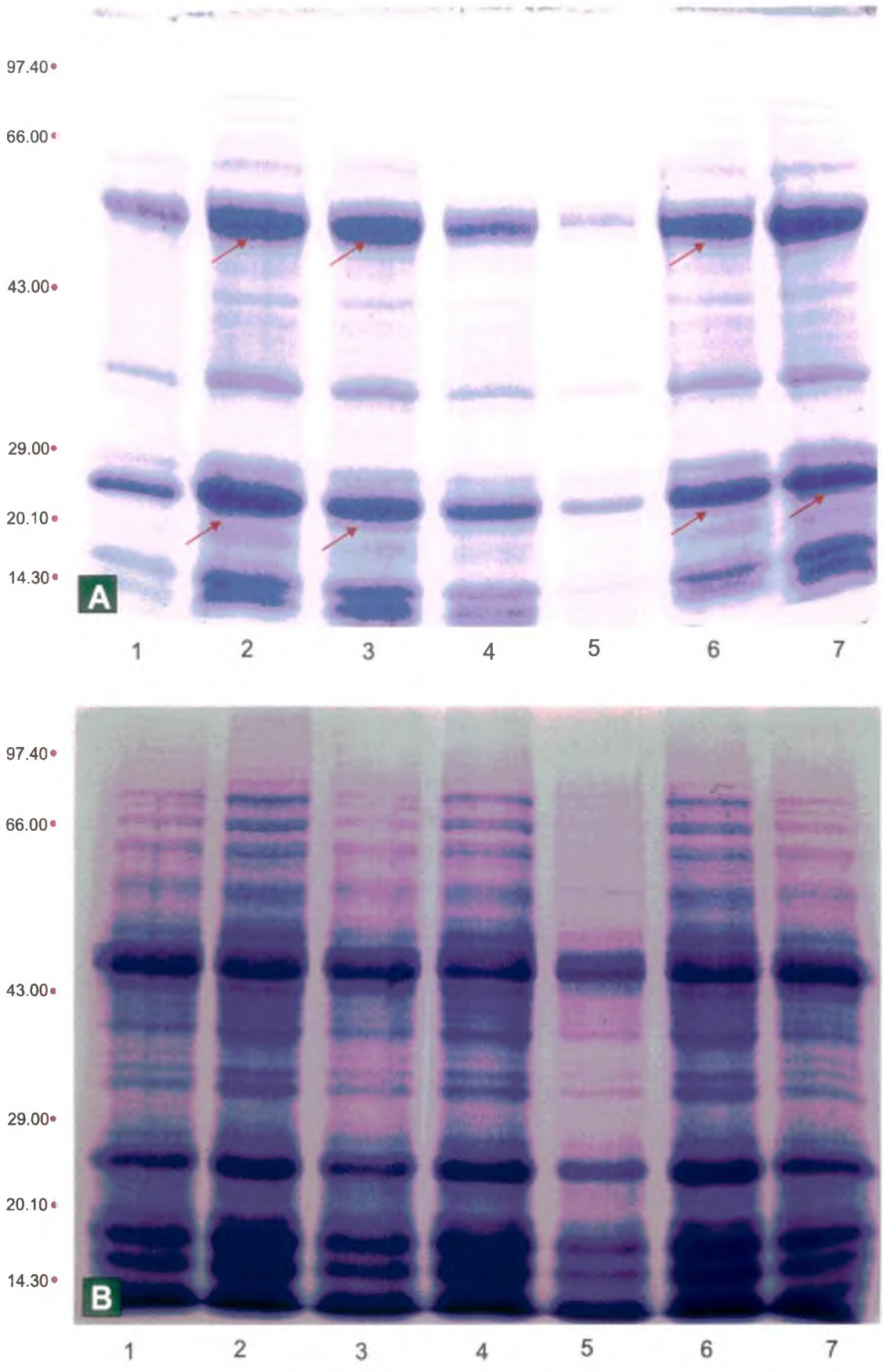


Plate XI (A&B) : SDS PAGE analysis of okra seeds treated with heavy metals A: Arka Anamika (Lane 1& 5: Control; 2 & 6: Cd; 3: Cu; 4: Hg and 7: Pb) B: Deepti (Lane 1 & 7: Control; 2 & 6: Cd; 3: Cu; 4: Hg; 5: Pb).

Table 31: SDS-PAGE analysis of soluble proteins from seeds of okra (cv.Arka Anamika) subjected to heavy metal treatments at 1000 μg ml^{-1}

Treatments	No. of bands	Molecular mass (kDa)
Control	14	83.18, 79.43, 72.44, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Cadmium	16	83.18, 79.43, 72.44, 66.07, 57.54, 47.86, 43.65, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 15.80, 14.79, 14.13
Copper	16	83.18, 79.43, 72.44, 66.07, 57.54, 47.86, 43.65, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 15.80, 14.79, 14.13
Mercury	16	83.18, 79.43, 72.44, 66.07, 57.54, 47.86, 43.65, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 15.80, 14.79, 14.13
Control	14	83.18, 79.43, 72.44, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Cadmium	15	83.18, 79.43, 72.44, 66.07, 57.54, 47.86, 43.65, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 15.80, 14.79, 14.13
Lead	13	83.18, 79.43, 72.44, 66.07, 57.54, 47.86, 43.65, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 15.80, 14.79, 14.13

Table 32: SDS-PAGE analysis of soluble proteins from seeds of okra (cv.Deepti) subjected to heavy metal treatments at 1000 μg ml^{-1}

Treatment	No. of bands	Molecular mass (kDa)
Control	14	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Cadmium	14	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Copper	14	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Mercury	14	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Lead	14	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Control	14	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Cadmium	14	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13

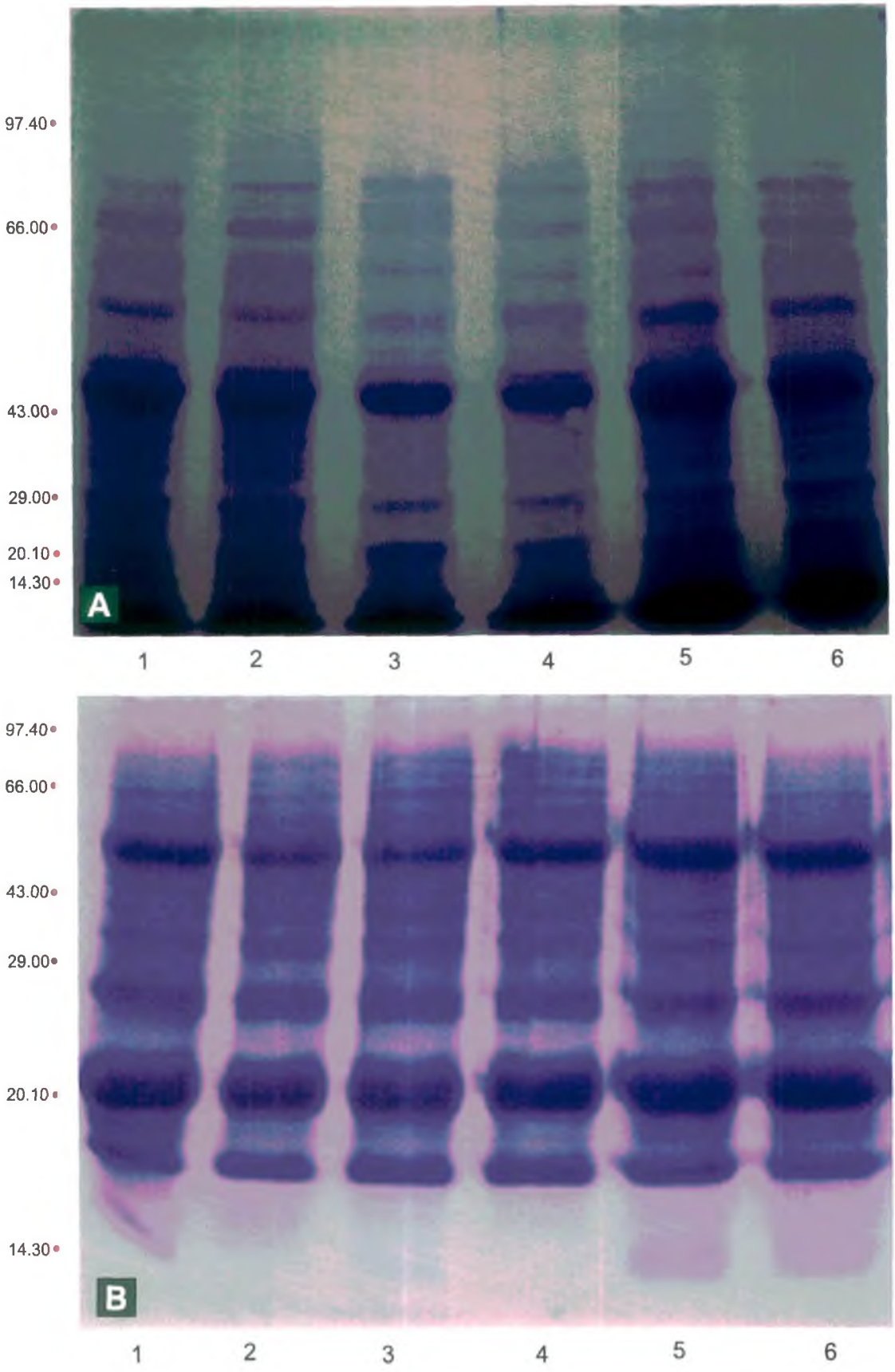


Plate XII (A&B): SDS PAGE analysis of okra seeds treated with heavy metals A: Parbhani Kranti (Lane 1 & 6: Control; 2 : Cd; 3: Cu; 4: Hg and 5:Pb) B: PB-57 (Lane 1 & 6:Control; 2 : Cd; 3 : Cu; 4: Hg and 5: Pb).

Table 33: SDS-PAGE analysis of soluble proteins from seeds of okra (cv.Parbhani Kranti) subjected to heavy metal treatments at 1000 μg ml^{-1}

Treatments	No. of bands	Molecular mass (kDa)
Control	15	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Cadmium	15	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Copper	15	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Mercury	15	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Lead	15	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Control	15	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13

Table 34: SDS-PAGE analysis of soluble proteins from seeds of okra (cv.PB-57) subjected to heavy metal treatments at 1000 μg ml^{-1}

Treatments	No. of bands	Molecular mass (kDa)
Control	14	83.18, 79.43, 72.44, 70.79, 66.07, 56.23, 50.12, 41.69, 36.31, 28.84, 22.39, 19.95, 15.85, 12.30
Cadmium	14	83.18, 79.43, 72.44, 70.79, 66.07, 56.23, 50.12, 41.69, 36.31, 28.84, 22.39, 19.95, 15.85, 12.30
Copper	14	83.18, 79.43, 72.44, 70.79, 66.07, 56.23, 50.12, 41.69, 36.31, 28.84, 22.39, 19.95, 15.85, 12.30
Mercury	14	83.18, 79.43, 72.44, 70.79, 66.07, 56.23, 50.12, 41.69, 36.31, 28.84, 22.39, 19.95, 15.85, 12.30
Lead	14	83.18, 79.43, 72.44, 70.79, 66.07, 56.23, 50.12, 41.69, 36.31, 28.84, 22.39, 19.95, 15.85, 12.30
Control	14	83.18, 79.43, 72.44, 70.79, 66.07, 56.23, 50.12, 41.69, 36.31, 28.84, 22.39, 19.95, 15.85, 12.30

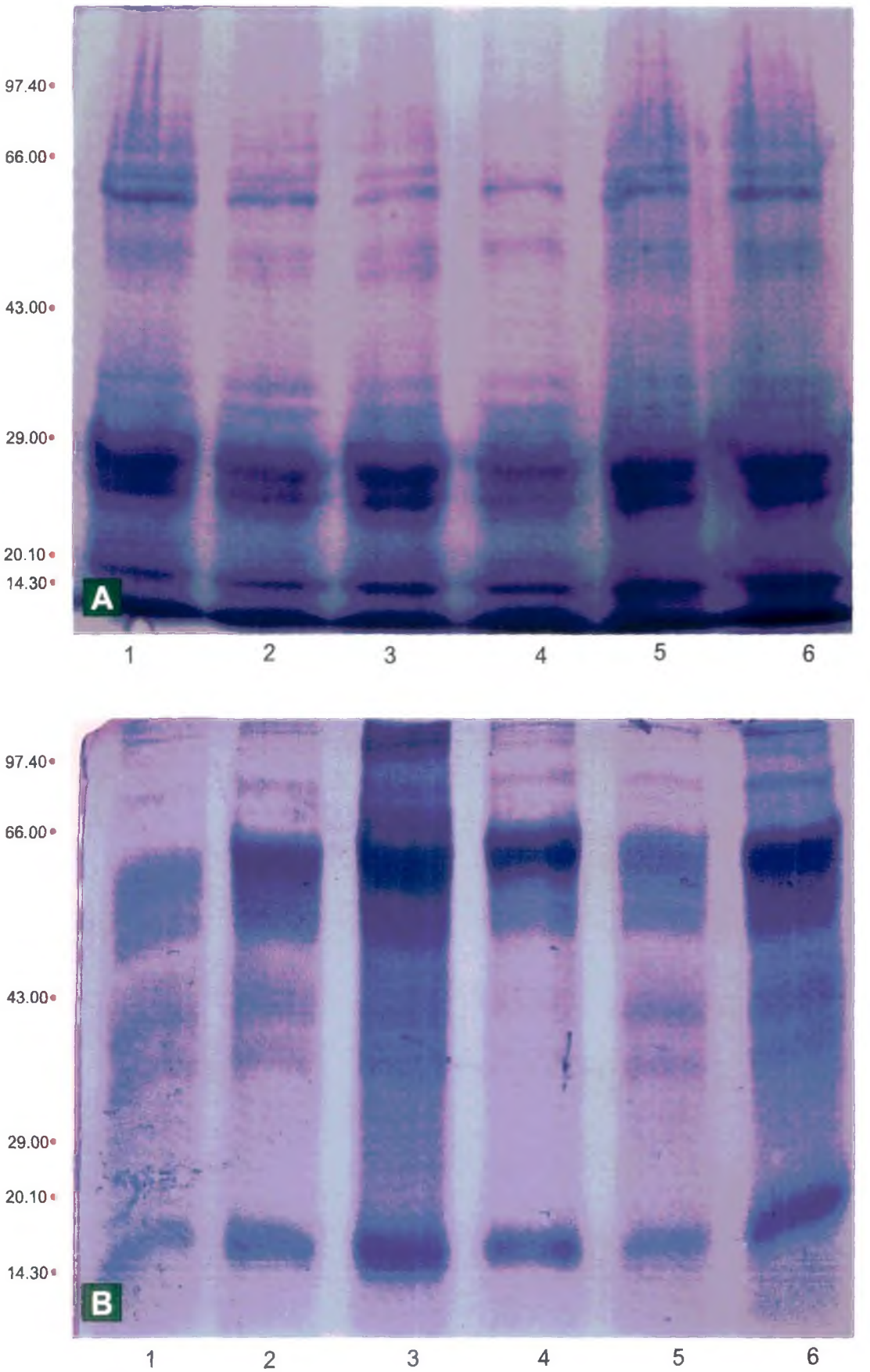


Plate XIII (A&B) : SDS PAGE analysis of A: Seeds of cv. Najuka - F1 treated with heavy metals (Lane 1 & 5: Control; 2: Cd; 3: Cu; 4: Hg and 6:Pb) B:Leaves of cv. Arka Anamika treated with heavy metals (Lane 1 & 5: Control; 2 & 6: Cd; 3: Cu and 4: Hg).

Table 35: SDS-PAGE analysis of soluble proteins from seeds of okra (cv.Najuka-F1) subjected to heavy metal treatments at 1000 μg ml^{-1}

Treatments	No. of bands	Molecular mass (kDa)
Control	13	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Cadmium	12	79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 23.99, 22.39, 17.38, 14.79, 14.13
Copper	12	79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 23.99, 22.39, 17.38, 14.79, 14.13
Mercury	11	79.43, 66.07, 57.54, 47.86, 41.69, 38.90, 23.99, 22.39, 17.38, 14.79, 14.13
Control	13	83.13, 79.43, 72.49, 66.07, 57.54, 47.86, 41.69, 38.90, 31.62, 23.99, 22.39, 17.38, 14.79, 14.13
Lead	11	79.43, 66.07, 57.54, 47.86, 41.69, 38.90, 23.99, 22.39, 17.38, 14.79, 14.13

Table 36: SDS-PAGE analysis of soluble proteins from leaves of okra (cv.Arka Anamika) subjected to heavy metal treatments at 1000 μg ml^{-1}

Treatments	No. of bands	Molecular mass (kDa)
Control	9	95.50, 87.10, 58.88, 50.12, 38.90, 34.67, 27.54, 24.55, 17.35
Cadmium	9	95.50, 87.10, 58.88, 50.12, 38.90, 34.67, 27.54, 24.55, 17.35
Copper	9	95.50, 87.10, 58.88, 50.12, 38.90, 34.67, 27.54, 24.55, 17.35
Mercury	9	95.50, 87.10, 58.88, 50.12, 38.90, 34.67, 27.54, 24.55, 17.35
Lead	9	95.50, 87.10, 58.88, 50.12, 38.90, 34.67, 27.54, 24.55, 17.35
Cadmium	9	95.50, 87.10, 58.88, 50.12, 38.90, 34.67, 27.54, 24.55, 17.35

4.7. Studies on antioxidative enzymes in leaves of okra plants subjected to heavy metal stress

4.7.1. Changes in enzymatic activities

Activities of antioxidative enzymes in leaves of different cultivars of okra subjected to heavy metal treatments at 1000 μg ml^{-1} of the salts were assayed. The enzymes included catalase(CAT), peroxidase(POX), ascorbate peroxidase(APOX), glutathione reductase(GR) and superoxide dismutase(SOD).

4.7.1.1. Catalase

Catalase activities in all the cultivars subjected to heavy metal stresses showed a decline. However, the degree of decrease varied with the cultivars and treatment. Cu induced maximum decrease in two cultivars (Arka Anamika and Paras Soumya), Hg in another two (Deepti and Najuka F1) while Pb and Cd in Parbhani Kranti and PB-57 respectively (Table 37). Analysis of the results revealed that Cd induced least decline of activity in five of the cultivars ranging from 10% to 57%. On the other hand highest inhibition was 82% which was induced by Cu in two of the cultivars.

Table 37: Catalase activity in leaves of okra cultivars following heavy metal treatment

Cultivars	Catalase activity (enzyme unit mg^{-1} protein)				
	Control	Cadmium	Copper	Mercury	Lead
Arka Anamika	2.24 ± 0.019	1.50 ± 0.009	0.40 ± 0.004	0.62 ± 0.014	0.71 ± 0.028
Deepti	1.29 ± 0.024	1.16 ± 0.014	0.76 ± 0.009	0.54 ± 0.005	0.68 ± 0.030
Najuka-F1	1.68 ± 0.009	1.52 ± 0.024	0.62 ± 0.014	0.59 ± 0.005	0.71 ± 0.019
Paras Soumya	1.95 ± 0.028	0.84 ± 0.033	0.35 ± 0.019	0.45 ± 0.019	0.40 ± 0.019
Parbhani Kranti	1.05 ± 0.033	0.85 ± 0.033	0.80 ± 0.033	0.60 ± 0.028	0.46 ± 0.028
PB-57	1.11 ± 0.028	0.45 ± 0.009	0.94 ± 0.024	0.55 ± 0.014	0.78 ± 0.033

Treatments were applied at 1000 μg ml^{-1} concentration of the salts
Values are mean of 3 replicates; ± = SEm

Table 38: Effect of heavy metals on peroxidase activity in leaves of okra

Cultivars	Peroxidase activity (Δ OD mg^{-1} protein min^{-1})				
	Control	Cadmium	Copper	Mercury	Lead
Arka Anamika	0.030 ± 0.001	0.048 ± 0.001	0.034 ± 0.001	0.073 ± 0.001	0.061 ± 0.003
Deepti	0.032 ± 0.0002	0.192 ± 0.003	0.061 ± 0.002	0.046 ± 0.002	0.117 ± 0.004
Najuka-F1	0.024 ± 0.0003	0.029 ± 0.002	0.086 ± 0.003	0.074 ± 0.002	0.071 ± 0.002
Paras Soumya	0.045 ± 0.002	0.075 ± 0.002	0.052 ± 0.003	0.180 ± 0.003	0.184 ± 0.002
Parbhani Kranti	0.038 ± 0.002	0.149 ± 0.003	0.114 ± 0.002	0.120 ± 0.004	0.185 ±0.005
PB-57	0.033 ± 0.001	0.064 ± 0.002	0.094 ± 0.002	0.051 ± 0.001	0.067 ± 0.002

Treatments were applied at 1000 μg ml^{-1} concentration of the salts
Values are mean of 3 replicates; ± = SEm

4.7.1.2. Peroxidase

Results presented in table 38 revealed that POX activity in all the cultivars was enhanced by the heavy metal treatments, though the degree of increase varied. Activities were enhanced by three to six times in the different treatments.

4.7.1.3. Ascorbate peroxidase

All the heavy metal stresses enhanced activities of APOX. Maximum enhancement of activity (68%) in Deepti was induced by Cu. Pb enhanced activities in four of the cultivars. In Najuka F1 the increase was 62% (Table 39).

4.7.1.4. Glutathione reductase

Glutathione reductase activity was expressed as the μM NADPH oxidised mg^{-1}protein min^{-1}. In this reaction the enzyme reduced glutathione with electrons from NADPH which was subsequently oxidised. In this case also it was observed that while the activities in control were quite low significant increases were recorded in the different treatments. Cu and Cd induced maximum activities in the different cultivars (Table 40).

Table 39: Effect of heavy metals on ascorbate peroxidase activity in leaves of okra

Cultivars	Ascorbate peroxidase (enzyme unit mg^{-1} protein)				
	Control	Cadmium	Copper	Mercury	Lead
Arka Anamika	0.104 ± 0.004	0.120 ± 0.004	0.137 ± 0.004	0.143 ± 0.005	0.123 ± 0.005
Deepti	0.076 ± 0.003	0.091 ± 0.004	0.128 ± 0.003	0.122 ± 0.003	0.119 ± 0.003
Najuka-F1	0.079 ± 0.004	0.090 ± 0.004	0.098 ± 0.004	0.103 ± 0.006	0.127 ± 0.002
Paras Soumya	0.071 ± 0.003	0.085 ± 0.004	0.092 ± 0.004	0.103 ± 0.002	0.115 ± 0.004
Parbhani Kranti	0.082 ± 0.004	0.097 ± 0.003	0.099 ± 0.003	0.112 ± 0.003	0.125 ± 0.003
PB-57	0.084 ± 0.004	0.098 ± 0.005	0.102 ± 0.004	0.100 ± 0.004	0.110 ± 0.003

Treatments were applied at 1000 μg ml^{-1} concentration of the salts
Values are mean of 3 replicates; ± = SEm

Table 40: Changes in glutathione reductase activity in leaves of okra subjected to heavy metal treatments

Cultivars	Glutathione reductase (μM NADPH oxidized mg^{-1} protein min^{-1})				
	Control	Cadmium	Copper	Mercury	Lead
Arka Anamika	0.152 ± 0.003	0.690 ± 0.005	0.950 ± 0.012	0.780 ± 0.011	0.755 ± 0.014
Deepti	0.171 ± 0.002	1.710 ± 0.014	1.560 ± 0.048	0.550 ± 0.001	1.260 ± 0.004
Najuka-F1	0.272 ± 0.001	1.540 ± 0.023	1.270 ± 0.014	0.484 ± 0.001	0.721 ± 0.007
Paras Soumya	0.240 ± 0.002	0.720 ± 0.013	0.780 ± 0.019	0.609 ± 0.012	0.627 ± 0.011
Parbhani Kranti	0.295 ± 0.071	0.716 ± 0.004	0.856 ± 0.008	0 683 ± 0.005	0.790 ± 0.005
PB-57	0.289 ± 0.003	0.688 ± 0.005	1.07 ± 0.038	0.676 ± 0.002	0.974 ± 0.003

Treatments were applied at 1000 μg ml^{-1} concentration of the salts
Values are mean of 3 replicates; ± = SEm

4.7.1.5 Superoxide dismutase

All the heavy metals increased activities of SOD in the different cultivars. Increase of activity was not same in all the treatments. Pb induced activity which was more than four times that of control in Parbhani Kranti, while in PB-57 the increase was only to a lesser degree (Table 41). Differences were also observed in the constitutive enzyme activities of the different cultivars.

Table 41: Changes in superoxide dismutase activity in leaves of okra subjected to heavy metal treatments

Cultivars	Superoxide dismutase (enzyme unit mg^{-1} protein)				
	Control	Cadmium	Copper	Mercury	Lead
Arka Anamika	0.066 ± 0.002	0.184 ± 0.003	0.016 ± 0.0005	0.034 ± 0.001	0.168 ± 0.006
Deepti	0.098 ± 0.001	0.161 ± 0.001	0.272 ± 0.003	0.127 ± 0.002	0.141 ± 0.002
Najuka-F1	0.148 ± 0.002	0.166 ± 0.003	0.164 ± 0.002	0.207 ± 0.003	0.117 ± 0.004
Paras Soumya	0.080 ± 0.002	0.100 ± 0.003	0.150 ± 0.003	0.180 ± 0.005	0.250 ± 0.004
Parbhani Kranti	0.063 ± 0.004	0.153 ± 0.004	0.194 ± 0.006	0.251 ± 0.009	0.290 ± 0.007
PB-57	0.118 ± 0.001	0.167 ± 0.001	0.155 ± 0.0004	0.122 ± 0.0005	0.171 ± 0.005

Treatments were applied at 1000 $\mu g\ ml^{-1}$ concentration of the salts
Values are mean of 3 replicates; ± = SEm

4.7.2. Changes in isozyme profile

Peroxidase isozymes were analyzed by PAGE as described in materials and methods. No significant differences were observed in isozymes of peroxidase extracted from the variously treated seeds (cv. Arka Anamika). Two isozymes of Rm values 0.31 and 0.34 were evident in control, Cd and Pb treated seeds whereas in Cu and Hg treated seeds only one isozyme of Rm value 0.31 was evident. Similar results were also obtained in leaves.

4.8. Changes in lipid peroxidation of membranes following heavy metal treatments

The degree of lipid peroxidation of membrane lipids gives an indication of cellular damage. In the present study this was measured. Lipid peroxidation was measured as the content of malondialdehyde (MDA) formed or the amount of thiobarbituric acid (TBA) reactive substance. Results presented in table 42 revealed significant increases in lipid peroxidation of leaves from treated plants in relation to control. Cd induced maximum lipid peroxidation in three cultivars (Arka Anamika, Parbhani Kranti and PB-57) and Pb in two (Deepti and Najuka-F1). In Arka Anamika treated with Cd MDA content was more than double of control.

Table 42: Effect of heavy metals on membrane lipid peroxidation of okra

Cultivars	Lipid peroxidation (MDA content)				
	Control	Cadmium	Copper	Mercury	Lead
Arka Anamika	1.20 ± 0.04	2.52 ± 0.05	1.58 ± 0.02	1.30 ± 0.06	1.71 ± 0.05
Deepti	1.37 ± 0.02	1.54 ± 0.01	1.61 ± 0.07	1.58 ± 0.01	1.68 ± 0.01
Najuka-F1	1.17 ± 0.01	1.65 ± 0.02	1.62 ± 0.03	1.54 ± 0.12	1.73 ± 0.01
Paras Soumya	1.57 ± 0.01	1.69 ± 0.01	2.05 ± 0.01	1.65 ± 0.01	1.77 ± 0.03
Parbhani Kranti	1.94 ± 0.03	2.48 ± 0.00	1.95 ± 0.04	1.99 ± 0.01	2.20 ± 0.15
PB-57	0.95 ± 0.01	1.53 ± 0.03	1.42 ± 0.05	1.31 ± 0.02	1.39 ± 0.01

Treatments were applied at 1000 $\mu g\ ml^{-1}$ concentration of the salts
Values are mean of 3 replicates; ± = SEm

Table 42A: Analysis of variance of data presented in Table 42

Source	D.F.	S.S.	M.S.	F	C.D.
Replication	2				
Variety	5	5.32	1.06	117.78	0.068
Treatment	4	2.99	0.75	83.33	0.064
Interaction	20	2.66	0.13	14.44	0.155
Error	58	0.52	0.009		
Total	89	11.49			

4.9. Analysis of flavonoids from fruits of okra subjected to heavy metal stress by HPLC analysis

Influence of heavy metals on flavonoid accumulation in three cultivars (Arka Anamika, Deepti and Najuka-F1) of okra were analysed by HPLC. Flavonoids were extracted from the fruits of three cultivars of okra subjected to the different heavy metal treatments. The results obtained have been presented in figures and tables. In case of Arka Anamika all treatments had 3 peaks whereas Pb had only 2 peaks (Fig. 10 and Table 43). The first peak in all the treatments was higher than the control. The second peak in control, Cd and Cu treatments were more or less similar but there was a decrease in the peak height in Hg and Pb treatments. There was not much difference in the third peak. In Deepti Hg affected the accumulation of flavonoids (Fig. 11 and Table 44). The first peak in all the treatments was more or less same. The peak height decreased in relation to control in all the treatments except Hg. Peak height in Hg treatment was greater than that of control. In case of Cu and Pb treatments the second peak showed greater height and area than the corresponding ones in control. Peaks obtained following Cd treatment were almost similar to that of control. In case of the third peak the peak height of Cd treatment was almost similar with respect to control, whereas the peaks in Cu and Pb treatment were much higher. In Najuka-F1 all treatments led to a decrease in peak number and peak height (Fig. 12 and Table 45). Control had 4 peaks, Cd, Cu and Hg showed 3 peaks and only a single peak was observed in Pb treatment. The third peak in control was observed at

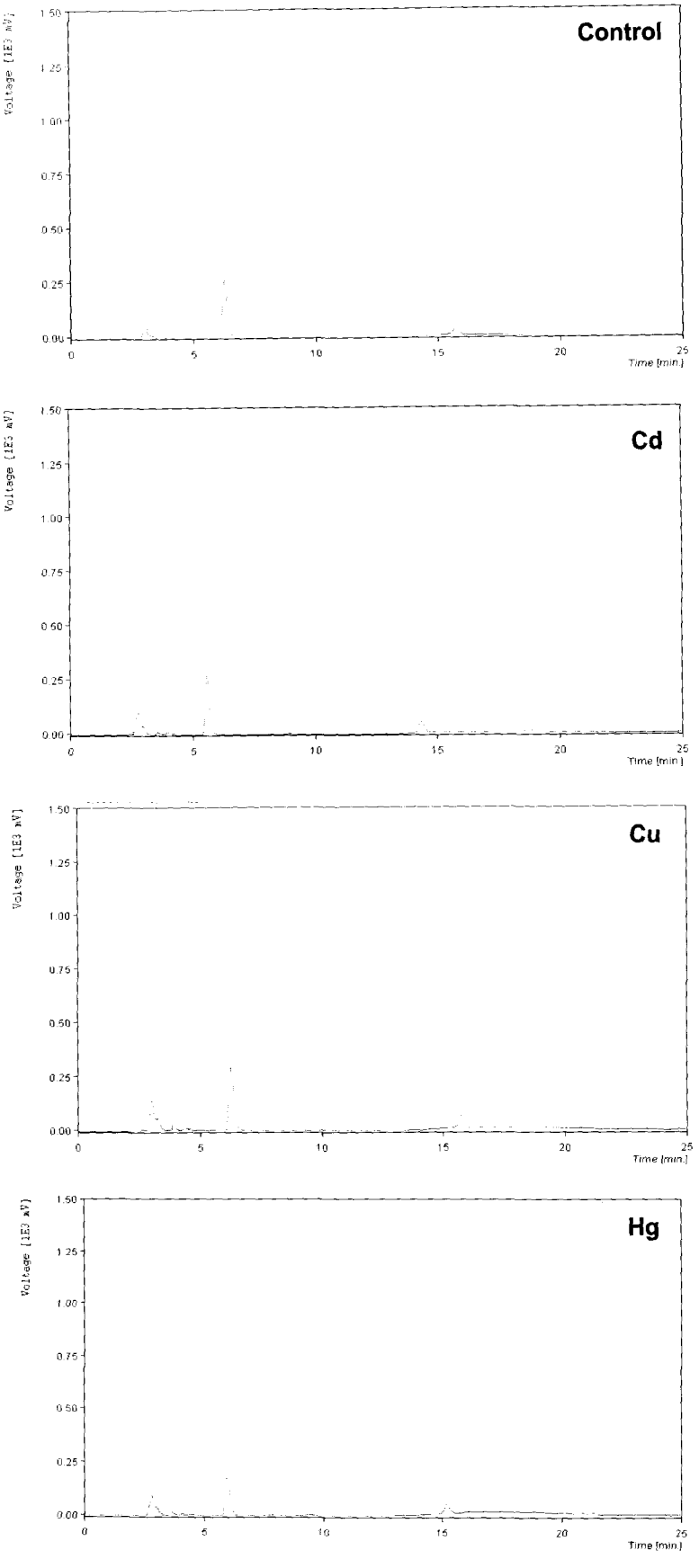


Fig.10. HPLC profile of flavonoids extracted from fruits of okra (cv. Arka Anamika) treated with different heavy metals

a retention time of 3.65 but in case of Cd, Cu and Hg treatments there was a slight shift in retention time which is 5.41. There was a general decline in peak height. Results thus reveal that Pb and Hg inhibited flavonoids accumulation depending on the cultivars while Cd treatment was more or less similar to control. Though no major changes were observed decrease or increase in accumulation were obtained in the different compounds as evidenced by change in peak height.

Table 43: HPLC analysis of flavonoids extracted from fruits of okra (cv. Arka Anamika) treated with different heavy metals

Treatment	Peak No.	Retention time (min)	Height (mV)	Area (mv.S)	Height (%)	Area (%)
Control	1	3.00	60.15	879.53	15.17	12.44
	2	6.25	268.83	3774.85	67.81	53.41
	3	15.72	58.31	1638.40	14.71	23.18
Cadmium	1	3.00	99.70	1862.04	18.48	17.10
	2	6.25	271.81	3567.77	50.37	32.75
	3	15.72	60.40	1194.99	11.19	10.97
Copper	1	3.00	144.23	2908.19	24.40	18.87
	2	6.25	306.91	4588.81	51.91	29.77
	3	15.72	76.15	2757.23	12.88	17.89
Mercury	1	3.00	103.98	1724.75	22.57	14.27
	2	6.25	182.72	2645.73	39.66	21.89
	3	15.72	54.18	1216.30	11.76	10.06
Lead	1	3.00	123.43	2061.26	45.27	22.21
	2	6.25	56.15	909.73	20.59	9.80

Treatments were applied at 1000 $\mu g\ ml^{-1}$ concentration of the salts

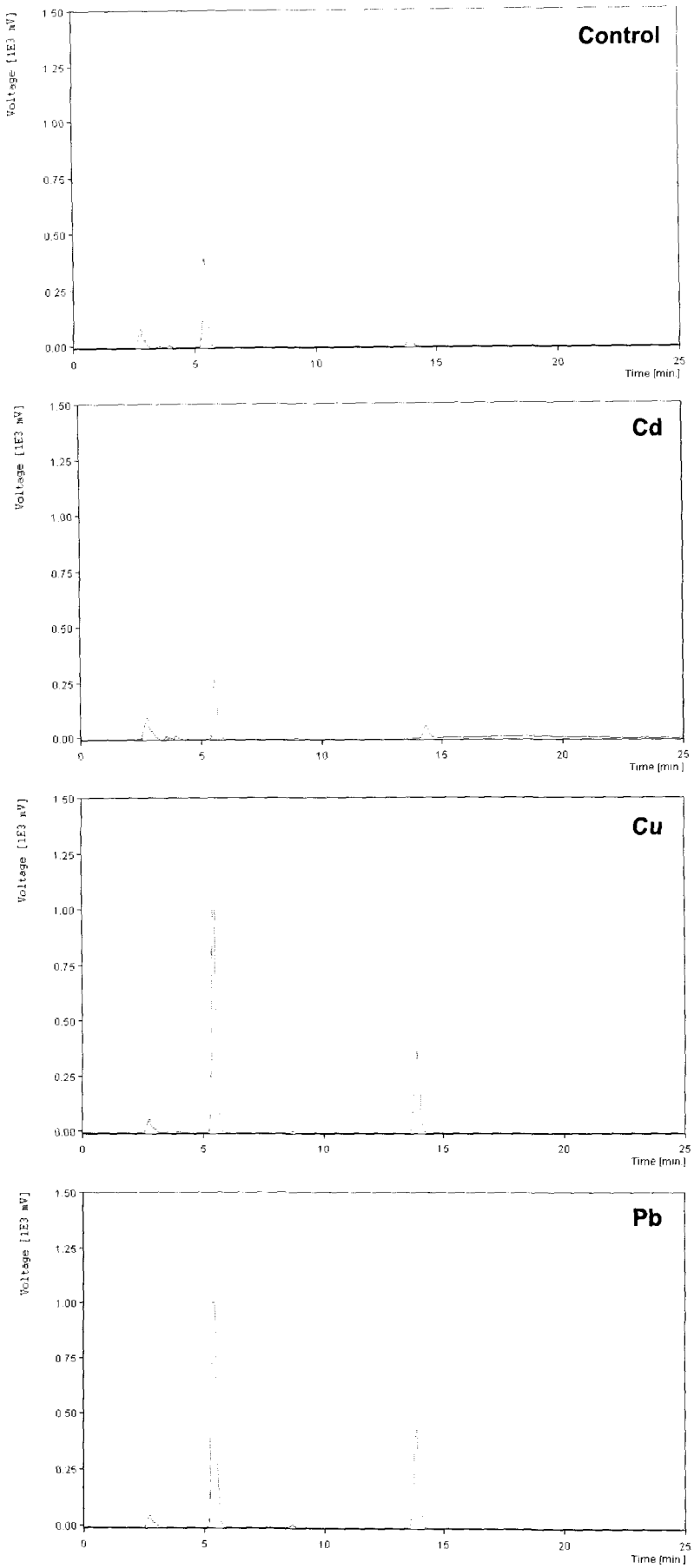


Fig. 11. HPLC profile of flavonoids extracted from fruits of okra (cv. Deepti) treated with different heavy metals

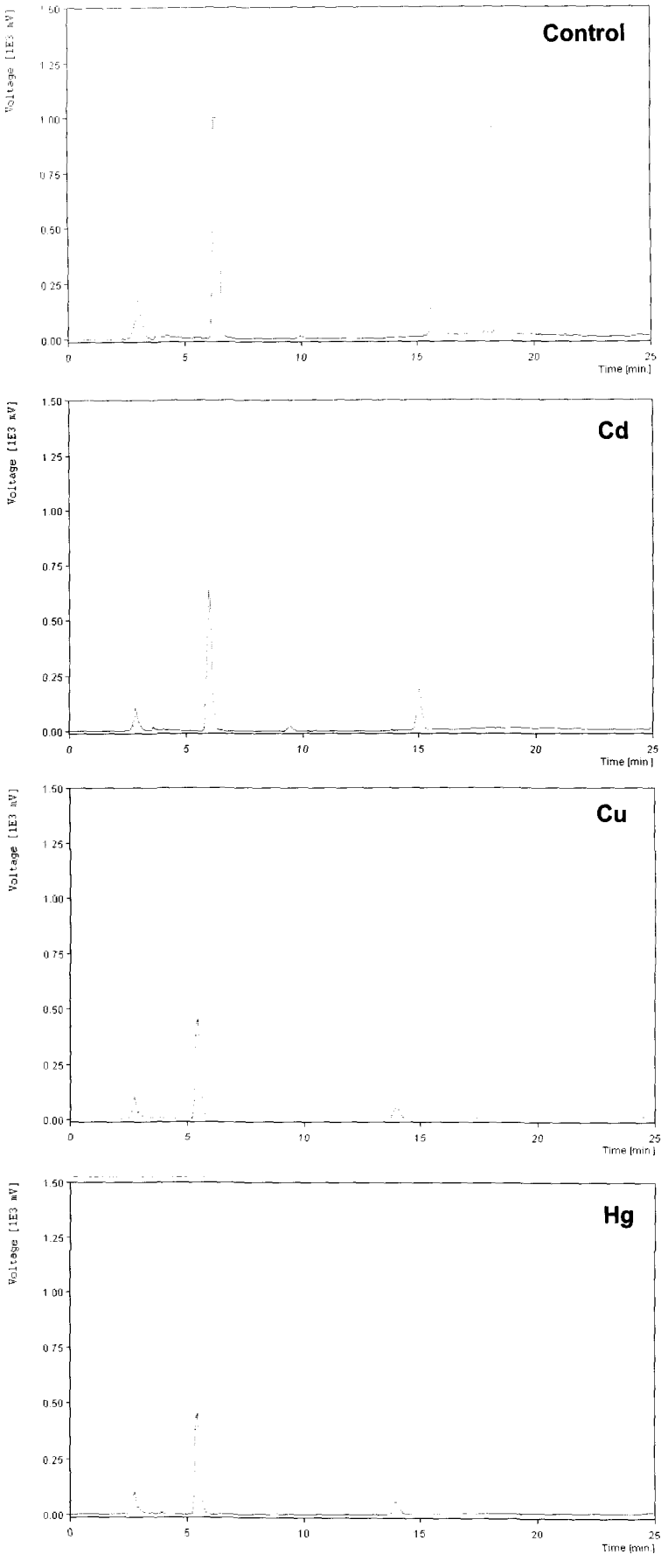


Fig. 12. HPLC profile of flavonoids extracted from fruits of okra (cv. Najuka-F1) treated with different heavy metals

Table 44: HPLC analysis of flavonoids extracted from fruits of okra (cv. Deepti) treated with different heavy metal

Treatment	Peak No.	Retention time (min)	Height (mV)	Area (mv.S)	Height (%)	Area (%)
Control	1	2.73	83.36	1221.03	14.86	17.01
	2	5.42	409.30	5081.26	72.95	70.77
	3	13.88	49.63	733.03	8.85	10.21
Cadmium	1	2.73	82.57	1552.31	14.95	15.14
	2	5.42	363.24	5304.05	65.77	51.72
	3	13.86	37.83	797.47	6.85	7.78
Copper	1	2.73	66.20	991.84	4.53	4.67
	2	5.42	1010.38	14271.08	69.13	67.24
	3	13.88	373.42	5873.32	25.55	27.67
Mercury	1	2.73	142.37	2424.55	54.95	39.13
Lead	1	2.73	54.62	825.28	3.60	3.59
	2	5.42	1010.39	15162.06	66.65	65.91
	3	13.86	439.39	6907.97	28.98	30.03

Treatments were applied at 1000 $\mu g\ ml^{-1}$ concentration of the salts

Table 45: HPLC analysis of flavonoids extracted from fruits of okra (cv. Najuka-F1) treated with different heavy metals

Treatment	Peak No.	Retention time (min)	Height (mV)	Area (mv.S)	Height (%)	Area (%)
Control	1	2.77	174.06	3714.55	10.25	10.50
	2	3.78	52.96	1229.41	3.12	3.48
	3	6.35	1000.51	17878.27	58.91	50.55
	4	15.75	338.12	6133.05	19.91	17.34
Cadmium	1	2.77	105.04	1642.28	10.20	9.17
	2	5.41	634.02	9310.03	61.55	51.99
	3	15.75	183.07	3032.87	17.77	16.94
Copper	1	2.77	151.98	2880.92	9.73	9.81
	2	5.41	991.45	14596.85	63.48	49.73
	3	13.96	234.37	4826.42	15.01	16.44
Mercury	1	2.77	99.88	1613.14	15.00	14.39
	2	5.41	454.64	6780.48	68.28	60.47
	3	13.96	55.47	1116.59	8.33	9.96
Lead	1	2.77	60.87	1172.21	34.80	29.35

Treatments were applied at 1000 $\mu g\ ml^{-1}$ concentration of the salts

4.10. Determination of heavy metal content

Having analysed all the changes brought about in okra plants following heavy metal treatments the accumulation of heavy metals in the tissues was determined. For this the fruits and roots of four cultivars (Arka Anamika, Deepti, Najuka F1 and PB-57) after soil application of Cd, Cu and Pb at 1000 μg ml^{-1} were determined as mentioned in materials and methods. Root showed maximum accumulation of all the heavy metals while in fruits the accumulation was much lower (Table 46). Traces of heavy metals were found in untreated control in all cases. In case of Cd fruit accumulation range from 0.117 to 0.186 ppm in all the different cultivars while for roots it ranged from 0.403 to 0.542 ppm. In Cu treatment the ranges for fruit and root were 0.081 to 0.293 ppm and 0.264 to 0.733 ppm respectively. Pb accumulation however ranged from 0.082 to 0.187 ppm in fruits and 0.138 to 0.464 ppm in the roots. Hence, mobilisation of Pb from the roots to the leaves seemed to be greater when compared to Cd and Cu. Differences in accumulation among the different cultivars were also evident.

Table 46: Heavy metal contents of fruits and roots of okra plants after heavy metal treatment

		Heavy metal content (ppm)					
Cultivar	**Part**	**Cadmium**		**Copper**		**Lead**	
		Control	Treated	Control	Treated	Control	Treated
Arka Anamika	**Fruit**	0.002	0.117	0.005	0.162	0.003	0.108
	Root	0.028	0.425	0.040	0.264	0.025	0.178
Deepti	**Fruit**	0.003	0.130	0.002	0.081	0.014	0.187
	Root	0.035	0.542	0.092	0.517	0.042	0.213
Najuka F-1	**Fruit**	0.004	0.148	0.008	0.195	0.004	0.082
	Root	0.029	0.473	0.134	0.733	0.023	0.138
PB-57	**Fruit**	0.007	0.186	0.017	0.293	0.011	0.128
	Root	0.018	0.403	0.159	0.659	0.064	0.464

Analysis were performed after 1 week of final application of heavy metal salts to soil

4.11. Microscopic studies

Cross sections of radicles from heavy metal treated seedlings of Arka Anamika were stained with Melzers reagent and observed under the light microscope. Significant differences were noticed especially in the cortical region with respect to starch accumulation. Intercellular space of cortical tissue showed some deposition. There were also changes in shape of the cortical cells. Maximum deposition was observed in Cu and minimum in Cd treatments (Plates XIV and XV). Further, alkaloid deposition in roots of okra plants grown in heavy metal treated soil sere also studied. For this cross section of roots and fruits of untreated and heavy metal treated (cv. Arka Anamika) stained with Wagners reagent was observed under light microscope. Accumulation of alkaloids in Cu and Cd treatments in roots were much higher than in control whereas, those of Pb and Hg were not significantly different. Maximum accumulation was observed in the cortical region and to some extent near the vascular bundles (Plates XVI and XVII). In case of fruits, sections from both basal portions and middle portions revealed changes in accumulation as well as in the cellular structure (Plates XVIII – XXI).

4.12. Effect of treatment with chemicals

Results of the various experiments related to the biochemical responses of different cultivars to the heavy metals showed alterations in metabolic processes within the plant. As certain previous reports are available on the influence of heavy metal stress ameliorating compounds on the stress imposed by heavy metals, in the present study a few experiments were conducted to determine how treatment with $CaCl_2$ or KNO_3 and subsequent imposition of the heavy metal stresses affects the plants response. For this, 3 cultivars were selected (Arka Anamika, Deepti and Najuka-F1) and seeds were treated as described in materials and methods. Germination percentage was calculated and analyses were made on protein content and antioxidant enzymes in the seedlings.

4.12.1. Germination

After 72 h of germination it was observed that while treatment with the heavy metal decreased germination markedly in relation to control, treatment with $CaCl_2$ and KNO_3 increased the germination to some extent which was however still lower

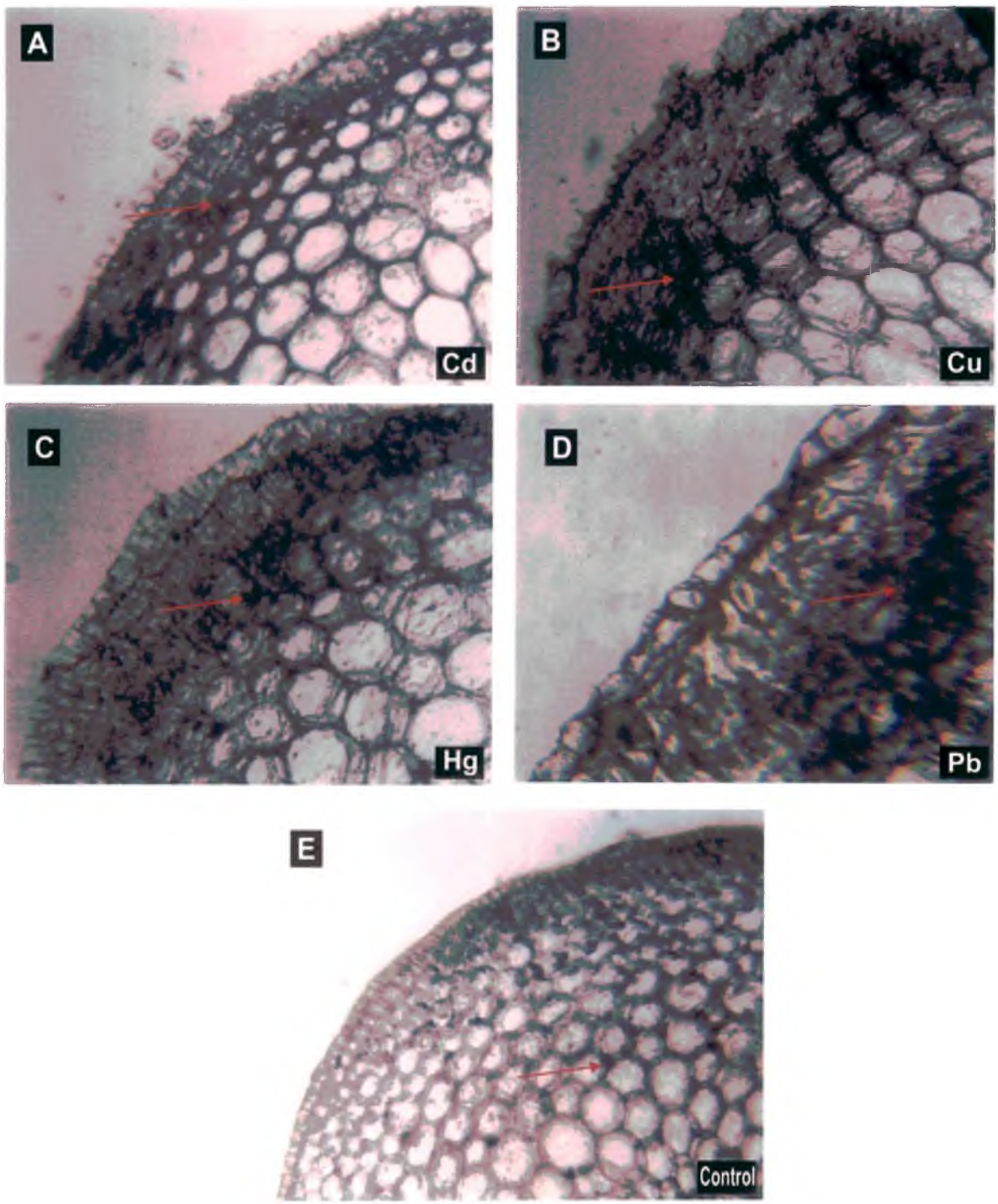


Plate XIV (A-E): Portion of transverse section (x10) of radicles of heavy metal treated okra seedlings. Sections were stained with Melzers reagent.

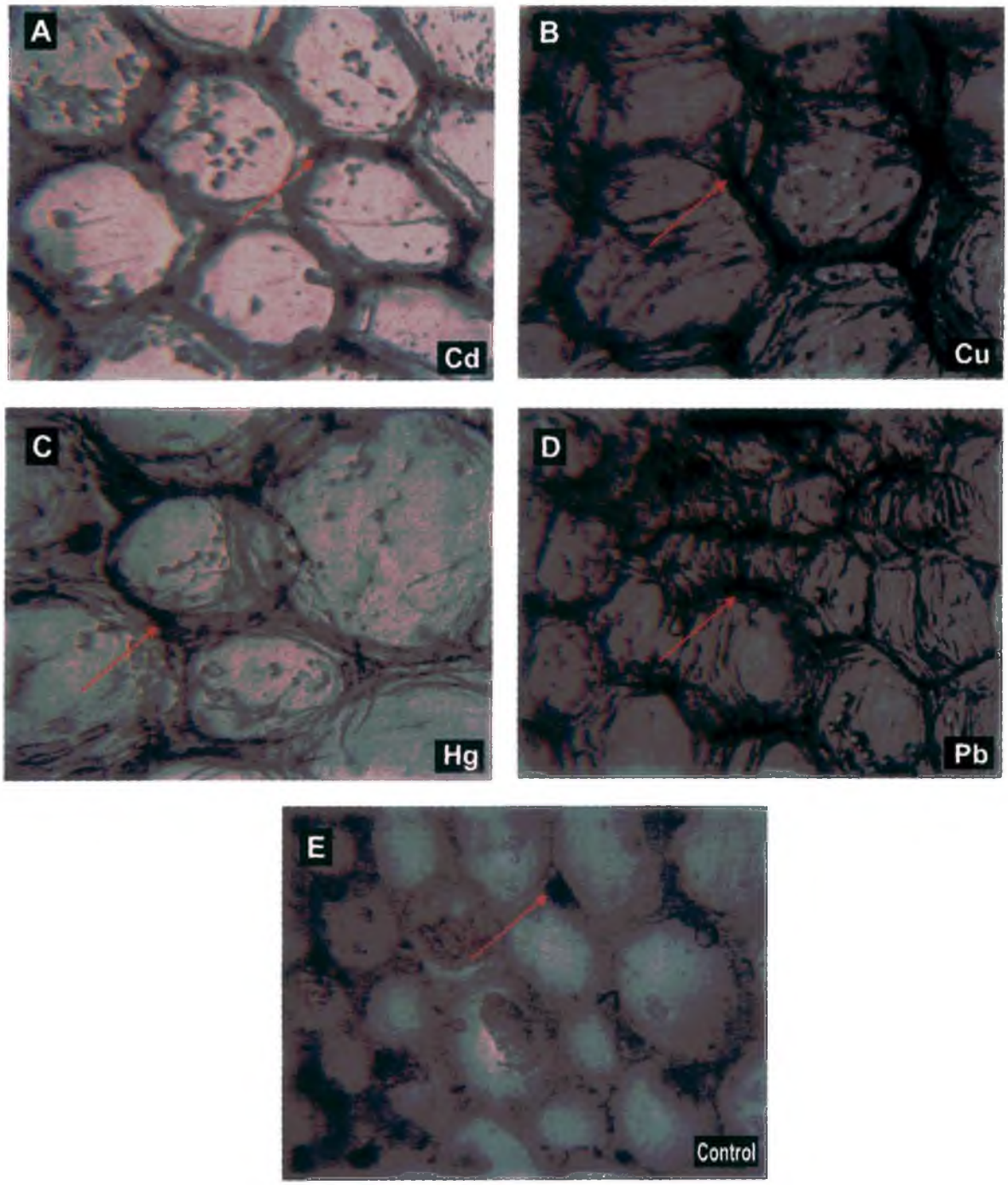


Plate XV (A-E): Portion of transverse section (x45) of radicles of heavy metal treated okra seedlings. Sections were stained with Melzers reagent.

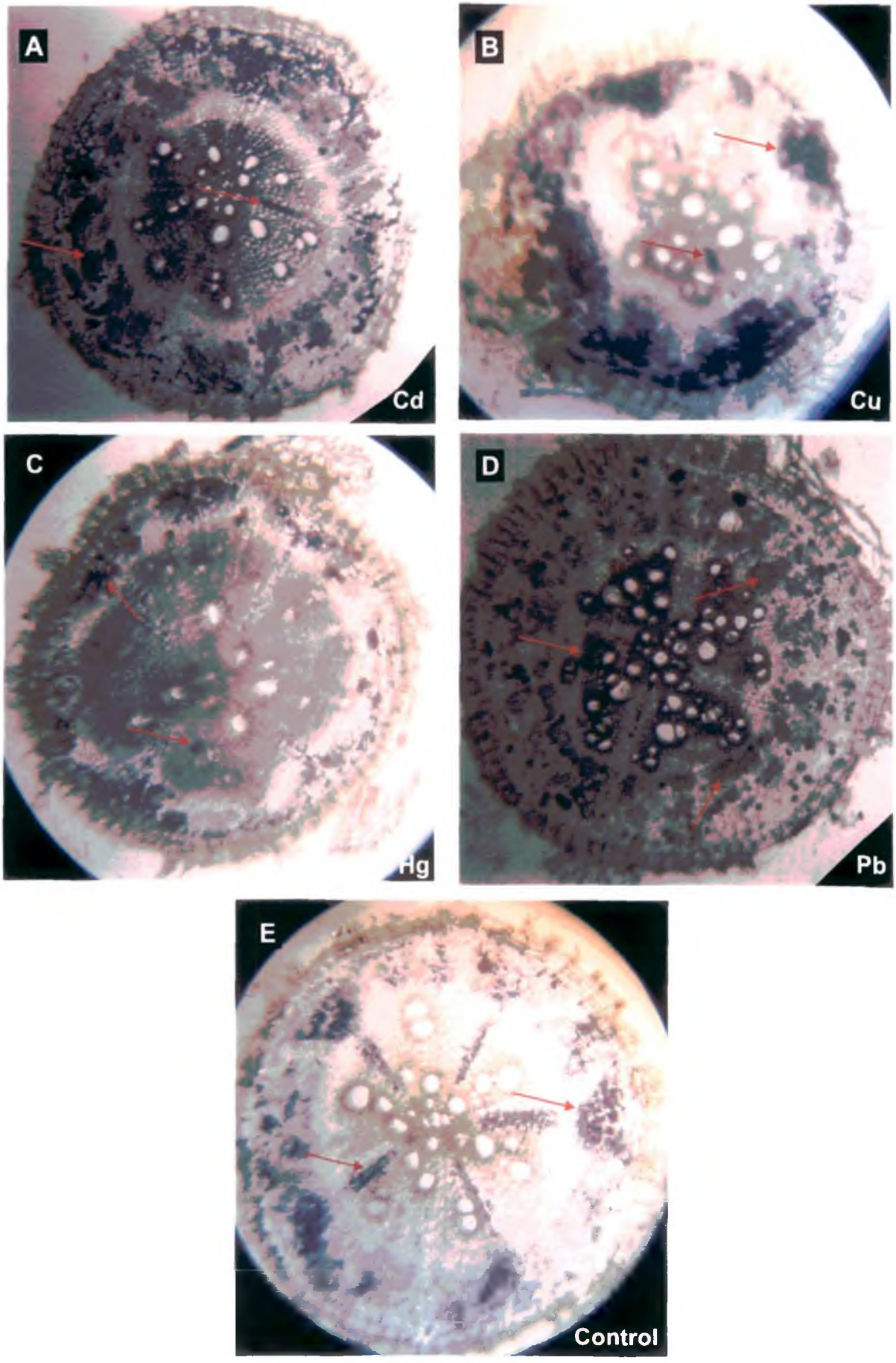


Plate XVI (A-E): Portion of transverse section (x10) of roots of heavy metal treated okra plants. Sections were stained with Wagners reagent.

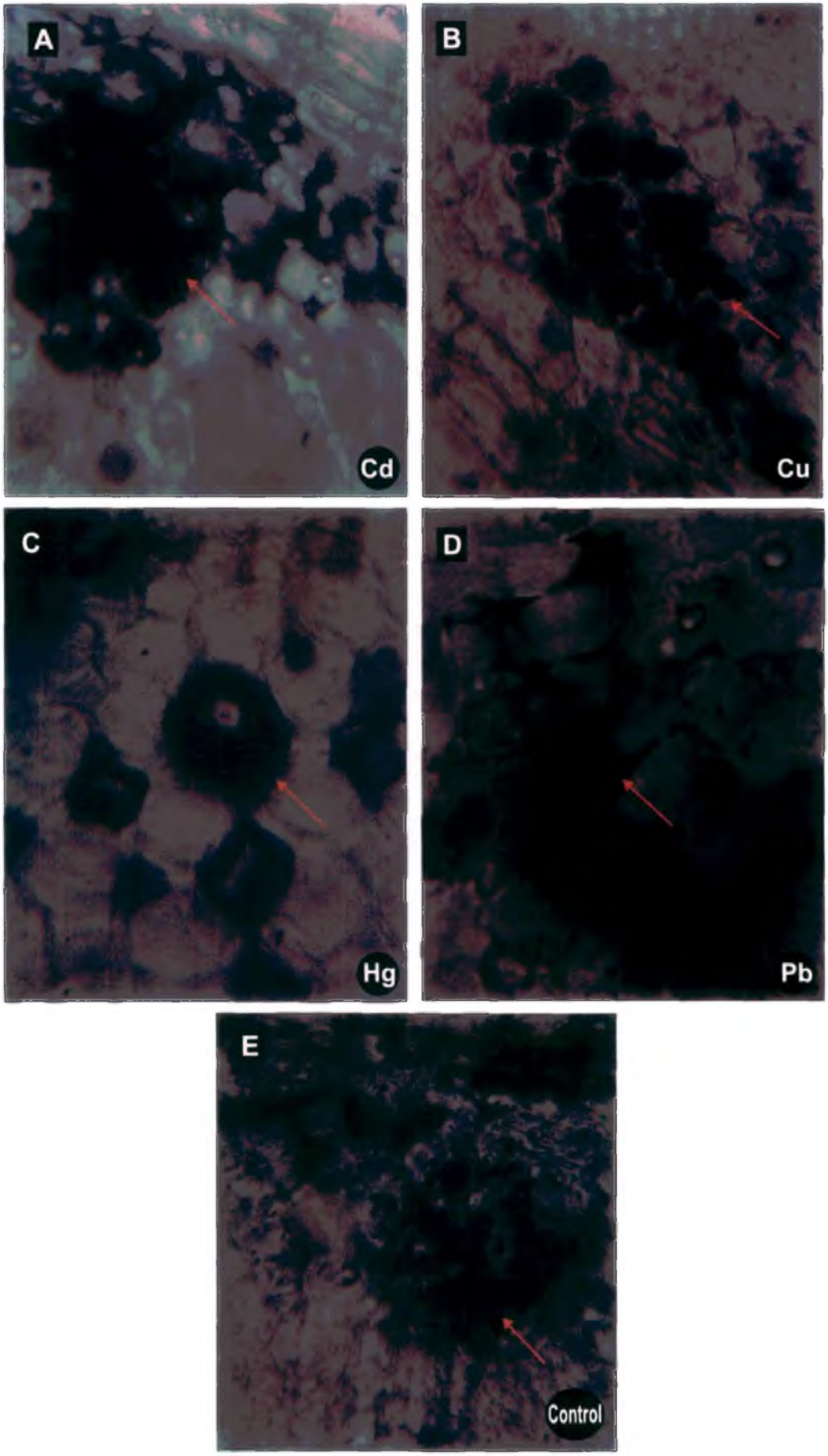


Plate XVII (A-E): Portion of transverse section (x45) of roots of heavy metal treated okra plants. Sections were stained with Wagners reagent.

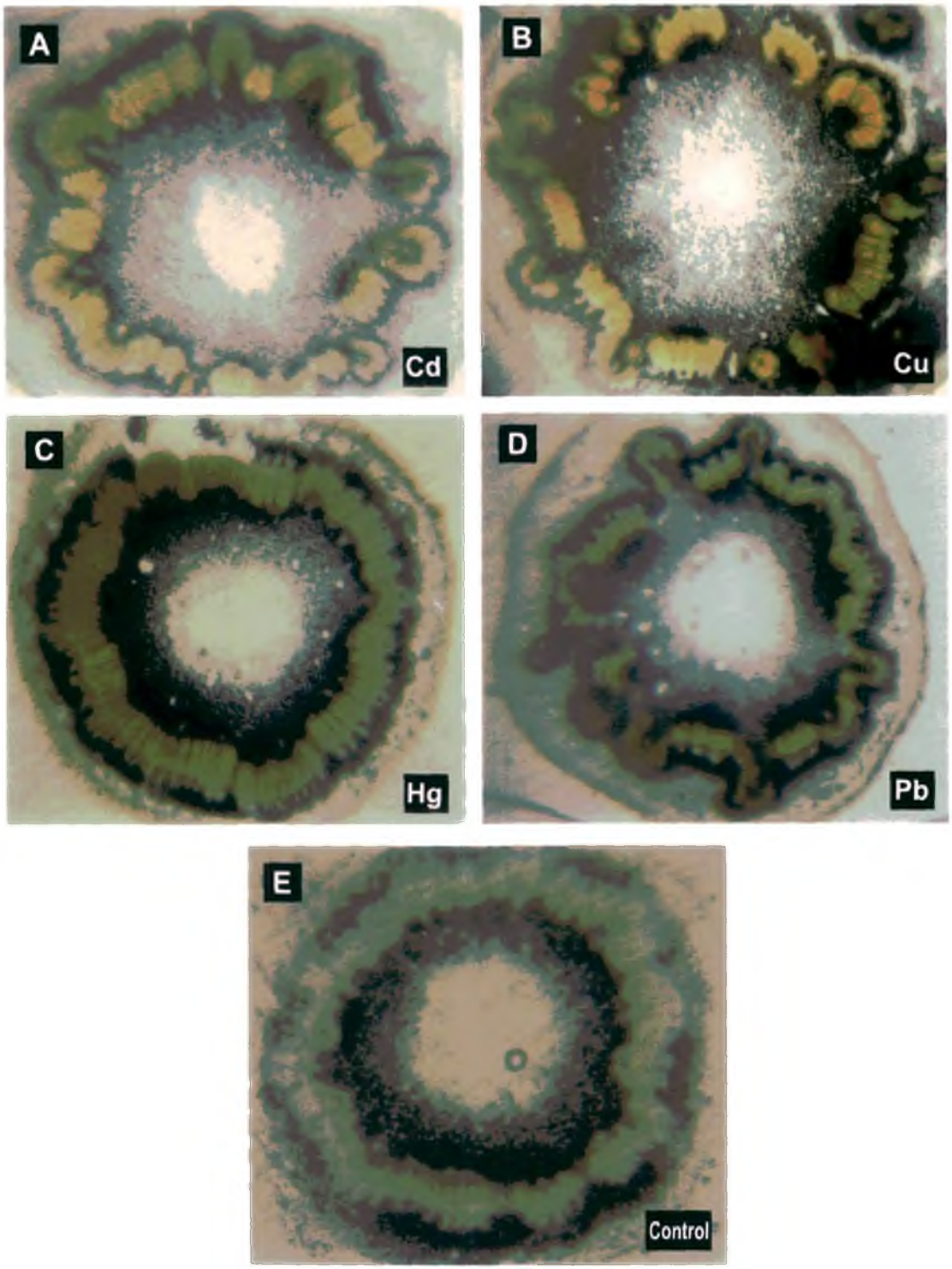


Plate XVIII (A-E): Cross sections of basal part of fruits of okra (cv. Arka Anamika) plants following heavy metal treatments, as observed under dissecting microscope (x5).

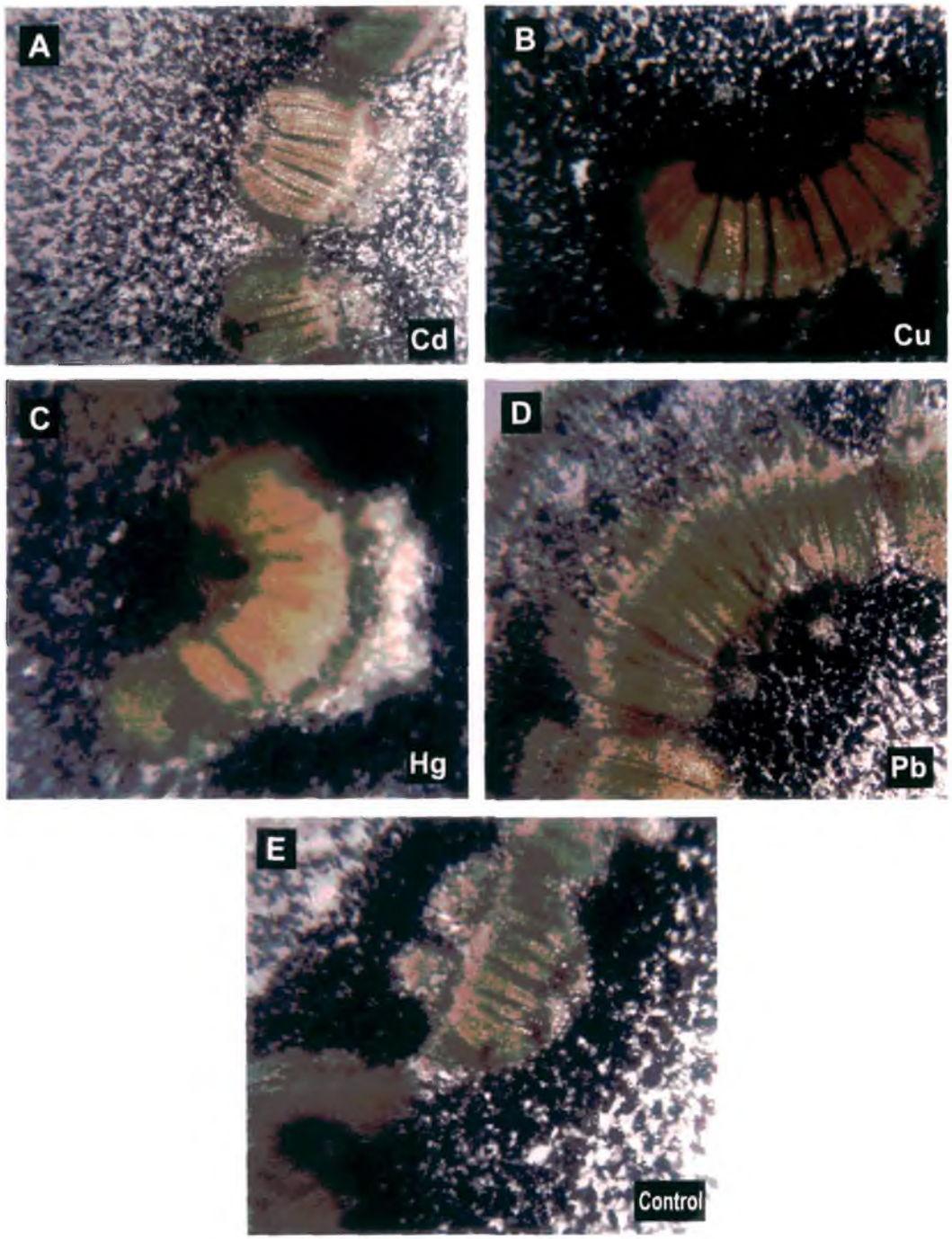


Plate XIX (A-E): Cross sections of basal part of fruits of okra (cv. Arka Anamika) plants following heavy metal treatments, as observed under light microscope (x10).

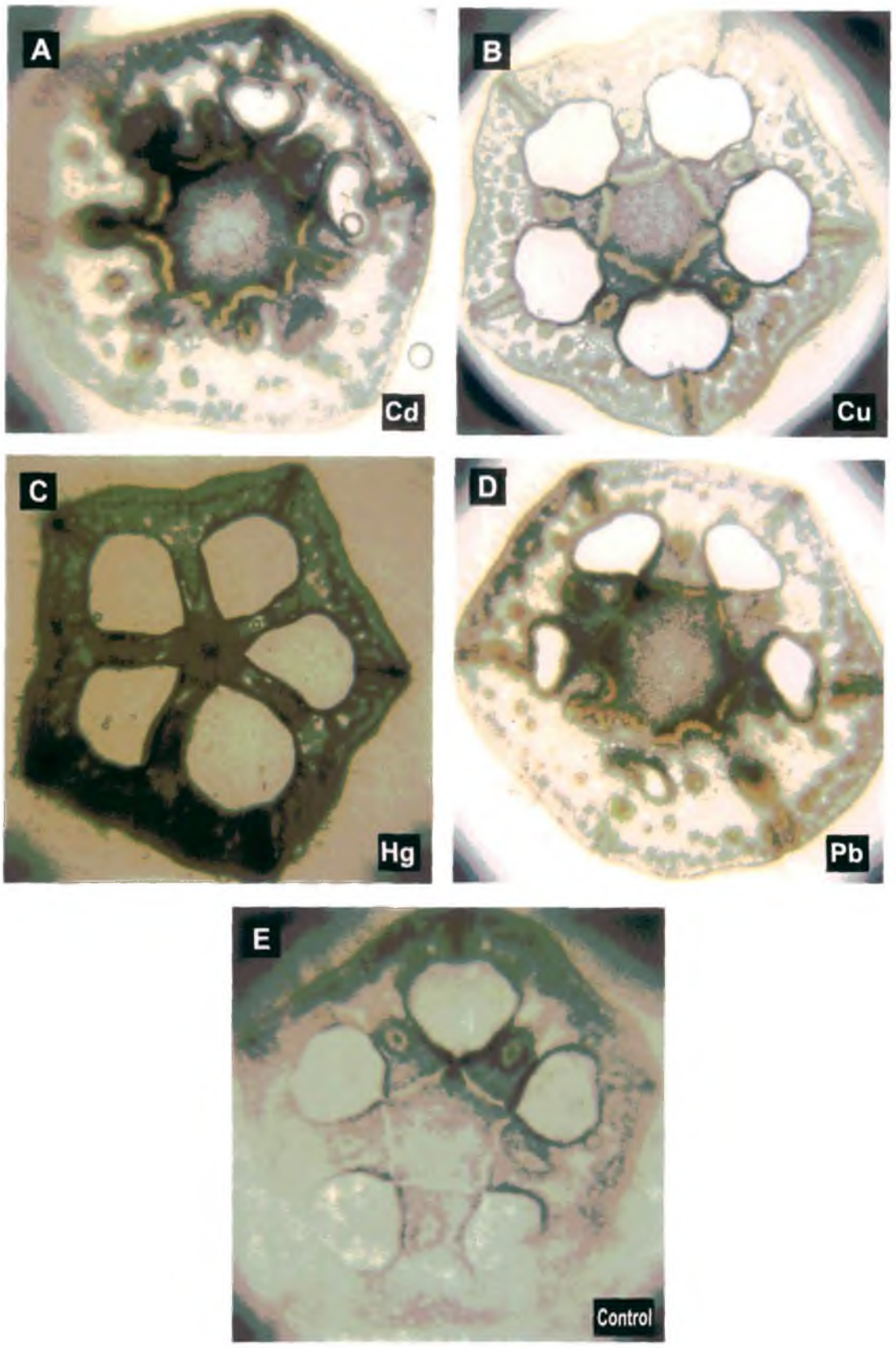


Plate XX (A-E): Cross sections of middle portion of fruits of okra (cv. Arka Anamika) plants following heavy metal treatments, as observed under dissecting microscope (x5).

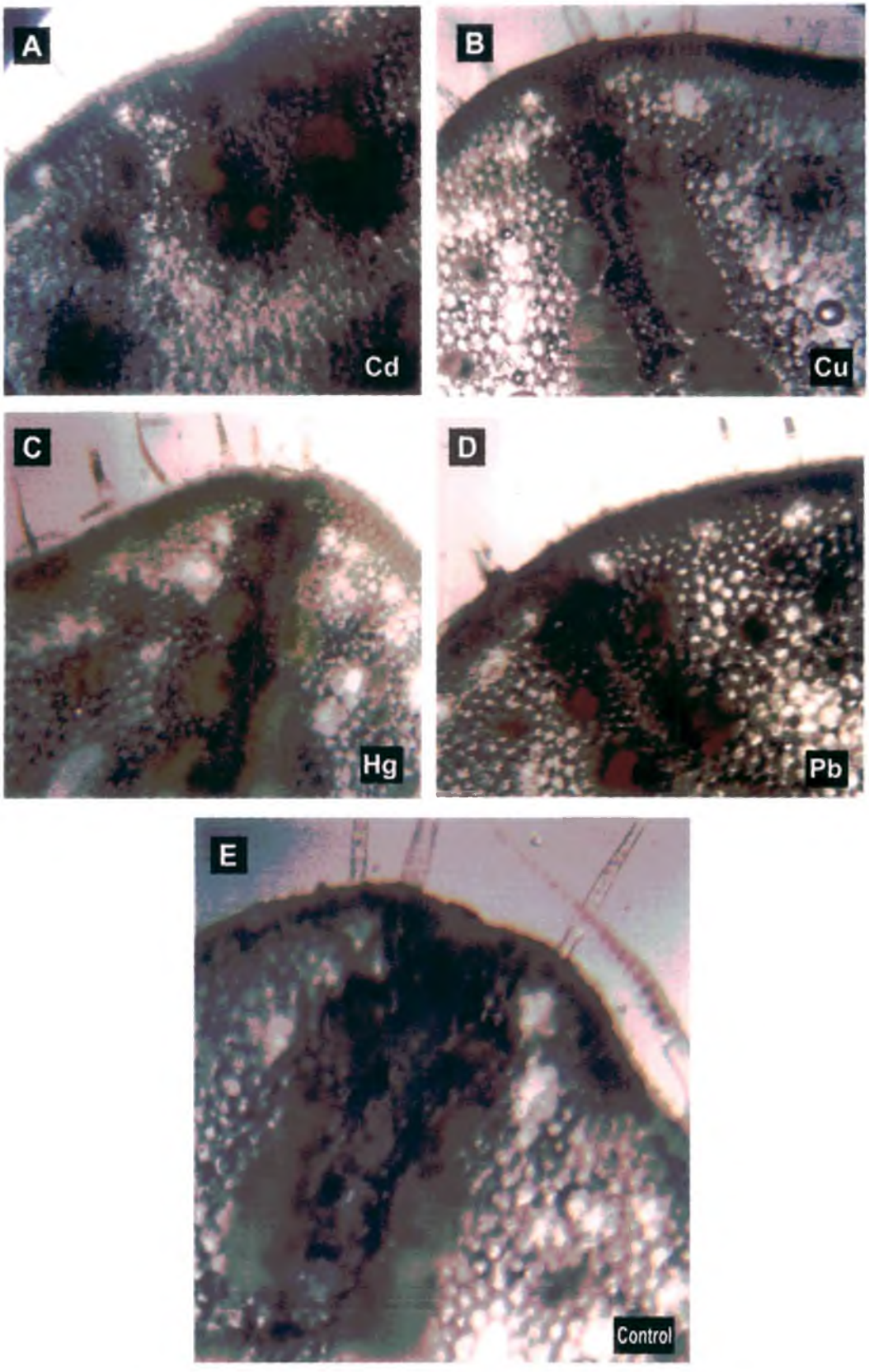


Plate XXI (A-E): Cross sections of middle portion of fruits of okra (cv. Arka Anamika) plants following heavy metal treatments, as observed under light microscope (x10).

than in the control. All the 3 cultivars responded in a more or less similar way (Table 47 and Plate XXII).

Table 47: Influence of treatment with chemicals on germination of okra seeds subjected to heavy metal treatments

		Germination per cent			
Amendments	Treatments	Arka Anamika	Deepti	Najuka-F1	PB-57
	Control	86.67 ± 2.72	93.33 ± 2.72	86.67 ± 2.72	83.33 ± 2.72
Control	$CaCl_2$	90.00 ± 4.72	93.33 ± 2.72	90.00 ± 0.00	93.33 ± 2.72
	KNO_3	96.67 ± 2.72	93.33 ± 2.72	90.00 ± 0.00	93.33 ± 2.72
	Control	60.00 ± 4.72	36.67 ± 2.72	50.00 ± 4.72	60.00 ± 4.72
Cadmium	$CaCl_2$	63.33 ± 2.72	86.67 ± 2.72	60.00 ± 4.72	63.33 ± 2.72
	KNO_3	76.67 ± 2.72	73.33 ± 2.72	63.33 ± 2.72	66.67 ± 2.72
	Control	63.33 ± 2.72	53.33 ± 2.72	53.33 ± 2.72	63.33 ± 7.21
Copper	$CaCl_2$	76.67 ± 2.72	73.33 ± 2.72	66.67 ± 2.72	70.00 ± 4.72
	KNO_3	86.67 ± 2.72	76.67 ± 2.72	63.33 ± 2.72	73.33 ± 2.72
	Control	36.67 ± 2.72	40.00 ± 0.00	33.33 ± 2.72	33.33 ± 2.72
Mercury	$CaCl_2$	50.00 ± 4.72	60.00 ± 4.72	46.67 ± 2.72	53.33 ± 2.72
	KNO_3	56.67 ± 2.72	50.00 ± 4.72	43.33 ± 2.72	53.33 ± 2.72
	Control	40.00 ± 4.72	53.33 ± 2.72	40.00 ± 4.72	36.67 ± 2.72
Lead	$CaCl_2$	53.33 ± 2.72	66.67 ± 2.72	53.33 ± 2.72	56.67 ± 2.72
	KNO_3	63.33 ± 2.72	66.67 ± 2.72	56.67 ± 2.72	60.00 ± 4.72

Treatments were applied at 1000 μg ml^{-1} concentration of the salts

Results obtained after 72 h ; Values are mean of 3 replicates; ± = SEm

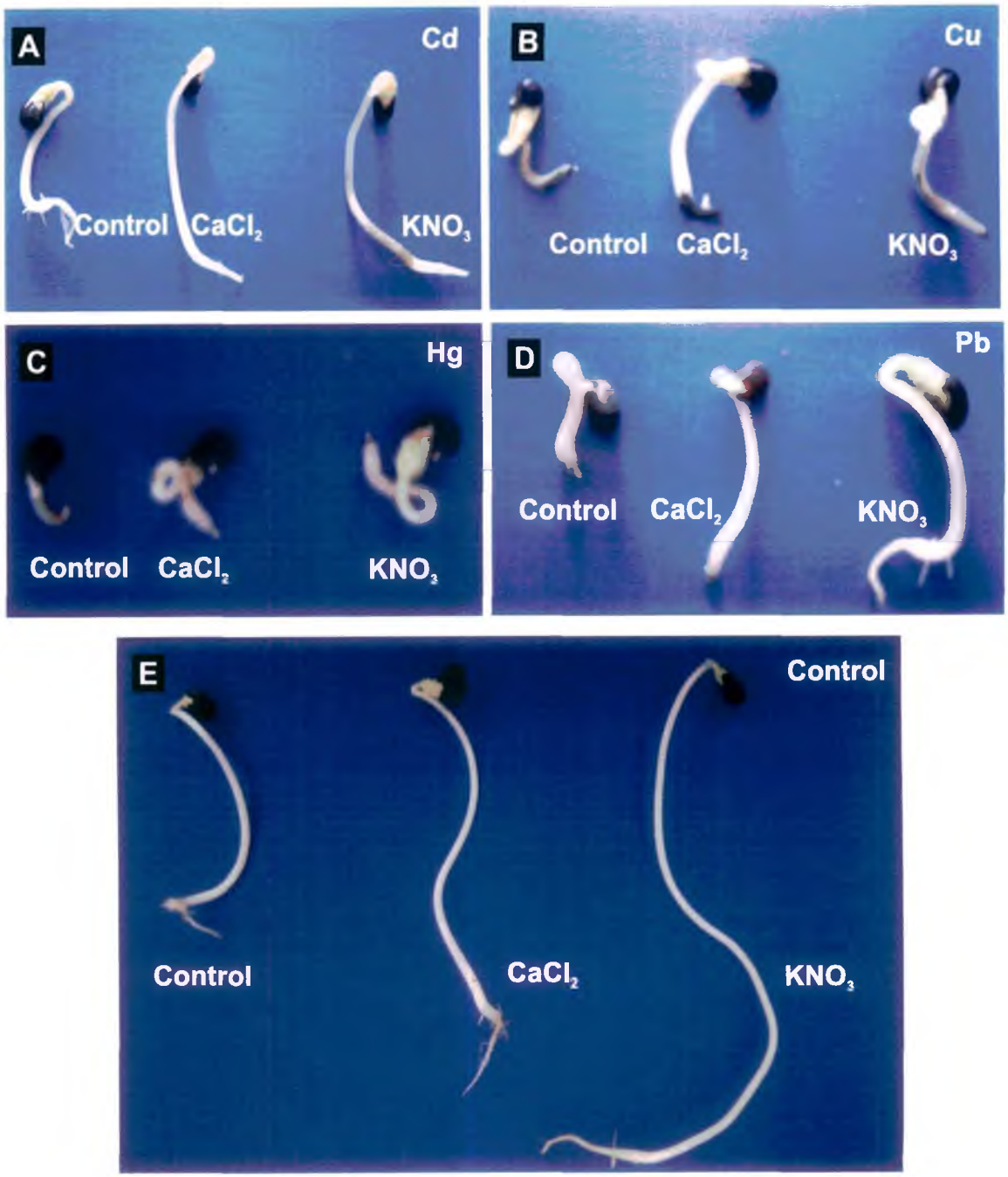


Plate XXII (A-E): Germination studies of okra after amelioration with $CaCl_2$ and KNO_3.

4.12.2. Protein content after amendment

Protein contents of seedlings from the various treatments were determined and results have been presented in Fig. 13. It was observed that in seedling protein content increased to some extent following heavy metal stress. Treatment with $CaCl_2$ and KNO_3 in combination with heavy metals also increased the protein content. However, interestingly it was observed that a combination of $CaCl_2$ and Pb treatment lead to a decrease in protein content in all the 3 tested cultivars. The increases however were not very significant in most of the cases.

4.12.3. Changes in enzyme activities after amendments

Activities of the various antioxidative enzymes tested previously i.e. CAT, POX, APOX, GR and SOD was also determined in the seedlings after combined treatment of heavy metals with $CaCl_2$ and KNO_3 as described earlier in materials and methods. CAT activity was found to decrease in all the cases in relation to untreated control. When compared to heavy metal treatments without ameliorating compounds it was observed that the percentage decrease in activity was greater in the seedlings which were exposed to the ameliorating compounds. However, in case of $CaCl_2$ treatment followed by Hg or Pb the decrease was lesser than the control (Fig. 14). In case of POX, GR and SOD the activities increased in most of the cases. It was observed that combined treatment with $CaCl_2$ and KNO_3 led to further increase in activity as compared to the seedlings subjected to only heavy metals (Fig. 15-17). In case of APOX decline was observed in all cases following heavy metal stress either with or without $CaCl_2$ and KNO_3 (Fig. 18). The above results have shown that $CaCl_2$ and KNO_3 have increased the antioxidant responses elicited by the heavy metal treatments.

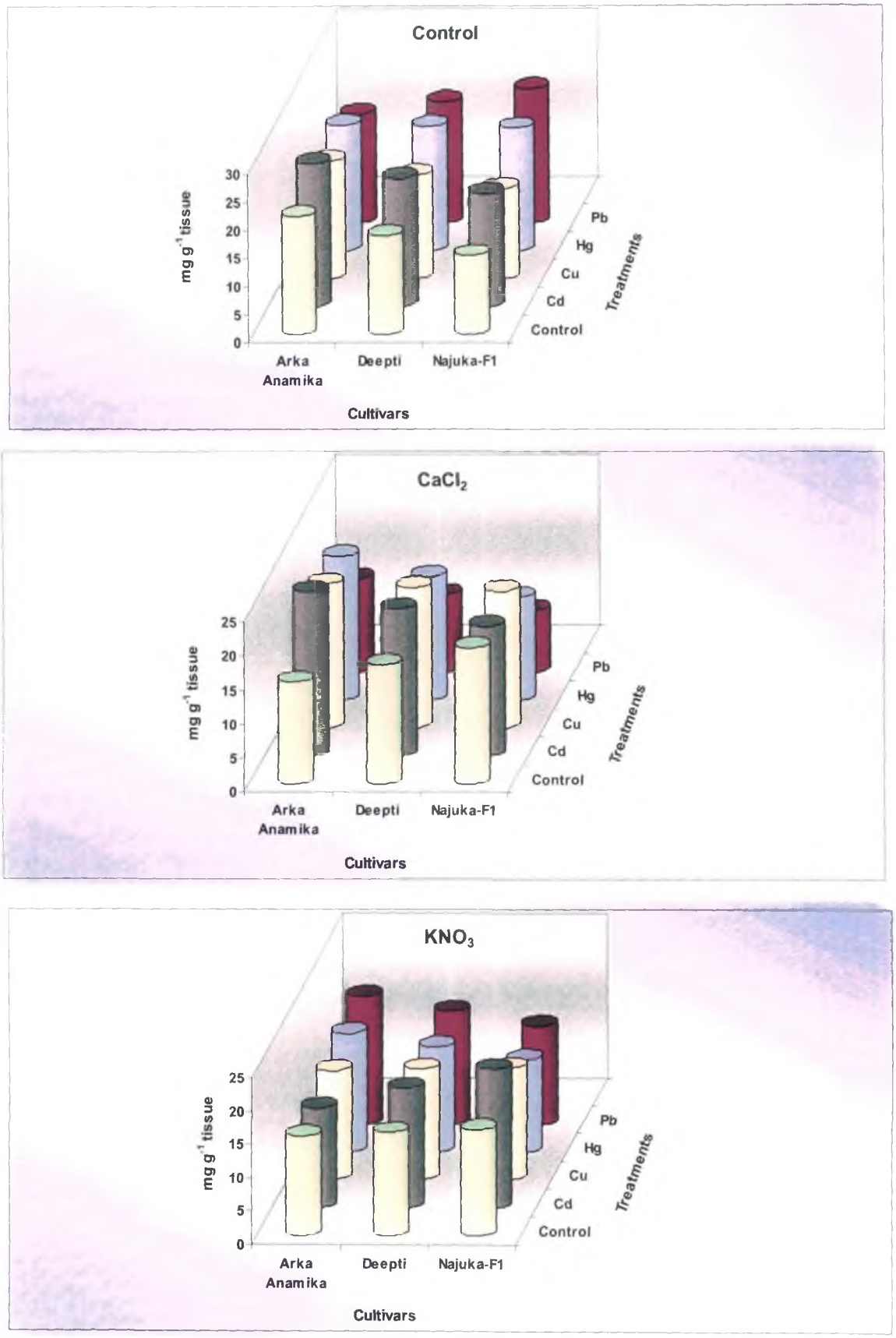


Fig.13. Effect of $CaCl_2$ and KNO_3 on protein contents of okra seedlings subjected to heavy metal stress.

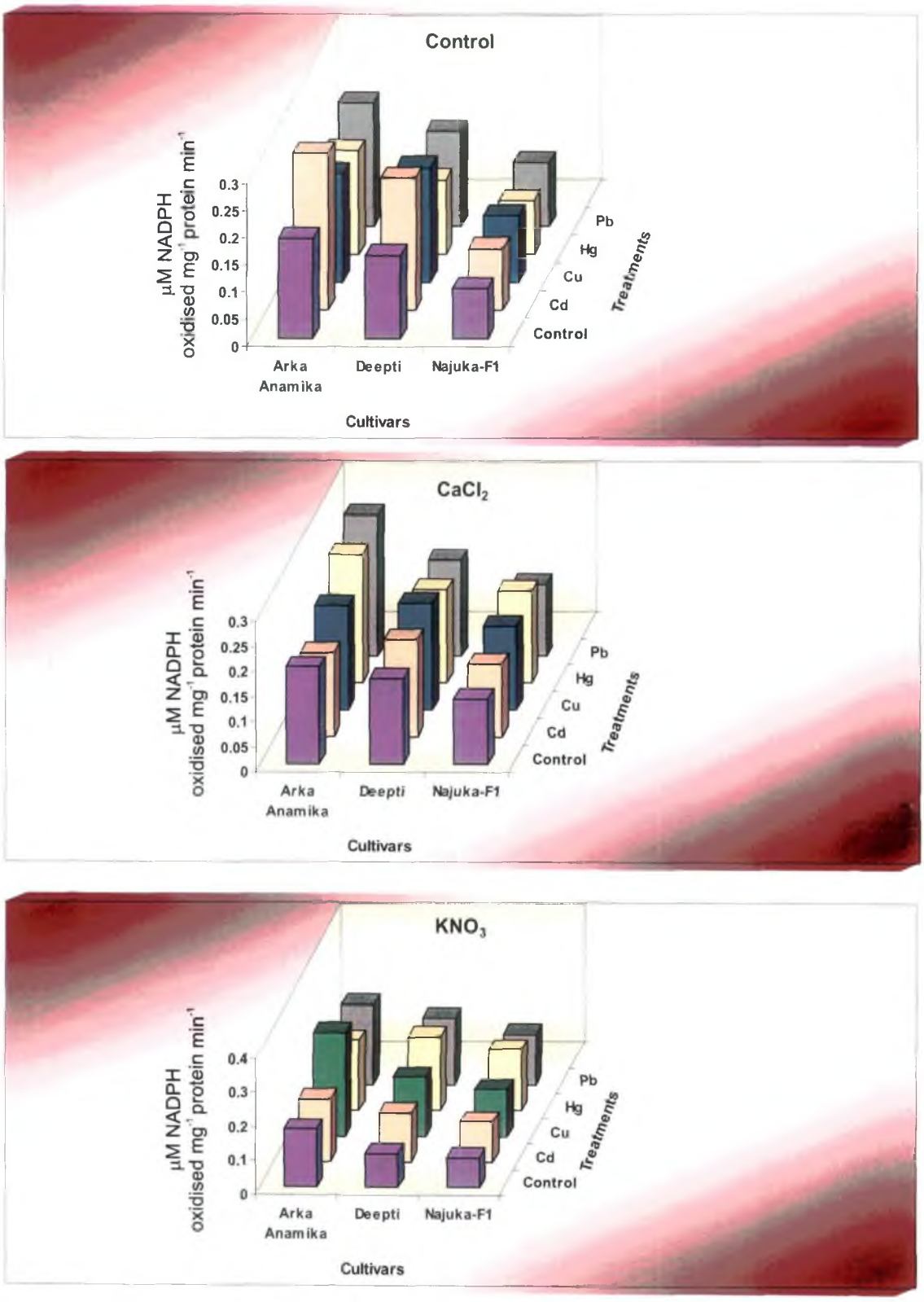


Fig.14. Catalase activities in okra seedlings following treatments with $CaCl_2$ or KNO_3 and heavy metals.

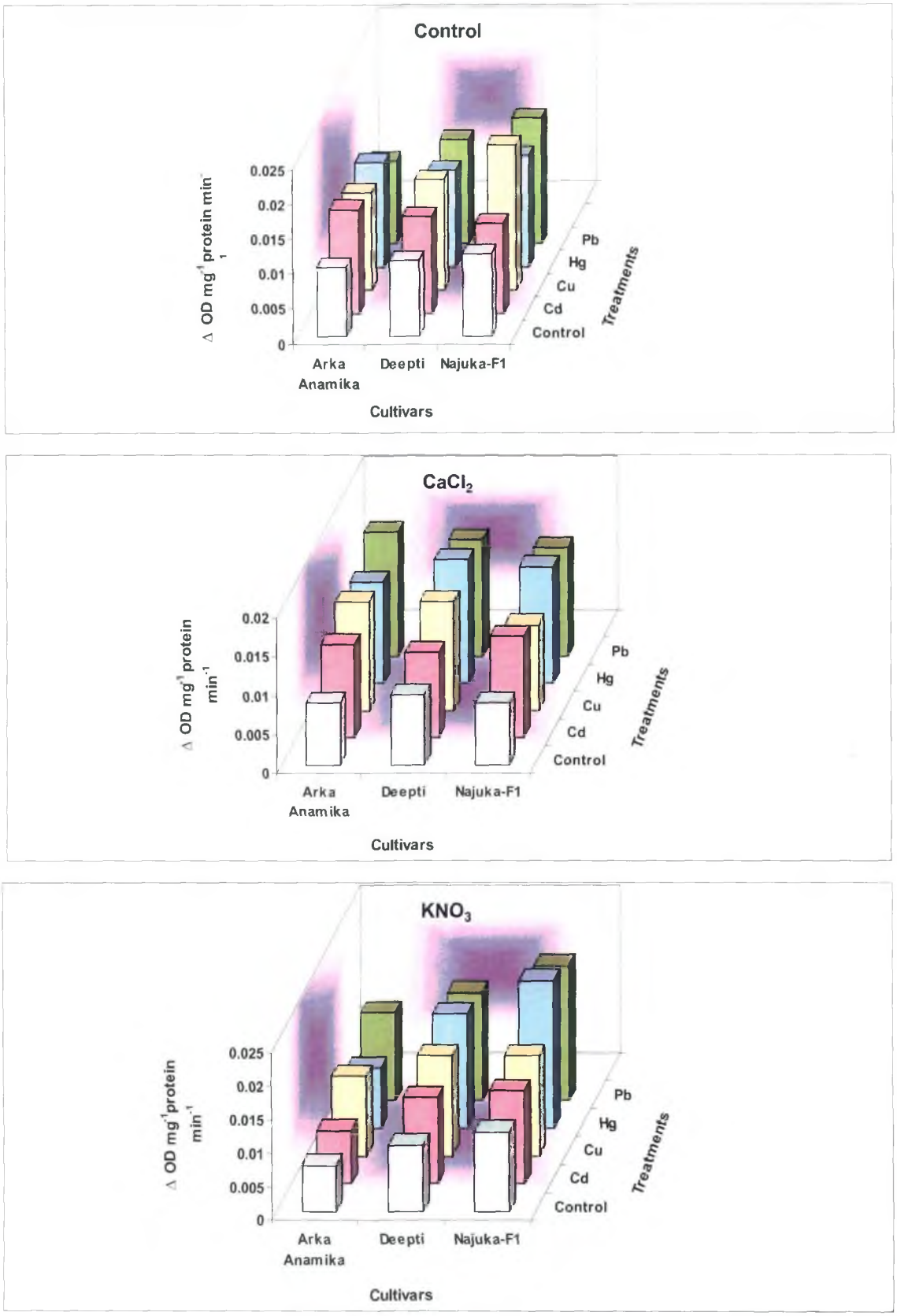


Fig.15. Peroxidase activities in okra seedlings following treatments with $CaCl_2$ or KNO_3 and heavy metals.

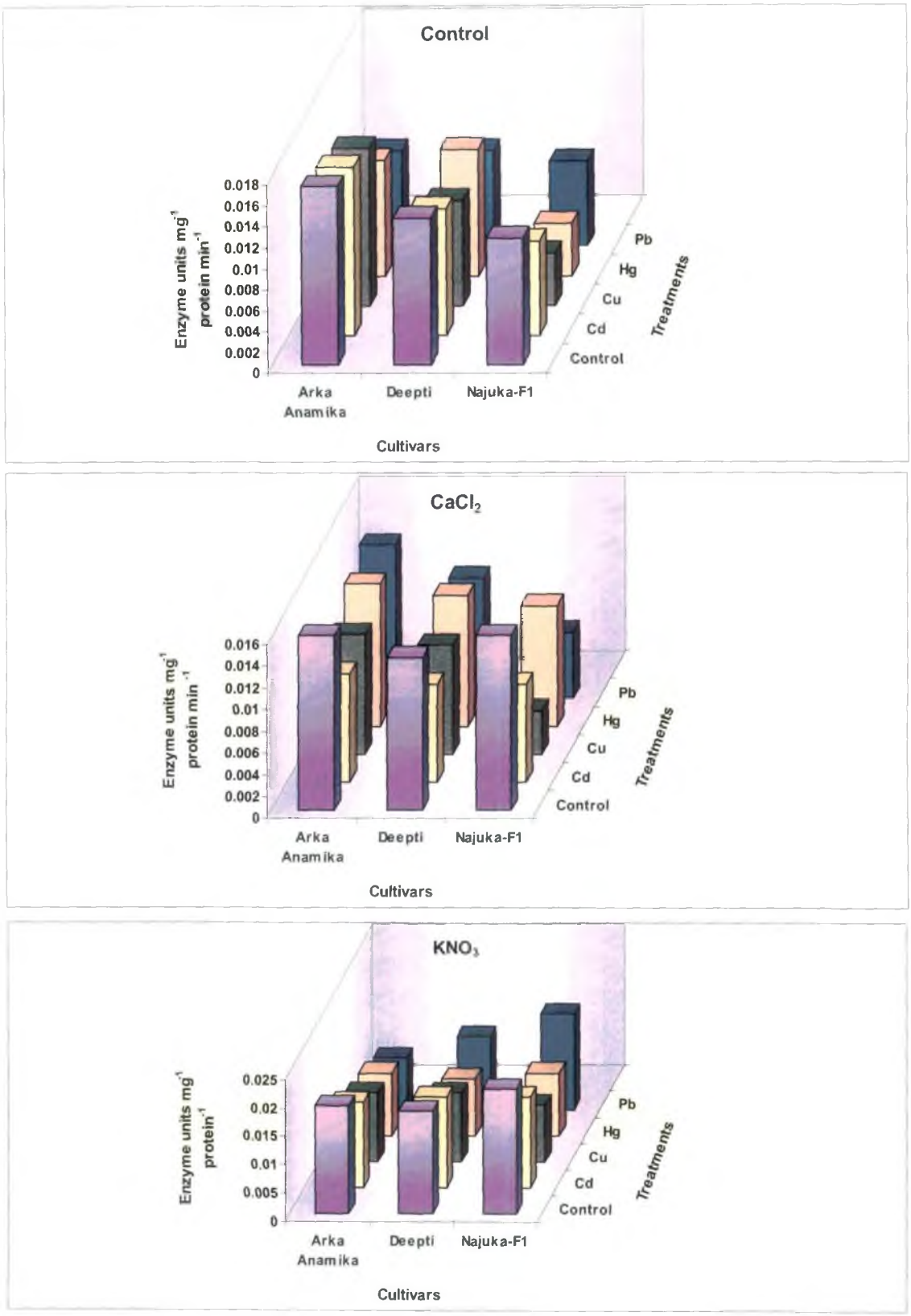


Fig.16. Glutathione reductase activities in okra seedlings following treatments with $CaCl_2$ or KNO_3 and heavy metals.

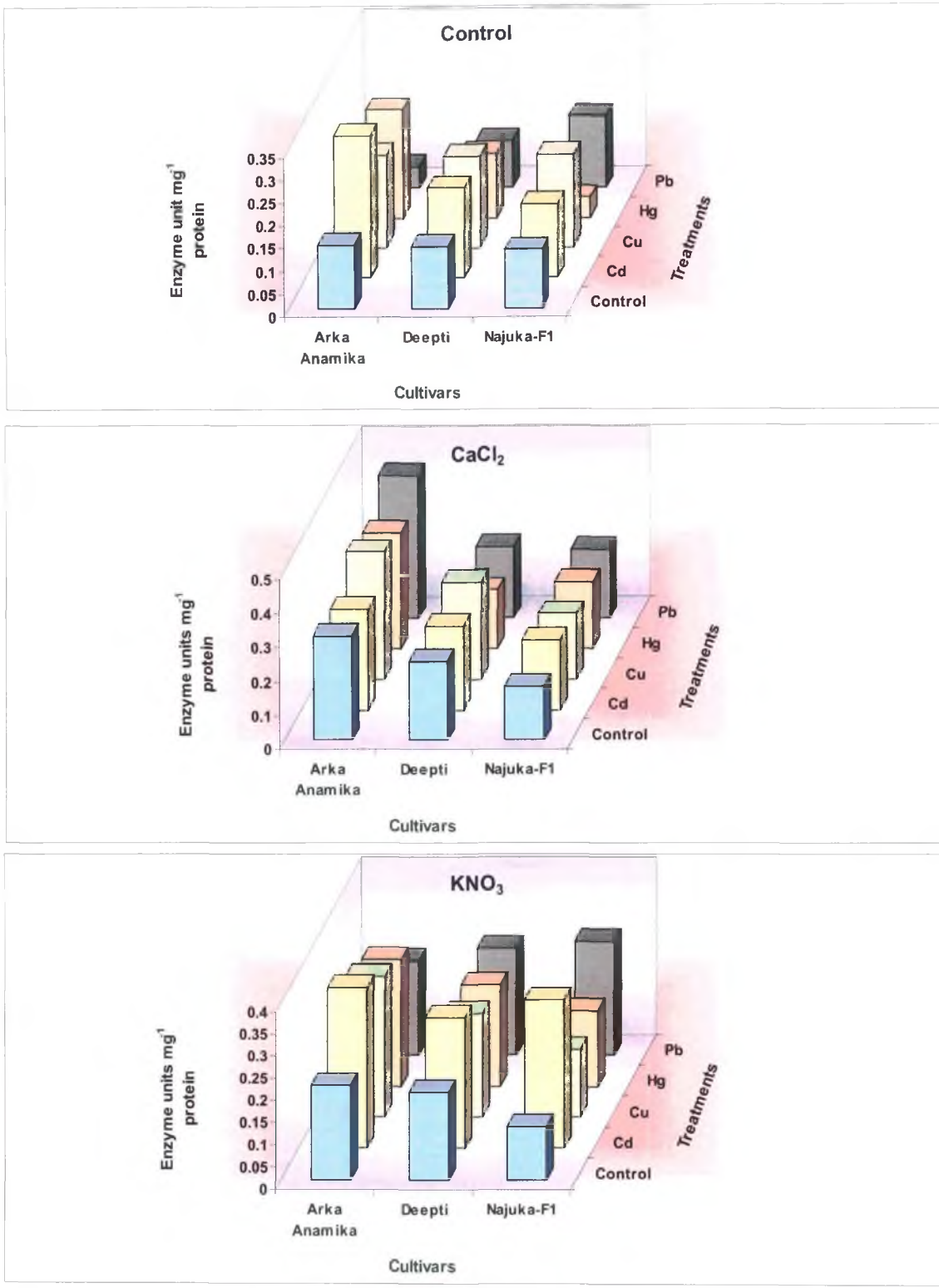


Fig. 17. Superoxide dismutase activities in okra seedlings following treatments with $CaCl_2$ or KNO_3 and heavy metals.

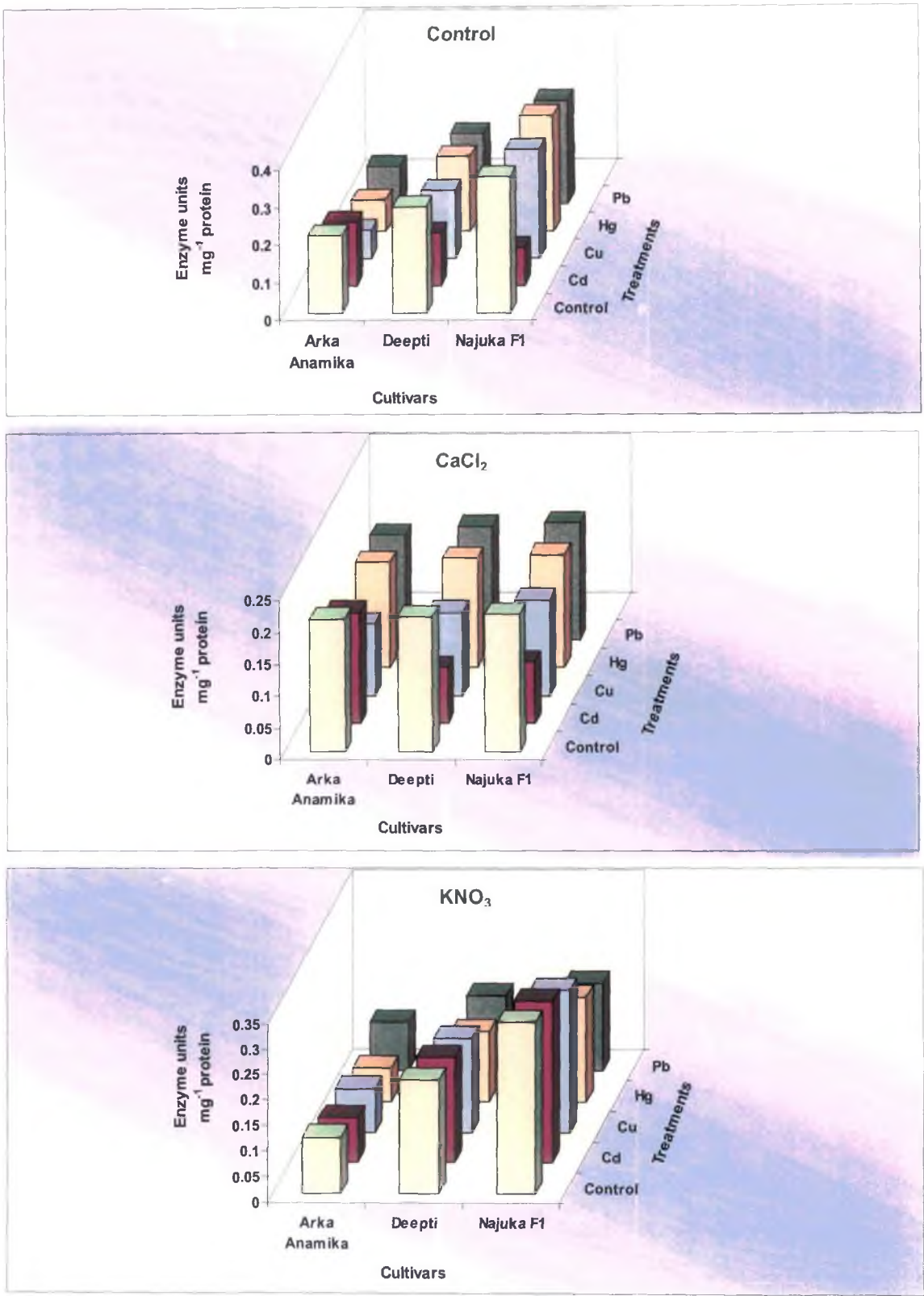


Fig.18. Ascorbate peroxidase activities in okra seedlings following treatments with $CaCl_2$ or KNO_3 and heavy metals.

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.

SUMMARY

1. A review of literature pertaining to heavy metal stress in plants has been presented.

2. The materials used and procedures followed to carry out the experiments are discussed in details in materials and methods.

3. Okra [*Abelmoschus esculentus* (L.) Moench] seedlings of different cultivars (Arka Anamika, Deepti, Najuka-F1, Paras Soumya, PB-57 and Parbhani Kranti) were selected for the present study.

4. The four heavy metals cadmium nitrate 4-hydrate [$Cd(NO_3)_2$ $4H_20$], copper(II) sulphate-5-hydrate [$CuSO_45H_2O$], mercury(II) chloride [$HgCl_2$] and lead(II) nitrate [$Pb(NO_3)_2$] used in the present study were applied in the form of their respective salts at 100 and 1000 $\mu g\ ml^{-1}$ concentration.

5. In most of the cultivars 1000 $\mu g\ ml^{-1}$ treatments with the heavy metal salts inhibited germination significantly. After 72 h different cultivars exhibited germination between 77% (Parbhani Kranti) and 100% (Arka Anamika) in control.

6. The tolerance index of the different heavy metal treated okra plants showed a decreasing trend. Hg had the most deleterious effect in all the cultivars.

7. There was a marked decrease in the leaf area of the heavy metal treated plants. There was a general decline in most of the growth parameters after heavy metal treatment.

8. Relative growth rate decreased with heavy metal application in most of the cases.

9. The heavy metal treatments showed an inhibitory effect on the specific leaf area. The inhibition was maximum with Hg treatment in Deepti, Paras Soumya and Parbhani Kranti.

10. Highly significant decrease in yield was observed in all the treatments and cultivars after the heavy metal treatments.

11. The results of quantification of reducing sugar at different stages following heavy metal treatments revealed that in all stages there was an increase of reducing sugar, although there was a decrease in the total soluble sugar content. Starch contents in roots, stems and leaves of okra plants following heavy metal treatments also showed a decreasing trend.

12. Proline content of leaves of all six cultivars was determined after heavy metal treatments at seedling, vegetative and reproductive stages. In all treatments heavy metal induced accumulation of proline. However, 100 $\mu g\ ml^{-1}$ treatments induced comparatively higher accumulation than 1000 $\mu g\ ml^{-1}$.

13. Total chlorophyll content of the leaves showed a general decline following heavy metal treatments. Among all the heavy metals Hg reduced total chlorophyll content to the greatest degree in all the six cultivars. Decreases in almost all the treatments were statistically significant.

14. Heavy metal treatments significantly reduced the carotenoid contents in all the cultivars.

15. Protein contents of the leaves and roots of okra cultivars at seedling, vegetative and reproductive stages were determined for all treatments. Leaves of both seedling and vegetative stage had slightly higher protein content than reproductive stage. In the leaves heavy metal treatments mostly increased the protein content though not very significantly.

16. SDS-PAGE analysis of the heavy metal treated seed proteins revealed accumulation of few new proteins which again varied with the cultivar. Similarly, no significant changes were observed in leaves.

17. Activities of antioxidative enzymes in leaves of different cultivars of okra subjected to heavy metal treatments at 1000 $\mu g\ ml^{-1}$ of the salts were assayed. Catalase activities in all the cultivars subjected to heavy metal stresses

showed a decline.POX activity in all the cultivars was enhanced by the heavy metal treatments, though the degree of increase varied.All the heavy metal stresses enhanced activities of APOX and SOD in the different cultivars.

18. A significant increase in lipid peroxidation of leaves from treated plants in relation to control was observed. Cd induced maximum lipid peroxidation in three cultivars (Arka Anamika, Parbhani Kranti and PB-57) and Pb in two (Deepti and Najuka F1).

19. Influence of heavy metals on flavonoid accumulation in three cultivars (Arka Anamika, Deepti and Najuka-F1) of okra were analysed by HPLC. In case of Arka Anamika all treatments had 3 peaks whereas Pb had only 2 peaks. The second peak in control, Cd and Cu treatments were more or less similar but there was a decrease in the peak height in Hg and Pb treatments. The peak height decreased in relation to control in all the treatments except Hg. Peak height in Hg treatment was greater than that of control. Peaks obtained following Cd treatment were almost similar to that of control. Control had 4 peaks, Cd, Cu and Hg showed 3 peaks and only a single peak was observed in Pb treatment.

20. Accumulation of heavy metals in roots and fruits was determined. Traces of heavy metals were found in untreated control in all cases. Maximum deposition was observed in Cu and minimum in Cd treatments.

21. The microscopic studies of cross section of radicles of seedlings revealed some deposition in the intercellular spaces of cortical tissue. Changes in the shape of the cortical cells were also noticed.

22. In case of fruits, sections from both basal and middle portions revealed changes in accumulation as well as in the cellular structure.

23. Treatment with $CaCl_2$ and KNO_3 along with the heavy metals increased the germination to some extent which was however still lower than control.

24. It was observed that in seedling protein content increased to some extent following heavy metal stress. Treatment with $CaCl_2$ and KNO_3 in combination with heavy metals also increased the protein content.

25. Treatment with $CaCl_2$ and KNO_3 increased the antioxidant responses elicited by the heavy metal treatments.

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