Studies on Anthropogenic Stresses in Tea (*Camellia sinensis* (L.) O. Kuntze)

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TO WHOM IT MAY CONCERN

This is to certify that Ms Gargi Saha is submitting her thesis entitled *"Studies on Anthropogenic Stresses in Tea (Camellia sinensis (L.)O.Kuntze "* on the basis of her work for the award of Ph.D degree from the University of North Bengal carried out under my supervision at the Department of Botany, University of North Bengal.

She bears a good moral character and I wish her all the best in life.

Usha Chakraborty

(Usha Chakraborty)

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CONTENT

Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze) is one of the most important evergreen perennial plantation crops of India and most popular hot drinks in the world over. Since very ancient time habitual tea drinking has been considered to be beneficial to health. Tea is known to have antimutagenic, anticarcinogenic, antibacterial, anticoagulant, and having potent antioxidative properties. Its cultivation, spread over more than 3,96,000 hectors of land divided into two distinct regions –the North Indian tea belt region located between 22°- 27° N and South Indian tea belt at 7° N. North- East India produces 75 % of the total Indian tea in three different land scapes –the hilly terrain of Darjeeling upto an elevation of 2000 m, yielding the world's finest quality of tea,the extensive riverine flat plains at the base of Himalayan range i.e. Terai Dooars, (Plate I) and the Brahmaputra valley of Assam located at 100m. above sea level, which is the largest flat plains of the world and which accounts for more than half of the Indian tea production (Jain, 1991).Since the plant grown in the tropical agroclimatic zone, pest, weed and disease causing organisms cause serious damage to the crops, for which excessive use of chemical has been continuing since long past. Besides, as most of the nutrients in the soil remain in unavailable form so use of chemical fertilizer has been necessary for the optimum productivity of the crop.But the use of chemical fertilizers and pesticides have caused a serious problem of pollution and the loss of land fertility (Bezbaruah *et al.* 1996). Abiotic stresses are known to cause oxidative stress in plants by producing reactive oxygen species (ROS) like superoxide radical , hydroxyl radical, hydroperoxyl radical, hydrogen peroxide, which are inevitable products of natural redox reactions in various cellular compartments (Zhang and Kirkham 1994; Alscher *et al.* 1997).

Soil contamination with heavy metals released from anthropogenic activities has become a world wide problem, leading to the loss of crop yield and health hazards as they enter into the food chain (Salt *et al.* 1995; Schlickler and Caspi, 1999). Indiscriminate use of chemicals and the presence of residue in the tea leaves are a major concern and more sensitive issue than other crops to avoid toxic hazards for the consumers as they are harvested at short intervals. So, it is necessary to keep the

Plate I: Tea Garden in plains of Dooars

residues much below the Maximum Residue Limit (MRL) stipulated by different international agencies. Heavy metals are defined as the metals with a density higher than 5 gm cm^{-3}. Fifty-three of the ninety naturally occurring elements are heavy metals (Weast, 1984).Heavy metals are dangerous because they tend to bioaccumulate. The main sources of contamination in agricultural soil are fertiliser impurity (Cd^{2+}) , use of refuge derived compost and sewage sludge (Cd^{2+},Ni^{2+},Pb^{2+},etc.) and to a lesser extent , mineral weathering (Hildebrand, 1989; Alloway , 1995). Cadmium can be found in soil because of insecticide, fungicide, sludge, and commercial fertilizers that are used in the agriculture field.Cadmium, the most toxic heavy metal pollutants for human beings, animals and plants as it enters in the environment mainly from industrial processes and phosphorous fertilizers and then get transferred to the food chain (Wagner, 1993). Agricultural plants represent an important pathway for the movement of potentially toxic trace elements from soil to human being. WHO (1984) has recognized health hazards of metal in food chain even at low concentrations (Nigam *et al.* 2002). When accumulated in the plant tissues, it causes alterations in catalytic efficacy of enzymes (Van Asseche and Clijsters, 1988; Somashekaraiah *et al.* 1992; Romero- Puertas *et al.* 1999.Piqueras *et al.* 1999), damage to the cellular membranes (Fu and Brouillette, 1997) and inhibits the root growth (Wilkins, 1978). These changes result in inhibition of chlorophyll biosynthesis and photosynthesis (Singh and Singh, 1997) and mineral nutrient uptake (Greger and Lindberg, 1987). Among various toxic metals, cadmium is recognized as the most hazardous element that is not essential for plant growth but easily taken up by plants (Nigam *et al.*2002).

Some of the heavy metals are essential for the life processes as trace elements, (copper, selenium zinc) to maintain the metabolism of the organism. Copper is a plant micronutrient that is an essential component of several enzymes and coenzymes involved in metabolic pathways of plants (Mourato *et al.* 2009). However, at high concentrations Cu can become phytotoxic affecting plant development due to direct or indirect interference with numerous physiological processes (Maksymiec 1997, Vangronsveld and Clijsters, 1994). At higher concentrations they interfere in plant growth and metabolism, induce perturbations in the root cell plasma membranes producing altered membrane structure and function, which result in nutrient deficiencies

in the leaf tissues (Palma *et al.* 1987, Demidchik *et al.*1997). Sources of copper toxicity in agricultural field are the use of copper containing fertilizers, fungicides and insecticides.

Stress is the physiological state of a plant when they are exposed to the extremely unfavourable conditions. The situation is counteracted by the alteration of the biochemical parameters. The extremely unfavourable conditions 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; Maclecka *et al.*2001; Shaw *et al.* 2004). The ROS is scavenged in plants by antioxidant enzymes like super oxide dismuttase, peroxidase, catalase, ascorbate peroxidase, glutathione reductase and non enzymatic (carotenoid, ascorbate, glutathione) (Scandalios,1993). Measurement of activities of antioxidant enzymes is helpful in indicating the oxidative stress in plants (Geebelen *et al.* 2002). Cell death occurs when the oxidative stress induced metabolic change exceed the scavenging capacity.

As the tea plants are grown under monoculture, provide favourable conditions for variety of pests and diseases which accounts for the potential croop loss (Plate II). The use of pesticide in the field to combat pests and diseases has been advocated, which contribute to major anthropogenic addition to the natural communities. Indiscriminate and excessive uses of these chemicals create environmental pollution, bioamplification of the residue in human system and resurgence of pests and destroy the ecological balance by damaging target and non target organisms. The application of pesticide alters the physiological responses in tea by altering biochemical processes which would give them greater adaptability.The chemical fungicide and insecticide application also casuses the great phytotoxic effect to the plant. It also alters the plant metabolism and causes the oxidative damage to the plant. Some fungicides used to mitigate or prevent pathogen attack may be involved in activating certain defensive responses of plants by influencing the key steps of the phenolic and oxidative processes (Gracia *et al.* 2003). The chemical application like endosulfan, methomyl, acphate and surfactant at seedling

Plate II: Insect infested tea bushes.

stage alter the photosynthetic rate (Haile *et al.* 2000). The progressive loss of enzymic and non-enzymic antioxidants in plants subjected to pesticide treatment indicated a loss of cellular protective machinery with an increase in oxidative damage by increase in lipid peroxidation (Panda and Patra, 2000). The pesticide treatment can impose an osmotic stress causing damage to membrane structure. Membrane proteins interact with pesticides which may affect the production of enzyme protein (Deshpande and Swami, 1990) .Organophosphorous pesticides used in agriculture, by virtue of their anti-acetylcholinesterase (AchE) activity block nerve transmission and thus pose threat to non-target organisms including human populations (Somara *et al.* 2002).

Considering the importance of the above, the present work was undertaken to determine how the different tea varieties respond to the different chemical induced stresses.The objectives of the present work were –

1. To study the biochemical responses of different varieties to the stress in terms of changes in proteins, carbohydrates, proline, phenols, chlorophyll carotenoids.
2. To determine the effect of stresses on antioxidative enzymatic activities like peroxidase, phenyl ammonia lyase, polyphenol oxidase.
3. To determine the specific expression of new protein(s) following stress.
4. To determine the flavour component changes due to application of insecticide, fungicide or heavy metal.

In order to achieve the above mentioned objectives standard methods for the analysis have been used which have been described in the following pages. A brief literature review in this area has also been provided.

Literature Review

2.1. Heavy metal stress

According to Burzynski (1989), treatment of 4 days old cucumber (*Cucumis sativus*) seedlings with $PbCl_2$ and $CdCl_2$ caused a significant increase in the accumulation of heavy metals by plants, especially in the roots. The accumulated Pb initially enhanced the uptake of phosphorous after the plant had been transferred to a nutrient medium (6,24hrs) but after 48 hrs the uptake was dropped to below control level. The plants treated with Cd exhibited a constant nitrate uptake and nitrate reductase activity. It is suggested by the authors that the reason for the decreased nitrate reductase activity lay rather in the lower nitrate uptake than in a direct effect of the heavy metals on the enzyme.

Experiments were carried out by Yamamoto (1990) to determine if Cd was taken up by and distributed within soyabean plant. Unnodulated 23day old soyabean plants were supplied with solution of varying Cd concentrations (0.00 to 0.66) for 4days under continuous intensity (ca.1200 lux) and constant temperature (25^{o}C). As the concentration of the solution was increased, all plant parts showed increased concentration of Cd. In the stems, the response was essentially linear over the entire range of Cd concentrations employed (r^2 =0.991), while in root a linear relationship was found up to 0.43 ppm (r^2=0.983). At all cadmium concentrations used, the roots contained by far the greatest part of the total plant cadmium content, although the roots predominantly decreased to some extent with increasing doses of cadmium. When expressed as concentration ratio which is the ratio of Cd concentration of each plant part to that of external solution, the ratios of 120-160, 14-26 were obtained for roots and stems respectively, over a concentrations range from 0.15-0.43 ppm of added Cd. Addition of Cd to external solution appeared to cause a release of Ca, Mg, Zn, and possibly Mo into the solution but not of Fe, Cu and B.

The effect of increasing doses of heavy metals (Cd, Cu, Ni, Pb and Zn) applied separately or together on the growth of Italian Ryegrass (*Lolium multiflorum*) and on the content of heavy metals in plants. Among the examined metals only Cu (in a dose of 320 mg/kg soil) and Zn (in a dose of 320 mg /kg soil) caused some disturbances in the development of the rye grass. As a result of these disturbances there occurred a decrease

in the yield of the first cut caused by Cu, and a decrease of the mass of roots caused by Cu and Zn. The toxicity of Cu and Zn considerably increased when all five investigated heavy metals were applied together. An effect of interaction of heavy metals on their absorption by plants was visible as regard as Cd, Ni, Pb and Zn. The contents of Cd, Ni, and Zn and Pb increased in ryegrass, while the investigated heavy metals did not considerably affect the uptake of Cu by the plant (Gorlach *et al.*,1990).

Wang and Yang (1990) studied the effect of copper pollution on wheat and rice in calcareous soil fertilized with sludge containing copper. Their results were revealed that high concentration of copper in soil affected the growth of the crops and the 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 absorption and accumulation of copper within the organs following the order of: root>stem>leaf>grain. The copper content in grains of wheat and rice was not higher than 20 ppm. The soil fertilized with sludge, the variation of available copper and soil capacity were also studied. They suggested that 130ppm of copper as critical value and 800ppm as a maximum permissible limit in sludge when it is fertilized to calcareous soil.

The toxicological effect of Cr (III) on some biochemical parameters in pepper both in soil culture and nutrient culture experiments were studied by Zhou *et al.* (1990). According to them heavy metal treatment decreased fresh weight and promoted senescence of pepper plant by decreasing chlorophyll content and activities of super oxide dismutase and catalase activity as well as increasing iron content and peroxidase activities over control values.

The single and combined effect of metals like lead, cadmium, nickel and UV and radiation on protein content and protein synthesis in leaves of barley seedlings was investigated by Bhattarcharya (1991). He reported that the treatment produced an increase in protein synthesis and total soluble protein content. Combined treatments produced changes depending on the stages of irradiation, metal content of the substratum and storage period of seeds after irradiation.

Leita *et al.* (1991) studied the distribution of cadmium and induced Cd – binding protein in roots, stems, and leaves of *Phaseolus vulgaris*. Roots, stems and

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

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

Moustakas *et al.*(1994) conducted a field experiment to evaluate the effects of Cu and Pb on photosynthesis and growth characteristics of oats. The plants grown on the site elevated levels of Cu-Pb were reduced in height and biomass, comparerd to control plants , and appeared chlorotic while the accumulations of both Cu and Pb in the above ground parts were in the range considered to be phytotoxic. Cu and Pb led to pronounced reduction (47%) of chlorophyll (Chl) (a+b) content, accompanied by

proportional changes in ribulose 1, 3-bisphosphate carboxylase /oxygenase (RuBPCO) activity. Hence, Cu and Pb effects did not result in the destruction of photosynthetic apparatus but in its coordinate reduction. Growth at the heavy metal contaminated site resulted in a decreased (7%) quantum yield of photochemistry in photosystem 2(PS 2) , as given by the ratio F-v/F-m measured in dark adapted leaves in the field.The half rise time (t-1/2) from the initial (F-O) to maximal (F-m) Chi flurescence was increased, suggesting that the amount of active pigments associated with the photochemical apparatus decreased and that the functional Chl antennae size of the photosynthetic apparatus was smaller compared to the control plants . Although, Cu and Pb affected the photosynthetic apparatus in multiple ways, the prevailing effect was that on RuBPCO activity, which in turn must have limited the overall photosynthetic activity.

Seven-day old seedlings of *Vigna catjang* Endl. were treated with distilled water or 10^{-5} M $PbCl_2$ or 10^{-5} $CdCl_2$ or (10^{-4} M $PbCl_2$+ 5 times 10^{-5} reduced glutathione (GSH) or [10^{-5} M $PbCl_2$ +5 times 10^{-5} buthionine sulfoximine (BSO) or (10^{-5} M $CdCl_2$+ 5 times 10^{-5} M GSH) or (10^{-5} $CdCl_2$+ 5 times 10^{-5}M BSO) for 6 days under open air conditions in a net house (Bhattacharyya and Choudhuri ,1995). They observed that the heavy metal treated plants showed significant decline in biomass, leaf area, root length, root metabolic activity, relative water content, pigment and protein content and there was a significant rise in MDA, alpha- NH^2, proline content and electrical conductivity of leaf leachate. 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. Author suggested that the possible involvement of phytochelatin like substances in the mitigation of metal induced damages.

Six rice genotypes *viz.*, Mahsuri, Pankaj, IET 66, TTB 101-14, Biraj and Khoram were taken by Baruah and Bharatnath (1996) and grown in sand in a green house with different levels of Fe in nutrient solutions *viz* control (2 ppm), 100 ppm and 200 ppm to observe the change in growth, ion uptake and metabolism of rice (*Oryza sativa* L.) seedlings at excess level of iron. 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 along with 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 when grown in higher Fe level. They concluded that Pankaj and TTB 101-114 are suitable for growing under higher toxic concentration of iron.

Six year field study was conducted by Gigliotti *et al.*(1996) to evaluate heavy metal accumulation in top 20cm of a clay-loam calcareous soil amended with urban waste compost and to determine heavy metal uptake and distribution in corn plants grown in the soil, compared with untreated soils. 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 in 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. The trace amount of Pb was found in the stalks in the last 3 years of experiment. The limited mobility of Pb was confirmed in a contemporary hydroponic greenhouse experiment. The values of the plant 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. They 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 the plants, animal or human health.

The effect of nickel toxicity in *Hyptis suaveolens* (L.).Poit and *Helianthus annus* L. was observed by Pillay *et. al.* (1996). Ni treatment of the plants resulted in an increase in Ni content of the leaves causing a disruption in their metabolism. The concentration of soluble nitrogen and protein decreased, wheareas reducing sugar and starch contents were markedly higher than the control in both the plants. Catalase activity decreased while peroxidase and polyphenol oxidase activity was increased .In sunflower reduction in catalase activity was 5 times and the increase in peroxidase and

polyphenol oxidase was 4 and 8 fold respectively. Decrease in P content was correlated with increased in the activity of acid phosphatase and ATP ase.

Mishra and Chowdhury (1997) conducted a study on seeds of rice (*Oryza sativa* L.cv.IR-36 and Ratna) subjected to heavy metal (Pb^{2+} and Hg^{2+}) stress .They observed inhibition in germination percentage , shoot and root length and in their fresh and dry mass after 7 days . Hg^{2+} was more effective than Pb^{2+} in inhibiting germination and IR-36 was more tolerant than Ratna to these heavy metals. Both Pb^{2+} and Hg^{2+} inhibited the starch hydrolysis due to inhibition of L-amylase. When the embryos were treated with these heavy metals and grown *in vitro*, 2% sucrose in the medium could overcome the inhibitory effect of Pb^{2+} on embryo and while same, could not erase the inhibitory effect of Hg^{2+} on embryo growth significantly. Thus, Hg^{2+} was shown to be potentially more lethal than Pb^{2+} in inhibiting germination of rice seeds as Pb^{2+} inhibited the germination of the seeds 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.

Cadmium and copper uptake and distribution as well as their effects on growth and lipid composition in 17 day old tomato seedlings (*Lycopersicon esculantum* Mill.cv.63/5 F1) grown in culture solution supplied with two concentrations of Cd or Cu (0, 5 and 50mu-M) were investigated by Ouariti *et al.* (1997). The accumulation of these metals considerably higher in roots than in primary leaves and accumulation of metals increased with external metal concentration. Biomass production of the growing roots and primary leaves was strongly depressed at high metal levels. Significant decrease in lipid classes and changes in 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. These metals reduced the phospholipid and neutral lipid contents more in roots than in leaves. Heavy metal treatment induced an alteration in the fatty acid desaturation processes, as it was observed that 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. Moreover, 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). It was established from their results that, metal induced proline accumulation depends on the development of the metal induced H_2O_2 deficit in the leaves.

Shah and Dubey (1997) studied the effect of cadmium on protein, amino acid and protease, amino peptidase and carboxy peptidase in rice seedlings. Extractable proteins, free amino acids and the activities of the enzymes protease, leucine aminopeptidase and carboxypeptidase were determined in seedlings of two rice culltivers , Roma and Jaya, raised in cadmium nitrate [$Cd(NO_3)_2$] containing medium. With 500 μM $Cd(NO_3)_2$, the protein level was increased by 1.7 to 3.0 times in roots and 0.23 to 1.8 times in shoots of 20 days grown seedlings. Also 15 days old plants contained 0.2 to 0.4 times higher amino acid level in roots 0.4 to 0.8 times higher in shoots compared to non stressed seedlings. Cd^{2+}treatments significantly reduced protease activity in roots and shoots. In vitro activity of protease was inhibited markedly at concentration in excess of 100μM $Cd^{2+}$and leucine aminopeptidase and carboxylase activities were inhibited by 48 to 68% respectively in roots, whereas in shoots the activity increased by 36% to 47% with 500μM Cd^{2+}concentration.

Keltjens and Van Beusichem (1998) studied the copper and cadmium uptake and induction of phyto-chelatins (PC) synthesis in hydroponically grown maize and wheat plants exposed to these metals. They observed a close positive relationship between the concentrations of cadmium and PC in the plant shoot material. A decreased shoot concentration of cadmium after addition of copper, due to metal competition at common root absorption sites, coincided with lower PC levels. Differences in metal uptake and xylary metal transport among the two plant species were reflected in corresponding difference in PC concentration. According to the authors, the use of biomarkers such as phyto-chelatins, non-protein thiols specially induced in plants upon exposure to heavy metals, can be diagnostics criteria in heavy metal research and practice.

To elucidate the role of proline in plant responses to heavy metal stress, Sharma *et al.* (1998) 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.

Jemal *et al.* (1998) reported that peper plants (*Capsicum annuum*), like many other plant species, respond when stressed with cadmium chloride by the synthesis of phytochelatins [$(Glu\text{-}Cys)_n$ Gly] (PCs) and desglycyl phytochelatins [$(Glu\text{-}Cys)_n$] , where n=2-4 . Higher molecular weight PCs with a chain length longer than four have also been detected;

Chugh and Sawhney (1999) conducted an experiment 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 nutrient solution containing 0, 2.5, 5, 7.5 and 10 mM Cd. The effect on various aspects of photosynthesis was investigated after 6 and 12 days of treatment. The rate of photosynthesis, chlorophyll content, activities of photosystem I (PS I) and II PS (II) and a few photosynthetic enzymes *viz.* ribulose -1, 5- bisphosphate carboxylase (EC 4.1.3.9), NADP – glyceraldehydes - 3phosphate dehydrogenase (EC 1.2.1.13), fructose- 1, 6- biphosphatase (EC 3.1.3.11) and NADP – malate dehydrogenase (EC 1.1.1.82) declined progressively with the 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} cholorophyll (chl), the rate of photosynthesis showed maximum decline on per plant basis. The rate of photosynthesis and activities of enzymes showed a further decline on extending the period of exposure to 12 days. Cd had a more pronounced effect on the activity of PS II during the initial stages; however, extending the exposure for 12 days, the function of PS I also affected equally. 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, the activity of PS II was inhibited by 46% whereas that of PS I remained unaffected. Activity of photosynthetic enzymes showed far greater inhibition 12 days after treatment compared to the effect on the rate of photosynthesis (mg^{-1} chl basis) and on photosystems.

Furting *et al.* (1999) carried out an experiment on reed plants fed with heavy metal concentrations of 100 µM $Cu^{2+}$and 10 mM Fe^{2+} under hypoxia. They found 1mg g^{-1} dry weight (DW) Cu^{2+} and 8 mg g^{-1} DW Fe^{2+} in roots of the 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 amount in rhizomes were only 0.06 mg Cu^{2+} g^{-1}DW and 2 mg Fe^{2+} DW . Increased contents of both the 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 the energy charge values remained above 0.85 in Cu^{2+} and 0.79 in Fe^{2+} treatments . The energy metabolism of the rhizomes was not affected. When roots were fed with 40 µM Cu^{2+} and 1mM Fe^{2+} respectively Cu and Fe contents of roots as well as rhizomes were high enough to induce oxidative stress . Authors concluded that increased, but environmentally attainable, amounts of copper and reduced iron ions disturb root energy metabolism, and root functioning and development .Latent injuries, based on oxidative stress, may be harmful for roots and rhizomes under long term exposure.

Cadmium sulphate ($3CdSO_4$, $8H_2O$) was applied as a foliar spray to mung bean at the concentration of 40, 60, 80and 150µM at weekly intervals. The activity of ammonia assimilating enzymes viz, glutamate dehydrogenase, and glutamine synthase and glutamate oxoglutarate amino trasferase were inhibited to variable extents in the root nodules. Of the enzymes, GDH and GOGAT registered maximum decline. The fine structure of chloroplasts in Cd treated plants was degenerated similar to the senescing leaves. The principal symptomps of Cd action were the presence of osmiophilic plastoglobuli and a disorganization of the lamellar structures, mainly grana stacks. In Cd treated nodules, the peribacteroid membranes around the bacteroids seen to degenerate. Probably considerable amount of Poly-B hydroxyl but β-hydroxybutyrate granules, inside the bacteroids renders the root nodules ineffective (Keshan, 1999)

Heavy metal accumulation in field receiving fly ash from a thermal power plant and subsequent uptake in the different parts of the crop plants growing in the respective field were investigated by Barman *et al.* (1999). The metal content (Cd, Cu, Zn, Fe, Ni, Cr and Pb) in the soil samples are higher than that in control soil and lower than that in background value. In case of Cd, Zn and Pb the concentration is either below or within the critical concentration. In the edible parts of the plant Cu, Zn and Pb concentration are within the recommended permissible limits, whereas Cd, Cr and Ni show a little higher concentration. Overall the results showed that the heterogeneous accumulation of metals in plants varies from species to species and also within the different parts of the same palnt.

Toppi *et al.* (1999) reported that production of stress ethylene in carrot plants was highly stimulated by 1mM Cd. Plants pretreated with buthionine sulfoximine (BSO) 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 30min. 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.

Leopold *et al.* (1999) investigated 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. 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 detoxifications, 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.*Silene vulgaris* grown under natural conditions on a mining dump synthesized low molecular weight heavy metal binding

compounds and no complexes of heavy metal ions to phytochelatins. The free heavy metal ions were not detectable in the extracts of all investigated plants and cell cultures. Author suggested that the induction of phytochelatins is a general answer of higher plants to heavy metal exposition, but some of the heavy metal ions are able to form stable complexes with phytochelatins. They also suggested that the investigation of tolerant plants from the copper mining dump showed the phytochelatins are not responsible for the development of the heavy metal tolerant phenotypes.

Lipid peroxidation in relation to senescence of detached rice leaves caused by excess copper was investigated by Cher (1999). Excess copper, which was found to promote senescence, increased the level of lipid peroxidation but not the level of H_2O_2. Catalase and glutathione reductase activities were reduced by excess copper. Super oxide dismutase and ascorbate peroxidase activities did not seem affected by excess copper. Free radical scavengers inhibited excess copper-promoted senescence and the lipid peroxidation, suggesting that, lipid peroxidation induced by excess copper is mediated through free radicals.

Panda and Patra (2000) studied the relationship between the toxicity of Cr (III) ions and the oxidative reactions in plant cells in wheat seedlings. Leaves from 7 and 9 days old wheat seedlings were incubated in various concentrations of Cr (III) ions containing solutions for 24 hr in light. Cholrophyll and carotenoid breakdown and increases 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 senescence parameters. Mannitol and sodium benzoate both were effective for the leaves of both ages. Catalase activity was increased in Cr (III) ions in younger leaves while the activity was decreased in older ones. Peroxidase activity decreased with increasing Cr (III) ion concentration. Superoxide dismutase activity was increased slightly 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 light, which accelerated the leaf senescence.

Peralta *et al.*(2000) have reported in preliminary studies 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. They used the doses of 0, 5, 10, 20 and 40 ppm for the study. The seed germination and the growth of the plant was affected significantly 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%. The shoot size was reduced by 16% as compared to control when it was exposed to 5 ppm of Cd (II). Whereas, 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.

Yadav and Srivastava (2000) carried out an experiment to evaluate the cadmium effect on foliar epidermal traits, five molar concentrations of cadmium chloride (10^{3} M - 10^{-7} M) were used for treating the seeds/seedlings of four commercially important crops (*Carthamus tinctorius* cv. Tara, *Cicer arietinum* cv. K-468, *Hordeum vulgare* cv.Jyoti and *Setaria italic acv.* Saket-4). It was observed that $CdCl_2$ treatment could increase /decrease the epidermal cells peer unit area and stomata per unit area of stomatal pore. Cadmium chloride treatment could also induce several types of stomatal anomalies like stomata with unequal guard cells /subsidiary cells, stomata with more or less than one guard cells / subsidiary cells, disintegration of guard cells /subsidiary cells. It was observed that *Cicer arietinum* has shown less susceptibility to cadmium as compared to the other crops.

The influence of cadmium on growth and development of *Vicia faba* L., broad bean was assed in pot cultures with cadmium iodide (CdI_2) in different concentrations ranging from 15 to 500 mg per kg of soil. There was a decline in plant height and total dry weight. Root size was decreased most significantly with a corresponding reduction in the frequency of root nodules. Total soluble protein in leaf, stem and root suffered a pronounced loss with increasing concentration of cadmium. Chlorophyll a was the most sensitive pigment followed by chlorophyll b and carotenoids. Nitrate reductase activity

too was adversely affected. Cadmium contamination induced abnormalities in stomata and trichomes (Bhatnagar *et al.* 2000)

Schiizendiibel *et al.* (2001) reported that unspecific peroxidase in pine roots were not inhibited by Cadmium, but increased slowly with the time pattern clearly distinct from that observed for the constituents of the SOD-ascorbate- glutathione pathway. Its activities were elevated in root tips which showed increased concentrations of phenolics and lignification in response to Cadmium.

Prasad *et al.*(2001) conducted an experiment to find out effect of heavy metals in aquatic plants .Several physiological responses of aquatic vascular plants like *Lemna trisulca* L.were investigated when they were exposed to the elevated concentrations of cadmium (up to 10mM) and copper (up to 50μM) . They found that Lemna fronds were able to accumulate the two heavy metals but copper treated plants showed toxic symptoms at concentrations 1000-fold lower in comparison to cadmium. *Lemna trisulca* could tolerate elevated levels of cadmium i.e. up to 10mM, without significant changes in photosynthetic pigment concentration. On the other hand copper concentrations 25 and 50 μM showed the significant pigment degradation. Total gas exchange and net photosynthesis were affected by Cd in *Lemna* fronds. On the contrary, the inhibition of total gas exchanges and net photosynthesis caused by Cu (2-50μM) correlated with Chl a and carotenoid concentrations decrease as well as with the decay of fluroscence from PS-II- . An increasing impact of respiration was observed in total oxygen exchange when the *Lemna* plants were treated with increasing concentrations of Cd (up to 5mM) and with Cu (2-50μM). In SDS PAGE analysis in Cd trated fronds , a dose-dependent accumulation of two polypeptides with apperant molecular weight 18 and 10 kDa , respectively as well as the appearance of two smaller polypeptides apparent molecular weights 8 and 7 kDa) was observed but in Cu-treated fronds neither accumulation of existing proteins nor any extra proteins were appeared .

Monni *et al.* (2001) measured chlorophyll, organic (citric and malic acids) and abscisic acid (ABA) contents and stem water potential on *Empetrum nigrum* L. (crowberry) to indicate the possible physiological effects of heavy metal deposition on the plant. 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. The results showed all the investigated parameters were clearly affected by heavy metal emissions. Chlorophyll content in leaves as well as organic acid contents in the leaves and stems was lower close to the emission source. On the contrary, ABA contents in stems and leaves in general, were higher in plants growing 0.5 km from the pollution source. The results showed that the plant exposed close to the smelter the stem water potential 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.

Diaz *et al.*(2001) reported that pepper (*Capsicium annum* 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. Peroxidase activity was also induced in roots, but the 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. The induction of both enzymatic activities was associated with the accumulation of soluble phenolics and lignin was observed in hypocotyls. The two SKDH isozymes present in the control hypocotyls (SKDH-3 and SKDH-4) increased in a similar proportion after Cu stress. In 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 control. The application of the chelator EDTA was able to counteract all the stress effect of the metal stated above.

In a study by Hegedus *et al.* (2001) with green and greening barley seedlings which represent two different stages of development to evaluate 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) . Although the roots were the main site of cadmium accumulation, 1.5- 3% of cadmium was translocated into leaves and causes the oxidative damage which was indicated by the reduced chlorophyll content and increased malondialdehyde content of the leaves. The APX activity was increased without any increase in the activity of POD in the roots of both types of seedlings

exposed to various cadmium concentrations. In leaves, however, elevated level of POD and APX was observed. In roots of green seedlings at high concentration of cadmium the APX activity was reduced on the fourth day of culture but no inhibition was found in the POD activity. CAT activity in leaf which mainly represented the peroxisomal enzyme activity did not showed any changes under cadmium stress. The results showed 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 the heavy metal stress.

The combined effect of Copper and Cadmium adversely affected the germination, seedling length and number of lateral roots in *Solanum melongena*, reported by Neelima and Reddy (2002).

The effects of various nickel levels (0.05, 0.5, 5.0 and 50μM) on photosynthesis and chlorophyll content in rapeseed (Brassica *campestris* var. Toria PT 303) was examined by Pratibha and Rathore (2002). The studies revealed that while low levels of nickel (upto0.5μM) enhance the photosynthetic rates, however high levels of 5μM had reverse effect. A decrease in the total chlorophyll content with increasing concentrations of nickel was also observed in the rapeseed plants.

The influence of Cd on pepper plant was also studied 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 the different physiological parameters , and antioxidative enzymatic activities of leaves. On the basis of growth parameters they found that 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 responses to the Cd^{++} stress were observed among cultivars, Abdera being the most resistant to cadmium stress, and Mondo and Herminio the most sensitive cultivars. The increase in activity of glutathione reductase and guaiacol peroxidase of most cultivars was observed in the cadmium concentrations of 0.5 mM, while catalase (CAT) and superoxide dismutase (SOD) were slightly depressed at that concentration. The analysis of SOD activity pattern by native-PAGE showed the presence of four SODs in most

cultivars which were identified as Mn-SOD, Fe-SOD, CuZn-SOD I and CuZn –SOD II. However, the CuZn-SOD s were absent in the Cd sensitive cv. Herminio. The growth of pepper plants with 0.5mM Cd inhibited the activity of CuZn-SODs in all cultivars, whereas, 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. The results suggest that in pepper plants the tolerance to Cd toxicity is more dependent on the availability of NADPH than its antioxidant capacity.

Perfus–Barbeoch *et al.* (2002) investigated the Cd^{2+} toxicity effects on plant water loss, gas exchanges and stomatal behavior in *Arabidopsis thaliana* L. Effects of 1week Cd^{2+}application in hydroponic condition ($CdCl_2$ 10-100 μM) were analyzed . No significant effects on the plant water relationship and carbon assimilation was observed at a10μM Cd $^{2+}$concentration. At higher 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 proved by fluorescence measurements. In epidermal strip bioassays, nanomolar Cd $^{2+}$concentrations reduced stomatal opening under light in *A.thaliana* , *Vicia faba* and *Commelina communis* .5mM ABA application limited the root to shoot translocation of cadmium. The cadmium induced stomatal closure was likely ABA independent, since a 5 day water treatment with 50 μM Cd^{2+}did not affect the plant relative water content. A similar cadmium induced stomatal closure was observed in the ABA insensitive mutant abi 1-1, which showed a higher transpiration rate than the wild type but did not accumulate more cadmium as cadmium uptake is not dependent only on the transpiration flow. Application of putative calcium channels inhibitors suppressed the inhibitory effects of cadmium in epidermal strip experiments, suggesting that cadmium can enter the guard cell trough calcium channels. Patch-clamp studies with *V.faba* guard cell protoplast 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.

Different clones of *Salix viminalis* with different resistances to Cd , Cu, and Zn were cultivated hydroponically in the presence of 7μmol L^{-1} Cd, 3 μmol L^{-1} Cu and 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 enzymatic activities of the aspartate peroxidase (APX), guaiacol peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT) were also analyzed. Salicylic acid was also measured since it is known to be involved in antioxidative activities. Some differences in sensitive and resistant clones could be observed .The SOD activity was higher in untreated resistant clones compared with the sensitive clones. However, under metal treatment, the SOD activity was similar. Moreover, TBA-rm was higher in shoots of the resistant clones compared to the sensitive ones, while the opposite was found in roots (Landberg and Greger, 2002)

Fediuc and Erdei (2002) studied the capability of common reed and cattail (*Phragmites australis* (Cav.) Trin.ex Steud. and *Typha latifolia* L.) to accumulate and translocate Cd^{2+}at the level of thiol metabolism and antioxidant enzyme activity. 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. Plants samples were taken for assays and analysis in 2 -4 weeks intervals. They reported that most of the Cd^{2+}taken up was retained in the roots in both species. *Typha* accumulated more cadmium in shoots than *Phragmites*.The increasing accumulation of cadmium in *Typha* had a positive correlation with the increase of thiol content while in *Phragmites* glutathione reductase, catalase and peroxidase activities were increased. Author concluded that different defence strategies operate in the two plants under cadmium stress. In *Typha*, this strategy relies more on thiol induction and metal binding leading to the heavy metal avoidance, while in *Phragmites* increased antioxidant enzyme activities and thus based on scavenging of active oxygen species.

Shu *et al.* (2002) reported that both Fankou and Leachang lead /zinc (Pb/Zn) mine tailings located at Guangdong Province contained high levels of total and DTPA extractable Pb, Zn and Cu. Different populations of the two grasses growing on the tailings were *Paspalum distichum* and *Cynodon dactylon.* Tillers of these populations

including those from an uncontaminated area were subjected to the following concentrations: 5, 10, 20, and 30mg 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 suggested that both grass populations showed a greater tolerance to the three metals than those growing in the uncontaminated area, which indicate the co-tolerant ecotypes have evolved in the two grasses. Among the two grass populations *P. distichum* collected from Fankou tailing had the highest tolerance to Cu while Lechang population had highest tolerance to Pb and Zn among the tested populations. The tolerance levels of *P. distichum* had a better growth performance than *C. dactylon* when they were grown together on the tailing sites. The tolerant populations of these species would serve as potential candidates for re-vegetation of wastelands contaminated with Pb, Zn and Cu.

The inhibition of plant growth and induction of visible symptomps of metal toxicity led to increased accumulation of the metals was reported by Pandey and Sharma (2002) in cabbage plants to excess (500 μM) of Co^{2+} , Ni^{2+} and Cd^{2+} in sand culture . In addition to chlorosis, Co^{2+} treated plants exhibited reddish purple coloration along the leaf margins, Ni^{2+} treated plants showed the black spots near the leaf margins and the plants treated with Cd^{2+} developed purple coloration along the leaf margins. At the equimolar concentration, inhibition of growth was most severe with excess Cd^{2+} and induction of visible symptoms was most severe with the application of excess Ni^{2+}. The uptake of Fe and its translocation to leaves decreased due to the exposure of excess concentrations of heavy metals. Exposure to each Co^{2+}, Ni^{2+} and Cd^{2+} decreased the 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+} exposure developed water stress by decreasing water potential and transpiration rate, associated with increase in diffusive resistance showing the water stress. The enhanced accumulation of proline in the leaves was further substained when the plants exposed to Co^{2+}, Ni^{2+} and Cd^{2+}.

Shrivastava *et al.* (2002) conducted a study to elucidate the heavy metal status in various morphological components of normal green and chlorotic plants of sugarcane variety CoLk8102 which indicated that chlorosis induced significant variation in Pb and Cr contents. Morpho-physiological components did not differ significantly with respect to any of the heavy metals. There was a marginal increase in transport index of heavy metals in chlorotic plants. Although chlorosis induced significant variation in Pb and Cr contents, on overall basis the partitioning of heavy metals followed a trend similar to that of partitioning of dry matter. The lead content was somewhat higher in the chlorotic plants as compared to the normal green plants on per unit plant weight basis.

Zornoza *et al.* (2002) studied cadmium stress in nodulated white lupin. White lupin plants (*Lupinus albus* L., cv.Multolupa) were grown hydroponically on perlite with different Cd concentrations in the nutrient solution (μM): 0, 18 and 45. Changes in growth, nodulation, nutrient concentrations, nutrient uptake and distribution, Cd bound to cell wall and some Cd stress indicators were studied in roots and shoots (leaves plus stem) of 35-d-old plants as a result of Cd uptake. A significant decrease in both root and shoot dry weight was found only when plants were grown on 45 μM Cd. Whereas nodulation and total N content decreased for both Cd levels. White lupin plant retained up to 88% of total Cd in the roots, showing some capacity of Cd translocation to the upper parts, but an important fraction of Cd was bound to cell wall. Cadmium addition reduced P, K, Fe, Mn and Zn concentrations in the shoot and Mn in the root. Despite the reduction in Mn concentration, Mn level in Cd-treated plants remained within normality, probably because white lupines a Mn accumulator. In Cd treated plants, the level of lipid peroxidation remained unmodified, whereas total thiols showed a pronounced increase in roots. Results obtained suggest that white lupin has developed several strategies of defense against Cd, as a high retention of Cd by cell walls and complexion by thiol groups, both contributing to diminish the level of free Cd . Moreover, the high Mn content of white lupin could contribute to the prevention of Cd damage on photosynthesis, since, chlorosis was not found.

Barman Roy and Bera (2002) studied the individual and combined effect of mercury and manganese on phenol and proline content in leaf and stem of mungbean seedlings. The mungbean (*Vigna radiata* L.Wilczek) cv. Pusa Baisakhi seedlings were

raised in individual (0, 1, 10, 100 and 1000 ppm) and combined solutions (1:1, 10:1, 1:10 ppm Hg: Mn) of mercury and manganese for 6 days. Phenol and proline were found to accumulate in leaves in response to treatments with heavy metals. The magnitude of accumulation was correlated with concentration of metals. However, a reverse trend was noticed in stem for phenol. Accumulation of phenol in response to heavy metal treatment was organ specific and occurred at higher rate in plant parts, which faced the stress mostly. However, accumulationof proline helped the plant to survive stress situation. In combined solutions, amelioration of mercurial toxicity by manganese was recorded.

The effect of aluminium on the growth and physiology of *Acacia nilotica* seedlings, a fast –growing tree legume was studied by Malathi *et al.*(2002) The toxic effect of aluminium in acid soils poses a major threat to plant species. The short term effects of Al in hydroponically grown *A.nilotica* seedlings were studied for 30 days. Al was supplemented to the nutrient solution in the form of AlK $(SO_4)_2$. Chlorophyll content showed a significantly decreasing trend with increasing Al. Stomatal conductance showed a similar trend as did nitrogen and phosphorous contents. Protein contents were correlated to that of nitrogen levels. The result showed that, the seedlings can not tolerate Al levels above 100 ppm.

It was reported the repeated use of copper (Cu) to control vine downy mildew, caused by *Plasmopara viticola* , is mainly responsible for the heavy increase of Cu concentration in the upper layers of vineyard soils. Brun *et al.*(2003) created an artificial soil gradient with Cu enrichments ranging from 0-400 mg kg-1for the determination of the effects of elevated soil Cu on the development of plant. On this gradient, five 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.,), were quantified for survival, growth and reproduction throughout one flowering season. They reported that the high concentrations of Cu in the soil resulted in low survival, low plant biomass, delay in flowering and fruiting, and low seed set. The effects differed among speices. Moreover, high soil Cu concentrations had contrasting effects on patterns of resource allocation depending on the plant speices.

Singh and Tiwari (2003) reported that excess of cadmium induced changes in oxidative scenario and water status of plants viz. total water content, specific water content, water saturations deficit(WSD) and transpiration of *Brassica juncea* grown in soil pot culture. Although lower and marginal level of excess cadmium (100 and 250 ppm) improved growth but higher levels (500 ppm) caused significant suppression of growth. Proline accumulation, an indicator of water stress, occurred at higher level of cadmium. Gradual increase in activities of certain antioxidative enzymes such as catalase and peroxidase along with increased lipid peroxidation were also observed. The excess levels of cadmium also decrease the concentration of soluble protein and chlorophylls and increase the ratio of chlorophyll a/b.

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

Survival and behavior of water hyacinth [*Eichhornia crassipes* (Mart.) Solms] under different conditions of heavy metal concentrations were studied by Soltan and Rashed (2003). Plants were grown in different media (distilled water, Nile water, waste water and different concentrations of heavy metals) and visual changes were also observed in plants during each experiment. The heavy metal concentrations (Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn), pH and conductivity of the media were measured before, during and at the termination of the experiments. The effects of different media on metal accumulation by the plants were analyzed with the plant samples after termination of the experiments. Their results showed that water hyacinth can survive in a mixture of heavy metal concentrations up to 3mg L^{-1} and in 100 mg Pb L^{-1} solutions, whereas higher concentrations of metals as mixture and 100 mg Cd L^{-1} led to rapid fading of the plants. Water hyacinth decrease the pH of the growth media as it has exhibited a deprotonation reaction during the uptake of the metal ions .The results indicated that the water hyacinth plays an outstanding role as a heavy metal decontaminator; but 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 the plants growing in Kima drain wastewater.

The effect of Cd on 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, Cu and Mg in response of the uptake and translocation in rice plant were investigated by Liu *et al.*(2003). They conducted a pot experiment on 20 rice cultivars of different genotypes and origins by adding 100 mg kg^{-1} of cadmium (Cd) to the soil. The results showed that the effect of Cd on rice growth and development were variety dependent; some cultivars were strongly tolerant to soil Cd stress, where others were very sensitive to the heavy metal. Differences were also observed 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 the rice plants. The effects of Cd on the concentrations of the mineral nutrient in the 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 in 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 correlation 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 the rice plants. The results revealed that the rice cultivars differed greatly in the growth and development to Cd and in absorption and translocation of different metals like Cd, Fe, Zn, Cu, Mn and Mg. The interactions of Cd and Fe, Zn, Cu are synergetic in uptake and translocation from root to shoot by rice plant.

Stolt *et al.* (2003) studied the PC accumulation in 12 days old seedlings of two cultivars of spring bread wheat (*Triticum aestivum*) and two spring durum wheat cultivars (*Triticum turgidum*) with different degree of Cd accumulation in the grains.

Shoots and roots were analyzed for dry weight, Cd and PC accumulation. The results showed neither significant differences between the species or the varieties in the growth responses to Cd, nor the distributions of PC chain length or PC isoforms. At 1μM external Cd, durum wheat had a higher total Cd uptake than bread wheat, though; the shoot-to-root Cd concentration ratio was higher in bread wheat. The results when comparing varieties within a species, the high grain Cd accumulators showed the lower rates of root Cd accumulation shoot Cd accumulation and root PC accumulations, but higher shoot-to-root Cd concentrations ratios. Intraspecific variation in grain Cd accumulation is apparently not only explained by differential Cd accumulation but rather by a differential plant-internal Cd allocation pattern. However, the higher average grain Cd accumulation in the durum wheat than the bread wheat is associated with a higher total Cd accumulation in the plant, rather than with differential plant internal Cd allocations. The root internal PC chain length distributions and PC-thiol-to Cd molar ratios did not differ significantly between species or varieties , suggesting that differential grain Cd accumulation is not due to is not due to differential PC based Cd sequestration in the roots.

The influence of cadmium (Cd^{2+}) on the wheat plant (*Triticumt aestivum* L.) was also studied by Shukla *et al.*(2003) . Cd^{2+}accumulation and distribution were analyzed in 3weeks 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 was studied in detail up to 21 days on biomass productivity, plant growth, protein, amino acids, photosynthetic pigments, starch, soluble sugars and essential nutrient uptake to explore the level up to which the plant can withstand the stress of heavy metal. Plants showed symptoms of heavy metal toxicity as observed by various morphological parameters which were recorded with the growth of the plants when treated with 0.5, 1.0, 2.5, and 5.0 mg L^{-1} of Cd^{2+} major biochemical constituents which plays a major role in plant metabolism such as chlorophyll,protein, free amino acid, starch and soluble sugars levels were altered by the heavy metal . The root, shoot-leaf length and the biomass progressively decreased with increasing Cd^{2+}concentration in the nutrient medium. The Cd^{2+}uptake and accumulation was found to be maximum

during the initial growth period ,it also interfere with the nutrients uptake, especially Ca^{2+}, Mg^{2+}, K^{+}, Fe^{2+} ,Zn^{2+} ,Mn^{2+} from the growth medium.

Panda and Khan (2003) 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 concentrations .Lipid peroxidation measured in terms of thiobarbituric acid reactive substance content increased in senescing leaves concurrently in total peroxide content. Ascorbate content showed higher in 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. The results showed acceleration senescence in rice under heavy metal toxicity stress.

Parida *et al.* (2003) conducted an experiment using an alkaline sandy loam equilibrated with the 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 investigate the Ni accumulation pattern and its influence on growth and micronutrient distribution in fenugreek plants. They reported that green and dry matter yields of fenugreek increased slightly upto 20 gm Ni kg^{-1} soil but decreased significantly with the application $\geq$ 40mg Ni kg^{-1} soil. The characteristics toxicity symptoms of interveinal chlorosis of the crops were observed in the the pots receiving $\geq$ 40mg Ni kg^{-1} soil. The increasing rates of Ni application increased consistently the total Ni content in the plant tissues. The roots accumulated much higher amount of this element compared to the shoots. The Fe content in the plants showed an increase whereas that of Cu and Zn experienced a decrease content with the rise in the applied Ni.

Skorzynska-Polit *et al.* (2003) studied the changes in the content of reactive oxygen species (ROS) and the activities of the antioxidant system in leaves of *Arabidopsis thaliana* (L.) Heynth exposed to Cd^{2+}. Mature plants growing in the nutrient solutions 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 the concentrations of 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 100muM Cd^{2+}. The Cd^{2+} treated

plants always showed the higher superoxide dismutase (SOD) activity than in control plants. Catalase (CAT) activity decreased with the 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 50muM Cd^{2+}.Strong reduction in ascorbate (AA) content and enhanced activity of monodehydraye ascorbate reductase (MDHAR) were observed at 25 muM Cd^{2+}. Glutathione reductase (GR) activity was always higher than in the control but decreased when the concentration of Cd^{2+} increased. However, it was accompanied by gradual content increase of SH groups.

The effects of copper and lead applied in the form of chloride salts on root, shoot, and leaf growth of the bean (*Phaseolus vulgaris* L.) was studied by Zengin and Munzuroglu (2004). It was observed that both heavy metals significantly prevented the growth of root, shoot and leaves of seedling. 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. Extension of the exposure time to heavy metals of seedling led to greater decrease of root, shoot and leaf growth. It was determined copper had highest toxic effect than lead.

Panda and Choudhury (2004) investigated the changes in nitrate reductase (NR) activity and subsequent induction of oxidative stress in *Polytrichum* under chromium toxicity. It was observed that exposure of Cr at different concentrations for 24 hr and 48 hr reduced the NR activity in moss cells with significant inhibition after 48 hr at 100mM of Cr. Reduction in total chlorophyll content was observed in moss cells after Cr treatment. High accumulation of Cr was seen after 24 hr and 48 hr. Cr prompted the malondialdehyde (MDA) production. 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 of all the enzymes was seen after 24 hr and 48 hr of Cr application. Increase in CAT, GR or SOD was highly significant with the increasing concentration and the duration of the metal treatment. The GPX activity was decreased after 48 hr of Cr exposure.

The changes in fresh weight, total protein contents , concentration of cadmium , and glutathione content in maize kernels cultivated for 5 days at three different Cd concentrations (0, 10 , 100 μmol L^{-1} $CdCl_2$) was studied by Klejdus *et al.* (2004) . 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 the concentration of cadmium. An intensive preservation of homeostasis of Cd^{2+} ions in the germinating plants by defending mechanisms might explain the differences of uptake rate of cadmium .The defending mechanisms might be triggered by the all studied concentrations of heavy metals at the time of exposure by linear increase of GSH content.

The effect of Cd, Pb, Al, or Cu on *Corchorus olitorius* plant was studied by Mazen (2004). When the plants were treated by 5μg cm^{-1} of Cd, Pb, Al or Cu in hydroponic culture for 6 days, they accumulated 190, 150, 350 and 325 μg g^{-1} (dm) of these metals in the leaves. The sharp rise in amino acid content in the leaf tissues were noted when the plants were exposed to the tested metals; 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 *Corychorus olitorius* plant to accumulate all the metals. The plant growth was significantly reduced by the treatment of all the metals except Cu and added cys, K_2SO_4 or SA alleviated the growth retarding effect of these metals.

The potential accumulation of Cd (II), Cr (VI) and Cu (II) in *Convolvulus arvensis* L. using an agar based medium was studied by Torresday *et al.* (2004). The result showed that shoots of the plants demonstrated the capability of accumulate more than 3800mg Cr, 1500 mg of Cd, and 560 mg of Cu kg^{-1} of dry tissues, when the plant was exposed to 20 mg L^{-1} of these heavy metals .This study and the field data reported previously showed 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 the plant could be considered as a potential Cr-hyperaccumulator.

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. They found that, the highest intracellular copper uptake from 2mM Cu media occurred within 4 hr in both strains, but significantly less accumulated in tolerant species by using inductively coupled plasma atomic emission spectrometry. The copper tolerant strain exhibited significantly more intracellular proline and less potassium efflux than the wild strain. By 24 hr differences between strains in intracellular Cu diminished, as concentrations in both strains reached their highest level. Proline accumulation was decreased significantly at the same time. High copper concentration in agar media after 2weeks of cultivation, showed decreased in growth, pigment content, chlorophyll a degradation and chlorophyll a fluorescence in wild –type of *T.erici*. Proline alleviated the toxic effects of Cu in both strains, but markedly so in case of the tolerant strain.

Alfalfa plants grown in soil at different stages were exposed to separate batches of Cr (VI) at 100 mgL^{-1} and Cd (II), Cu (II), Ni (II), or Zn (II) at 500 mgL^{-1} (Videa *et al.* 2004). Four days after germination, all metals, except Zn (II), had lethal effects on the seedlings. Furthermore, when the heavy metals were 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 populations. While approximately 90% of the plants exposed to Cd (II), Cu (II) and Zn (II) were able to grow without apparent negative effects on 20 days after germination, but Cr (VI) and Ni (II) still showed lethal effects. The heavy metal concentration in shoot dry tissues was 1209 mg kg^{-1} for Cd, 887 mg kg^{-1} for Cu and 645 mg kg^{-1} for Zn. The results demonstrated that the tolerance of alfalfa plants to Cd, Cu and Zn was positively correlated with the age of the plants. From these results possibility of using alfalfa plants, via transplant, to clean up soils was opened, where the concentration of Cd, Cu or Zn is high enough to avoid the alfalfa seed germination.

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

Several complementary studies were carried out by Viard *et al.* (2004) to asses the contamination induced by traffic at the vicinity of a highway (A31, France), 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 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 decreased with increasing distance from the highway. The study revealed that, among the three metals lead indicated the best metal to evaluate road transport contamination.

Barazani *et al.* (2004) conducted an experiment in which the ability of *Allium schoenprasum* L. (chives) to accumulate and tolerate cadmium in aqueous Hogland

medium at 50μM and 250 μM were tested under continuous growth or several successive harvests of shoots. After 28 days of continuous growth, chives accumulated the heavy metal up to 0.2% and 0.5% of its dry weight, when grown in 50 μM and 250 μM, respectively. The leaves were harvested in every 16 days; there were no obvious stress symptoms after six harvests during a period of 96 days at 50 μM Cd, whereas, at 250 μM, after 64 days and four harvests, inhibition of growth occurred. A total of 1.2g kg^{-1} DW and 2.4 kg^{-1} DW was accumulated in the leaves, respectively in each treatment. Total SH compound 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 sulpher 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 commonly edible plants.

Ajasa *et al.* (2004) conducted an experiment in which the concentration levels (ppm) of selected toxic 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 used for the estimation of heavy metals on 10 plant species collected from different locations within Ogbomoso. The plants used for the study 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 (51340±21) were found in *Morinda lucida*. Their results also showed that *Ocimum canum* had the highest amount of K (36600± 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).

Cucumis sativus (cucumber) was tested to assess an ecotoxicity in soils contaminated by heavy metals copper, cadmium and lead separately and in

combinations. The growth of the plant was the toxicity endpoint, 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 ($EC50_{mix}$<1 TU), and antagonistic ($EC50_{mix}$>1 TU), responses. Ternary combination of Cu+Cd+ Pb produced an antagonistic response for the growth of *Cucumis sativus*. Bioaccumlations of the heavy metals like Cu, Cd and Pb were observed in *Cucumis sativus*, the accumulation 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 (An *et al.* ,2004).

Cadmium extraction potential and degree of resistance to Cd stress was determined in ten *B.juncea* cultivars (V_1- V_{10}) commonly grown in India by Quadir *et al.*(2004). Ten days old seedlings of *B.juncea* cultivars were exposed to various levels of cadmium chloride (0.0 – 2.0 mM) for 72 hr in hydroponics culture and the leaf samples were analyzed at 24, 48 and 72 hr 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 in *B.juncea* cv. Pusa Jai Kisan (V_5) showed the least increase in the lipid peroxidation rate but accumulated higher levels of biomass, Cd and glutathione contents among the studied cultivars. The 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 indicates its

possible incorporation in synthesis of the phytochelatins and metallothioneins to sequester Cd and to combat Cd stress.

Growth responses were analyzed in *Prunus cerasifera*, a peach root stock after exposure to various copper concentrations, by Lombardi and Sebastiani (2005). The plantlets tolerated Cu concentrations up to 50μM and showed improved Fe uptake unexpectedly under low to moderate concentrations (from 0.1 to 50μM). At 100 μM of Cu, plantlets reduced relative growth rate for both fresh and dry weight and severe browning were developed which progressed to necrosis. CAT and SOD activity levels and the modulation of transcription of catalase and superoxide dismutase genes were analyzed in the plant after exposure to various concentrations of copper. The total catalse and superoxide dismutase and simultaneous induction of gene expression of *Sod* and *Cat* were observed under Cu toxicity. The result demonstrated that, the plant is quite tolerant to the metal and mobilizes catalase and supeeroxide dismutase in order to mitigate Cu-stress damages.

The giant reed (*Arundo donax* L.) plant which grew on the surface soil and irrigated with mixed heavy metal solutions of Cd (II) and Ni (II) were tested to study the impact of these heavy metals on growth and photosynthesis by Papazoglou *et al.* (2005). 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 stem height and diameter, no of nodes, fresh and dry weight of leaves, and net photosynthesis (Pn) were examined, the results indicated that the parameters were not affected by the heavy metals and thus indicating that plants tolerate the high concentrations of Cd and Ni. They suggested from the study that, the giant reed plants are very promising energy plants, and they can be cultivated in contaminated soils to provide biomass for energy production purposes.

Plants of two mungbean genotypes MH 85-111 and MH 98-6 were exposed to different levels of cadmium 28 days after sowing. Plants exposed to 3.0 and 4.0 mM Cd^{2+} did not survive and died before entering into the reproductive phase (Kumar and Dhingra, 2005). Cadmium induced reduction in the number of flowers and *in vitro* pollen germination but did not affect the pollen viability. However, it stimulated the

growth of the tube. Cadmium although did not affect the pistil length, it decreased number of ovules/pistil. Ovules were morphologically normal and receptive. *In vivo* stylar studies revealed all the ovules were not penetrated by pollen tube and number of unpenetrated proximal ovules was increased by Cd^{2+} and cv. MH 85-111 was affected more adversely than MH 98-6. Cadmium inhibited the number of pods, seeds, seed weight / plant and 100 seed weight, inhibition being more in MH 85-111 than MH 98-6. Cadmium treatment did not affect starch content but increased protein content in physiologically mature seeds. Accumulation of Cd^{2+} was maximum in the roots and least in the seeds. Cadmium accumulation, in general was higher in MH 85-111 than MH 98-6 and stem of MH 85-111 accumulated four times Cd^{2+} than MH 98-6. Seed cadmium however, was comparable in both the genotypes.

Rai *et al.* (2005) studied the effect of different concentration of Cd on *Phyllanthus amarus* Schum. and Thonn.; because *P. amarus* is mostly grown as weed in agricultural and waste lands and is a reputed medicinal plant used in Indian indigenous system of medicine with hepatoprotective, diuretic, stomachic properties and is recently being used for the treatment of hepatitis B. Result showed that, Cd causes significant decrease in fresh and dry weight, length of root and shoot, protein, chlorophyll, caritenoid and and starch content was increased. Moreover, ultra morphological changes were also observed in stomatal opening and wax deposition on both the surfaces of leaves. They noted that, the therapeutically active compounds –phyllanthin and hypophyllanthin, enhanced at certain levels of Cd due to abiotic stress.

In an experiment by Chaoui and Ferjani (2005) 12 days old seedlings of pea were treated for 4 days by 20 and 100µM of Cd $(NO_3)_2$ or $CuSO_4$. The result showed 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. Though no change in antioxidant capacities were observed in plants treated with the same metal concentration. The Cd–reduced growth could be associated to an elevation in the activities of IAA oxidase and of lignifying peroxidases was observed at the same dose. Increase of these latter, with the loss in antioxidant

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

An experiment with radish seedlings exposed to 0.25 and 1.0 mM of $CdCl_2$ for 24 hrs was carried out by Vitoria *et al.* (2005). Result exhibited the structural changes of the chloroplasts, mitochondria and nuclei when compared to non-treated control plants. Changes in the organelle shape, an increase in the stroma volume and a deposition of electron-dense material in the double membrane of the chloroplast was observed when the plant was exposed to Cd^{2+} stress. The changes in the chloroplast membranes were not so 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. No alterations were observed in the vacuoles of radish seedlings grown at different Cd^{2+} concentrations for the period tested.

In order to understand the difference between Zn, an essential micronutrient and Cd, a non-essential element, Cd-10μM and Zn supplemented (10, 50, 100 and 200 μM) Cd 10 μM treated *Ceratophyllum demersum* L.(Coontail),a free floating freshwater macrophyte was chosen for as study conducted by Aravind and Prasad (2005) Cadmium at 10 μM concentration decreased thiol content , enhanced oxidation of ascorbate (AsA) and glutathione (GSH) to dehydroascorbate (DHA) and glutathione disulfide (GSSG), respectively, a clear indication of oxidative stress. Zinc supplementation to Cd (10 μM) treated plants effectively restored thiols, inhibited oxidation of AsA and GSH maintaining the redox molecules in reduced form. Cd 10 μM slightly induced ascorbated peroxidase (APX, E.C.1.11.1.11) but inhibited monodehydroascorbate reductase (MDHAR, E.C.1.6.5.4), dehydroascorbate reductase (DHAR, E.C. 1.8.5.1) and glutathione reductase (GR, E.C. 1.6.4.2), enzymes of ascorbate-glutathione cycle (AGC).Zn supplementation restored and enhanced the functional activity of all the AGC enzymes (APX, MDHAR, DHARand GR). Glutamylcysteine synthatase (GCS, E.C. 6.3.2.2) was not affected by Cd as well as Zn, but Zn supplements increased glutathione –S-transferase (GST, E.C.2.5.1.18) activity to a greater extent than Cd and simultaneously restored glutathione peroxidase (GSH-PX,

E.C.1.11.1.9) activity impaired by Cd toxicity. Zn-alone treatments did not change above investigated parameters. These results clearly indicate the protective role of Zn in modulating the redox status of the plant system through the antioxidant pathway AGC and GSH metabolic enzymes for combating Cd induced oxidative stress.

Smeets *et al.* (2005) did an experiment in which oxidative stress has been shown to be of great importance in the toxicity of several metals. The relationship of cadmium phytotoxicity and antioxidative reaction in bean (*Phaseolus vulgaris* L.) plants was investigated. Eleven days old seedlings were exposed to an environmentally realistic concentration of cadmium (2 μM $CdSO_4$). The antioxidative defence mechanism was significantly activated after 24 hr of cadmium exposure. Some enzymes capable of quenching reactive oxygen species (syringaldazine peroxidase, EC 1.11.1.7; guaiacol peroxidase, EC 1.11.1.7) as well as enzymes important in the reduction of NAD (P)$^+$ (isocitrate dehydrogenase , EC 1.1.1.42: malic enzyme, (EC 1.1.1.40) were significantly elevated by cadmium exposure. Furthermore, the ascorbatte-glutathione cycle appeared to be a very important mechanism against cadmium –induced oxidative stress. In leaves, significant increase of ascorbate peroxidase (EC 1.11.1.11) and glutathione reductase (EC 1.6.4.2) and significant changes in the ascorbate and glutathione pool were observed. Morphological and other biochemical parameters (lipid peroxidation) were significantly enhanced 48 hr after the start of the cadmium exposure. At the end of the experiment (72h after the start of the metal treatment), even visual effects, such as chlorosis, were observed. The result showed that, cadmium, like other metals, induces cellular redox disequilibrium suggesting that an environmentally realistic concentration of cadmium can cause oxidative stress.

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 doses; such accumulation was more important in roots than in leaves reported by Youseef *et al.* (2005). Biomass production was severely inhibited, even at low cadmium concentration. They also reported that the metallic ion seemed to affect selectively chloroplastic membranes due to an inhibition of polyunsaturated fatty acid biosynthesis. Moreover, a lipid peroxidation occurred due to the spectacular increase of malondialdehyde (MDA) content observed in cadmium treated leaves.

Soyabean seedlings treated with 6 mg kg^{-1} Cd during 72 hr induced a slight growth inhibition in roots, stems and leaves as observed by Drazic and Mihailovic (2005). A significant desiccation of cotyledons and leaves with a decrease in chlorophyll content in leaves were observed. Application of 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 effect on the significant decrease in roots. Under the influence of Cd, Fe content was decreased in roots and increased in leaves, while SA removes the effect. Magnesium content was substantially decreased in root and stem under the influence of Cd, SA attenuated the the effect of Cd only in roots, while in leaves it induces a significant increase of the content of this element. Cd uptake was not decreased by SA, but changes its distribution in plant organs depending on the concentration of added Cd. The result indicated that, the influence of SA on the alleviation of toxic effects of Cd was indirect, through a development of general antistress response of the seedlings which includes also the regulation of K and Mg distribution.

Panda and Choudhury (2005) reported the toxicity of chromium in plants. Chromium is known to be a toxic metal that can cause the severe damage to plants and animals. Chromium induced oxidative stress involves induction of lipid peroxidation in plant that cause the severe damage to cell membranes. Oxidative stress induced by chromium initiates the degradation of photosynthetic pigments causing decline in growth. High chromium concentration can disturb the chloroplast ultra structure thereby disturbing the photosynthetic process. Like Cu and Fe, Cr is also a redox metal and its redox behavior exceeds that of other metals like Co, Fe, Zn, Ni etc. The redox behavior can thus be attributed to the direct involvement of chromium in inducing oxidative stress in plants. Cr can affect the antioxidant metabolism in plants. Antioxidant enzymes like SOD, CAT, POX and GR are found to be susceptible to Cr resulting in a decline in their catalytic activities. However, both metallothioneins and organic acids are important in plants as components of tolerance mechanisms and are also involved in detoxification of this toxic metal.

Singh and Agarwal (2005) reported the effect of different heavy metal salts on the growth, yield and metal accumulation pattern of wheat (*Triticum aestivum*) cv. HD

2285. The studies revealed that, application of heavy metals in soil before sowing caused varying extent of reduction in yields of wheat. Mercury caused maximum reduction in biological as well as economic yields followed by copper, lead and cadmium, while zinc did not affect the growth and grain yield of wheat markedly. The number of spikes/pot and grains / spike were reduced, while 1000grain weight increased significantly by the application of copper, lead, and cadmium in soil. The heavy metal stress, however did not affect the harvest index of wheat plants. The content of all the tested metals increased both in straw and grain by their application in the soil, but their accumulation was much higher in vegetative shoots (straw) than in reproductive shoot (grain). However, zinc registered higher content in grain than in straw of wheat plants. The content of metals in wheat shoots was in the order of Zn > Cu > Cd > Pb. The larger proportion of both essential (Cu) and toxic metals (Pb and Cd) absorbed by wheat plants thus remained in straw and small proportion of the same only transported to edible part (grains).

Metwally *et al.*(2005) carried out an experiment to evaluate the correlation between selected biochemical responses to toxic C;d and the degree of Cd sensitivity in a set of pea (*Pisum sativum* L.) genotypes. Ten genotypes were analyzed that differ in their growth response to Cd when expressed as root or shoot tolerance indices (T/s). Concentrations of non protein thiols (NPTs) and malondialdehyde (MDA) , activity of chitinase ,peroxidase (POX), and catalase significantly increased in all genotypes of pea treated with Cd; Cd sensitivity genotypes was correlated with relative increase in MDA concentrations as well as activities of chitinase and POX , suggesting similar Cd stress effects. Activities of ascorbate peroxidase (APX) decreased, but concentrations of glutathione (GSH) increased in the less Cd-sensitive genotypes. Differences in root leaf contents of Cd revealed no change with TI, metabolic parameters, and enzyme activities in Cd treated plants, respectively, except that, shoot Cd concentration positively correlated with shoot chitinase activity. Toxic Cd levels inhibited the uptake of nutrient elements such as P, K, S, Ca, Zn, Mn, and B by plants in an organ and genotype specific manner. Cd sensitivity was significantly correlated with decreased root Zn concentrations. The results showed that both the similarities, as well as distinct features, in Cd toxicity expression in genotypes of one species, suggesting that independent and

multi-factorial reactions modulate Cd sensitivity on the low- tolerance level of plants. The study illustrates the biochemical basis of earlier detected genotypic variation in Cd response.

Ranieri *et al.* (2005) indicated that, the bread wheat (*Triticum aestivum* L.) cv. Albimonte can be defined as "shoot cadmium excluder" – by comparing the cadmium (Cd) content in leaves and roots and by calculating the shoot –to –root cadmium concentration ratio. Furthermore, they evaluated whether the excess Cd exposure could generate oxidative stress in leaves and roots of this cv., in terms of hydrogen peroxide (H_2O_2) accumulation , NAD(P)H oxidation rate , and variations in reduced glutathione (GSH) content and peroxidase (POD, EC 1.11.1.7) activity. Finally they surveyed possible quali-quantitative differences in thiol –peptide compound pattern between roots and leaves, in order to verify whether phytochelatins (PCs) and related thiol-peptides could contribute in limiting Cd –induced oxidative stress. Unambiguous characterization of PCs and related forms present in the root samples was obtained by electrospray ionization mass spectrometry (ESI-MS) and ESI –tandem MS (ESI-MS/MS). The result revealed that, in leaves the stress generated by the low accumulation of Cd (due to a moderate translocation in plant) seems to be counteracted by the antioxidant response and by the PC biosynthesis. On the contrary, in roots, in spite of the elevated presence of PCs and related thiol-peptide –compounds, the excess of Cd causes a decline in the antioxidant protection of the organ, with the consequent generation of considerable amounts of H_2O_2, which is a direct agent of the oxidative stress.

The influence of two heavy metal salts lead and cadmium ($Pb^{2+}$and Cd^{2+}) on plants, including plant and root size, plant genome stability as well as global genome expression were analyzed by Kovalchuk *et al.*(2005). Metal uptake was measured and showed that, there was a significantly higher incorporation of Cd than of Pb, 0.6 and 0.15uM 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 the contrary, 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 lead to a dose dependent increase of homologous recombination whereas Pb had no effect on the

same. Analysis of the global genome expression of plants chronically exposed to 50uM of Cd and Pb revealed 65 and 338 up and down regulated genes by Cd and 19 and 76 by Pb, respectively. The result indicated interestingly that, half of the genes that changed their expression in Pb- treated plants also changed their expression in Cd- treated plants. The greater number of genes regulated by Cd reflects generally higher genome instability of plants as well as higher uptake as compared to Pb.

Treatment of different concentrations of both hexavalent and trivalent chromium on *Azolla pinnata* resulted in significant biochemical and oxidative aberrations was investigated by Upadhyay and Panda (2005) .Total peroxide was increased, while lipid peroxidation decreased, as indicated by malondialdehyde formation after 48 hr of treatment. Ascorbate and glutathione contents increased under chromium treatment. Catalase activity was decreased whereas guaiacol peroxidase, glutathione reductase and superoxide dismutase were increased along with the period of metal treatment. These results suggest that, acute chromium toxicity induces oxidative damage in the plant.

In a study by Aina *et al.*(2006) rice seedlings were exposed to a range of Cd concentrations (0.1μM, 1 μM, 10 μM, 100 μM and 1mM) for 15 days and a combination of different molecular approaches were used to evidence Cd effects and to asses the plants ability to counteract metal toxicity. Only the highest concentration of Cd (1mM) caused a complete plant growth inhibition, whereas, the lowest concentrations seemed to stimulate the growth. At the 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 revealed the absence of preferential mutation sites. Plant responses were analyzed by measuring the levels of glutathione (GSH) and phytochelatins (PCs), the thiol –pepetide involved in heavy metal tolerance mechanisms. Result showed a progressive increase of GSH up to 10μM, of Cd treatment, whereas a significant induction only PC3 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 10μM, 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.

Page *et al.*(2006) carried out an experiment on seedlings of wheat (*Triticum aestivum* L.) and white lupin (*Lupinus albus* L.) radiolabelled for 24 hr with 65 Zn , 109 Cd, 54Mn, and 57Co via one seminal root (wheat) or via the main root (lupin). The plants were grown on rhizoboxes containing soil and the samples were collected throughout the experiment and was analysed afterwards for their radionuclide contents. A strong retention in the labeled part of the root was observed for 57Co in wheat and lupin and for 109 Cd in lupin, while 65 Zn and 54 Mn were transported to the shoot in both plants. 65 Zn was redistributed via the phloem from older to younger leaves, 54 Mn accumulated in the first leaves and no major redistribution within the shoot was observed. 109Cd was present in the shoot of lupin. The redistribution of 65Zn, 109Cd, 54Mn and 57 Co in phloem differed between wheat and lupin. The 65Zn content in the wheat roots appearing after the labeling phase represented 34% of the total content in the plant at the end of the experiment and less than 3% remained in the labeled root, while a high percentage of 65Zn was retained in the originally labeled part of the main root of lupin. The root system of wheat and lupin accumulated smaller quantities of 109 Cd, 54Mn and 57 Co. 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 labeled part of the roots. 65Zn was present in large quantities in the rhizosphere soil close to all parts of the root system of wheat. 65Zn, 109Cd, 54Mn, and 57Co were found in the bulk soil for both plants, 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 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.

The effects of different levels of industrial wastes on growth traits and metal accumulation in aerial portions of *Populus* x *euramericana* clone I-214 were evaluated by Giachetti and Sebastiani (2006) .The experiment was started in April 2003. Scions of *Populus* x *euramericana* clone I-214 were grown outdoor near Pisa (Italy), in lysimeters

filled with soil naturally present in the land around the experimental site. The climatic parameters were recorded daily throughout the whole experiment and growth relieves were performed during the growing season. The 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. After six months since the plantation of the scions, aerial portions of every plant were harvested for biomass and metal content analysis. The results indicated that the waste exerted beneficial effects on poplars mainly through a general increase of growth traits and that the nutrient relocation is the mechanisms were involved in modulating the growth rate. The concentration and the amount of the mineral elements were analyzed (N, P, K, Na, Ca, Mg, S, B, Fe, Mn, Cu, Zn, Cr) changed the determinately among treatments, organs and position. They concluded from the study that, phytoremediation strategies of tannery wastes might be possible and sustainable for the polar plantations in soil amended with non-hazardous levels of industrial waste, which maintain total heavy metals concentration close to the background levels.

Labra *et al.* (2006) carried out an experiment 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 germination of seeds was observed starting from 100-300 ppm up to 1500 ppm. The heavy metal Cr uptake was dependent on the concentration of the metal in the medium. They reported that the metallothioneins, involved in the binding of heavy metal, were measured by capillary electrophoresis (CE) , and showed a dose-response induction . Differential expressions of several proteins were analyzed by two dimensional gel electrophoresis for the protein profile study.Their results showed that, proteins induced by heavy metal exposure are principally involved in oxidative stress tolerance or in other stress pathways. Inductions of proteins were implicated in sugar metabolism. They inferred that the identification of factors involved in plant responses 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 different heavy metal stresses of Cu^{2+}, Zn^{2+}, Cd^{2+},and Pb^{2+} at different concentrations were studied by sand culture. The results showed that, with Cu^{2+}, Zn^{2+},

Cd^{2+} concentrations reaching 100 mg L^{-1}, both the seed germination rates and young – seedling heights of *Poa pratensis* declined to some extent and their decrements increased as the heavy metal concentration increased . Pb^{2+} did not show the significant effect on these two indexes. Cu^{2+} significantly inhibited the root and above ground biomasses and the growth of the root was also inhibited. Cu^{2+}concentrationof 600 mg L^{-1}, the root length was decreased by as high as 96.67%, compared with those in control. With Zn^{2+}, Cd^{2+} and Pb^{2+}concentrations going above 200 mg L^{-1} , the root and the above ground biomasses of the plant appeared to be inhibited , and the inhibitory effects became intensified as the concentration increased. The four heavy metals appeared undifferentiated in the action pattern of chlorophyll, i.e. they enhanced chlorophyll synthesis with their concentrations standing below 200 mg L^{-1} and the chlorophyll content was declined as their concentration continued to increase after reaching 200 mg L^{-1}.

One of the adverse effects of heavy metals on plants is the generation of harmful active oxygen species, leading to oxidative stress (Michalak, 2006). The results obtained by the authorshowed that, during the heavy metal stress phenolic compounds can act as metal chelators and on the other hand phenolics can directly scavenge molecular species of active oxygen. It was concluded from the study that, phenolics, especially flavonoids and phenylopropanoids, are oxidized by peroxidase, and act in H_2O_2 scavenging, phenolic/ASC/POX system.

The efficiencies of neutral salts, strong acids, and chelates for extracting cadmium from three paddy soils were examined by Makino *et al.*(2006). The test results showed that, higher the selectivity of 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 content 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 substantially decreased soil levels of exchangeable and acid –soluble Cd, which are the major forms of bioavailable Cd for rice (*Oryza sativa* L.).

Copper (Cu) from various anthropogenic and natural sources plays one of the major heavy metal contaminants in the environment was reported by Xiong *et al.*(2006). Cu induced nitrogen (N) metabolism damage in the popular vegetable Chinese cabbage (*Brassica pekinensis* Rupr.) were studied in aquatic culture experiments with this plant were done. For the aquatic culture experiment two Cu levels [0.3 μ mol L^{-1} (control) and 10.3 μ mol L^{-1}] and two N levels (0.05- and 1 fold Hogland's solution) were used. The results demonstrated the adverse effect of Cu on N metabolism and plant growth. Cu exposure elevated Cu concentration in the roots and shoots. The root length was also shortened and fewer leaves were produced and the biomass was lowered by the Cu exposure. The results were also demonstrated effects of N deficiency on N metabolism and plant growth. N deficiency increased the ratio of root/shoot 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 of N metabolism. The study revealed that, Cu exposure decreased nitrate reductase (NR) activity in the roots and shoots; the total chlorophyll content was also reduced. Treatment increased the total free amino acid content in the leaves and decreased the nitrate contents and NR activity in roots and leaves. In addition the interactive effects between Cu exposure and N level on chlorophyll and nitrate content in the leaves.

Pendergrass and Butcher (2006) conducted an experiment where carrots, lettuce, and tomatoes were cultivated in a greenhouse in control soil and soil with elevated levels of lead and arsenic. The samples were analysed 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±11μg g^{-1}, which represents a bioconcentration factor (BCF) of 0.03.

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. The total metal concentrations of plants and the associated soil samples were analyzed. 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. The 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. *Phyla nodiflora* was the most efficient in the accumulation of heavy metals like, Cu and Zn in 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). Three metal uptakes were highly correlated, whereas translocation of Pb was negatively correlated with Cu and Zn though translocation of Cu and Zn were correlated. From the result they concluded that, native plant species growing on contaminated sites may have the potential for phytoremediation.

In an experiment to study the effect of low levels of heavy metals on plant growth, biomass turnover and reproduction for *Hieracium pilosella*, plants were grown for 12 weeks on substrates with different concentrations of heavy metals obtained by diluting contaminated soils with silica and sand. The result showed that, the more metal-contaminated soil the substrate contained, the lower the leaf production rate and plant mass. The phonological development was also delayed. The flowering phenology was very sensitive to heavy 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 (Ryser and Sauder, 2006).

Stephen *et al.* (2006) conducted a study 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. Five plant species, bean (*Phaseolus vulgaris* L.), broccoli (*Brassica oleracea* L.), carrot (*Ducus carota* L.), pepper (*Capsicum annum* L.) and tomato (*Lycopersicon esculantum* 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 plants grown in sediment showed significantly greater biomass and yield as compared to plants from the reference soil. The elemental study of the tissues showed that Zn and Mo were only 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 result revealed that, this reclaimed sediment can be utilized for the production of vegetables intended for human consumption. The result also suggest that, sediment material with similar physicochemical characteristics and elemental concentrations that fall within the pertinent regulatory guidelines should also be a suitable safe medium for vegetable production.

Heavy metal contamination of soil resulting from waste water irrigation is a cause of serious concern. A potential health impact on consuming contaminated product was reported by Sharma *et al.* (2007). They analyzed the impact of waste water irrigation on heavy metal contamination of *Beta vulgaris*, a highly nutritious leafy vegetable that is widely cultivated and consumed in urban India. A field study was conducted at three major sites that were irrigated by either treated or untreated waste water in the suburban areas of Varanasi, India according to normal practice. Samples of irrigation water, soil, and the edible portion of the (*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. The result showed that, heavy metals in irrigation water were below the internationally recommended (WHO) maximum permissible limit set for agricultural use for all heavy metals except Cd at all the sites. 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 value. During summer, in the edible portion of the plant the Cd concentration was higher than the permissible limits of the Indian standards, 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 concentrations in the vegetable samples and in showed that, Zn in soil had a positive significant

relationship with vegetable contamination during winter. Cd, Cu, and Mn concentrations in soil and plant showed a significant positive relationship only during summer. Concentration of Cr and Pb during winter season and Zn and Ni during summer season showed significant negative relationship between soil and plant contamination. From the study they concluded that, the use of treated and untreated waste water for irrigation increased the contamination of Cd, Pb, and Ni in the edible portion of vegetables causing a potential health risk in the long term from this practice. They also noted that, adherence to standards for heavy metal contamination of soil and irrigation water does not ensure safe food.

Gianazza *et al.* (2007) conducted an experiment with seedlings of *Lepidium sativum* (L.). Exposure of the plant to increasing concentrations of Cd resulted in the growth inhibition and 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 the seeds. Analysis by ESI-MS alter two–dimensional electrophoresis showed that these proteins exibit sequences similar to those of storage proteins from various Cruciferae sp.According to the author 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 were present in higher amounts in plantlets exposed to Cd heat-shock, in agreements with literature data , and jasmonate like inducible protein are related to cellular stress and another two (LEAs or late embryogenesis abundant) are involved in embroyogenesis. Changes in protein expression can be detecded by two-dimentional 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.

The accumulation and distribution of arsenic and cadmium by tea plants were studied by Shi *et al.* (2008). The field investigation and pot trial, they found the low mobility of arsenic and cadmium in tea plants. Most arsenic and cadmium absorbed were fixed in feeding roots and only small amount was transported to the above-ground parts. Distribution of arsenic and cadmium, based on their concentrations of unit dry matter, in tea plants grown on un-contaminated soil was in the order: feeding

roots>stem> main roots>old leaves>young leaves. When tea plants were grown on polluted soils arsenic and cadmium were transported less to the above-ground parts. The concentration of cadmium in soil significantly and negatively correlated with chlorophyll content, photosynthetic rate, and transpiration rate and biomass production of tea plants.

Insecticide/ Fungicide

Photosynthesis inhibition of soybean leaves by insecticides was studied by Reheem *et al.* (1991). Field grown soybean cv. Williams-82 plants were sprayed with malathion or carbaryl formulations at 30, 60 and 90 days after planting. Net photosynthesis (PN) was measured in the control (water-sprayed) and pesticide-treated plants, 1, 3 and 7 days after treatment, with a LICOR 6200 Portable Photosynthesis System. After the first application the pesticide-treated plants showed a significant reduction (24% with malathion and 20% with carbaryl) in PN. The 60-day spray treatment PN suppression on day 1 and day 3 after treatment was the same as after the first application; but PN reached the same level as that of the water-sprayed control 7 days after treatment. After the 90-day treatment no change in PN was observed with the pesticide-treated plants compared to the control. These data indicate that malathion and carbaryl formulations may exert a detrimental influence on soybean physiology.

Pesticides (Brominal, Cuprosan and Fenvalerate) at 10 and 50 ppm suppressed growth, respiration and nitrogenase activity of*Azotobacter chroococcum, Azospirillum brasilense* and*Azospirillum lipoferum*. The inhibitory effect on respiration of *A. lipoferum* was most pronounced after 3 and 4 days of the pesticide application, studied by Omar and Alla (1992).

Phytotoxic effects of Benzimidazole fungicide on bedding plants were evaluated by Iersel (1996). Benzimidazoles are effective and widely used fungicides, but they may be phytotoxic. The effects of a single drench application of six benzimidazoles and one acetanilide fungicide on photosynthetic gas exchange, growth, development, and nutrient levels of four species of bedding plants in twenty growth –chambers and four greenhouses were studied. Daily carbon gain and carbon - use efficiency were calculated from continuous crop gas exchange measurements in the growth chambers.

The maximum labeled rate of Benlate DF caused a 7-to 10 day decrease in net photosynthesis and daily carbon gain in transplants of all species. It also caused pronounced interveinal chlorosis and a 2-to 3-day delay in flowering. Growth of Benlate DF –treated plants was reduced more at high (90%) than at low (60% to 80%) relative humidity. Benlate DF had severe effects on 2 week old petunia (*Petunia* x *hybrida*) seedlings in plug flats, reducing photosynthesis 25 % to 57%. Cleary's 3336 WP decreased photosynthesis in some traials Benlate DF reduced photosynthesis within 24 hr, but 3336 WP effects did not become apparent until 1 week after the treatment. This indicates different modes of inhibition. 3336 WP also caused leaf-tip and marginal chlorosis in impatiens (*Impatiens wallerana*). Mertect 340-F was extremely phytotoxic but is not labeled for drench applications (it was included because of its chemical similarities to other benzimidazoles). The only benzimidazole fungicide that did not reduce photosynthesis was Derosal, but it caused slight interveinal chlorosis in some studies with petunia. Leaf Ca levels decreased by Benlate DF and Derosal .Subdue (or metalaxyl), an acetanilide fungicide, did not affect photosynthesis or cause any visual symptoms. The results indicate that some benzimidazole fungicides can cause growth reductions and visual damage in bedding plants.

An experiment was carried out by Sudandara *et al.* (1996) to evaluate the genotoxic effect of an organophosphorous pesticide on *Allium* root meristems in *vivo*. The organophosphorous pesticide malathion not only induces damage to the chromosome but also reduce the frequency of cell division. The root tip cell of *Allium* exposed to malathion when post treated with *Phyllanthus* extract and distilled water could neither restore the normal mitotic index nor bring about reduction in the mitotic irregularities. However, the residual analysis of the treated and post treated cells showed the absence of pesticidal residues.

Fungicide action is generally assumed to be dependent on an antibiotic effect on a target pathogen, although a role for plant defense mechanisms as mediators of fungicide action has not been excluded. It was demonstrated by Molina *et al.* (1998) that in *Arabidopsis*, the innate plant defense mechanism contributes to the effectiveness of fungicides. In NahG and *nim1* (for noninducible immunity) *Arabidopsis* plants which normally exhibit increased susceptibility to pathogens, the fungicides metalaxyl, fosetyl,

and $Cu(OH)_2$ are much less active and fail to control *Peronospora parasitica*. However, the effectiveness of these fungicides is not altered in *Arabidopsis* mutants defective in the ethylene or jasmonic acid signal transduction pathways. Application of the systemic acquired resistance activator benzothiadiazole (BTH) in combination with these fungicides results in a synergistic effect on pathogen resistance in wild-type plants and an additive effect in NahG and BTH-unresponsive *nim1* plants. BTH treatment normally induces long-lasting pathogen protection; however, in NahG plants, the protection is transient. These observations suggest that BTH treatment can compensate only partially for an impaired signal transduction pathway and support the idea that pathogen defense mechanisms are under positive feedback control. These observations are strikingly reminiscent of the reduced efficacy of antifungal agents in immunocompromised animals.

Effect of herbicides on nodulation, symbiotic nitrogen fixation, growth and yield of pea (*Pisum sativum*) was studied by Sing and Wright (1999). Two pot experiments were performed to study the effects of three pre-emergence herbicides (terbutryn/ terbuthylazine, trietazine/simazine and prometryn) and a post-emergence herbicide (bentazone) on nodulation, symbiotic nitrogen fixation, growth and yield of pea (*Pisum sativum* L.) grown in perlite under nitrogen-free conditions. Decreased nodulation, total nitrogenase activity, net photosynthesis, leaf area, root and shoot dry weight, nitrogen content and seed yield of peas were observed in all pre-emergence herbicide treatment. The effects of herbicides increased with increase in rate of application. Among the herbicides tested, terbutryn/terbuthylazine and trietazine/ simazine had the greatest adverse effects. Pea plant biomass (root plus shoot) was correlated with plant nitrogen content but not total nitrogenase activity. The experiments suggested that the decreased growth of herbicide-treated plants was due to direct effects of the herbicides on peas and not due to indirect effects of the herbicides on rhizobia.

Effect of endosulfan and methylparathion on hydrolytic enzymes in germinating seed of jowar was investigated by Sabale and Misal (2000). A varied response of jowar (*Sorghum bicolor* L.) seeds was recorded under the influence of endosulfan and methylparathion with respect to the level of some hydrolytic enzymes during germination. The result revealed that lower dose of endosulfan (0.05, 0.1% v/v)

stimulated alpha amylase, protease, acid phosphatase and alkaline phosphatase activities.Wherease, methylparathion treatment suppressed amylase activity but markedly increased protease level at lower concentrations. Toxic concentrations of both the pesticides shifted the peaks of enzyme activities towards early hours of germination. In general treatment of methylprathion imposed a severe osmotic stress during germination of jowar seeds as compared to endosulfan.

Bisen and Hajra (2000) investigated the persistence and degradation of some insecticides in Darjeeling tea. A field trial was conducted during dry and wet seasons to understand the occurrence of residues, persistence, dissipation rate and half life values of six widely used insecticides at recommended doses / dilutions *viz.* Monocrotophos 36% SL, Malathion 50% EC , Fenvalerate 20% EC , Dimethonate 30% EC , Quinalphos 20% AF and Dicofol 18.5% EC in processed tea. The insecticides were applied as aqueous solutions at the dilution of 1:400 for all the insecticides except Fenvalerate which was applied @ 1: 4000 during dry and wet seasons. The initial deposits (4hrs) of different insecticides except Fenvalerate were found to be higher in dry season than wet season. No residue of monocrotophos after 4 hrs of its application was detected. The residue of malathion, fenvalerate, dimethonate on 5^{th} day after application were found below the permissible maximum residue limit.Wherease, in case of quinalphos it was observed on 7^{th} day after application. The residue of dicofol on 7^{th} day during wet season (0.03 ppm) was found below the tolerance limit. Dissipation followed a first order reaction in all cases and the half life values varied from 0.24 to 2.73 days. The results also indicate that, one round of plucking may be discarded in dry season when quinalphos and dicofol are applied on the tea bushes of Darjeeling.

Gupta and Tripathy (2000) studied the oxidative stress in cucumber (*Cucumis sativus* L) seedlings treated with acifluorfen. Treatment of diphenyl ether herbicide acifluorfen-Na (AF-Na) to intact cucumber (*Cucumis sativus* L cv.Poinsette) seedlings induced over accumulation of protoporphyrin IX in light (75 μmole $m^{-2}s^{-1}$). The extra – plastidic accumulation of protoporphyrin IX during the light exposure disappeared within two hrs transfer of acifluorofen –treated seedlings to darkness. This was due to re-entry of migrated protoporphyrin IX into the plastid and its subsequent conversion to protochlorophyllide. In light, protoporphyrin IX acted as a photosensitizer and caused

generation of active oxygen species. The latter caused damage to the cellular membrane lipids that resulted in production of malondialdehyde. Damage to the plastidic membranes resulted in damage to photosystem I and photosystem II reactions. Dark-incubation of herbicide-sprayed plants before their exposure to light enhanced photodynamic damage due to diffusion of the herbicide to the site of action. Compared to control, in treated samples the cation-induced increases in variable fluroscence maximum fluroscence ratio and increase in photosystem II activity was lower due to reduced grana stacking in herbicide-treated and light-exposed plants.

Residues and persistence of chloropyriphos in processed black tea was investigated by Manikandan *et al.* (2001). Field experiments were conducted in wet (September) and dry (February) seasons in 1998 and 1999 at Valparai (TamilNadu , India) to determine the residues of chlorophyriphos in black tea. Residue levels at different harvest intervals, persistence, and dissipation pattern and half-life values were calculated. The initial deposit of chloropyriphos residues on tea leaves was higher in wet season than in dry season. Residues of chloropyriphos dissipated exponentially after spraying during both the seasons and reached below the European Union tolerance limit of 0.1 ppm on 10^{th} day after application during wet season and 12^{th} day after application during dry season. Regression lines drawn for chloropyrifos showed that it followed the first order dissipation. Half –life values varied from 1.62-1.68 days for chloropyrifos and a safety harvest interval of 12 days is suggested.

Kalam and Mukherjee (2001) studied the influence of hexaconazole, carbofuran and ethion on soil microflora and dehydrogenase activities in soil and intact cell. The total microbial count was highly affected (up to 61% at 1000µg level) in presence of hexaconazole and persisted upto 21days. Bacteria were more susceptible than actinomycetes. Carbofuran and ethion were moderately toxic to soil microflora. Inhibitory effects of all the three pesticides gradually decreased after 21days as was evident by increase in total microbial count except carbofuran. GDH activity in soil was also affected initially (up to 14 days) by all the three pesticides (60.3% in hexaconazole at 1000µg level) and inhibition gradually decreased to zero except carbofuran (15-20% toxicity persisted up to 35 days). GDH and LDH activity in presence of hexaconazole was strongly affected in intact cells of some standard culture of bacteria like *Rhizobium*

sp.(host *Dolichos* sp., 32.1 and 72.5%) , *Bacillus subtilis* Cohn (86.75 and 76.5%), *Azotobactor* sp. (36.9 and 55.4%) and *B.sphaericus* (67.6% GDH) respectively. Carbofuran inhibited the enzyme activity in B.subtilis (55.55 and 35.3 %) and to some extent in *B.sphaericus*. Ethion moderately inhibited LDH activity in *Rhodococcus* sp. AK1 (17.1 and 33.3%), *Rhizobium* (27.6% LDH), *E.coli* HB 101(34.2% LDH) as evidenced by formazan formation. From the result it might be concluded that among the three pesticides tested hexaconazole strongly inhibited the dehydrogenase system in bacteria including nitrogen fixing bacteria of soil and thus may affect soil fertility. It was concluded that hexaconazole was more toxic than ethion to dehydrogenase enzymes.

Beneficial effects of fungicide seed treatments for soybean cultivars with partial resistance to *Phytophthora sojae* was investigated by Dorrance and Mc Clure (2001). *Phytophthora sojae* is a yield-limiting soybean pathogen in areas where soils remain saturated for long periods of time. *P. sojae* has been successfully managed with single dominant resistance genes (*Rps* genes). The proportion of fields with populations of *P. sojae* capable of causing susceptible interactions with many of the *Rps* genes has increased in number. The fungicides metalaxyl and mefenoxam have been used both as in-furrow and seed treatments to provide protection against damping-off caused by *P. sojae*. To determine the plant age when partial resistance and *Rps* genes are effective against *P. sojae*, author evaluated a greenhouse assay in which soybean seeds were planted and inoculated with a zoospore suspension to compare the disease reaction of soybean seeds and seedlings. Efficacy of different fungicide rates also was evaluated using the cultivar with partial resistance with this inoculation technique. Seeds and seedlings of a cultivar with high levels of partial resistance were susceptible to infection by *P. sojae* while those of a cultivar with an *Rps* gene were resistant. For the cultivar with partial resistance, reductions in percent emergence and the number of damped-off seedlings were significantly higher for plants inoculated at the day of planting compared to inoculations of plants with unifoliates present (5 days after planting). Results also indicated that fungicide seed treatment on cultivars with partial resistance may be beneficial when the environmental conditions that favor *P. sojae* infections occur prior to soybean emergence. This greenhouse assay appears to be useful in examining overall

fungicide efficacy; however, it did not detect consistent and quantifiable differences in rates of seed treatment fungicides.

Somara *et al.* (2002) conducted an experiment for the localization of identical organophosphorus pesticide degrading (*opd*) genes on genetically dissimilar indigenous plasmids of soil bacteria : PCR amplification , cloning and sequencing of *opd* gene from *Flavobacterium balustinum* . Plasmid borne organophosphorus degrading (*opd*) gene of *Flavobacterium balustinum* has been amplified using polymerase chain reaction (PCR) and the resulting PCR product (1.25 kb) was cloned in puc18. Further, a detailed restriction map was determined to PCR product and subcloned as overlapping restriction fragments. The nucleotide sequence was determined for all subclones to obtain complete sequence sequence of of PCR amplified fragment. The sequence showed 98% similarities to *opd* genes cloned from other soil bacteria isolated from diversified geographical regions. The protein sequence predicted from the nucleotide sequence was almost indentical to parathion hydrolase, a triesterase involved in hydrolysis of triester bond found in variety of op-pestisides. The signal sequence of parathion hydrolase contained recently discovered twin arginine transport (*tat*) motif. It appears *tat* motif plays a critical role in membrane targeting of parathion hydrolase.

Effects of systemic fungicides on protein, carbohydrate, amino acids and phenolic contents of susceptible (Mexipak) and resistant (Povan) Varieties of *Triticum aestivum* L.was studied by Siddiqui and Ahmed (2002). Application of systemic fungicides caused a significant ($P<0.001$) decrease in total protein and carbohydrate content compared to the control. MexiPak (susceptible) was more adversely affected than Povan (resistant). A substantial increase in total phenol was observed in the two varieties tested. Among the amino acids, proline, methionine, tyrosine and tryptophane were found in appreciable amounts.

Impact of fungicides on active oxygen species and antioxidant enzymes in spring barley (*Hordeum vulgare* L.) exposed to ozone were investigated by Wu and Tiedenmann (2002). Two modern fungicides, a strobilurin, azoxystrobin (AZO), and a triazole, epoxiconazole (EPO), applied as foliar spray on spring barley (*Hordeum vulgare* L. cv. Scarlett) 3 days prior to fumigation with injurious doses of ozone

(150–250 ppb; 5 days; 7 h/day) induced a 50–60% protection against ozone injury on leaves. Fungicide treatments of barley plants at growth stage (GS) 32 significantly increased the total leaf soluble protein content. Additionally, activities of the antioxidative enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate-peroxidase (APX) and glutathione reductase (GR) were increased by both fungicides at maximal rates of 16, 75, 51 and 144%, respectively. Guiacol-peroxidase (POX) activity was elevated by 50–110% only in AZO treated plants, while this effect was lacking after treatments with EPO. This coincided with elevated levels of hydrogen peroxide (H_2O_2) only in EPO and not in AZO treated plants. The enhancement of the plant antioxidative system by the two fungicides significantly and considerably reduced the level of superoxide ($O_2^{\cdot-}$) in leaves. Fumigation of barley plants for 4 days with non-injurious ozone doses (120–150 ppb, 7 h/day) markedly and immediately stimulated $O_2^{\cdot-}$ accumulation in leaves, while H_2O_2 was increased only after the third day of fumigation. Therefore, $O_2^{\cdot-}$ itself or as precursor of even more toxic oxyradicals appears to be more indicative for ozone-induced leaf damage than H_2O_2. Ozone also induced significant increases in the activity of antioxidant enzymes (SOD, POX and CAT) after 2 days of fumigation in fungicide untreated plants, while after 4 days of fumigation these enzymes declined to a level lower than in unfumigated plants, due to the oxidative degradation of leaf proteins. This is the first report demonstrating the marked enhancement of plant antioxidative enzymes and the enhanced scavenging of potentially harmful $O_2^{\cdot-}$ by fungicides as a mechanism of protecting plants against noxious oxidative stress from the environment. The antioxidant effect of modern fungicides widely used in intense cereal production in many countries represents an important factor when evaluating potential air pollution effects in agriculture.

Effect of herbicides on growth and development of *Oxalis latifolia* was studied by Pandey and Singh (2003). The efficacy of trifluralin and oxadiazon each at 0.5, 1.0 and 1.5 kg/ha as pre-emergence, as well as glyphosate and 2,4-D each at 0.5 kg/ha applied 30 days after sowing (DAS) and each at 1.0 kg/ha applied 30 and 45 DAS was evaluated on *O. latifolia* in pots under greenhouse condition. Trifluralin at all levels and oxadiazon at 1.5 kg/ha inhibited the formation of bulbils up to 60 days stage. Glyphosate at 0.5 or 1.0 kg/ha applied 30 DAS completely killed the foliage and there

was no formation of any leaf, inflorescence and bulbils till 60 days stage, whereas glyphosate at 1.0 kg/ha applied 45 DAS controlled the formation of bulbils and inflorescence up to 105 and 150 days stages, respectively. Glyphosate at 1.0 kg/ha applied 45 DAS proved the most effective in controlling the growth and development of *O. latifolia*. The higher rates of all the herbicides were more effective than the lower rates in reducing the growth and development of *O. latifolia*.

Physiological stress responses of *Vitis vinifera* L. to the fungicides fludioxonil and pyrimethanil was investivated by Saladin *et al.*(2003) The effects of the fungicides fludioxonil and pyrimethanil were evaluated on grapevine leaves using in vitro-grown plantlets, fruiting cuttings, and plants grown in vineyards. *In vitro*, both water content and osmotic potential decreased in treated leaves. Moreover, carbohydrate accumulated, suggesting that plantlets could react to the stress through an active osmoregulation process by uptaking sugars from the medium. Besides, pyrimethanil stimulated the accumulation of proteins, whereas no significant effect was observed using fludioxonil. The cuttings exhibited similar responses than *in vitro* though they appeared to be more tolerant since half of the studied parameters recovered 10 days after treatment. In vineyard, both fungicides modified leaf water content and carbohydrate levels, whereas nitrogenous compounds accumulated transiently. These results suggest that in vineyard-grown plants, a strong sugar translocation from mature leaves to sink organs occurs transiently, as well as a protein synthesis and a stimulation of soil nitrogen uptake.

Water stress and glyphosate treatments to glyphosate-resistant (GR) cotton (*Gossypium hirsutum* L.) can cause abscission of young bolls although the interaction of these factors is not well defined. Studies were conducted by Pline *et al.* (2003) to quantify the effects of water stress and glyphosate treatments on fruit retention, fruit placement, and carbohydrate partitioning in GR and conventional cotton varieties grown in a phytotron environment. Glyphosate-resistant plants treated with glyphosate at the four-leaf stage, postemergence (POST), and at the eight-leaf stage, POST-directed (PDIR), had fewer first-position bolls after 0 and 1 d of water stress than nontreated GR and conventional plants but did not differ after 2 and 3 d of water stress. Glyphosate-treated GR plants reached first bloom 3 to 4 d later than nontreated plants. Five-day-old bolls from plants of one genotype, SG 125RR, treated with glyphosate had lower

fructose content than bolls from nontreated plants. Subtending leaf carbohydrates and boll sucrose, glucose, and starch content did not differ after glyphosate treatments. Increasing water stress caused reductions in subtending leaf glucose, sucrose, and starch content, as well as reductions in boll starch and sucrose content. Reductions in boll starch and sucrose content in response to water stress may indicate the potential for abscission. Water stress and glyphosate treatments to GR cotton do not alter carbohydrate profiles in boll or leaf tissues in a like manner. Differences in carbohydrate profiles of young bolls and leaves from glyphosate-treated and water-stressed cotton plants suggest that water stress and glyphosate treatments may promote fruit abscission in different manners.

The effects of triazole and strobilurin fungicide programmes on nitrogen uptake, partitioning,remobilization and grain N accumulationin winter wheat cultivars was syudied by Ruske *et al.*(2003). Field experiments were conducted over 3 years to assess the effect of a triazole fungicide programme, and additions of strobilurin fungicides to it, on nitrogen uptake, accumulation and partitioning in a range of winter wheat cultivars. Commensurate with delayed senescence, fungicide programmes, particularly when including strobilurins, improved grain yield through improvements in both crop biomass and harvest index, although the relationship with green area duration of the flag leaf (GFLAD) depended on year and in some cases, cultivar. In all years fungicide treatments significantly increased the amount of nitrogen in the above-ground biomass, the amount of nitrogen in the grain and the nitrogen harvest index. All these effects could be linearly related to the fungicide effect on GFLAD. These relationships occasionally interacted with cultivar but there was no evidence that fungicide mode of action affected the relationship between GFLAD and yield of nitrogen in the grain. Fungicide treatments significantly reduced the amount of soil mineral N at harvest and when severe disease had been controlled, the net remobilization of N from the vegetation to the grain after anthesis.Fungicide maintained the filling of grain with both dry matter and nitrogen. The proportionate accumulation of nitrogen in the grain was later than that of dry matter and this difference was greater when fungicide had been applied. Effects of fungicide on grain protein concentration and its relationship with GFLAD were inconsistent over year and cultivar. There were several instances where

grain protein concentration was unaffected despite large (1.5 t/ha) increases in grain yield following fungicide use. Dilution of grain protein concentration following fungicide use, when it did occur, was small compared with what would be predicted by adoption of other yield increasing techniques such as the selection of high yielding cultivars (based on currently available cultivars) or by growing wheat in favourable climates.

The effective durations of pesticide-induced susceptibility of rice to brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae), and physiological and biochemical changes in rice plants following pesticide applications, were studied. The effective duration of the herbicide butachlor on the susceptibility of rice variety Zhengdao 2 to BPH exceeded 16 days. The difference in damage rating between rice plants with pesticide treatments and control plants gradually decreased with days after treatment (DAT). There was a significant correlation between damage rating and DAT. The number of rice tillers at 3 - 10 DAT and plant heights at 7-10 DAT declined on Zhengdao 2 with the butachlor treatment. On the other hand, there were no effects of butachlor on damage rating, number of rice tillers and plant height for Xiushui 63. This might be related to tolerance of Xiushui 63 to butachlor. For Zhengdao 2, the fungicide jingganmycin and the insecticide bisultap had a significant influence on BPH damage, number of rice tillers and plant height. In contrast to butachlor, jingganmycin and bisultap had a significant effect on BPH damage to Xiushui 63. However, these two pesticides had no significant effect on the number of rice tillers and plant height of Xiushui 63. In general, the effective duration of butachlor on rice plants was greater than jingganmycin and bisultap. Oxalic acid content and photosynthesis in rice plants declined significantly after jingganmycin and bisultap treatments. These findings are consistent with those of pesticide-susceptibility of rice to BPH. Pesticide-induced susceptibility of rice resistance to BPH counteracts the role of varietal resistance in integrated pest management, (Wu *et al.* 2004).

Effects of fungicide and insecticide mixtures on apple tree canopy photosynthesis, dark respiration and carbon economy were investigated by Untiedt and Blanke (2004). Fungicide/insecticide mixtures were applied at times and doses commonly used in commercial orchard practice. Their effects on photosynthesis and

dark respiration were evaluated in two seasons with respect to the potential stress they impose on an apple tree using cv. 'Elstar'. The mixtures included the fungicides mancozeb, flusilazol and dithianon, and the insecticides oxydemeton-methyl or pirimicarb. A new technology was employed to continuously examine photosynthesis, dark respiration and carbon balance of apple trees based on six canopy chambers, which enclosed apple trees under natural conditions in the field, with on-line measurements and continuousanalysis of CO_2 exchange and automated data acquisition.The fungicides mancozeb and flusilazol combined with the insecticide oxydemeton-methyl reduced whole tree canopy CO_2 assimilation mostly at midday and, using hourly means, by an averaged 7.4% on the day of its application. This reduction in whole canopy photosynthesis declined with time, restoring most of the original photosynthetic potential within 3–5% in 3 days, hence, indicating acceptable phytotoxicity. This fungicide/insecticide mixture overproportionally, in relation to the changes in photosynthesis, increased dark respiration by up to 72% in the night after application, thereby drastically affecting the tree's carbon balance in an adverse way.In contrast; the fungicide dithianon combined with the insecticide pirimicarb decreased dark respiration by 15–21% with reductions in canopy photosynthesis in the order of 6–9%. Because the decrease in dark respiration exceeded that in photosynthesis, the apple tree overall gained carbon in a balance.Overall, effects on photosynthesis were smaller than on dark respiration. The effects of the pesticide combinations on photosynthesis are attributed to the CO_2-independent Hill reaction in photosynthesis and to uncoupling the photosynthetic electron flow from phosphorylation, thereby inhibiting energy, viz. ATP formation or its transfer, rendering dissociation of ATP into ADP and P_i.

Two-way effect of pesticides on zeatin riboside content in both rice leaves and roots were studied by Hua Qiu *et al.* (2004). Cytokinins zeatins including zeatins riboside (ZR) play a vital regulation role in growth, development, physiology and biochemistry of rice plant. The effect of four commonly used pesticides in paddy fields on the ZR contents in rice leaves and roots was investigated using Enzyme-Linked Immunosorbent Assays (ELISA). Experimental rice plants were grown under hydroponics culture conditions, and subjected to a foliar spray or root treatment with different concentrations of these pesticides. Zeatin riboside content in rice leaves

decreased significantly three days after foliar sprays (3 DAS) with 150 and 300 ppm buprofezin, 30 and 60 ppm imidacloprid, 200 ppm jinganmycin, and 480 ppm triazophos. At 7 DAS a significant reduction occurred irrespective of the pesticide concentration. The ZR content in rice roots did not change so dramatically as in rice leaves. At 3 DAS, it was reduced significantly only in the plants subjected to a foliar spray with 100 ppm jinganmycin or significantly increased in the plants sprayed with 480 ppm triazophos, while at 7 DAS, there were no significant differences in ZR content under all circumstances. When subjected to root treatment with these pesticides, rice plants were extremely sensitive to triazophos and even wilted three days after the treatments (3 DART). Root treatment with 150 ppm buprofezin, 100 ppm jinganmycin, 60 ppm imidacloprid, respectively, caused a significant reduction in ZR contents in rice leaves; however, all treatments except with triazophos did not reduce ZR contents in rice roots significantly. Seven days after foliar sprays with the pesticide mixtures, i.e. triazophos+imidacloprid and triazophos+buprofezin. ZR content significantly reduced in rice leaves but not in roots.

Herbicidal and antioxidant defense responses of transgenic rice plants that overexpressed *Myxococcus xanthus* protoporphyrinogen oxidase gene was studied by Jung and Back (2005). Leaf squares of the wild-type incubated with oxyfluorfen were characterized by nectrotic leaf lesions and increase in conductivity and malonyldialdehyde levels, whereas transgenic lines M4 and M7 did not show any change with up to 100μM oxyfluorfen. The wild -type had decreased F_v/F_m and produced a high level of H_2O_2 at 18 hr after foliar application of oxyfluorfen, whereas transgenic lines M4 and M7 were unaffected. In response to oxyfluorfen, violaxanthin, β-carotene, and chlorophylls (Chls) decreased in wild –type plants, whereas antheraxanthin and zeaxanthin increased. Only a slight decline in Chls was observed in transgenic lines at 48 hr after oxyfluorfen treatment. Noticeable increases of Cu/Zn – superoxide dismutase, peroxidase isozymes 1and 2, and catalase were observed after at 48hr of oxyfluorfen treatment in the wild-type. Non-enzymatic antioxidants appeared to respond faster to oxyfluorfen-induced photodynamic stress than did enzymatic antioxidants. Protective responses for the detoxification of active oxygen species were induced to counteract photodynamic stress in oxyfluorfen-treated, wild type plants.

However, oxyfluorfen-treated, transgenic plants suffered less oxidative stress, confirming increased herbicidal resistance resulted from dual expression of *M.xanthus* Protox in chloroplasts and mitochondria.

Phytotoxicity of copper fungicides viz. Bordeaux mixture , stabilized Bordeaux mixture and copper oxychloride were found phytotoxic to guava fruits , while carbendazim,benomyl and mancozeb were non phytotoxic . Bordeaux mixture was highly phytotoxic and caused heavy russetting, followed by stabilized Bordeaux mixture and then copper oxychloride. The higher concentrations of these fungicides were more toxic than their lower doses. The russetted fruits failed to attain normal size and thus reduced the quality of fruits to a greater extent, Gaikwad and Nimbalkar (2005).

Changes of antioxidants levels in two maize lines following atrazine, a photosynthetic herbicide were studied by Alla and Hassan (2005). Growth and antioxidants levels of shoot of 10-d-old maize lines (*Zea mays* L.Hybrid 351 and Giza 2) differentially responded to atrazine treatment at the recommended field dose (RFD) during the following 20 d. Atrazine significantly reduced shoot fresh and dry weights but significantly accumulated H_2O_2 , lipid peroxides and carbonyl groups in Giza 2 during the whole experiment ; an effect that, prolonged with either elapse of time or increasing the herbicide dose. Mean while , ascorbic acid (AsA) and reduced glutathione (GSH) contents were significantly decreased along with significant inhibitions in activities of superoxide dismutase (SOD; EC 1.15.1.1) , catalase (CAT; EC 1.11.1.6) , ascorbate peroxidase (APX; EC 1.11.1.7) , guaiacol peroxidase (GPX; EC 1.11.1.7) , and glutathione-S-transferase (GST ; EC 2.5.1.18). Similar responses were observed in Hybrid 351 only during the first 12 d, and seemed to be overcome thereafter. These results indicate that an induced oxidative stress in maize following atrazine treatments. Such state appeared to be counterbalanced in Hybrid 351 but continued in Giza 2 concluding Giza 2 as more susceptible to atrazine than Hybrid 351. Therefore, the differential susceptibility of Giza 2 to atrazine is related to deficiency in antioxidant levels.

Effect of Metasystox Application on Cottonseeds Quality was evaluated by Osman *et al.* (2006). Two field experiments were carried out at the Agricultural Farm of the Faculty of Agriculture, University of Khartoum to study the effect of the insecticide Metasystox on cottonseed quality of two local cultivars, Barakat-90 and Barac-67. Three levels of concentrations of this insecticide recommended dose, 1.5 of the recommended dose and 2 fold of the recommended dose were applied on field grown cotton. Oil, protein, phytic acid, and minerals content of cottonseeds were determined. The results showed significant increase in cottonseed oil of Barakat-90 and Barac-67, as influenced by different levels of treatments. Protein content increased significantly in cottonseeds of both cultivars. In contrast, the results of phytic acid, showed no significant difference in Barakat-90 cultivar. However, significant reduction was observed in Barac-67 cultivar. The value of mineral content of both cultivars has no consistent pattern of change.

The effects of glyphosate on protein metabolism, mesophyll cell ultrastructure and nodule ultrastructure and functioning of *Lupinus albus* cv. was investigated by Maria *et al.*(2007). Multolupa inoculated with *Bradyrhizobium* sp. (*Lupinus*) were investigated by them. Young leaves and nodules were especially affected because these organs act as sinks of herbicide. The alterations on nodular and chloroplast ultrasturcture varied depending on herbicide concentration and the time of exposure. After 3 days of 2.5 mM glyphosate application some toxic effects were detected. The most important alterations on nodules were the progressive cellular degradation of plant and bacteroidal cytosol and the rupture of bacteroidal membrane, whilst the peribacteroid membrane of the symbiosomes was preserved. This is the first report on the effect of glyphosate on legume-nodule ultrastructure. Glyphosate inhibited *B.*sp.(*Lupinus*) growth at concentrations higher than 62.5μM . In the mesophyll cells, gradual disorganization of grana and intergrana was observed, loosing the parallel alignment with the chloroplast axis. As in nodules, degradation of membrane systems was observed, with the deformation, and even the rupture, of the tonoplast. These progressive effects were similar to those described in senescence process. The adverse effects produced on infected zone can be due both to a direct effect of the herbicide on microsymbiont and to an indirect effect of glyphostate action on photosynthetic

apparatus. Glyphossate produced changes in nodule cytosol and bacteroid proteins content and polypeptide pattern of leaves and nodules. With respect to proteins related to the oxygen diffusion mechanism, a large decrease in leghemoglobin and glycoproteins (recognized by antibodies MAC 236 and MAC 265) content was detected, which suggests that the oxygen diffusion mechanisms were also affected by glyphosate.

Strobilurin fungicides induce changes in photosynthetic gas exchange that do not improve water use efficiency of plants grown under conditions of water stress was studied by Nason *et al.* (2007). The effects of five strobilurin (beta-methoxyacrylate) fungicides and one triazole fungicide on the physiological parameters of well-watered or water-stressed wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and soya (*Glycine max* Merr.) plants were compared. Water use efficiency (WUE) (the ratio of rate of transpiration, E, to net rate of photosynthesis, A_n) of well-watered wheat plants was improved slightly by strobilurin fungicides, but was reduced in water-stressed plants, so there is limited scope for using strobilurins to improve the water status of crops grown under conditions of drought. The different strobilurin fungicides had similar effects on plant physiology but differed in persistence and potency. When applied to whole plants using a spray gun, they reduced the conductance of water through the epidermis (stomatal and cuticular transpiration), g_{sw}, of leaves. Concomitantly, leaves of treated plants had a lower rate of transpiration, E, a lower intercellular carbon dioxide concentration, c_i, and a lower net rate of photosynthesis, A_n, compared with leaves of control plants or plants treated with the triazole. According to the authors The mechanism for the photosynthetic effects is not known, but it is hypothesised that they are caused either by strobilurin fungicides acting directly on ATP production in guard cell mitochondria or by stomata responding to strobilurin-induced changes in mesophyll photosynthesis. The latter may be important since, for leaves of soya plants, the chlorophyll fluorescence parameter F_v/F_m (an indication of the potential quantum efficiency of PSII photochemistry) was reduced by strobilurin fungicides. It is likely that the response of stomata to strobilurin fungicides is complex, and further research is required to elucidate the different biochemical pathways involved.

Materials & Methods

3.1. Plant Material

3.1.1. Collection

Fresh tea clones were collected mainly from two experimental stations of different geographical locations in India, which were maintained in the Germplasm Bank at Department of Botany, North Bengal University and were used for experimental purposes. The clonal cuttings were collected from a) Tocklai Experimental Station, Jorhat, Assam , and b) Darjeeling Tea Research Centre, Kurseong.West Bengal.

3.1.2. Propagation

Tea plants are generally propagated by cuttings or by seeds. For propagation by cuttings, plants were raised from the shoots of elite mother plants. Tea cuttings with good mature leaf and having a stem size of 2.5cm to 3.5cm were selected for planting. As soil preparation is the most important part in propagation technique, so immense care was taken to prepare soil appropriately.

Sandy soil (sand 75% and soil 25%) with a pH ranging from 4.5- 4.8 was used for propagation of tea plants by cuttings. Soil pH was adjusted by treating the soil with 2% aluminium sulphate solution. It was followed by applying two watering to remove the excess aluminium sulphate .The treated soil was heated between 60^0-80 ^{0}C in fire on a metal sheet to kill eelworms, if present in the soil.

Polythene sleeves (8"x6") were filled up with the prepared soil and stacked in rows in a bed and sprinkled with water thoroughly. All cuttings were planted directly into the sleeves after dipping them in rooting hormone. These cuttings (Plate III) were covered with a polethene cloche and watered every 3rd or 4th day as per requirement until the appearance of new leaf. The whole setup was kept under a green agro house.

3.1.3. Plantation

Experimental plots were prepared before plantation.Simazine@75gm /20lit water and Glyophosphate@1:200were used for weed control (Borpujari and Bannerjee, 1994). Then pits (.45m X .45 m X .45m) were dug at the intervals of 5cm between plants and 9 cm between rows to row. Planting mixture were prepared in the ratio of 4.5 kg well

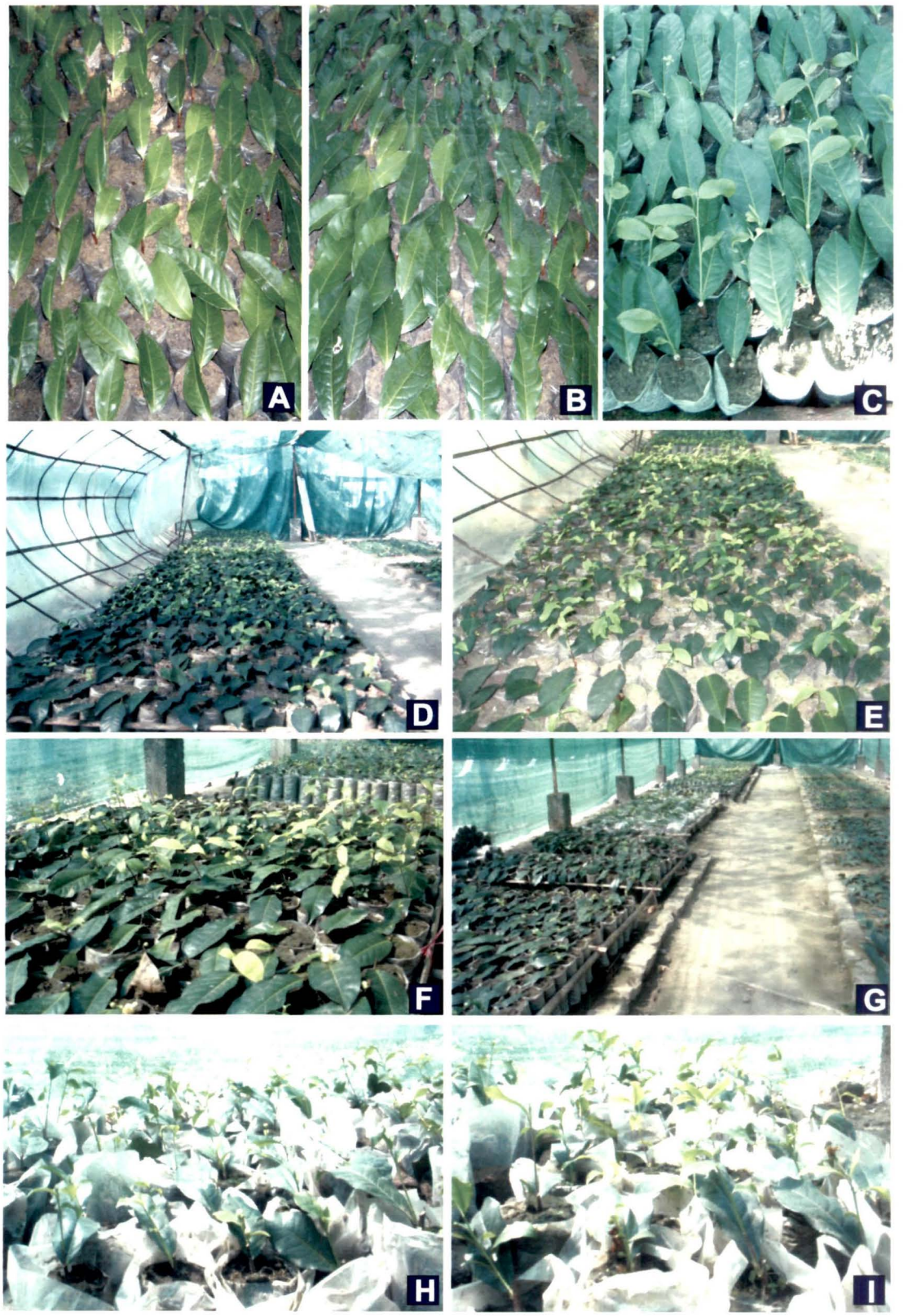


Plate III (A-I): Tea Nursery showing different stages of propagation.

rotten dry cattle manure, 30kg rock phosphate, 30kg superphosphate, and 2.5 gm phorate [O,O-diethyl S- (ethylthiomethyl) phosphorodithoioate]. Rock phosphate was placed at the bottom of each pit following which half portion was covered with cattle manure and soil mixture. Phorate was mixed with a portion of excavated soil and was applied approximately 5cm below the ground level.

Following the soil conditioning, the plants were inspected, selected and brought to the experimental field and planted in the prepared soil and pits were refilled with the conditioned soil upto the ground level. Well drained, deep and friable loam, heavily mulched rich in organic matter having pH 4 to 6, low in calciuim and generally rich in iron and manganese is considered ideal soil for the tea plantation.

Tea plants of different varieties were also grown in earthen pots (one plant per pot of 30cm diameter) each pot containing 5kg of prepared soil mixture (soil:planting mixture1:1).

All the pots were maintained in the glass house under natural condition (PlateIV).

Ten months old seedlings with well developed shoot and root system were transferred from the sleeves to the pots. Careful attention was paid to the seedlings to produce healthy plants.These were then maintained both in glasshouse and experimental garden under natural condition with regular watering.

3.1.4. Maintenance

Polythene cloche were removed from every bed when new shoots begin to appear, then they were treated with manure (aluminium phosphate 8 parts by weight, ammonium phosphate sulphate 16:20 -35 parts by weight, magnesium sulphate and zinc sulphate 3 parts by weight) was done after rooting following the method of Ranganathan and Natesan (1987) and continued upto 12 months once in 15 days. The mixture was dissolved @30gm in 1 lit of water and applied @50ml/plant.

The mature plants (1year and above) were maintained by using a manure of N,P,K consisting of 10 kg urea (46%N), 20kg ammonium phosphate (11% P_2O_5), 8kg muriate of potash (60%K_2O) in the soil at a regular interval . Miraculin (7ml /10lit) was

Plate IV (A-B): Tea varieties being grown in pots for experimental purposes

Plate V: Healthy tea bushes in experimental garden

sprayed in the field for the better growth of the bush. Watering was also applied at a regular interval. (Plate V).

In young plants of 3yrs tipping was done once in a year to promote lateral branching; but in case of mature plants two year of deep pruning cycle was maintained.

3.2. Application of chemicals

3.2.1. Heavy metals

A) Selection of heavy metals

The heavy metal compounds selected for the study were Copper sulphate 5-hydrate [$CuSO_4$, $5H_2O$] and Cadmium nitrate 4-hydrate [Cd (NO_3), 4 H_2O].

B) Application of chemicals

Solution of $CuSO_4$, $5H_2O$ and Cd (NO_3), 4 H_2O were prepared at concentration of 100µg / ml, 500 µg / ml and 1000 µg / ml. These solutions were applied in two ways either to detached shoots of well grown bushes i.e. *in vitro* or to the young intact seedlings i.e. *in vivo* of tea plants of different varieties at different intervals. For control plants water was applied.

3.2.1.1. *In vitro*

Heavy metal treatments were given to 10 varieties of tea i.e. TV-27, TV-23, TV-26, TV-30, TV-29, TV-28, TV-22, TV-18, HV-39, and T-78 collected from Tocklai Experimental Station, Jorhat, Assam, and Darjeeling Tea Research Centre; Kurseong.West Bengal.Very young shoots (first four leaves) were collected from healthy bushes of the experimental garden of Department of Botany and immeserd immediately with the different concentration of heavy metals and studied at 48 hr interval.

3.2.1.2. *In vivo*

Intact young plants maintained in pot (two year old nursery seedlings) of 8 varieties of TV-27, TV-23, TV-26, TV-30, TV-29, TV-28, HV-39, and T-78 were subjected to different concentration of heavy metal solutions at a definite time interval.

3.2.2. Fungicide/ insecticide

A) Selection of Chemicals

One each of commonly used fungicide and insecticide applied in tea garden were selected for the study.

Fungicide

Hexaconazole is the one of the most common fungicide applied in the tea garden for the prevention of various types of fungal diseases.

Insecticide

Acephate is one of the most commonly used organophosphate foliar insecticides in tea garden for the prevention of different kind of insecticidal problems like, aphids, leaf miners, caterpillars, sawflies and thrips.

B) Application of chemicals

Fungicide

Hexaconazole ,the common fungicide applied at 0.1% (normally applied by the planters in tea garden) concentration in intact young plants maintained in pot (two year old nursery seedlings) of 8 varieties of TV-27, TV-23, TV-26, TV-30, TV-29, TV-28, HV-39, and T-78 with mist sprayer at an interval of 7 days.

Solutions were also sprayed at the full grown bushes of two varieties TV-23 and, HV-39 with mist sprayer at an interval of 7days.

Insecticide

Acephate was sprayed at 1:400 ratios on the potted plants and full grown bushes of the said varieties in the same way as mentioned above.

3.3. Extraction and quantification of phenols

3.3.1. Extraction

Phenols were extracted from tea leaves following the method of Mahadevan and Sridhar (1982). 1gm of leaf tissues were cut into pieces and immersed immediately into 10ml boiling absolute alcohol for 15min. After boiling it was cooled and crushed in

mortar with pestle. The extract was passed through two layers of cheesecloth and then filtered through Whatman No.1 filter paper. Final volume was adjusted with 80% ethanol (5ml / gm fresh weight of leaves). The total extraction procedure was done in dark to prevent any light induced degradation of phenol.

3.3.2. Estimation

3.3.2.1. Total phenol

The total phenol estimation was done following the method of Mahadevan and Sridhar (1982). To 1ml of alcoholic extract, 1ml of 1 N Folin- Ciocalteau reagent and 2ml 20% sodium carbonate solution was added in a test tube. The test tube was shaken and heated in boiling water bath for 1min. After cooling the reaction mixture, volume was raised to 25ml. Absorbance of the blue coloured solution was measured at 650nm in a systronic photoelectric colorimeter Model 101. Quantity of the total phenol was estimated using caffeic acid as standard.

3.3.2.2. O-dihydroxy phenol

The O-dihydroxy phenol was estimated following the method of Mahadevan and Sridhar (1982). 1ml of alcholic extract was mixed with with 2ml of 0.05 N HCl , 1ml of Arnow's reagent ($NaNO_2$ -10gm, Na_2MO_4 -10gm , distilled water -100ml) ,and 2ml of 1N NaOH were mixed thoroughly, following which the volume of the reaction mixture was raised to 10ml. Absorbance of pink coloured solution was recorded at 515nm . Quantity of the O-dihydroxy phenol was estimated by using caffeic acid solution as standard.

3.4. Extraction and estimation of free proline

3.4.1. Extraction

For the extraction of free proline the method of Bates *et al.* (1973) was followed. 1gm of plant tissue was crushed with 3% sulphosalycylic acid in mortar with pestle. The slurry was then filtered through Whatman No.1 filter paper at room temperature. The filtrate was collected and stored at 4^0C for further analysis.

3.4.2. Estimation

Proline content of the extract was estimated by following the method of Bates *et al* (1973) with some modification. To 1ml of extract, 3ml of distilled water and 1ml of Ninhydrin solution (2gm in 50ml acetone and water mixture) were added. Then the mixture was kept in a boiling water bath for 15min. After cooling the reaction mixture was poured in a separating funnel and 5ml of toluene was added and mixed vigorously. Lower colour layer was taken and O.D values were measured at 520nm. Quantification was done from a standard curve of proline.

3.5. Protein analysis

3.5.1. Quantification of protein

Extraction

Soluble protein was extracted from healthy and treated leaves, following the method of Chakraborty *et al.* (1995).Plant tissues (1gm) were ground in liquid nitrogen and crushed with 0.05M Sodium phosphate buffer (pH 7.2) containing 10mM $Na_2S_2O_5$,0.5 mM $MgCl_2$, 2mM soluble Polyvinyl pyrolidone (PVPP 10,000M), and 2mM poly methyl sulphonyl fluoride (PMSF),sea sand , insoluble PVPPwas added during crushing in mortar with pestle at ice cold condition .The homogenate was centrifuged at 4^0C for 20min at 10,000 r.p.m. The supernatant was used as crude protein extract and stored immediately in ice cold condition for the further analysis.

Estimation

Soluble protein content was estimated by following the method of Lowry *et al* (1951).To 1ml test solution 5ml of alkaline reagent (1ml of 1% $CuSO_4$, and 1ml of 2% sodium potassium tertarate, was added to 100 ml of 2% Na_2CO_3 in 0.1N NaOH) was added . This mixture was incubated at room temperature for 15 min then 0.5 ml of 1N Folin Ciocalteau reagent was added and mixed thoroughly and was measured at 720nm. Soluble protein content was estimated from the standard curve made with BSA (bovine serum albumin).

3.5.2. SDS- PAGE analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for detail analysis of protein profile. Total soluble proteins were extracted in 0.05 M sodium phosphate buffer, used as crude protein extract for 10% SDS-PAGE analysis following the method of Sambrook *et al.*(1989). Protein samples were loaded on the well of the gel and run for 3hrs at 200V and 15-20mA current. After completion of electrophoresis the gel was kept in fixer, stained in Coomassie Brilliant Blue (R-250) solution and finally kept in destain solution of methanol, glacial acetic acid and water (4.5: 4.5: 1).

3.5.2.1. Preparation of stock solution

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

(A) Acrylamide and N'N'-methelene bis acryl amide

A stock solution containing 29% acrylamide and 1% bisacrylamide was prepared in water. As both of the chemicals are slowly deaminated to acrylic and bisacrylic acid by alkali and light the pH of the solution was kept below 7.0.The stock solution was filtered through Whatman No. 1 filter paper, was kept in brown bottle and stored at 4^0 C and used within one month.

(B) Sodium Dodecyl sulphate (SDS)

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

(C) Tris buffer

a) 1.5 M Tris buffer was prepared for resolving gel. The pH of the solution was adjusted to 8.8 with conc. HCl and stored at 4^0C for further use.

b) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was made 6.8 by using conc HCl and stored at 4^0 C.

(D) Ammonium Persulphate (APS)

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

(E) Tris –Glycine electrophoresis buffer

Tris –Glycine running buffer consists of 25mM Tris base, 250mM Glycine (pH8.3) and 0.1% SDS. A 1X solution was made by dissolving 3.02gm Tris base, 18.8 gm Glycine and 10 ml of 10%SDS in 1L of distilled water.

(F) SDS gel loading buffer

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

3.5.2.2. Preparation of gel

For the analysis of protein patterns by SDS-PAGE mini slab gel (plate size 8cm X 10 cm) was prepared. For the preparation of gel first two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and dried. Then 1.5 mm thick spacers were placed between the glass plates at three sides and sealed thoroughly with high vacuum grease and clipped tightly to prevent any leakage of the gel solution during pouring. Resolving and stacking gel solution were prepared by mixing compounds in the following order and the solution were poured by Pasture pipette leaving sufficient space for comb in the stacking gel(comb + 1cm).

Composition of solutions

10% resolving gel

Name of the compound	Amount (ml)
Distilled water	2.85
30% acrylamide	2.55
1.5 M Tris (pH8.8)	1.95
10% SDS	0.075
10% APS	0.075
TEMED	0.003

5% stacking gel

Name of the compound	Amount (ml)
Distilled water	2.10
30% acrylamide	0.50
1.5M Tris (pH6.8)	0.38
10% SDS	0.030
10% APS	0.030
TEMED	0.003

After pouring the resolving gel solution, it was overlaid immediately with isobutanol and kept 2hrs for polymerization .After complete polymerization of resolving gel the overlay 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 overlaid with water. Finally the gel was kept for polymerization for 30-45min. After polymerization the comb was removed carefully and washed thoroughly with water. The gel was then mounted in the electrophoresis apparatus. Tris –Glycine buffer was added sufficiently in both upper and lower reservoir of the gel apparatus. Any bubble trapped at the bottom of the gel was removed very carefully with a bent syringe.

3.5.2.3. Sample preparation

Sample solutions were prepared by mixing the sample protein with 1xSDS gel loading buffer (final volume 40 µl). All the samples were floated in a boiling water bath for 3min. After boiling 40 µl of each sample was loaded in a predetermined order into the bottom of the well carefully with a micropipette.

3.5.2.4. Electrophoresis

Electrophoresis was performed at constant 15mA current for a period of 3hrs until the dye front reached the bottom of the gel.

3.5.2.5. Fixing and staining

After electrophoresis the gel was removed carefully from the glass plates and then stacking gel was cut off from the resolving gel and finally kept in fixer solution of glacial acetic acid: methanol: water (10: 20: 70) for over night. The gel was removed from fixer and stained with Coomassie blue for 4 hrs at 37^{O} C with constant shaking at low speed. The stain was prepared by dissolving 250mg of Coomassie brilliant blue (Sigma R 250) in 45ml of methanol .After dissolving the stain completely ,45ml of water and 10ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No.1 filter paper.

After staining the gel was finally destained with destaing solution,containing methanol,water and acetic acid (4.5:4.5:1) with continuous shaking at 40^0 C until the background became clear.

3.6. Extraction and quantification of pigments

3.6.1. Chlorophyll

Chlorophyll was extracted from leaves following the method of Harborne (1973). Leaf tissues (1gm) were crushed by using 80% alcohol in a mortar with pestle in the dark to prevent the photo oxidation of chlorophyll. The extract was then filtered through Whatman No.1 filter paper by adding 80% acetone from the top till the the residue became colourless. The filtrate was collected and the final volume was made up to 25ml,following which the chlorophyll content was measured by taking the O.D values at 645nm and 663nm respectively in a UV-VIS spectrophotometer (DIGISPEC-200GL) and calculation was done by using the following formulae described by Arnon(1949).

Total chlorophyll: (20.2 A_{645} +8.02 A_{663}) µg/ml

Chlorophyll a: (12.7 A_{663}- 2.69 A_{645}) µg/ml

Chlorophyll b: (22.9 A_{645}-4.68 A_{663}) µg/ml

3.6.2. Carotenoids

Carotenoids were extracted and estimated by following the method of Lichtenthaler (1987). 1gm leaf tissue were crushed in a mortar with pestle in O.D. values of the filtrate were taken at 480nm , 645nm, 663nm in a UV- VIS spectrophotometer and carotenoid content was estimated by using the following the standard formula.

A_{480}- (0.114X A_{663}) – 0.638(A_{645})µg /mlfresh weight.

3.7. Extraction and quantification of carbohydrates

3.7.1. Extraction of total Sugar

For extraction of total soluble sugars method of Harborne (1973) was followed. Fresh leaf tissues were crushed with 95% ethanol and filtered. Then the alcoholic

fraction was evaporated on boiling waterbath.The aqueous fraction was centrifuged by using table centrifuge and supernatants were collected. Finally the volume was made up with double distilled water.

3.7.2. Estimation total sugar

Total sugar estimation was done following using of anthrone, (Plummer 1978). To 1ml of test solution 4ml of Anthrone reagent was added and mixed thoroughly. Then the mixture was placed in a boiling water bath for 10min. The reaction mixture was cooled under running tap water. The absorbance was measured in Systronic photoelectric Colorimeter Model 101 at 570nm. Total sugar was then calculated from the standard curve of Dextrose solution.

3.7.3. Estimation of reducing sugar

For the estimation of reducing sugar Somogyi method as described by Plummer (1978) was followed.1ml of alkaline copper tartarate solution (prepared by dissolving 4gm $CuSO_4.5H_2O$,24 gm anhydrous Na_2CO_3, 16gm of Sodium Potasium tartarate and 180 gm anhydrous Na_2SO_4 in distilled water and made the volume upto 1liter.) was added to 1ml of test solution .After mixing the reaction mixture was kept in a boiling water bath for 15min.The reaction mixture was cooled under running tap water and then1ml of Nelson's ArsenoMolybdate reagent and 2ml distilled water were added . Absorbance was measured in a colorimeter at 540nm .Finally; concentration of reducing sugar was determined by plotting the O.D values on the standard curve of dextrose solution.

3.8. Extraction of enzymes

3.8.1. Phenylalanine ammonia lyase (PAL, EC:4.3.1.5)

For the extraction of phenylalanine ammonia lyase (PAL) method of Chakraborty *et al.* (1993) was followed. Leaves (1gm) were ground to powder in liquid nitrogen and extracted in 0 .1M Sodium borate buffer (pH8.8) containing 2mM β-mercaptoethanol under ice cold condition. The slurry was centrifuged at 15000 rpm for 20min at 4^0C. After centrifugation the supernatant was collected, the final volume was meuasured and used immediately for assay or stored for the further use at 20^0C.

3.8.2. Peroxidase (POX; EC.1.11.1.7)

For the extraction of peroxidase the method of Chakraborty *et al.* (1993) was followed. The leaves (1gm) were powdered in liquid nitrogen and extracted in 0.1M sodium borate buffer (pH 8.8) in a mortar with pestle in an ice cold condition. The homogenate was centrifuged immediately at 15000rpm for 20min at 4^0C. After centrifugation the supernatant was collected, the volume was measured and used immediately for assay or stored at 20^0C.

3.8.3. Polyphenol oxidase (PPO, EC: 1.14.18.1)

For the extraction of PPO the method of Mahadevan and Sridhar (1982) was followed with some modifications. Leaf tissues (1gm) were powdered in liquid nitrogen and extracted with 0.2M Sodium phosphate buffer (pH 6.6). The slurry was centrifuged at 4000 rpm for 30min at 4^0C. After centrifugation the supernatant was collected, volume was measured and the enzyme assay was done immediately or stored at-20^0C.

3.9. Assay of enzyme activities

3.9.1. Phenylalnineammonia lyase

PAL activity was determined spectrophotometrically by measuring the production of cinnamic acid from L-phenylalanine. The reaction mixture contained 0.3ml 300µM Sodium borate buffer (pH8.8), 0.3ml 30µM L-phenylalanine and 0.5ml of supernatant in a total volume of 3ml. Reaction mixture was incubated at 40^0C for 1hr the absorbance was read at 290 nm against the blank i.e. assay mixture with out the enzyme. The enzyme activity was measured as µg cinnamic acid produced in 1min / g fresh leaf tissue.

3.9.2. Peroxidase

For the estimation of peroxidase enzyme activity, the reaction mixture was prepared by mixing 1ml of 0.2M Sodium phosphate buffer (pH 5.4), 100µl of 4mM H_2O_2 , 100µl of O-dianisidine(5mg /ml methanol) ,1.7ml distilled water and 100µl of freshly prepared enzyme extract was added . Peroxidase activity was estimated spectrophotometrically in UV –VIS spectrophotometer (DIGISPEC-200GL) at 460 nm by monitering the oxidation of O-dianisidine in presence of H_2O_2 (Chakraborty *et.al.*, 1993). Specific activity was expressed as the increase of absorbance at 460nm/g tissue/min.

3.9.3. Polyphenol oxidase

Polyphenol oxidase activity was measured by the method of Mahadavan and Sridhar *et al.* (1982) with slight modification. 1ml of freshly prepared enzyme extract was mixed with 2ml of 0.2M sodium phosphate buffer (pH6.0) and 0.01M pyrogallol was added to it in the dark. Reading was noted every 1min interval at 495nm. The blank was set with 3ml of phosphate buffer. PPO activity was measured at $\triangle$ O.D.A_{495} /g tissue/min.

3.10. Isozyme analysis by polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) for isozyme analysis of different enzymes the method of Davis(1964) was followed, using 8% resolving gel and 5% stacking gel in Tris-glicine buffer (pH8.3) . The different solutions were prepared for the analysis as follows:

3.10.1. Preparation of the stock solution

Solution A : Acrylamide stock solution (Resolving gel)

Acrylamide stock solution was prepared for the resolving gel , by dissolving 28g of acrylamide and 0.74 g of N'N'methelene bisacrylamide dissolved in100ml warm distilled water. The stock solution was filtered with Whatman No.filter paper in dark and stored in dark bottle at 4^{O}C.

Solution B: Acrylamide stock solution (stacking gel)

For the preparation of acrylamide stock solution for stacking gel 10gm of acrylamide and 2.5g of bis-acrylamide was dissolved in 100ml warm distilled water. The stock solution was then filtered withWhatman No.1 filter paper and stored at4^{O}C in a dark bottle.

Solution C: Tris-HCl (Resolving gel)

Tris-HCl buffer was prepared by dissolving 36.6.g of Tris base in distilled water and 0.25ml of TEMED was added. The pH of the solution was adjusted to 8.9 with conc. HCl. The final volume of the solution was made upto 100ml with distilled water. The solution was then stored at 4^{O}C for further use.

Solution D: Tris –HCl (Stacking gel)

5.98g of Tris base was mixed with distilled water and 0.46 ml of TEMED was added to it .Finally the pH of the solution was adjusted to 6.7 with conc. HCl. The final volume of the solution was made upto 100ml with distilled water. The solution was stored at 4^{O} C for further use.

Solution E: Ammonium persulphate solution (APS)

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

Solution F: Riboflavin solution

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

Solution G: Electrode buffer:

Fresh electrode buffer was prepared by dissolving 0.6g of Tris base and 2.9gm of Glycine in 1L distilled water.

3.10.2. Prepartion of gel

For native anionic PAGE mini slab gel was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease from the plates and then dried. 1.5mm thick spacers were placed between the glass plates on three sides and were sealed with high 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: distilled water in the ratio of 1:1:4:1 by pasture pipette leaving sufficient space for (comb +1cm) the stacking gel. This resolving gel was immediately overlayed with water and kept for polymerization for 2hrs. After complete polymerization of the resolving gel, the overlay was poured off and washed with water to remove any unpolymerized acrylamide.

The stacking gel solution was made by mixing solution B: D: F: distilled water in the ratio of 2:1:1:4. The stacking gel solution was poured over the resolving gel and comb was inserted immediately and overlayered with water. Finally the gel was kept for

30-45min in strong sunlight for polymerization. After polymerization the comb was removed carefully and washed carefully with water. The gel was then finally mounted in a electrophoresis apparatus. Tris –Glycine buffer was poured sufficiently in the both upper and lower reservoir. Any air bubble, was trapped at the bottom of the gel was removed very carefully with a bent syringe.

3.10.3. Sample preparation

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

3.10.4. Electrophoresis

Electrophoresis was performed at a constant 15mA current for a period of 3-4 hrs at $4^{O}C$ until the dye font reached at the bottom of the gel.

3.10.5. Fixing and Staining

After electrophoresis the gel was removed from the glass plates and then the stacking gel was cut off from the resolving gel and finally stained with suitable dye.

3.10.5.1. Peroxidase

Extraction for peroxidase isozyme analysis was done by grinding 1gm of leaf tissues in liquid nitrogen in pre-chilled mortar and pestle and finally extracted with 0.1M Sodium phosphate buffer (pH 7.0) as described by Davis (1964). The staining of the gel for the peroxidase isozyme pattern was performed following the method of Reddy and Gasber (1973). The gel was stained with the solution of Benzidine dye in acetic acid water mixture consisting of Benzidine (2.08gm), Acetic acid (18 ml), 3% H_2O_2 (100ml) for 5min . The reaction was stopped with 7% acetic acid after the appearance of clear blue coloured bands. Analysis of isozyme was done immediately.

3.10.5.2. Polyphenol oxidase

Extraction for polyphenol oxidase isozyme analysis was done by powdering 1gm of leaf tissues in liquid nitrogen in pre-chilled mortar and pestle and was extracted with 0.1M sodium phosphate buffer (pH 7.0) as described by Davis(1964). After the electrophoresis the gel was equilibrated in 0.1% p-phenylenediamine in 0.1M potassium phosphate buffer (pH 7.0) for 30min.This was followed by the addition of 10mM Catechol solution in the same buffer. The gel was shaken in this buffer until the appearance of brown discreate band, analysis of the isozyme pattern was done immediately.

3.11. Extraction of catechins from tea leaves

Catechin was extracted from tea leaf tissues following the method of Obanda and Owuor (1994) with some modifications. 10gm leaf samples were taken and extracted with 100ml of 80% acetone and kept in a water bath at 45^{O}C for 30 min. Extract was then filtered through Whatman No.1 filter paper. Acetone extract was concentrated to dryness and the residue was dissolved in 20ml of distilled water. Water solution was separated with equal volume of chloroform for four times in a separating funnel. The pH of the final extract i.e. water layer was adjusted to 2 by adding 2N of HCl and finally extracted with methyl isobutyl ketone. Finally the extract was concentrated to dryness and dissolved in 3ml of 2% acetic acid. The samples were finally filtered through milipore filter (Milipore 0.4 µm HA filter paper).

3.12. HPLC analysis of catechins

High performance liquid chromatography (HPLC) of the extract was performed for the anlysis of catechin. The HPLC analysis was done on a Shimadzu Advanced VP Binary gradient system with a C-18 hypersil column using 50% acetonitrile as the mobile phase, in isocratic mode. Injection volume was 20µl and the flow rate 1ml min^{-1}. Detection was done at 278 nm and the total run time was 25min.

3.13. Determination of heavy metal contents in tea leaf

Heavy metal content was estimated with the help of Atomic Absorption Spectrophotometer (AAS). Tea leaves 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 gm of leaf was digested at a temperature of 180^{O}Cfor 15min. After complete digestion the volume was made upto 100ml with distilled water and the AAS (Perklin Elmer A Analyst 200) was done and the reading was noted.

3.14. Statistical Analysis

Standard error of mean was calculated in all cases. Significance of difference between mean was analysed by ANOVA.

Experimental

In the present study experiments were carried out to determine the biochemical responses of tea plants to various anthropogenic stresses.The plants were subjected to different abiotic stresses, both heavy metals and insecticide and fungicide application separately. Results of all the experiments conducted in the present study are presented in the following pages. All experiments were done by using standard procedures as mentioned under materials and methods. In case of potted plants or bushes spraying with insecticide/fungicide resulted in healthier looking plants without any insect damage (Plate VI).

In case of *in vitro* study by heavy metal i.e. Cu and Cd some morphological changes were observed at the higher concentration i.e browning of leaf at the higher concentrations of Cu and Cd. The changes were more pronounced in case of Cu treatment at 500 µg/ml and 100 µg/ml (Plates VII). In seedlings treated with the same metals , no remarkable changes were observed even after 2nd treatment.

Since spraying with insecticide and fungicide is the common practice in tea garden to combat with pest and diseases, acephate an insecticide and hexaconazole a fungicide popularly used in tea garden were taken for the study as anthropogenic stress inducers. Biocemical studies were conducted regarding heavy metal and pesticide mediated changes in leaves following materials and methods.

4.1. Effect of different stresses on phenolics in tea leaves

Phenolics perform a wide range of physiological role in plants. Total and O-dihydroxy phenols, were extracted and estimated following the method described earlier in materials and methods.

4.1.1. Heavy metals

For *in vitro* studies on phenolics, young shoots (first four leaves) were taken from the well grown bushes and immersed immediately with different concentrations of heavy metal solutions. Estimation of total phenol revealed significant differences among the different varieties in cut shoots. It was observed that in case of cut shoots the total phenol content ranged from approxmimately 23.87-47.5mg/g tissue. Cu treatment led to an increase in phenol contents at 100 µg/ml in all Tocklai varieties.

Plate VI: Potted tea plants before(A & C) and after (B & D) spraying with insecticide/fungicide.

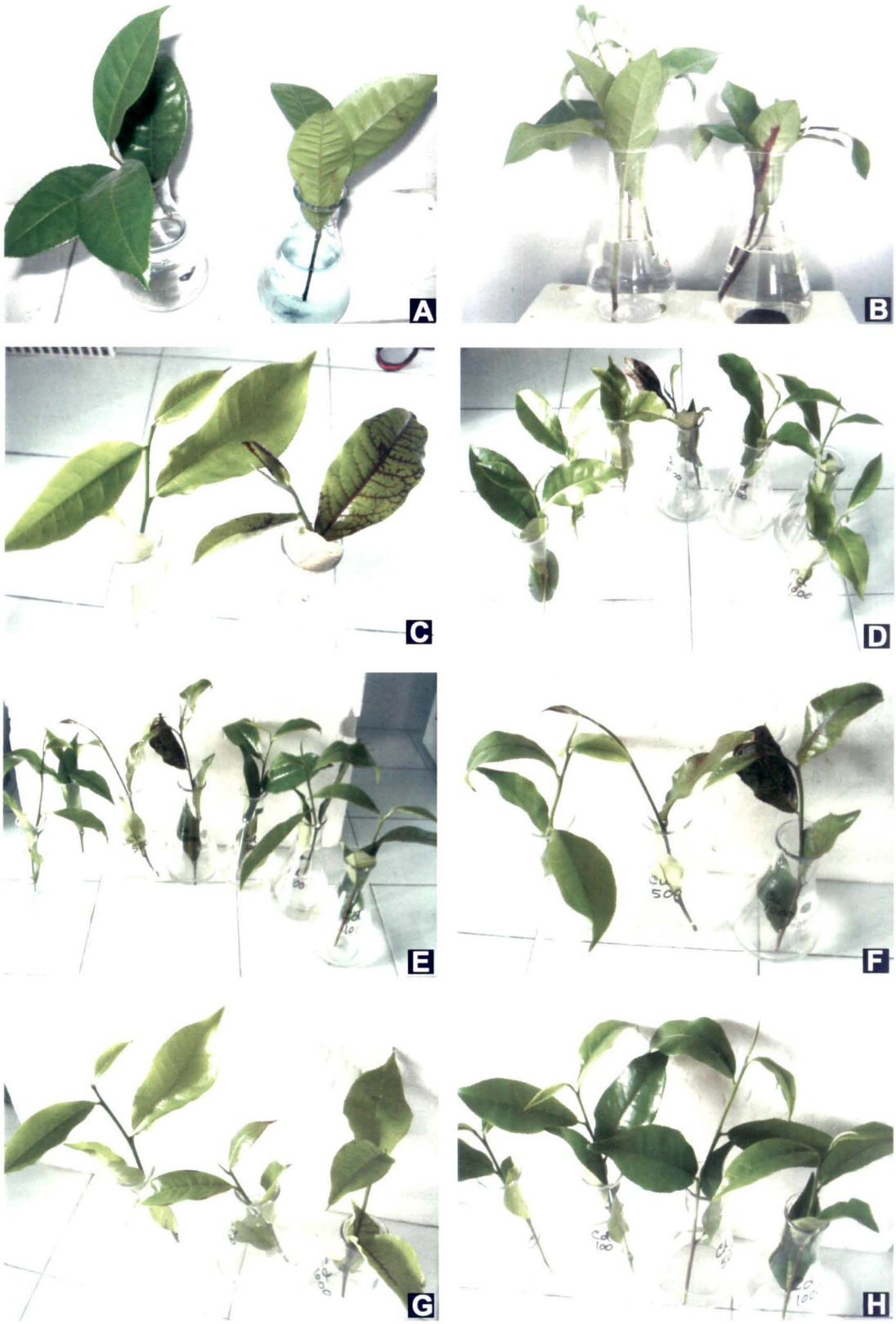


Plate VII (A-H): Treatment of cut-shoots of tea in heavy metal solutions.

Whereas, in two Darjeeling varieties i.e. T-78 and HV-39 total phenol content were reduced. At 500 µg/ml there was a decline in total phenol content in most of the cases. A significant reduction in total phenol content at 1000µg/ml was noticed (Table 1&2). Orthodihydroxyphenol content also revealed the same pattern.

Table1: Total phenol contents in tea leaves following Cu stress.

Varieties	Total phenol content (mg/g tissue)			
	Concentration of Cu (µg/ml)			
	0	**100**	**500**	**1000**
TV-18	26.0±0.57	32.87±1.44	47.0±1.15	29.81±0.40
TV-22	30.28±0.48	43.81±0.75	27.37±0.94	27.1±0.52
TV-23	27.5±0.28	38.6±0.34	56.1±0.63	22.5±1.44
TV-26	43.5±0.86	56.1±0.34	40.5±1.44	32.5±1.44
TV-27	47.5±0.28	63.0±1.15	51.0±0.57	31.0±1.15
TV-28	26.51±0.61	35.08±0.96	31.71±0.08	21.0±0.57
TV-29	30.1±1.79	47.5±1.44	33.6±1.81	30.88±0.39
TV-30	23.87±0.91	29.25±0.72	22±1.15	15.22±0.39
T-78	25.97±0.45	22.25±1.12	15.28±0.53	20.10±0.63
HV-39	35.87±0.50	29.25±0.43	25.72±0.16	19.6±0.49
CD Treatment (P=0.05) =6.467386; CD Varieties (P=0.05) =10.22583				

Values are mean of 3 replicates; ± = SEm

Table 1A: Analysis of variance of data presented in Table 1.

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	1150.48066	3	383.4935533	7.719932	0.000703	2.960351
Columns	2644.7294	9	293.8588222	5.915537	0.000147	2.250131
Error	1341.24574	27	49.67576815			
Total	5136.4558	39				

Table 2: Changes in O-dihydroxy phenol contents of tea leaves following application of Cu stress in cut shoots.

Varieties	O-dihydroxy phenol content (mg/g tissue)			
	Concentration of Cu (µg/ml)			
	0	100	500	1000
TV-18	7.55±0.14	7.94±0.82	9.88±0.46	6.41±0.34
TV-22	9.21±0.17	13.34±0.52	11.03±0.28	10.31±0.16
TV-23	1.68±0.06	1.93±0.03	2.37±0.26	2±0.01
TV-26	6.23±0.26	6.97±0.23	9.4±0.16	4.77±0.13
TV-27	6.75±0.12	7.45±0.24	9.43±0.13	6.71±0.02
TV-28	5.50±0.23	6.62±0.18	7±0.15	4.17±0.23
TV-29	6.82±0.01	7.12±0.12	5.35±0.18	4.32±0.11
TV-30	5.15±0.02	5.63±0.23	7.12±0.03	4.0±0.04
T-78	4.82±0.17	4.42±0.09	4.54±0.21	3.35±0.20
HV-39	7.75±0.05	6.28±0.16	5.69±0.17	4.12±0.25
CD Treatment (P=0.05) 0.881135; CD Varieties (P=0.05) =1.393196				

Values are mean of 3 replicates; ± = SEm

Table 2A: Analysis of variance of data presented in Table 2.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	29.4154475	3	9.805149167	10.63366	8.61E-05	2.960351
Columns	200.1220025	9	22.23577806	24.11464	1.12E-10	2.250131
Error	24.8963275	27	0.922086204			
Total	254.4337775	39				

In Case of Cd treatment only two varieties i.e. TV-28 and TV -29 showed the lower phenol content at 100 µg/ml, whereas, all other six varieties showed increase in phenol content (Table 3).

Table 3: Effect of application of Cd to cut shoot of tea on total phenol content in leaves.

Varieties	Total phenol content (mg/g tissue)			
	Concentration of Cd (µg/ml)			
	0	100	500	1000
TV-18	26.0±0.57	28.13±0.39	35.08±0.96	20.75±0.43
TV-22	30.28±0.74	42.5±1.44	35.5±0.28	24.82±1.72
TV-23	27.5±0.28	46±0.57	37.25±1,29	22.5±1.44
TV-26	43.5±0.69	51.25±0.72	40±1.55	31.87±1.12
TV-27	47.5±0.52	54.75±1.29	31.93±0.19	28.15±1.24
TV-28	26.51±0.61	22.5±1.15	20.15±1.64	18.05±0.55
TV-29	35.1±0.9	30.85±0.66	32.21±0.36	25.42±0.80
TV-30	23.87±0.91	28.12±0.68	18.44±1.55	15±1.12
T-78	25.97±0.85	34±0.69	30±1.2	20±1.12
HV-39	35.87±0.5	48.06±0.95	29.85±1.12	21.7±1.01
CD Treatment (P=0.05)=4.605687; CD Varieties (P=0.05) =7.282231				

Values are mean of 3 replicates; ± = SEm

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

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1261.634508	3	420.5448358	16.69308	2.47E-06	2.960351
Columns	1704.075403	9	189.3417114	7.515717	2E-05	2.250131
Error	680.2047675	27	25.19276917			
Total	3645.914678	39				

In case of O-dihydroxy phenol content the trend was similar to that of total phenol content (Table 4).

Table 4: Changes in O-dihydroxy phenol content in tea leaves following Cd stress imposed in cut shoots.

Varieties	O-dihydroxy phenol content (mg/g tissue)			
	Concentration of Cd (µg/ml)			
	0	100	500	1000
TV-18	7.55±0.20	8.7±0.13	7.23±0.21	5.37±0.25
TV-22	9.21±0.72	14.82±0.66	10.75±0.23	7.89±1.55
TV-23	1.68±0.90	2.50±0.10	1.83±0.12	1.33±0.55
TV-26	6.23±0.17	6.07±0.52	8.28±0.36	5.19±0.80
TV-27	6.75±0.90	7.10±0.95	6.36±1.64	5.12±1.55
TV-28	5.50±0.28	7.47±0.66	6.12±0.82	4.25±0.80
TV-29	6.82±0.69	5.66±0.80	4.45±0.95	3.17±1.24
TV-30	5.15±0.02	8.32±0.12	7.36±0.14	4.12±0.04
T-78	4.82±0.46	6.81±0.20	4.02±0.21	2.65±0.02
HV-39	7.75±0.52	8.79±0.30	4.15±0.20	3.78±0.18
CD Treatment (P=0.05)= 0.955959; CD Varieties (P=0.05) =1.511504				

Values are mean of 3 replicates; ± = SEm

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

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	63.8413	3	21.28043333	19.60718	5.94E-07	2.960351
Columns	175.92059	9	19.54673222	18.0098	3.12E-09	2.250131
Error	29.30415	27	1.085338889			
Total	269.06604	39				

All the eight varieties of tea seedlings exposed to different heavy metal concentrations of Cu and Cd showed changes in phenol content. After 1st treatment of all the concentrations of Cu i.e. 100, 500, 1000 µg/ml there was an increase in total and O-dihydroxy phenol content .Two Darjeeling varieties showed lower phenol content at highest concentration of Cu (Table 5&6).

Table 5. Effect of different concentration of Cu on total phenol contents of tea leaves after 1st application in potted tea seedlings.

Varieties	Total phenol content (mg/g tissue)			
	Concentration of Cu (µg/ml)			
	0	100	500	1000
TV-23	35.74±0.13	42.7±0.31	49.67±0.24	61.48±0.19
TV-26	24.25±0.17	36.1±0.20	46.43±0.23	56.66±0.02
TV-27	32.66±1.20	37.85±0.96	45.32±0.53	50.67±0.31
TV-28	35.37±1.18	41.66±1.20	49.21±0.56	57.30±0.47
TV-29	20.50±0.26	23.85±0.32	46.48±0.22	61.42±0.08
TV-30	26.0±1.20	32.98±2.10	48. 37±0.32	56.97±0.98
T-78	25.15±0.17	42.85±0.13	55.6±0.09	50±0.19
HV-39	41.15±0.24	60.7±0.26	63.25±0.02	57.75±0.03
CD Treatment (P=0.05)= 5.87053; CD Varieties (P=0.05) =8.302183				

Values are mean of 3 replicates; ± = SEm

Table 5A: Analysis of variance of data presented in Table 5.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	3280.334734	3	1093.444911	34.30423	2.8E-08	3.072467
Columns	845.6324219	7	120.8046317	3.789958	0.008211	2.487578
Error	669.3734906	21	31.87492813			
Total	4795.340647	31				

Table 6: Effect of different concentration of Cu on O-dihydroxy phenol contents of tea leave after 1st application in potted tea seedlings.

Varieties	O-dihydroxy phenol content (mg/g tissue)			
	Concentration of Cu (µg/ml)			
	0	100	500	1000
TV-23	10.60±0.11	11.25±0.13	12.5±0.23	15.6±0.10
TV-26	8.65±0.09	10.5±0.21	12.0±0.13	14.30±0.12
TV-27	6.5±0.15	9.0±0.07	13.6±0.03	14.99±0.21
TV-28	10.31±0.23	12.0±0.19	14.5±0.10	16±0.86
TV-29	3.50±0.45	6.56±0.82	10.5±0.12	13.57±0.17
TV-30	4.55±0.52	6.31±0.50	9.13±0.12	11.50±0.20
T-78	4.25±0.12	9.5±0.46	14.0±0.13	12.0±0.17
HV-39	6.25±0.24	8.36±0.26	11.5±0.03	8.56±0.13
CD Treatment (P=0.05)= 2.160194; CD Varieties (P=0.05) =5.196434				

Values are mean of 3 replicates; ± = SEm

Table 6A: Analysis of variance of data presented in Table 6.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	237.8608094	3	79.28693646	18.37052	4.4E-06	3.072467
Columns	106.7070219	7	15.24386027	3.531953	0.011536	2.487578
Error	90.63571563	21	4.315986458			
Total	435.2035469	31				

There was an increase in total phenol and o-phenol content following 2nd treatment also in 100 and 500 µg/ml of Cu in most of all the Tocklai varieties. Two Darjeeling varieties showed some different trend i.e highest phenol accumulation was noticed at 100 µg/ml, after which the phenol content declined. However, in all varieties of Cu treatment with the highest concentration i.e.1000 µg/ml showed reduced phenol content in all potted plants, after 2nd application (Table 7 & 8).

Table 7. Effect of different concentration of Cu stress after 2nd application in total phenol content.

Varieties	Total phenol content (mg/g tissue)			
	Concentration of Cu (µg/ml)			
	0	100	500	1000
TV-23	36.36±0.58	51.25±0.56	55.81±0.49	42.85±0.40
TV-26	26.57±0.43	41.25±0.23	54.52±0.88	36.25±0.92
TV-27	29.70±1.28	37.96±1.15	41.92±1.02	57.87±0.93
TV-28	30.25±1.03	58.53±1.18	65.55±1.11	49.60±0.92
TV-29	29.81±0.58	40.66±0.74	50.82±1.50	33.5±0.09
TV-30	32.81±1.17	43.89±1.20	53.75±1.50	46.35±0.08
T-78	28.22±0.69	66.50±1.20	51.67±1.50	44.62±1.10
HV-39	39.40±1.11	62.86±1.15	52.60±1.50	46.77±0.58
CD Treatment (P=0.05)= 7.433378; CD Varieties (P=0.05) =10.51238				

Values are mean of 3 replicates; ± = SEm

CD Varieties (P=0.05) =10.51238

Table 7A: Analysis of variance of data presented in Table 7.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	2213.671859	3	737.8906198	14.43859	2.5E-05	3.072467
Columns	615.6704719	7	87.95292455	1.721009	0.158205	2.487578
Error	1073.214066	21	51.1054317			
Total	3902.556397	31				

Table 8. Effect of different concentration of Cu stress after 2nd application in O-dihydroxy phenol content.

Varieties	O-dihydroxy phenol content (mg/g tissue)			
	Concentration of Cu (µg/ml)			
	0	100	500	1000
TV-23	9.56±0.34	12.6±0.04	14.56±0.06	12.35±0.20
TV-26	7.51±0.18	9.75±0.24	12.50±0.26	8±0.12
TV-27	8.50±0.45	9±0.16	14.5±0.22	15.0±0.20
TV-28	9.0±0.18	13.6±0.15	16±0.05	10.35±0.82
TV-29	6.58±0.14	8.5±0.18	9.52±0.82	7.0±0.25
TV-30	5.75±0.70	7.3±0.11	11.24±0.20	9.17±0.11
T-78	5.0±0.23	11.56±0.07	9.98±0.08	10.6±0.07
HV-39	7.5±0.86	9.5±0.48	8.25±0.10	6.56±0.90
CD Treatment (P=0.05)= 1.751428; CD Varieties (P=0.05) =2.476893				

Values are mean of 3 replicates; ± = SEm

Table 8A: Analysis of variance of data presented in Table 8.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	87.48793438	3	29.16264479	10.27893	0.000228	3.072467
Columns	100.9210719	7	14.41729598	5.081651	0.0017	2.487578
Error	59.57969063	21	2.837128125			
Total	247.9886969	31				

When seedlings in potted condition were treated similarly with Cd increase in total phenol content was observed in most of the varieties.Reduction of total phenol content was noticed at 1000 µg/ml.after 2nd application of Cd solution in seedlings . Among the six varities TV-23 and HV-39 showed decline phenol content at even 500 µg/ml. O-dihydroxy phenol also showed similar trends (Figs. 1 &2).

4.1.2. Fungicide / Insecticide

Both total and O-dihydroxy phenol contents were increased significantly after fungicide as well as insecticide spray. Maximum accumulation was observed following. 2nd spray which was similar for the eight tested potted varieties (Table -9).

The highest phenol accumulation was observed in TV- 30. Orthodihydroxy phenol content also showed similar trend. The result is presented in Table 10.

The two varieties of bushes revealed the same trend in total and orthodihyroxy phenol content. The inherent total phenol content in bush was higher than the potted plants. Result revealed that, insecticide treatment showed the greater accumulation of phenolics than that of fungicide. The O-dihydroxy phenol content was revealed the similar trend as total phenol (.Fig.3).

Results of all experiments on phenols reveal that plants respond to the tested anthropogenic stresses by an increased accumulation of phenolics. However, when the concentration of the compounds are very high, or more prolonged application is done, the phenol contents gradually decline.

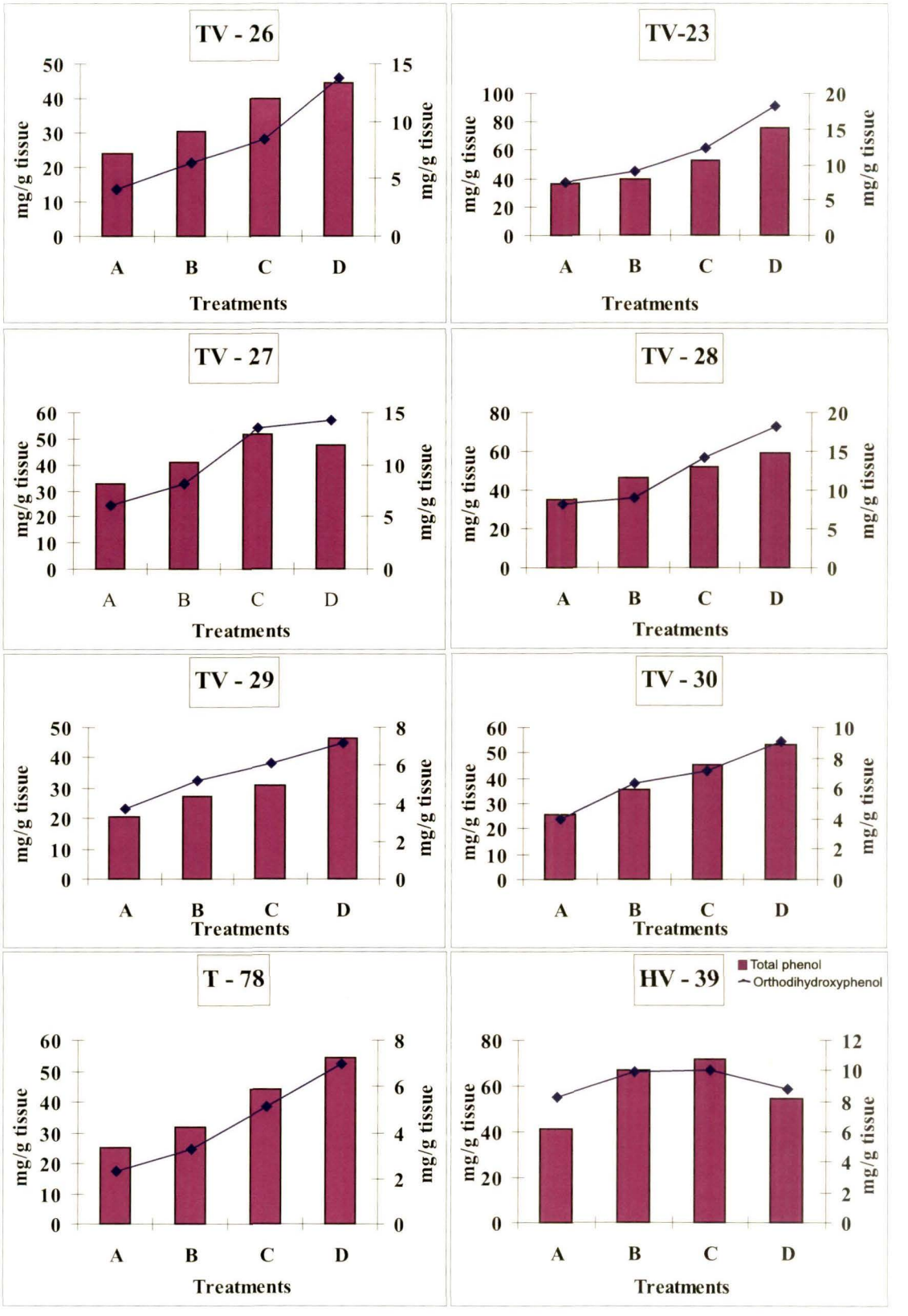


Fig.1: Effect of 1st application of different concentrations of Cd on phenol content of leaves of young tea plants.

A=Control, B= 100, C= 500, D= 1000 µg/ml.

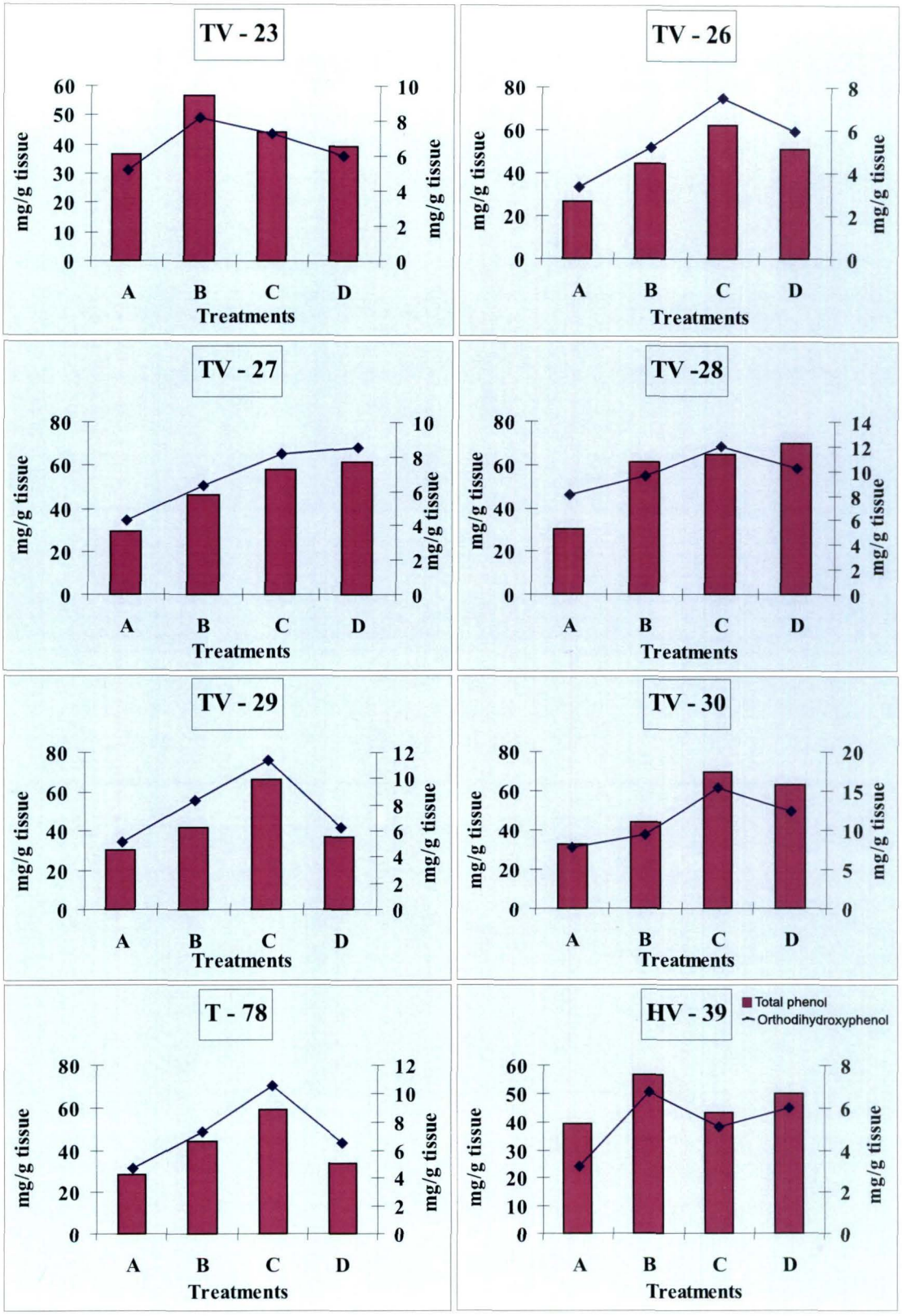


Fig.2: Effect of 2nd application of different concentrations of Cd on phenol content of leaves of young tea plants.

A=Control, B=100, C= 500,D=1000 µg/ml.

Table 9: Effect of Hexaconazole and Acephate spray on total phenol content of tea leaves in potted tea seedlings.

Varieties	Total phenol content (mg/g tissue)					
	1st treatment			2nd treatment		
	Control	Insecticide	Fungicide	Control	Insecticide	Fungicide
TV-23	25.69 ±0.03	35.41 ±0.21	32.50 ±0.08	20.41 ±1.25	45.90 ±1.12	43.70 ±0.98
TV-26	16.25 ±0.58	32.10 ±0.59	28 ±1.40	15 ±0.98	38.05 ±1.11	33.92 ±1.25
TV-27	24.90 ±0.90	41.16 ±0.86	35.41 ±0.88	25.99 ±0.23	49.66 ±0.22	40.50 ±1.11
TV-28	24.50 ±1.23	38.40 ±1.73	30.50 ±1.22	24 ±0.56	47 ±1.12	36 ±1.17
TV-29	18 ±2.10	26.40 ±2.17	22.50 ±1.50	18 ±1.20	27.90 ±0.23	37.87 ±0.28
TV-30	39.75 ±1.20	56.75 ±0.30	51.75 ±0.58	33.75 ±0.80	63.25 ±0.88	58.75 ±0.78
T-78	37.50 ±0.23	40 ±0.27	46.50 ±0.28	30 ±0.99	57.35 ±1.30	65.62 ±1.22
HV-39	33.40 ±0.25	45.33 ±1.15	46 ±1.29	35.41 ±0.86	50.83 ±0.89	51.40 ±0.90
CD Treatment (P=0.05)= 4.012528 CD Varieties (P=0.05) =6.552432				CD Treatment (P=0.05)= 5.427623 CD Varieties (P=0.05) =8.863271		

Values are mean of 3 replicates; ± = SEm

Table 9A: Analysis of variance of data presented in Table 9 (1st treatment)

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	624.4531	2	312.2266	34.8169	3.68E-06	3.738892
Columns	1718.096	7	245.4423	27.36967	3.94E-07	2.764199
Error	125.5474	14	8.967673			
Total	2468.096	23				

Table 9B: Analysis of variance of data presented in Table 9 (2nd treatment)

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	2454.294	2	1227.147	47.90551	5.47E-07	3.738892
Columns	1783.87	7	254.8386	9.94842	0.000168	2.764199
Error	358.6238	14	25.61599			
Total	4596.788	23				

Table 10: Changes in O-dihydroxy phenol content of tea leaves following Hexaconazole and Acephate treatment in potted tea plants.

Varieties	**O-dihydroxy-phenol content (mg/g tissue)**					
	1st treatment			**2nd treatment**		
	Control	**Insecticide**	**Fungicide**	**Control**	**Insecticide**	**Fungicide**
TV-23	4.07±0.13	5.98±0.14	4.95±0.86	5.50±0.70	8.20±0.25	7.58±0.25
TV-26	8.01±0.90	10.48±0.07	9.90±0.90	7.55±0.20	12.40±0.25	11.00±0.33
TV-27	7.90±0.92	10.40±1.31	8.75±1.20	8.70±1.32	13.86±1.01	12.90±1.11
TV-28	4.20±0.52	6.50±0.69	5.30±0.52	4.40±1.50	8.50±0.86	7.50±0.14
TV-29	6.82±0.09	8.55±1.18	9.10±0.32	6.82±0.98	10.85±0.08	12.50±0.53
TV-30	2.50±0.53	4.75±0.23	3.50±1.20	2.66±1.70	7.58±0.28	6.93±0.56
T-78	7.50±1.20	12±0.26	14±0.98	6.25±0.08	13.75±0.47	16.75±0.18
HV-39	9.77±0.52	13.37±0.30	12.35±0.20	8.74±0.46	16.47±0.88	15.95±0.49
CD Treatment (P=0.05)= 1.062841 CD Varieties (P=0.05) =1.735612				CD Treatment (P=0.05)= 1.557603 CD Varieties (P=0.05) =2.543554		

Values are mean of 3 replicates; ± = SEm

Table 10A: Analysis of variance of data presented in Table 10 (1st treatment)

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	31.7161	2	15.85805	16.14438	0.000232	3.738892
Columns	190.4668	7	27.20954	27.70083	3.65E-07	2.764199
Error	13.7517	14	0.982264			
Total	235.9346	23				

Table 10B: Analysis of variance of data presented in Table 10 (2nd treatment)

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	138.3279	2	69.16396	32.78497	5.22E-06	3.738892
Columns	176.038	7	25.14828	11.92074	6.11E-05	2.764199
Error	29.53474	14	2.109624			
Total	343.9007	23				

4.2. Effect of heavy metals and chemicals on proline content of tea leaves

4.2.1. Heavy metal

Proline plays a significant role in plant's stressed condition as an osmoregulator. Accumulation of proline was observed in various heavy metals, insecticide and fungicide treatments.In the present investigation accumulation of proline in response to the different stresses has been determined. Wide variation in proline content was noticed among the different varities of treated plants.

In *vitro* study of heavy metals maximum accumulation of proline was noticed at 500µg/ml. of Cu. Among the ten varieties HV -39 showed the highest accumulation, whereas, TV-29 the minimum accumulation was observed (Table 11).

Table 11: Effect on proline contents in tea leaves following application of Cu stress in cut shoots.

Varieties	**Proline content (µg/g tissue)**			
	Concentration of Cu (µg/ml)			
	0	**100**	**500**	**1000**
TV-18	266±5.48	372.75±9.09	500.62±16.23	479.50±8.66
TV-22	292.87±7.14	402.50±5.05	527.25±8.22	534.12±1.36
TV-23	155.31±3.78	292.50±6.50	357±8.66	256.56±5.08
TV-26	276.75±1.29	353±9.09	460±7.14	306±6.13
TV-27	207±6.63	391±4.90	520.62±4.90	322.75±5.05
TV-28	361±5.48	485±8.66	525±14.43	550.12±7.14
TV-29	169.62±6.13	230.75±7.14	158.62±12.76	195±8.66
TV-30	260±5.77	481.25±7.21	568.75±14.43	412.50±8.08
T-78	230±2.88	346.87±5.40	451.1±9.75	255±2.88
HV-39	319.37±12.62	342.00±5.19	510.62±9.70	639.62±6.13
CD Treatment (P=0.05)= 61.68524; CD Varieties (P=0.05) =97.53292				

Values are mean of 3 replicates; ± = SEm

Table 11A: Analysis of variance of data presented in Table 11.

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	218689.8845	3	72896.62817	16.13087	3.32E06	2.960351
Columns	302216.8305	9	33579.64783	7.430647	2.2E-05	2.250131
Error	122015.019	27	4519.074778			
Total	642921.734	39				

When shoots were dipped at different concentrations of Cd solutions comparatively at 500µg/ml highest accumulation was observed. In 1000µg/ml significant decline of proline content was evident in both the tested chemicals. The reduction was more pronounced in Cd (Table 12).

Table 12: Effect of proline contents in tea leaves subjected to Cd stress in cut shoots

Varieties	**Proline content (µg/g tissue)**			
	Concentration of Cd (µg/ml)			
	0	**100**	**500**	**1000**
TV-18	266±6.31	445.5±6.49	624±13.13	355±8.08
TV-22	292.87±7.14	507.5±12.76	688.75±8.93	446.25±14.43
TV-23	155.31±7.57	368±9.09	415.62±5.19	427.5±4.33
TV-26	276.75±6.49	373.5±7.21	507.5±8.08	296.25±6.35
TV-27	207±5.05	471.75±6.63	633.62±6.35	412.25±2.30
TV-28	361±6.92	595±4.33	695±8.22	455±7.14
TV-29	169.62±9.99	273±10.79	315.37±4.76	216.75±9.09
TV-30	260±6.92	537.5±4.83	612.5±7.57	418.75±4.76
T-78	230±8.66	387±5.19	481.25±9.00	299±9.38
HV-39	319.37±3.60	583±5.05	638.25±6.90	424.37±5.19
CD Treatment (P=0.05)= 46.43419;CD Varieties (P=0.05) =73.4189				

Values are mean of 3 replicates; ± = SEm

Table 12A: Analysis of variance of data presented in Table 12.

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	504220.442	3	168073.4807	65.63514	1.59E-12	2.960351
Columns	266527.8194	9	29614.20216	11.56478	3.43E-07	2.250131
Error	69139.55298	27	2560.724185			
Total	839887.8144	39				

In case of young seedlings proline content was enhanced till the second application of heavy metal solutions. The highest accumulation was noted at500µg/ml in both the tested metals. At 1000 µg/ml a declining trend was noted in all cases (Fig 4) The effect was more in Cd treatment than in Cu. (Fig.5)

4.2.2. Fungicide / Insecticide

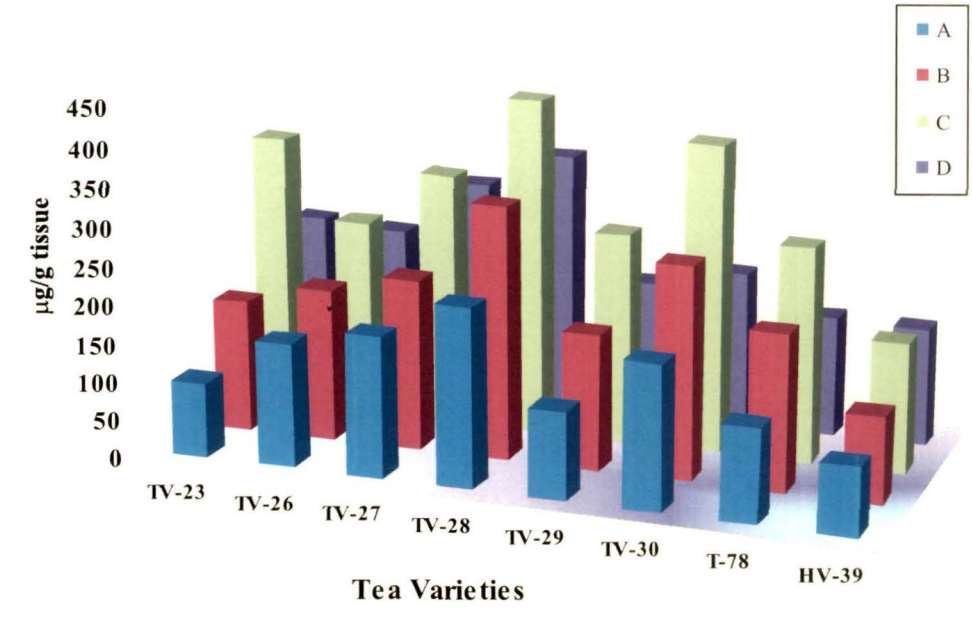
In case of potted plants and bushes the fungicide and insecticide treatments enhanced the proline accumulation significantly in all tested varieties. A great variation was found among different varieties. In seedlings proline content varied from 77-156.25 µg /gm tissue. Effect was more pronounced after the 2nd treatment as compared to the 1st treatment (Table13).

Bush showed the greater amount of proline content than potted plants in control. The diference of proline accumulation was more pronounced in pot than in bush (Fig 6a).

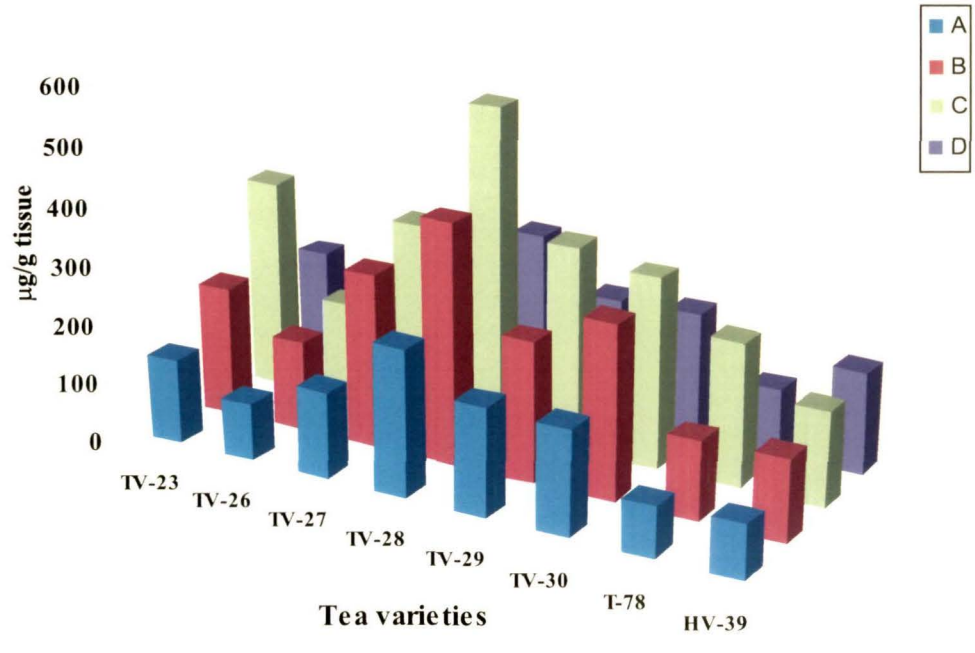
Proline contents of all varieties, in general showed an initial increase as a response to the different stresses, after which there was a decline.

4.3. Effect of different stresses on proteins of tea leaves

Changes in protein metabolism are the most important and immediate stress response in plants. Stress can induce changes in protein accumulation and the protein being synthesized. Effect of various anthropogenic stresses on both the protein content as well as the protein pattern of tea leaves were determined by following the standard methods mentioned in materials and methods. Results have been presented below.

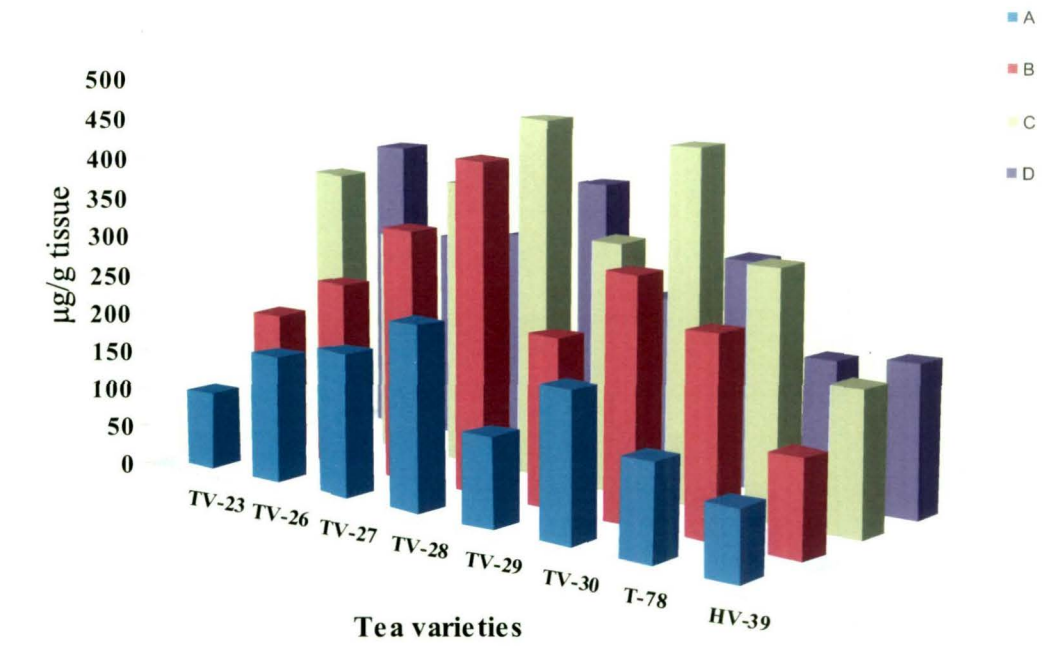


a

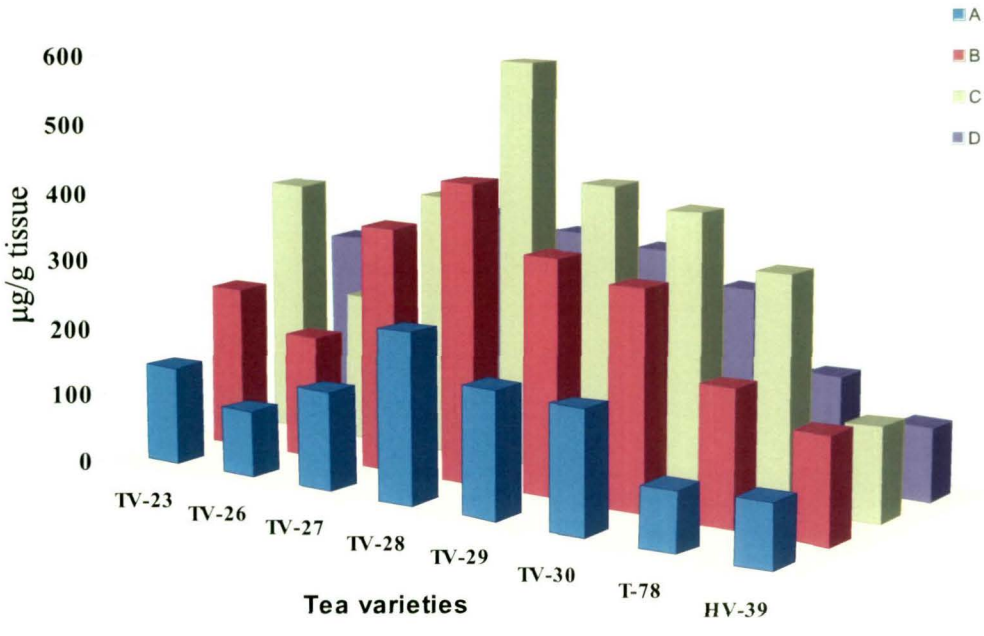


b

Fig.4: Changes in proline content of tea leaves of young plants following 1st (a) and 2nd (b) application of Cu stress.
Legend: A=Control, B=100, C=500 and D=1000 µg/ml



a



b

Fig.5: Changes in proline content of tea leaves of young plants following 1st (a) and 2nd (b) application of Cd stress.
Legend: A=Control, B=100, C=500 and D=1000 µg/ml

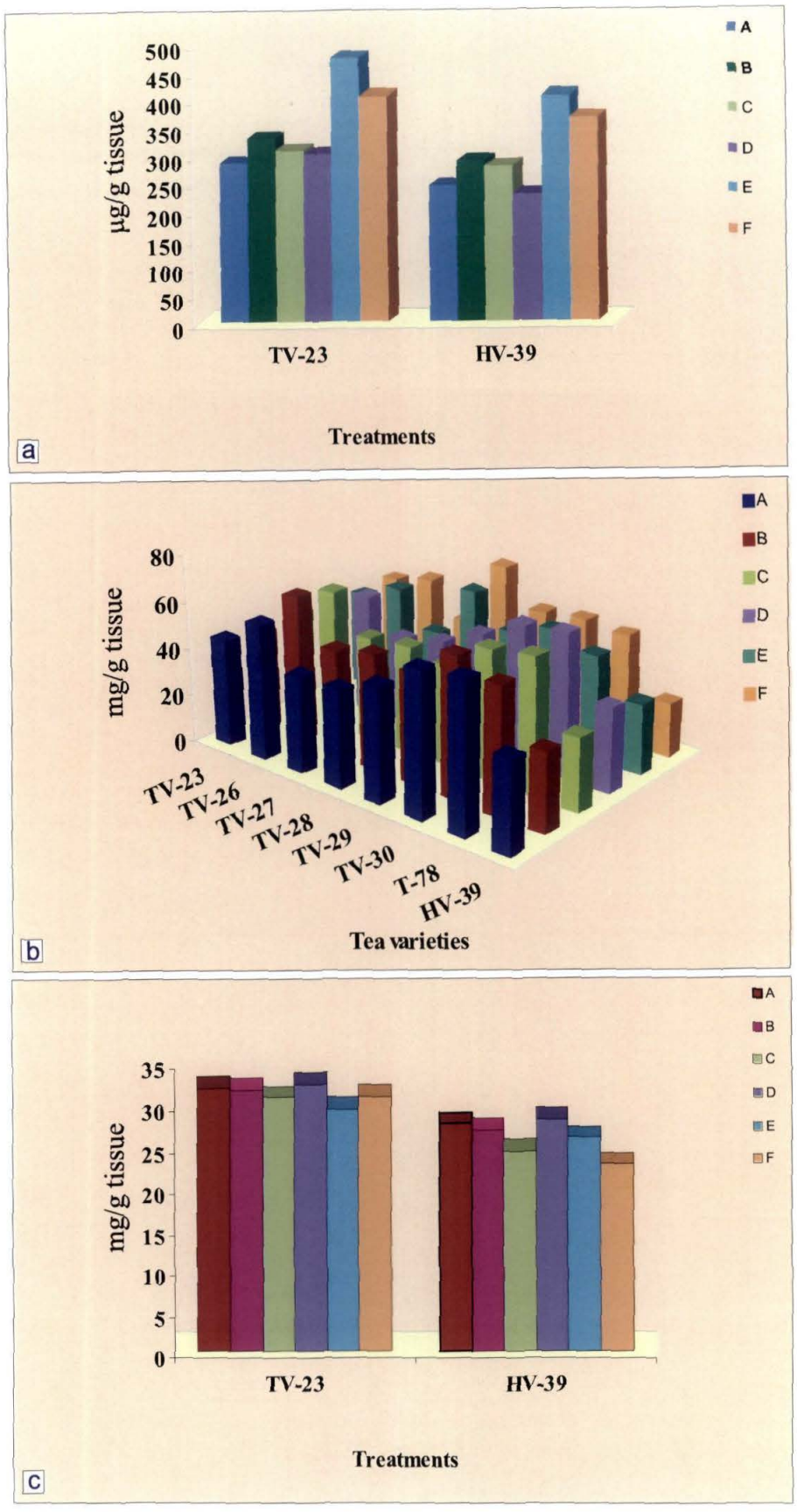


Fig.6 (a-c): Changes in biochemical components of tea induced by spraying of in fungicide or insecticide.
Proline content of leaves of bushes (a); Protein content of leaves of (b) and bushes (c)
Legend: (A-C) 1st application and (D-F) 2nd application
A&D=Control, B&E=Insecticide and C&F=Fungicide treatments

Table 13: Changes in proline content of tea leaves following fungicide and insecticide treatment in potted seedlings.

Varieties	Proline content (µg/g tissue)					
	1st treatment			2nd treatment		
	Control	Insecticide	Fungicide	Control	Insecticide	Fungicide
TV-23	129.50 ±6.23	268 ±2.10	230.60 ±2.34	156.25 ±1.86	358.75 ±1.82	337.50 ±2.55
TV-26	90.37 ±8.93	131.25 ±7.55	157.00 ±12.76	106.20 ±14.74	279.75 ±9.09	285 ±3.52
TV-27	99 ±7.55	136.62 ±7.55	126.50 ±11.65	89 ±5.67	205.37 ±9.00	210 ±3.72
TV-28	96.87 ±6.33	190.62 ±9.01	206.25 ±7.30	106.86 ±7.10	384.75 ±7.50	361 ±7.50
TV-29	109.50 ±6.32	209.37 ±2.68	237.50 ±8.23	102.50 ±3.22	376.87 ±9.50	333.75 ±5.38
TV-30	71 ±7.05	131 ±9.55	167.75 ±6.53	77.50 ±9.09	354.37 ±3.62	209 ±6.23
T-78	93.75 ±3.56	198.12 ±5.30	164.25 ±8.90	107.50 ±6.50	305 ±8.91	340 ±11.38
HV-39	83.75 ±11.38	239.58 ±8.93	164.00 ±12.50	117.17 ±7.57	359.37 ±8.50	284.37 ±11.65
CD Treatment (P=0.05)= 29.06471 CD Varieties (P=0.05) =47.6247				CD Treatment (P=0.05)= 41.34461 CD Varieties (P=0.05) =67.51547		

Values are mean of 3 replicates; ± = SEm

Table 13A: Analysis of variance of data presented in Table 13 (1st treatment)

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	41634.12453	2	20817.06226	28.33973	1.2E-05	3.738892
Columns	21151.15543	7	3021.593633	4.113508	0.011735	2.764199
Error	10283.75441	14	734.5538863			
Total	73069.03436	23				

Table 13B: Analysis of variance of data presented in Table 13 (2nd treatment)

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	225600.7235	2	112800.3618	75.88935	3.06E-08	3.738892
Columns	33580.7891	7	4797.255585	3.227477	0.029528	2.764199
Error	20809.31169	14	1486.379407			
Total	279990.8243	23				

4.3.1. Protein content

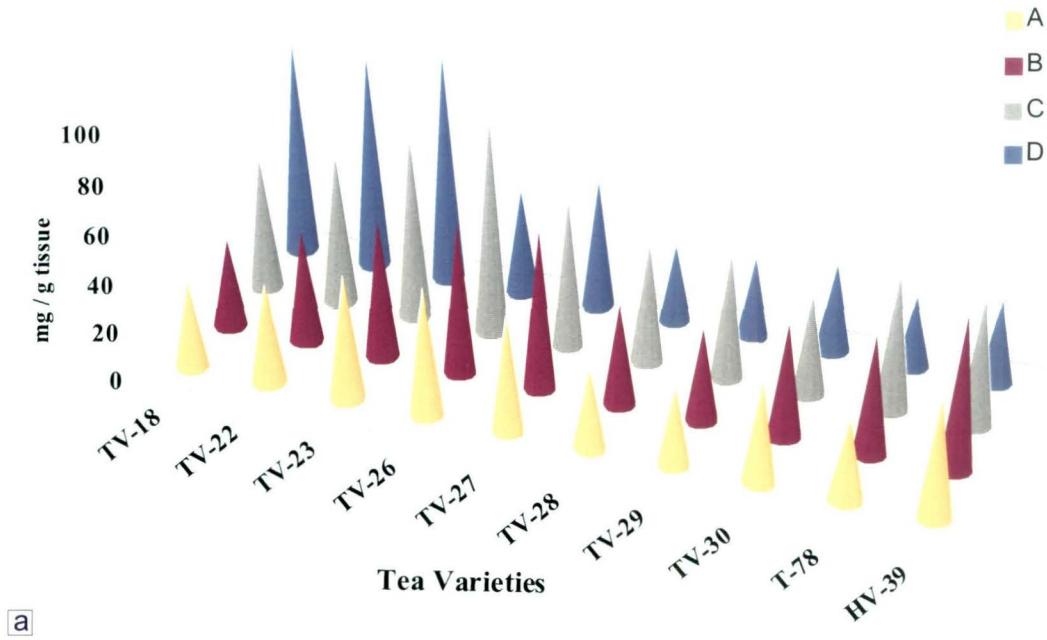
4.3.3.1.1. Heavy metal

Young shoots of ten varieties were subjected to heavy metal stress and then protein content was estimated. Results (Fig 7a) revealed a significant difference in protein content. Cu enhanced the protein content at lower concentration in every case, whereas, at higher concentration i.e.500µg/ml in some of the varieties there was a decline. Cu at 1000µg/ml further enhanced protein content in only three varieties of Tocklai i.e. TV-18, TV-22,23 and the other seven varieties protein content decreased. In case of Cd, higher accumulation of protein was also evident but at 1000µg/ml protein content was reduced (Fig 7b).

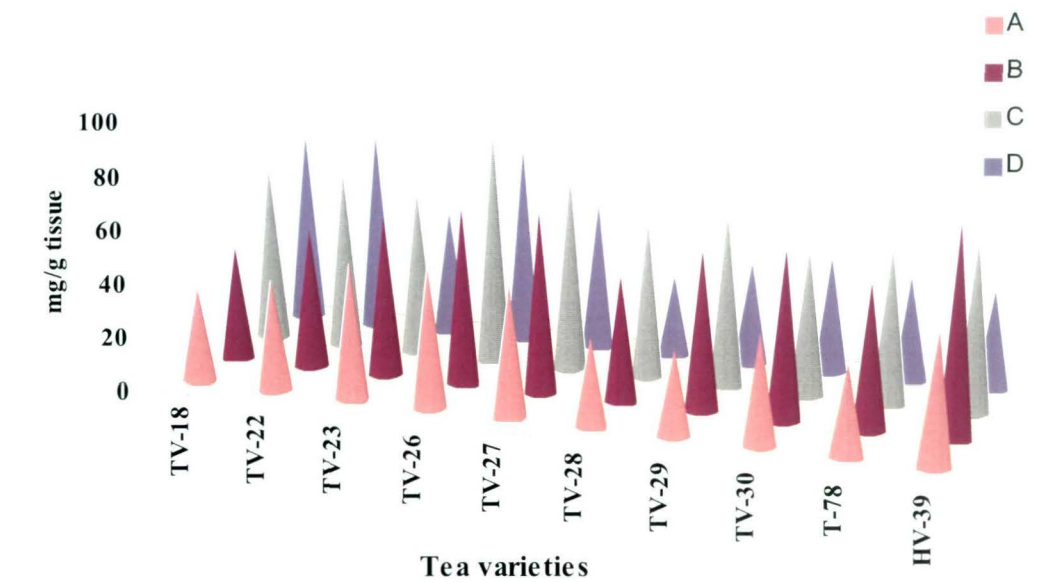
When the whole plants were subjected to different heavy metal stress changes were noticed in the protein content. There was significant increase in protein after 1st application of heavy metal and after 2nd treatment maximum protein content were found at 500 µg/ml, beyond that concentration protein content was reduced in most of the varieties (Figs. 8&9).

4.3.1.2. Fungicide/Insecticide

Effect of spraying with insecticide and fungicide was determined in seedlings and full grown mature bushes. Protein content was overall reduced in most of the seedlings (Fig.6b) after each application. There was slight reduction of protein at the first application of insecticide as well as fungicide but protein content was further reduced after the second application of these chemicals.But the changes are not very



a



b

Fig.7: Effect of Cu (a) and Cd (b) on protein content in tea leaves of cut shoots
Legend: A=Control, B=100, C=500, D=1000 µg/ml

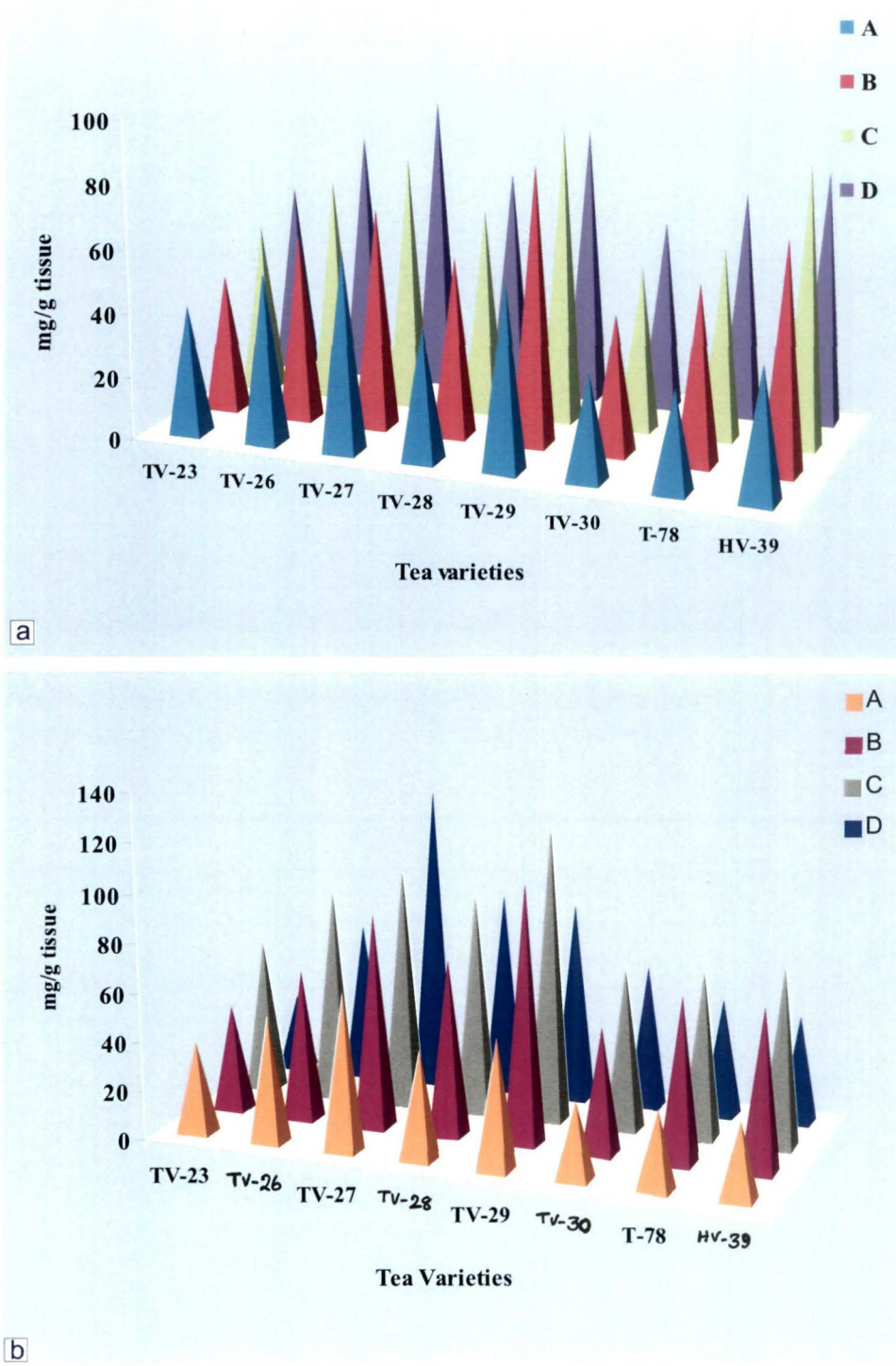
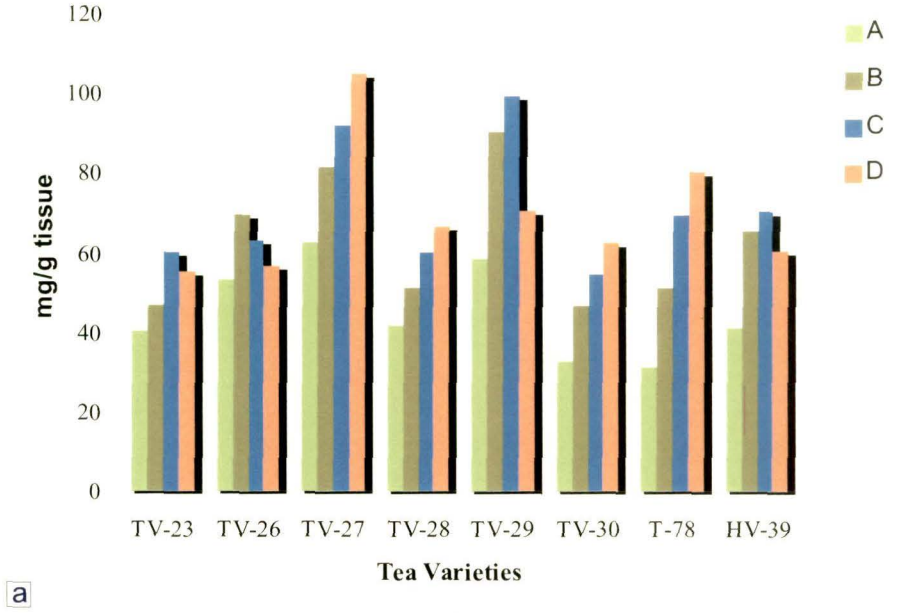
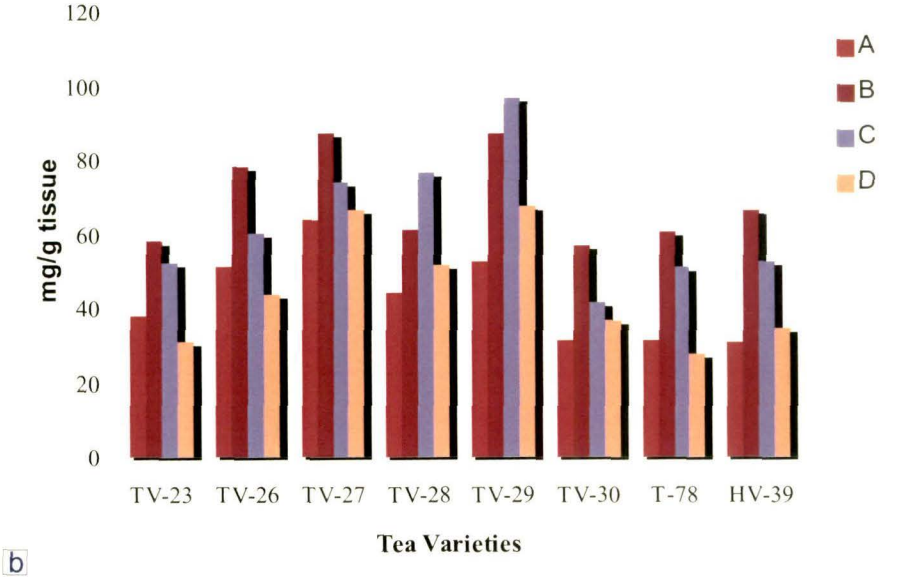


Fig.8: Changes in protein content of tea leaves of young plants following 1st (a) and 2nd (b) application of Cu stress
Legend: A=Control, B= 100, C=500, D=1000 µg/ml



a



b

Fig.9: Changes in protein content of tea leaves of young plants following 1st (a) and 2nd (b) application of Cd stress
Legend: A=Control, B= 100, C=500, D=1000 µg/ml

significant. In mature bushes of TV-23 and HV-39 protein content after 1st application of chemicals was not altered, but after 2nd application protein content was reduced (Fig 6c).

4.3.2. Protein pattern

SDS –PAGE analysis

In order to determine changes in protein profile following anthropogenic stresses SDS-PAGE analysis of soluble protein was carried out. Proteins were extracted from tea leaves of different varieties of treated and control. SDS–PAGE gel revealed a number of bands ranging from molecular weight 14-200 KD.

The treatments did not significantly alter the protein pattern but in some cases there was a apperence of new bands while in others some proteins were missing in relation to control as was evident in SDS PAGE analysis. In TV-23 (Plate VIII, fig A) young shoots treated with different heavy metal solutions, little changes of protein pattern were noticed. Two bands of approx 40 KD and 37 KD were absent in both Cd1000 and Cd 500. Similarly in Cu 500 and Cu1000 treatment of one band approx 40 K.D was over expressed. In Cd 1000 and Cu 500 there was overexpression of 35 KD and 30 KD band . In T-78 , (Plate VIII, fig. B) treatment with Cu at1000µg/ml led to significant change in protein pattern . In Cd at1000µg/ml overall protein expression did not show much difference from control but a band was missing. In Cd100 µg/ml two new bands of medium range were expressed. In TV-29, however, none of the concentration led to an overall decline in protein, but Cd at1000µg/ml there was disapperence of two high molecular weight bands (Plate IX, fig A). There was an overexpression of most of the proteins were found in all the varieties. When bushes were sprayed with insecticide/ fungicide in TV-23 expression of protein bands were enhanced, especially in case of fungicide treatment (Plate IX, fig B).

4.4. Changes in pigment content of tea leaves following different stresses

4.4.1. Chlorophyll

Chlorophyll is the most important plant pigment as it controls the photosynthesis and determines the productivity of a plant. Plant changes the chlorophyll content in

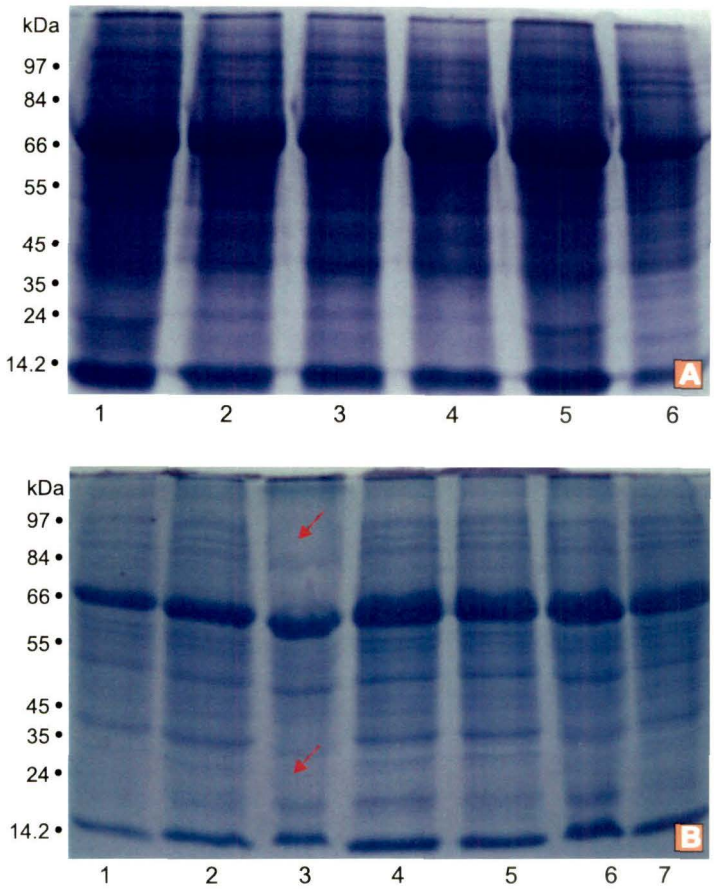
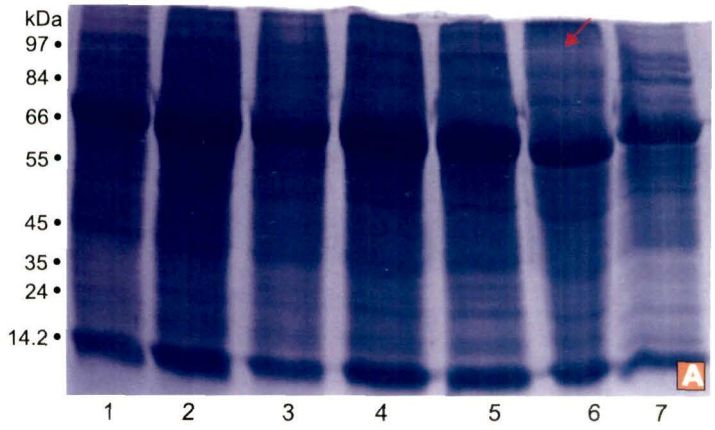


Plate VIII (A & B): SDS PAGE analysis of protein from leaves of tea plants subjected to heavy metal stress.

A:TV-23, (Lane 1: Cu 500 µg/ml; Lane 2: Cu 1000 µg/ml; Lane 3: Cd 100 µg/ml; Lane 4: Cd 500 µg/ml; Lane 5: Cd 1000 µg/ml; Lane 6: Control).

B: T-78, (Lane 1: Cu 100 µg/ml;Lane 2: Cu 500 µg/ml; Lane 3: Cu 1000 µg/ml; Lane 4: Cd 100 µg/ml; Lane 5: Cd 500 µg/ml; Lane 6:Cd 1000 µg/ml;Lane 7:Control).



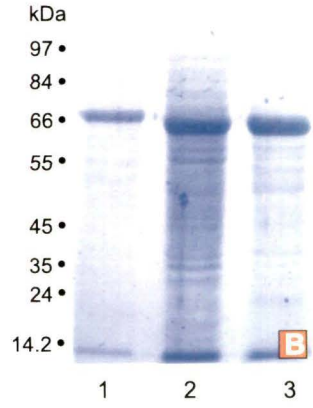


Plate IX (A-B): SDS PAGE analysis of protein from leaves of tea plants subjected to heavy metal (A) and insecticide/fungicide treatments (B).

A: TV-29- Lanes 1-3: Cu 100, 500 and 1000 μg/ml, respectively; Lanes 4-6: Cd 100, 500 and 1000 μg/m, respectivelyl;Lane 7:Control).

B: TV-23-Lane 1: Control; Lane 2: Fungicide; Lane 3: Insecticide.

stressed condition and the photosynthetic activity is altered. So, keeping this important phenomenon in mind total chlorophyll content, chl a, chl b was determined at various stresses.

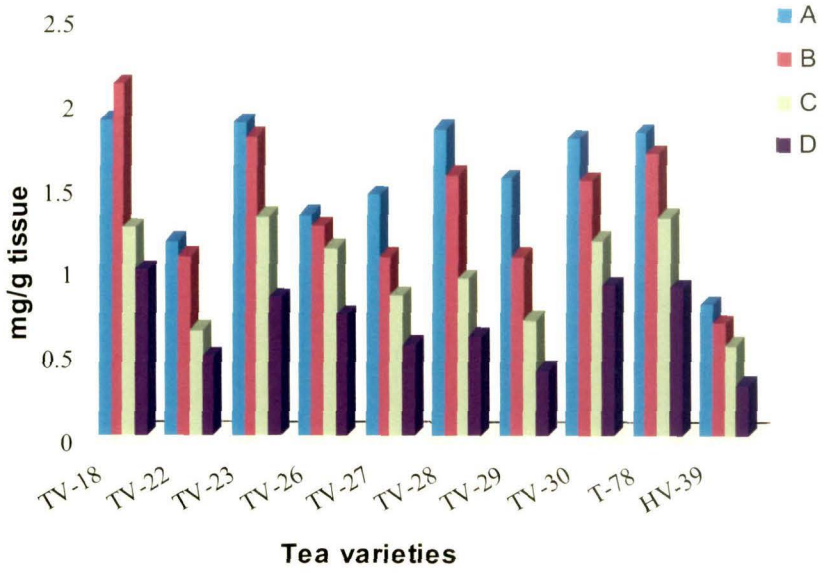
Total chlorophyll content, chl a, and chl b were determined in leaves of cut shoots following treatment with the heavy metals at different concentrations. Result showed that chlorophyll content declined significantly at the higher concentration of both the tested metals. When shoots were dipped in different concentration of Cu solutions changes of chlorophyll content was noticed. The result is presented in (Fig 10).

When the shoots were immersed in Cd solutions chlorophyll content were also declined . The highest decline of chlorophyll content was noted at 1000µg/ml in both the tested chemicals (Fig 11).

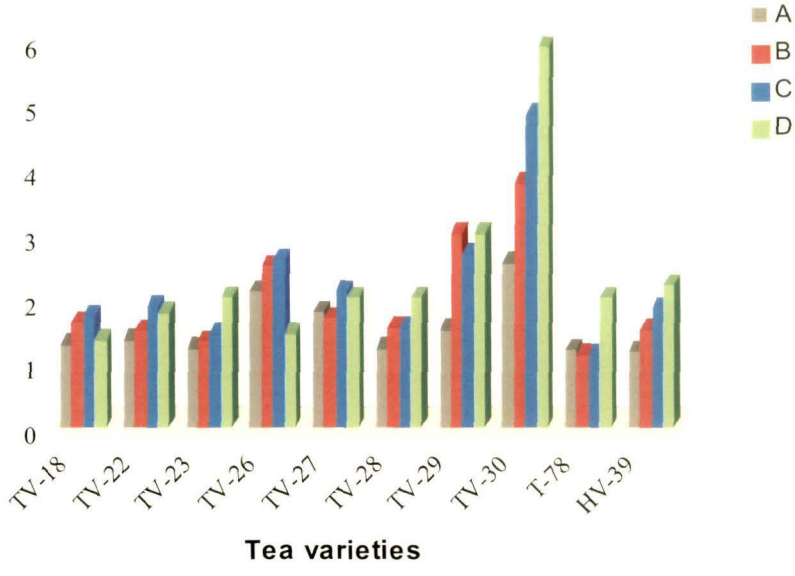
The responses of different stresses of heavy metal concentration on the seedlings were also studied. When seedlings were treated with different concentration of Cu solutions, the changes in total chlorophyll, chlorophyll a, chlorophyll b was recorded after each application. After 1st treatment at 100 µg/ml total chlorophyll content was increased in all varieties .Increasing a/b ratio in most of the cases also indicate the great loss of chlorophyll b than chlorophyll a at the identical condition (Table 14).

The reduction of total chlorophyll, chlorophyll a, chlorophyll b content was evidentat at 500µg/ml . In case of highest concentration maximum reduction was noted. The a/b ratio was also increased . After 2nd treatment reduced chlorophyll content was noted even at the lower concentration (100 µg/ml) (Table 15).

In case of Cd treatment TV-26, TV-27 ,TV-29,TV-30 varieties showed little increased chlorophyll content after 1st treatment at the lowest coentration, at the higher concentration decreased in total chlorophyll, chlorophyll a, chlorophyll b was noted.The result have been presented in (Table 16).

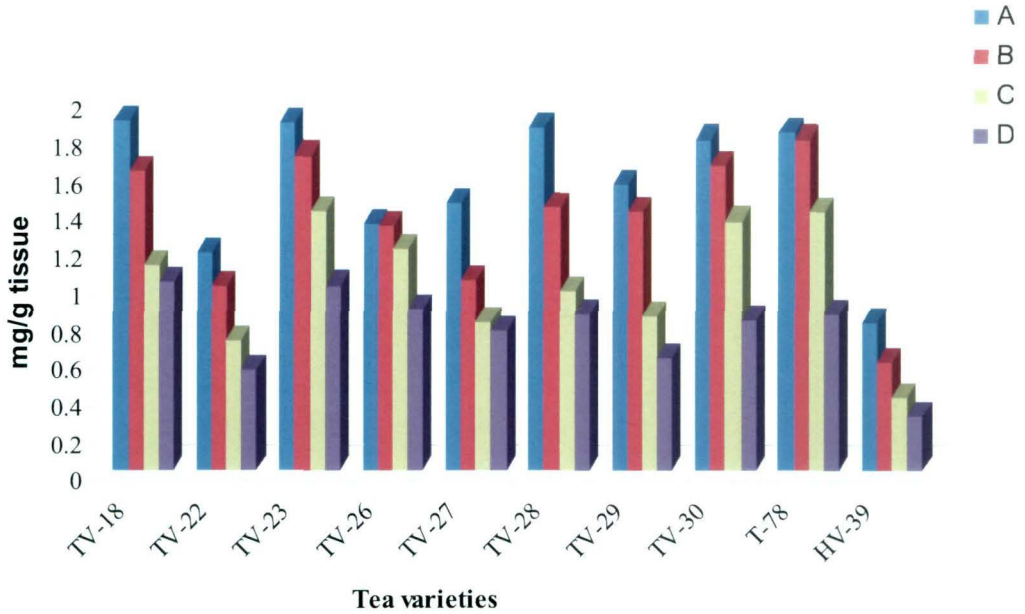


a

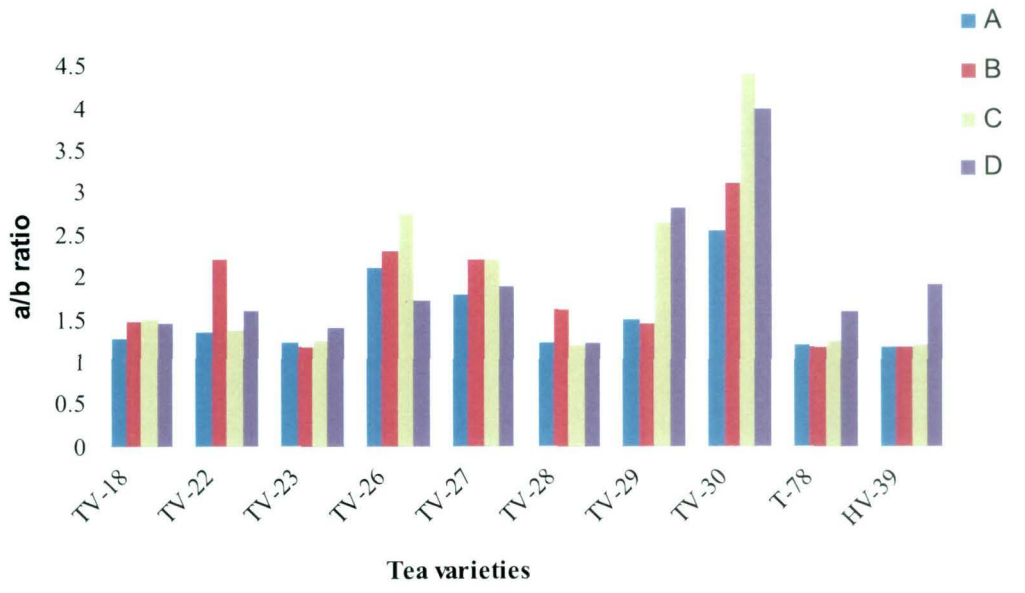


b

Fig.10: Effect of Cu stress on total chlorophyll content (a) and chlorophyll a/b ratio (b) of tea leaves in cut shoots.
Legend: A=Control, B= 100, C=500, D=1000 μg/ml



a



b

Fig.11: Effect of Cd stress on total chlorophyll content (a) and chlorophyll a/b ratio (b) of tea leaves in cut shoots.
Legend: A=Control, B= 100, C=500, D=1000 µg/ml

Table 14: Changes in cholorophyll content in young tea seedlings following 1st application of different concentrations of Cu.

Varieties	Chlorophyll content (mg/g tissue)							
	Concentration of Cu (µg/ml)							
	0		100		500		1000	
	T	a/b	T	a/b	T	a/b	T	a/b
TV-23	2.32 ±0.10	1.93 ±0.17	2.88 ±0.02	1.30 ±0.02	2.17 ±0.02	2.10 ±0.08	2.00 ±0.08	2.33 ±0.02
TV-26	1.37 ±0.01	2.51 ±0.09	1.53 ±0.07	2.23 ±0.05	1.30 ±0.03	2.68 ±0.04	1.11 ±0.01	2.92 ±0.15
TV-27	1.50 ±0.03	1.90 ±0.03	1.47 ±0.03	2.00 ±0.11	1.31 ±0.01	2.19 ±0.04	1.18 ±0.01	2.80 ±0.11
TV-28	1.87 ±0.07	1.10 ±0.04	2.03 ±0.13	1.18 ±0.06	1.77 ±0.01	1.07 ±0.01	1.52 ±0.03	1.15 ±0.03
TV-29	1.13 ±0.02	2.11 ±0.07	1.22 ±0.02	1.85 ±0.12	1.00 ±0.05	2.22 ±0.05	0.88 ±0.02	2.34 ±0.06
TV-30	1.56 ±0.02	1.56 ±0.06	1.74 ±0.03	1.55 ±0.01	1.37 ±0.08	1.64 ±0.09	1.20 ±0.01	1.72 ±0.05
T-78	1.48 ±0.05	2.50 ±0.02	1.56 ±0.02	2.10 ±0.07	1.26 ±0.05	2.57 ±0.08	1.07 ±0.01	3.11 ±0.01
HV-39	0.74 ±0.06	1.74 ±0.05	0.79 ±0.02	1.54 ±0.01	0.59 ±0.02	2.47 ±0.01	0.41 ±0.01	3.00 ±0.03
CD Treatment (P=0.05)= 0.096031; CD Varieties (P=0.05) =0.135808								

Values are mean of 3 replicates; ± = SEm

Table 14A: Analysis of variance of data presented in Table 14.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1.017109375	3	0.339036458	39.74955	7.71E-09	3.072467
Columns	7.059296875	7	1.008470982	118.2359	2.01E-15	2.487578
Error	0.179115625	21	0.008529315			
Total	8.255521875	31				

Table 15: Changes in cholorophyll content in young tea seedlings following 2nd application of different concentrations of Cu.

Varieties	Chlorophyll content (mg/g tissue)							
	Concentration of Cu (µg/ml)							
	0		100		500		1000	
	T	a/b	T	a/b	T	a/b	T	a/b
TV-23	2.71 ±0.24	2.00 ±0.02	2.67 ±0.03	2.12 ±0.01	2.49 ±0.01	2.19 ±0.03	1.29 ±0.15	1.82 ±0.10
TV-26	1.46 ±0.27	3.70 ±0.05	1.32 ±0.04	2.35 ±0.01	1.06 ±0.10	2.58 ±0.01	0.96 ±0.11	3.17 ±0.04
TV-27	1.82 ±0.50	1.96 ±0.04	1.50 ±0.03	1.90 ±0.01	1.16 ±0.11	2.17 ±0.05	0.98 ±0.06	2.23 ±0.04
TV-28	1.69 ±0.02	1.34 ±0.01	1.44 ±0.03	1.32 ±0.02	1.10 ±0.02	1.75 ±0.06	0.88 ±0.04	2.66 ±0.02
TV-29	1.07 ±0.02	2 ±0.03	1.15 ±0.14	2.59 ±0.02	0.98 ±0.09	2.59 ±0.13	0.72 ±0.07	2.55 ±0.09
TV-30	1.85 ±0.01	1.33 ±0.11	1.80 ±0.15	1.68 ±0.01	1.11 ±0.05	1.87 ±0.15	0.66 ±0.10	3.78 ±0.30
T-78	1.31 ±0.13	2.71 ±0.04	0.92 ±0.7	2.28 ±0.01	0.78 ±0.11	2.85 ±0.11	0.70 ±0.01	2.33 ±0.08
HV-39	0.91 ±0.20	2 ±0.02	0.79 ±0.03	1.54 ±0.04	0.66 ±0.15	1.05 ±0.10	0.49 ±0.10	1.57 ±0.11
CD Treatment (P=0.05)= 0.226096; CD Varieties (P=0.05) =0.319749								

Values are mean of 3 replicates; ± = SEm

Table 15A: Analysis of variance of data presented in Table 15.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	2.736534375	3	0.912178125	19.2929	3.04E-06	3.072467
Columns	6.293896875	7	0.899128125	19.01689	9.2E-08	2.487578
Error	0.992890625	21	0.047280506			
Total	10.02332188	31				

Table 16: Chlorophyll content in tea leaves following 1st application of Cd solution to potted tea varieties.

Varieties	Chlorophyll content (mg/g tissue)							
	Concentration of Cd (μg/ml)							
	0		100		500		1000	
	T	a/b	T	a/b	T	a/b	T	a/b
TV-23	2.32 ±0.10	1.93 ±0.05	2.23 ±0.04	2 ±0.15	2.20 ±0.18	2.09 ±0.08	2.01 ±0.02	2.58 ±0.20
TV-26	1.37 ±0.15	2.51 ±0.05	1.54 ±0.09	2.08 ±0.11	1.41 ±0.15	2.33 ±0.11	1.20 ±0.03	1.79 ±0.02
TV-27	1.50 ±0.11	1.90 ±0.17	1.57 ±0.03	2.34 ±0.12	1.33 ±0.11	1.60 ±0.08	1.08 ±0.04	1.89 ±0.01
TV-28	1.87 ±0.10	1.10 ±0.15	1.82 ±0.04	2.27 ±0.01	1.67 ±0.10	1.03 ±0.05	1.49 ±0.12	1.12 ±0.01
TV-29	1.13 ±0.09	2.11 ±0.11	1.24 ±0.09	2.44 ±0.02	0.95 ±0.09	2.27 ±0.60	0.79 ±0.17	2.59 ±0.03
TV-30	1.56 ±0.08	1.56 ±0.16	1.65 ±0.11	1.5 ±0.03	1.55 ±0.08	1.58 ±0.12	1.16 ±0.09	1.87 ±0.04
T-78	1.48 ±0.09	2.50 ±0.02	1.45 ±0.12	2.06 ±0.01	1.21 ±0.02	3.48 ±0.15	0.99 ±0.02	6.00 ±0.03
HV-39	0.74 ±0.10	1.74 ±0.01	0.62 ±0.17	2.10 ±0.02	0.60 ±0.11	1.76 ±0.11	0.36 ±0.07	2.88 ±0.09
CD Treatment (P=0.05)= 0.071417; CD Varieties (P=0.05) =0.101								

Values are mean of 3 replicates; ± = SEm

Table 16A: Analysis of variance of data presented in Table 16.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.735759375	3	0.245253125	51.98893	6.82E-10	3.072467
Columns	6.201921875	7	0.885988839	187.8125	1.76E-17	2.487578
Error	0.099065625	21	0.004717411			
Total	7.036746875	31				

After 2nd treatment there was a great loss of total chlorophyll, chlorophyll a, and chlorophyll b in all the varieties. The ratio of chlorophyll a/b showed an increase over control (Table 17).

Table 17: Chlorophyll content in tea leaves following 2nd application of Cd solution to potted tea varieties.

Varieties	Chlorophyll content (mg/g tissue)							
	Concentration of Cd (µg/ml)							
	0		100		500		1000	
	T	a/b	T	a/b	T	a/b	T	a/b
TV-23	2.71 ±0.06	2.0 ±0.01	2.48 ±0.06	2.17 ±0.04	2.47 ±0.09	2.22 ±0.11	1.86 ±0.01	2.38 ±0.02
TV-26	1.46 ±0.15	3.70 ±0.25	1.16 ±0.01	4.0 ±0.08	1.15 ±0.03	3.25 ±0.17	0.94 ±0.35	6.07 ±0.02
TV-27	1.82 ±0.09	1.96 ±0.15	1.32 ±0.03	2.35 ±0.03	1.13 ±0.24	2.64 ±0.20	1.04 ±0.40	2.85 ±0.13
TV-28	1.69 ±0.2	1.34 ±0.02	1.59 ±0.04	1.30 ±0.09	1.08 ±0.12	1.78 ±0.02	0.8 ±0.30	2.76 ±0.20
TV-29	1.07 ±0.15	2 ±0.10	1.03 ±0.10	2.67 ±0.09	0.90 ±0.35	2.5 ±0.03	0.76 ±0.04	2.3 ±0.02
TV-30	1.85 ±0.09	1.33 ±0.10	1.68 ±0.12	1.51 ±0.10	1.18 ±0.12	2.37 ±0.08	0.99 ±0.44	2.0 ±0.01
T-78	1.31 ±0.11	2.71 ±0.09	1.02 ±0.14	3.08 ±0.10	.0.79 ±0.03	2.59 ±0.35	0.67 ±0.35	2.47 ±0.21
HV-39	0.91 ±0.10	2 ±0.08	0.85 ±0.24	1.93 ±0.20	0.62 ±0.05	1.3 ±0.30	0.44 ±0.30	1.58 ±0.09
CD Treatment (P=0.05)= 0.142164; CD Varieties (P=0.05) =0.20105								

Values are mean of 3 replicates; ± = SEm

Table 17A: Analysis of variance of data presented in Table 17.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1.973096094	3	0.657698698	35.1847	2.25E-08	3.072467
Columns	7.198211719	7	1.02831596	55.0115	4.34E-12	2.487578
Error	0.392547656	21	0.018692746			
Total	9.563855469	31				

Analysis of total chlorophyll, chl a, chl b contents of leaves were done after each treatment of insecticide and fungicide to the seedlings and the bushes. Result indicates that the 1st spray of both the chemicals did not significantly alter the chlorophyll content (Table 18).

Table 18: Changes in chlorophyll content in tea seedlings following insecticide/fungicide treatment

Varieties	Chlorophyll content content (mg/g tissue)											
	1st treatment						2nd treatment					
	Control		Insecticide		Fungicide		Control		Insecticide		Fungicide	
	T	a/b	T	a/b	T	a/b	T	a/b	T	a/b	T	a/b
TV-23	1.45 ±0.10	1.48 ±0.20	1.25 ±0.33	1.60 ±0.30	1.53 ±0.30	1.46 ±0.25	1.19 ±0.04	1.87 ±0.02	1.14 ±0.15	1.47 ±0.02	1.09 ±0.50	1.92 ±0.01
TV-26	1.29 ±0.98	1.48 ±0.90	1.06 ±0.45	1.89 ±0.55	1.08 ±0.03	1.89 ±0.09	1.27 ±0.03	1.51 ±0.09	0.99 ±0.35	1.45 ±0.30	1 ±0.09	1.51 ±0.02
TV-27	1.41 ±0.19	1.50 ±0.25	1.19 ±0.10	1.78 ±0.30	1.44 ±0.25	1.48 ±0.35	1.27 ±0.03	1.54 ±0.03	1.12 ±0.28	1.43 ±0.15	1.02 ±0.45	1.81 ±0.44
TV-28	0.96 ±0.11	1.20 ±0.09	1.06 ±0.50	1.16 ±0.35	0.97 ±0.29	1.20 ±0.41	0.85 ±0.05	1.47 ±0.06	0.81 ±0.11	2 ±0.17	1.08 ±0.35	1.30 ±0.30
TV-29	0.76 ±0.13	1.33 ±0.08	0.96 ±0.14	0.84 ±0.20	0.82 ±0.02	1.00 ±0.42	0.64 ±0.08	1.56 ±0.11	0.66 ±0.38	2.0 ±0.35	0.85 ±0.25	2.10 ±0.45
TV-30	1.51 ±0.27	1.60 ±0.13	1.55 ±0.17	1.73 ±0.22	1.48 ±0.03	1.82 ±0.09	1.45 ±0.11	1.48 ±0.02	1.25 ±0.3	2.18 ±0.25	1.31 ±0.25	1.70 ±0.20
T-78	1.82 ±0.11	2 ±0.09	1.55 ±0.18	1.50 ±0.09	1.32 ±0.09	1.69 ±0.08	2 ±0.17	1.24 ±0.01	1.54 ±0.09	1.52 ±0.02	1.40 ±0.09	2.00 ±0.15
HV-39	1.05 ±0.35	1.56 ±0.30	0.96 ±0.01	1.56 ±0.09	1.03 ±0.10	1.68 ±0.08	1.70 ±0.10	1.85 ±0.05	0.98 ±0.20	1.94 ±0.10	1.30 ±0.15	2.60 ±0.18
CD Treatment (P=0.05)= 0.139708; CD Varieties (P=0.05) =0.228142							CD Treatment (P=0.05)= 0.181705 CD Varieties (P=0.05) =0.51394					

Values are mean of 3 replicates; ± = SEm

Table 18A: Analysis of variance of data presented in Table 18.

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	0.033058333	2	0.016529167	0.973907	0.40178	3.738892
Columns	1.479716667	7	0.211388095	12.45509	4.76E-05	2.764199
Error	0.237608333	14	0.016972024			
Total	1.750383333	23				

Table 18B: Analysis of variance of data presented in Table 18 (2nd treatment)

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.232933333	2	0.116466667	4.056726	0.040766	3.738892
Columns	1.678429167	7	0.239775595	8.351779	0.000428	2.764199
Error	0.401933333	14	0.028709524			
Total	2.313295833	23				

But decrease in total chlorophyll content, chl a, chl b were observed after 2nd spray. Both varieties of bushes i.e.. TV-23 and HV-39 showed a decrease in chlorophyll content (Fig 12a & b).

4.4.2. Carotenoid

Carotenoid, a non enzymatic antioxidant, plays a vital role in photoprotection of chlorophyll molecule.

In vitro heavy metal treatment in all the tested varieties showed a general decline in carotenoid content. In Cu treatment enhanced carotenoid content was noted in few varieties at the lowest concentration but the changes were not significant ,whereas, the higher concentrations decreased the carotenoid content. Similar trend was also evident in Cd (Fig 13).

In vivo heavy metal treatment also altered carotenoid content in both the tested metals. Cu induced an initial increased in carotenoid content after 1st treatment at the lowest concentration, though, after that, reduction was noted. After 2nd treatment greater loss of carotenoid was evident in all the varieties of seedlings (Fig 14). In Cd treated seedlings there was a little change of carotenoid content at the lowest concentration after 1st treatment. But significant reduction was noted at the higher concentration. Maximum loss was found at 1000µg/ml. Highest reduction of carotenoid content was noted afer 2nd application of each concentration (Fig 15a &b).

Analysis of carotenoid content after spraying with fungicide and insecticide in seedlings and to the mature full grown bushes revealed that carotenoid increased significantly after 1st and the 2nd application. In seedlings slight increase of carotenoid

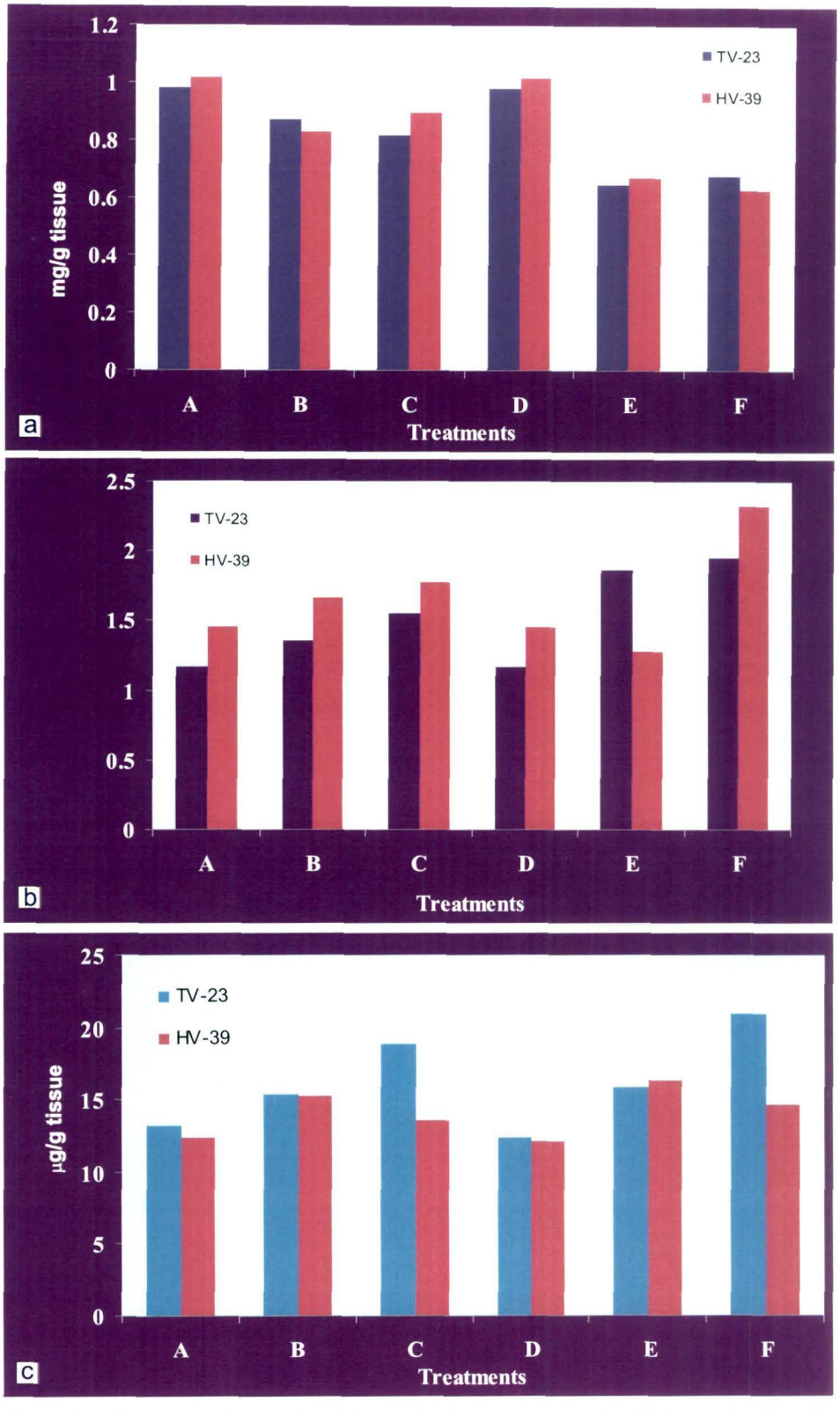
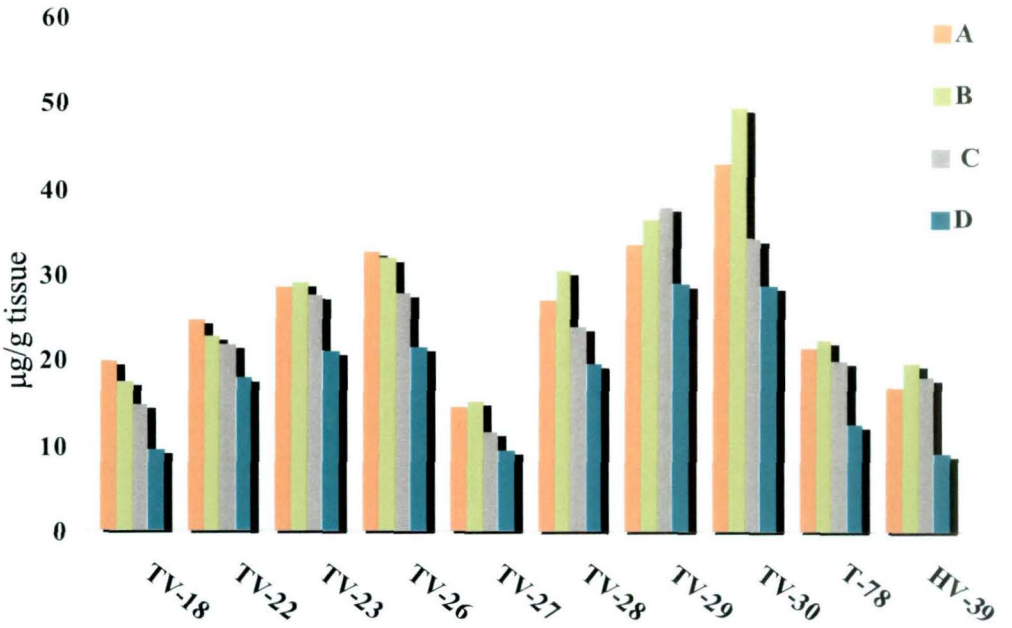
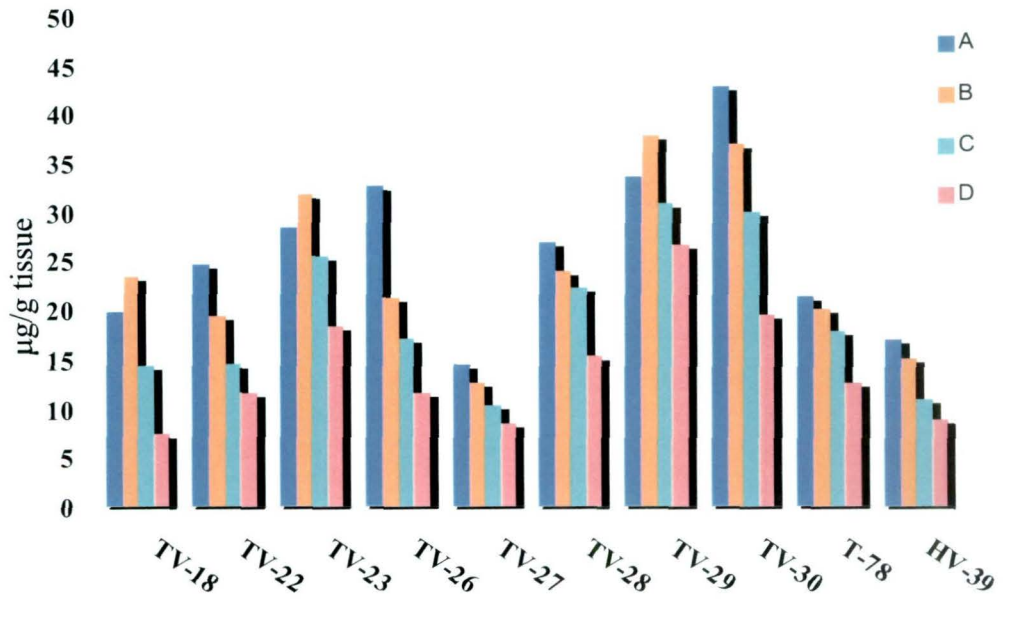


Fig.12: Changes in total chlorophyll content (a); Chlorophyll a/b ratio (b); and carotenoid content (c) of leaves of tea bushes induced by insecticide or fungicide spray.
(A-C): 1st application, (D-F) 2nd application A&D=Control; B&E=Insecticide and C&F=Fungicide treatments

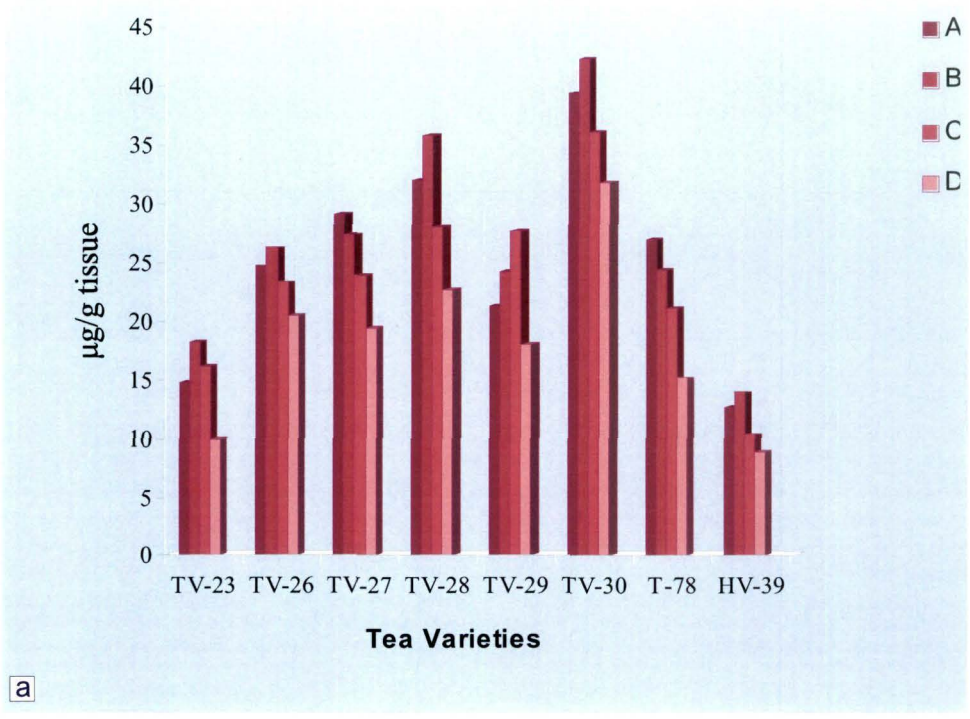


a



b

Fig.13: Effect of Cu (a) and Cd (b) stress on carotenoid content of tea leaves of cut shoots.
Legend: A=Control, B= 100, C=500, D=1000 µg/ml



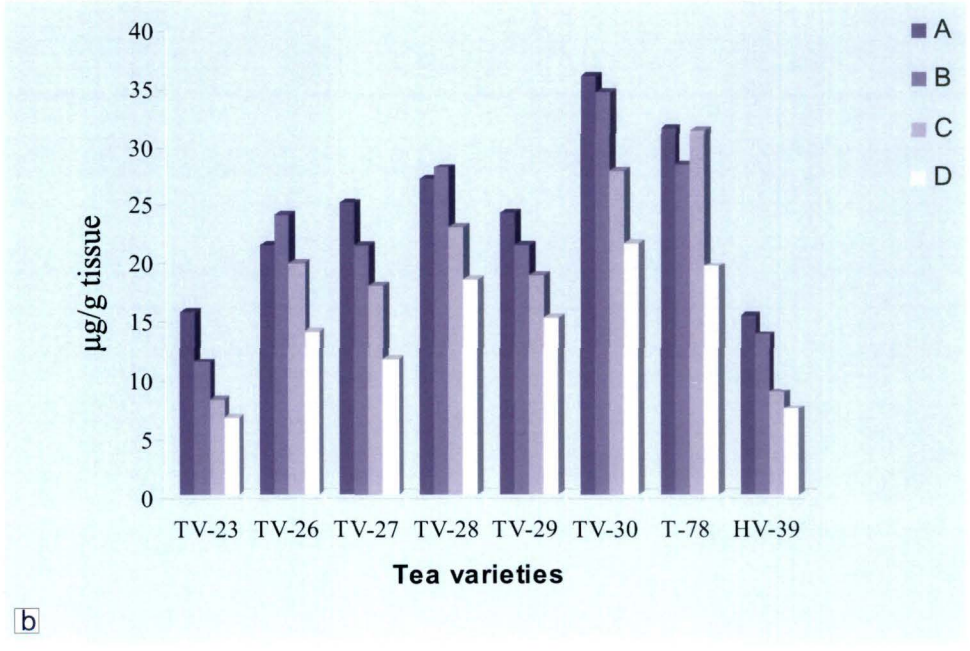


Fig.14: Effect of 1st application (a) and 2nd application (b) of Cu on carotenoid content in leaves of potted tea seedlings.
Legend: A=Control, B= 100, C=500, D=1000 µg/ml

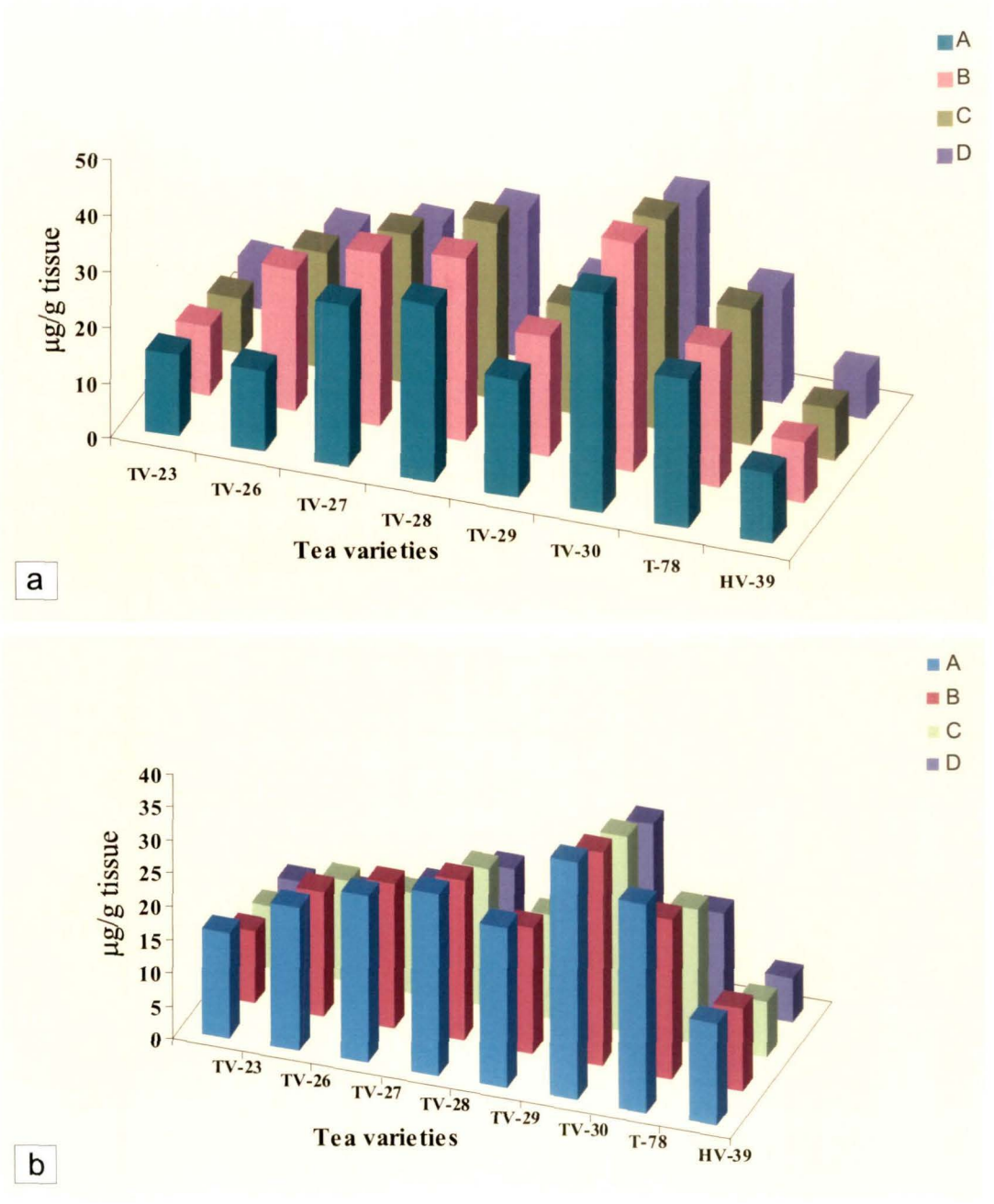


Fig.15: Effect of 1st application (a) and 2nd application (b) of Cd on carotenoid content in leaves of potted tea seedlings
Legend: A=Control, B= 100, C=500, D=1000 µg/ml

content was found after 1st application, whereas after 2nd application of these chemicals there was a significant increase of carotenoid content (Table 19).

Table 19: Changes in carotenoid content of tea leaves in seedlings following insecticide/fungicide spray.

Varieties	Carotenoid content (μg/g tissue)					
	1st treatment			2nd treatment		
	Control	Insecticide	Fungicide	Control	Insecticide	Fungicide
TV-23	10.95 ±0.50	18.72 ±0.56	15.20 ±0.50	11.31 ±0.30	20.12 ±0.25	16.90 ±0.50
TV-26	14.85 ±0.19	15.30 ±0.20	16.65 ±0.30	13.95 ±0.50	18.15 ±0.80	16.80 ±0.66
TV-27	12.77 ±0.44	14.35 ±0.25	13.30 ±0.50	12.25 ±0.10	15.57 ±0.25	19.42 ±0.15
TV-28	21.87 ±0.15	28.12 ±0.17	23.75 ±0.30	22.25 ±0.60	32.52 ±0.5	33.90 ±0.80
TV-29	10.35 ±0.10	13.20 ±0.40	16.50 ±0.50	10.20 ±0.53	16.65 ±0.66	17.35 ±0.72
TV-30	15.50 ±0.19	18 ±1.12	19.77 ±0.80	14.87 ±0.65	20.65 ±0.25	23.80 ±0.60
T-78	21.40 ±0.55	22.96 ±0.30	24.17 ±0.53	22.00 ±0.60	26.62 ±0.50	28.40 ±1.55
HV-39	10.50 ±0.15	16.10 ±0.17	13.75 ±0.66	12.15 ±0.36	18.50 ±0.45	17.75 ±0.17
CD Treatment (P=0.05)= 1.842609 CD Varieties (P=0.05) =3.008967				CD Treatment (P=0.05)= 1.846528 CD Varieties (P=0.05) =3.015367		

Values are mean of 3 replicates; ± = SEm

Table 19A: Analysis of variance of data presented in Table 19. (1st treatment)

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	60.3783	2	30.18915	10.2257	0.00183	3.738892
Columns	410.0131958	7	58.57331369	19.84	2.97E-06	2.764199
Error	41.33196667	14	2.952283333			
Total	511.7234625	23				

Table 19B: Analysis of variance of data presented in Table 19 (2nd treatment)

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	232.2186	2	116.1093	39.16189	1.84E-06	3.738892
Columns	626.1291	7	89.44702	30.16911	2.11E-07	2.764199
Error	41.50797	14	2.964855			
Total	899.8557	23				

When insecticide or fungicide was sprayed to bushes, there was increased carotenoid accumulation after each application (Fig 12c).

4.5. Studies on carbohydrate in tea leaves subjected to antropogenic stresses

4.5.1. Total soluble sugar and reducing sugar

Carbohydrates are the building blocks of cells and highly responsible for the plant's overall response to stress environments.

Influence of heavy metals on carbohydrate metabolism was analysed quantitatively after every treatment to determine the soluble total sugar, reducing sugar content. *In vitro* study of ten varieties with two metals revealed changes of total sugar- there was an overall decrease in total sugar, but at 100µg/ml Cu treatment in TV-18, TV-23, TV-28, total sugar was enhanced. The reducing sugar quantification following heavy metal treatments, revealed increased quantity at lower concentration of Cu, but at the highest concentration reducing sugar content was lowered in most of the cases (Fig 16). Cd treated shoots also showed higher total sugar content at lowest concentration in some varieties but, at the higher concentration showed declining trend, an increased reducing sugar content was noted in most of the varieties at lower concentration, though at the highest concentration it was declined (Fig 17).

In seedlings treated with the same concentration of Cu, there was accumulation of a higher total sugar content after 1st and 2nd treatment at lower concentration. The quantum of increase was not same in all varieties.The reducing sugar content was enhanced after 1st treatment with Cu upto 500 µg/ml. Reducing sugar content was declined after 2nd treatment at higher concentration except T-78&HV-39(Fig 18 & 19).

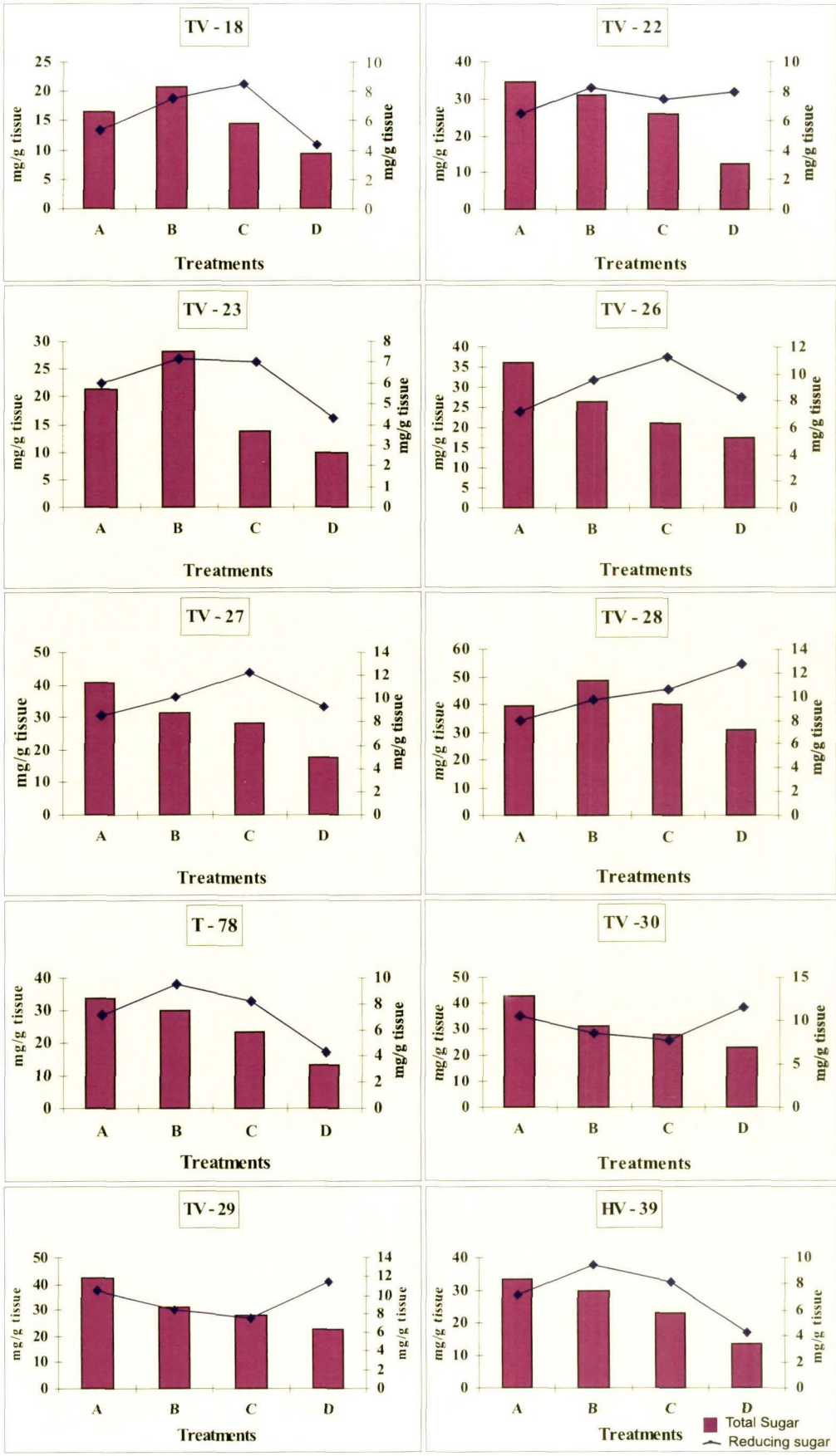


Fig.16: Changes in total and reducing sugar content in tea leaves of cut shoots following Cu stress

A=Control, B= 100, C=500, D=1000 µg/ml

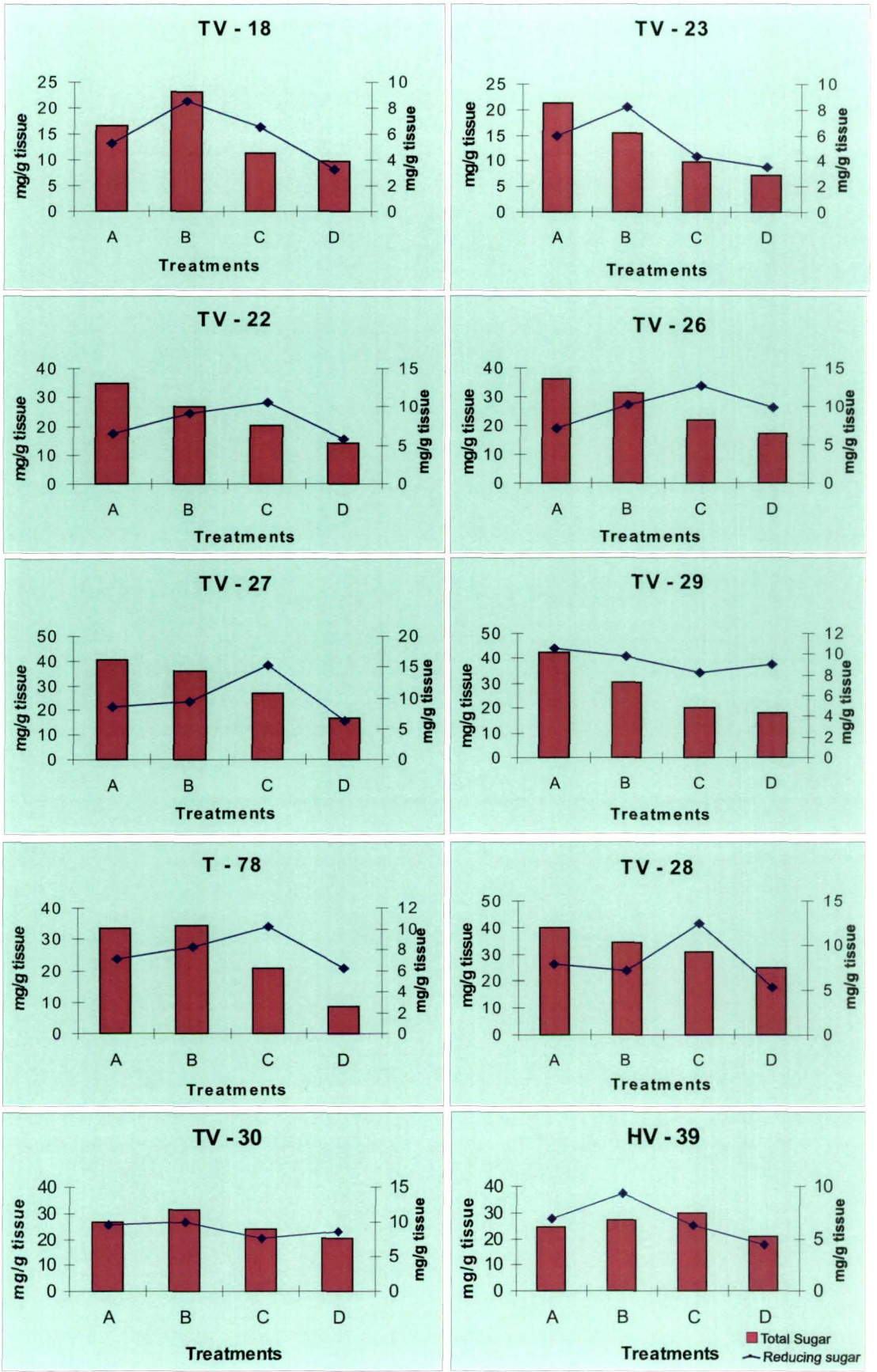


Fig.17: Changes in total and reducing sugar content in tea leaves of cut shoots following Cd stress

A=Control, B= 100, C=500, D=1000 µg/ml

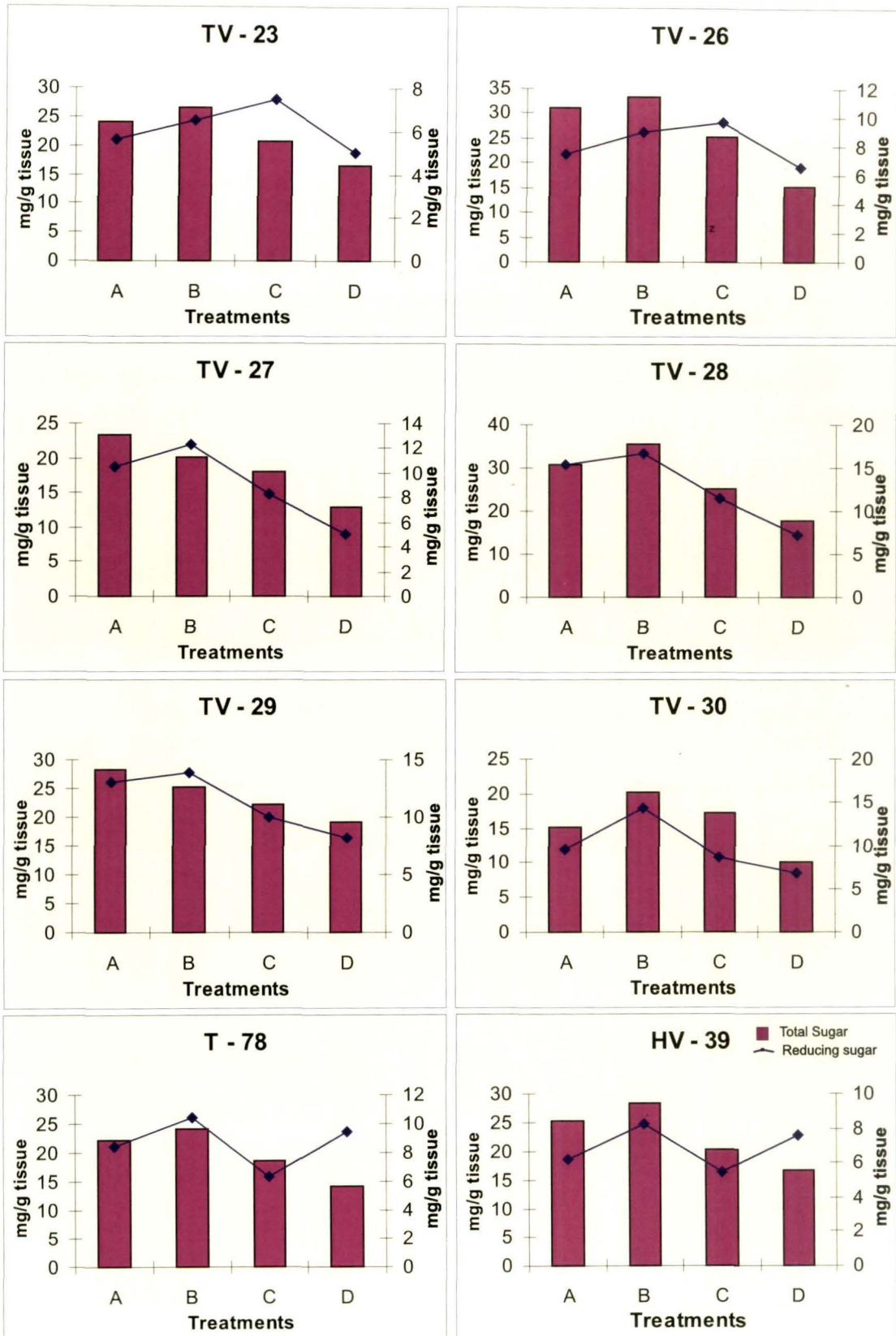


Fig.19: Total and reducing sugar content in leaves of potted tea seedlings subjected to Cu stress. (2nd).

A=Control, B= 100, C=500, D=1000 µg/ml

When seedlings were treated with Cd a significant change in total and reducing sugar content was noticed. While the lower concentration enhanced the total and reducing sugar content at the higher concentration sugar content delined.(Fig 20 & 21).

In case of potted plants subjected to insecticide/ fungicide treatement, some changes in total and reducing sugar content were observed. After 1st applications all the seedlings except TV-29 showed the reduction in total sugar content, though changes were not very marked after the 1st application. Total sugar level after the 2nd application of the insecticide and fungicide was reduced. (Fig 22). Reducing sugar level also showed similar declining trend after 1st and 2nd application of the tested chemicals.

The two varieties of bushes showed the decline sugar level after both the treatment.Changes were more pronounced after 2nd treatment. (Fig 23).

Results of studies of the anthropogenic stresses reveal that in most cases, sugar metabolism resulted in a reduction of carbohydrate content, though in some cases, there was an initial increased accumulation.

4.6. Studies on enzyme activities in the tea leave following stresses

Activities of 3 enzymes–phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POX) all of which are related to phenol metabolism, were analysed after imposition of the different stresses and results have been presented below.

4.6.1. Phenyl alanine ammonia lyase

Phenyl alanine ammonia lyase enzyme is an important enzyme which plays a vital role in phenol metabolism.There was an increment of PAL activity was noticed in all varieties in cut shoots dipped in Cu. When shoots were dipped in Cd solution PAL activity was also increased. In most of the Tocklai varieties PAL activity increased at lower concentration, but declined at higher concentration. The quantum of increase was higher in Cd than that of Cu at lower concentration (Fig. 24a & b).

In vivo study with different heavy metal concentrations revealed that PAL activity was enhanced after every treatment of Cu and Cd.(Fig 25 &26).

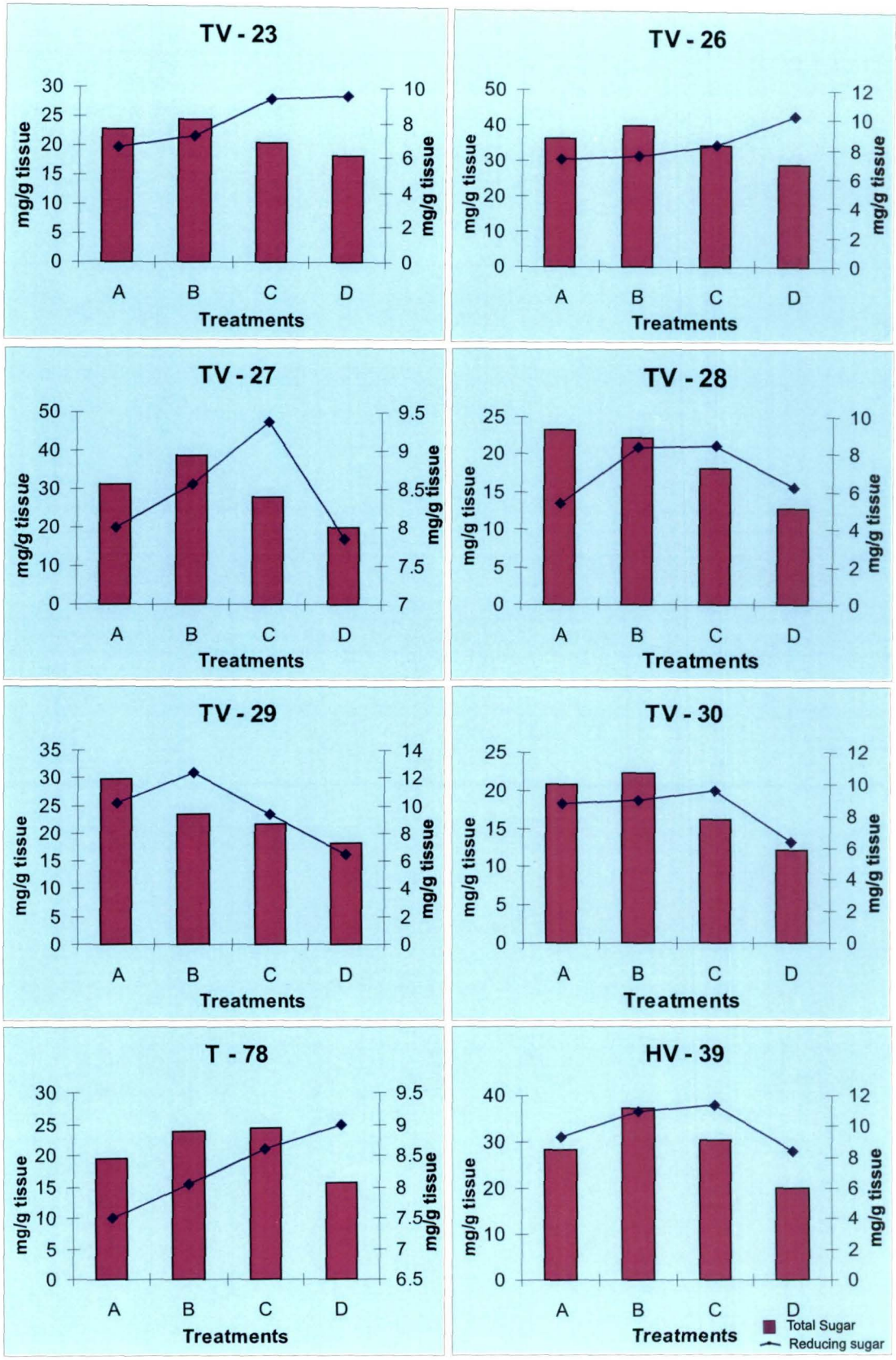


Fig.20: Total and reducing sugar content in leaves of potted tea seedlings subjected to Cd stress. (١٥٤).
A=Control, B= 100, C=500, D=1000 µg/ml

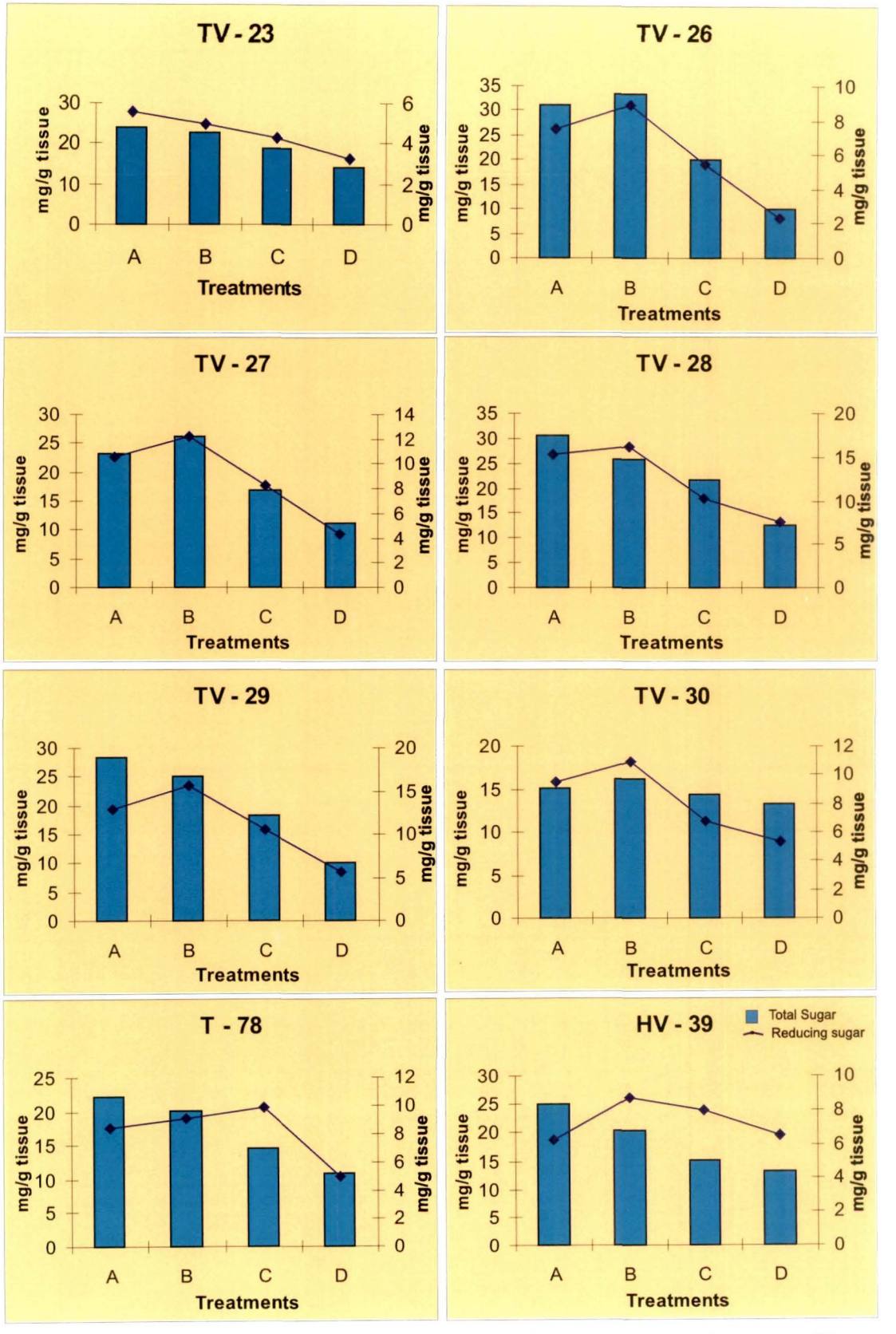


Fig.21: Total and reducing sugar content in leaves of potted tea seedlings subjected to Cd stress (2nd).

A=Control, B= 100, C=500, D=1000 µg/ml

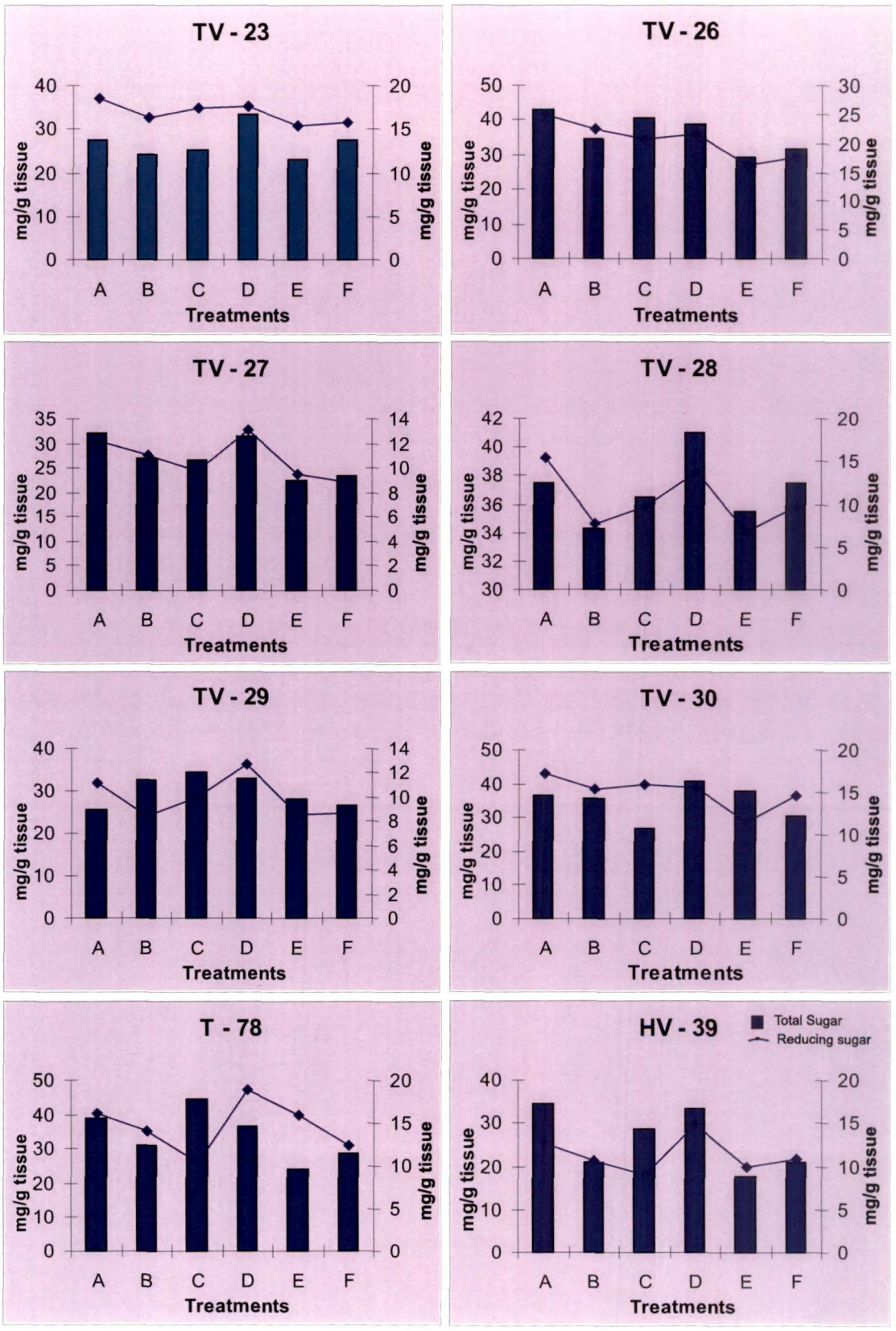


Fig.22: Changes in total and reducing sugar contents in leaves of potted seedling following insecticide/fungicide application.
(A-C): 1st application and (D-F) 2nd application
A&D=Control;B&E=Insecticide;C&F=Fungicide treatments

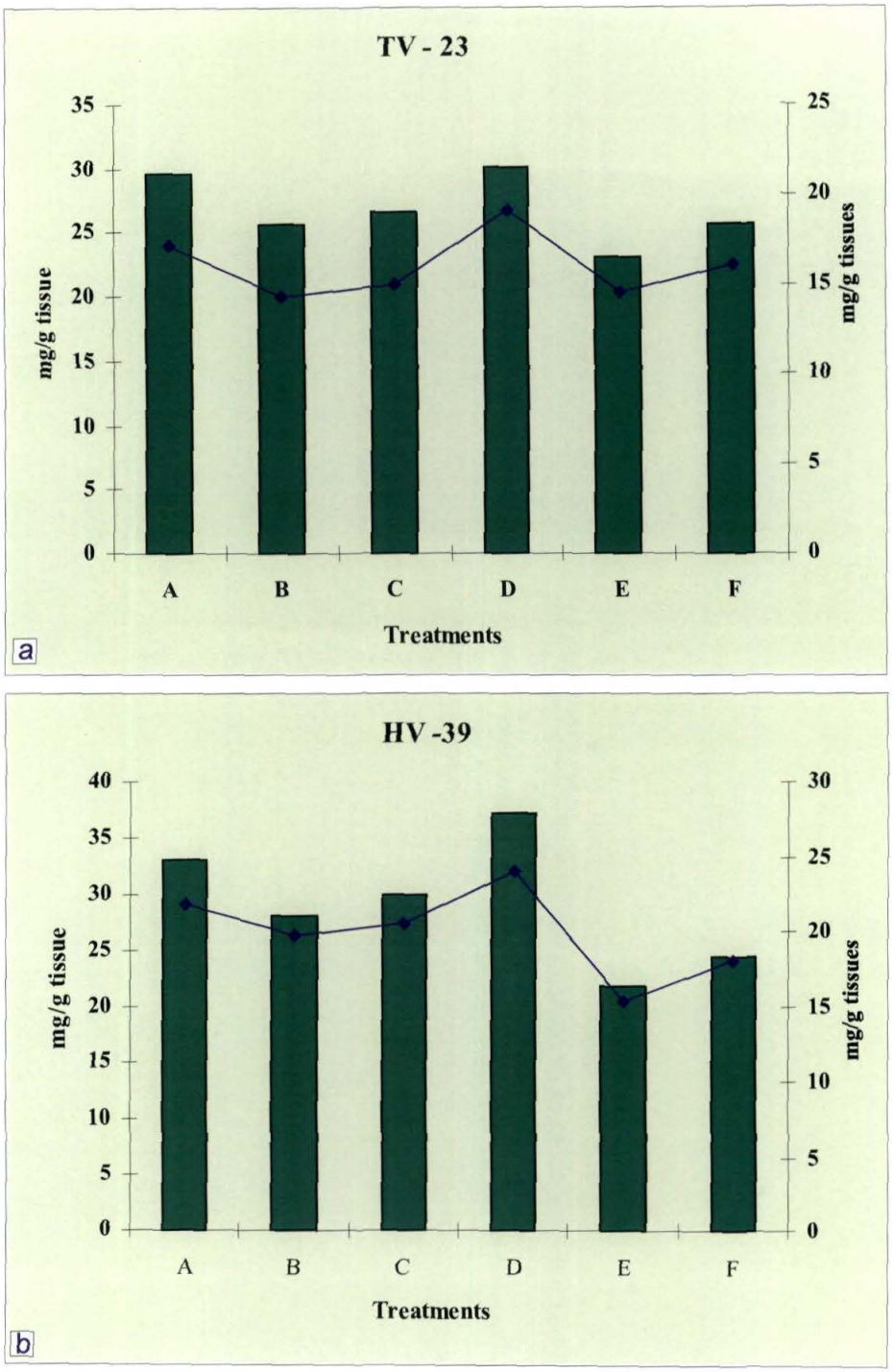
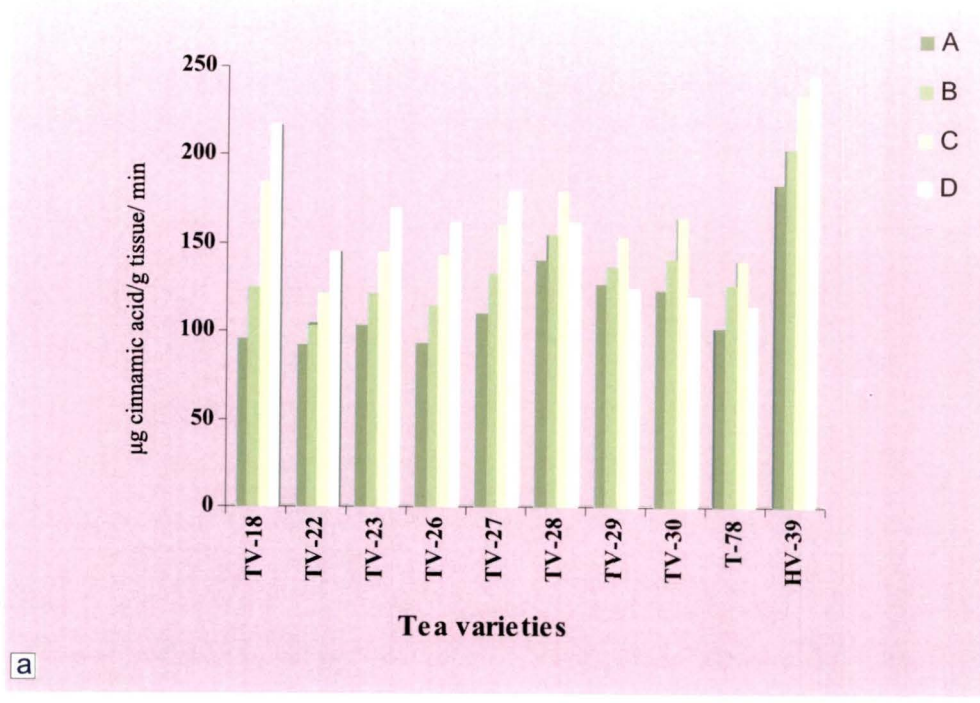


Fig.23: Effect of insecticides/fungicide spray on total and reducing sugar content of tea leaves of tea bushes.
(A-C): 1st application and (D-F) 2nd application
A&D=Control;B&E=Insecticide;C&F=Fungicide treatments



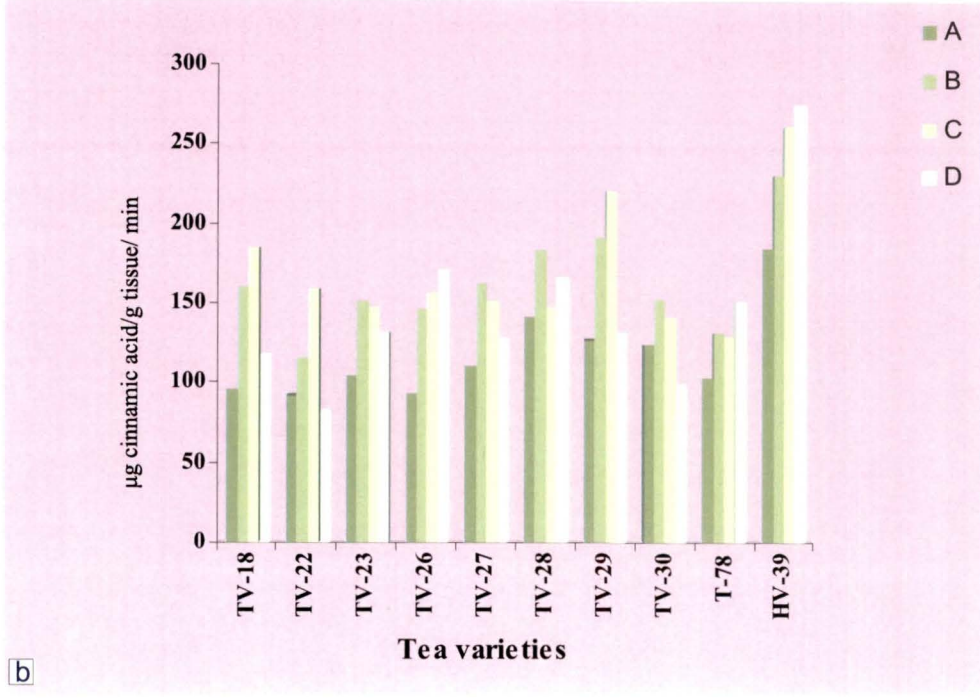


Fig.24: Changes in PAL activity of tea leaves of cut shoots subjected to Cu (a) and Cd (b) stress.
Legend: A=Control, B= 100, C=500, D=1000 µg/ml

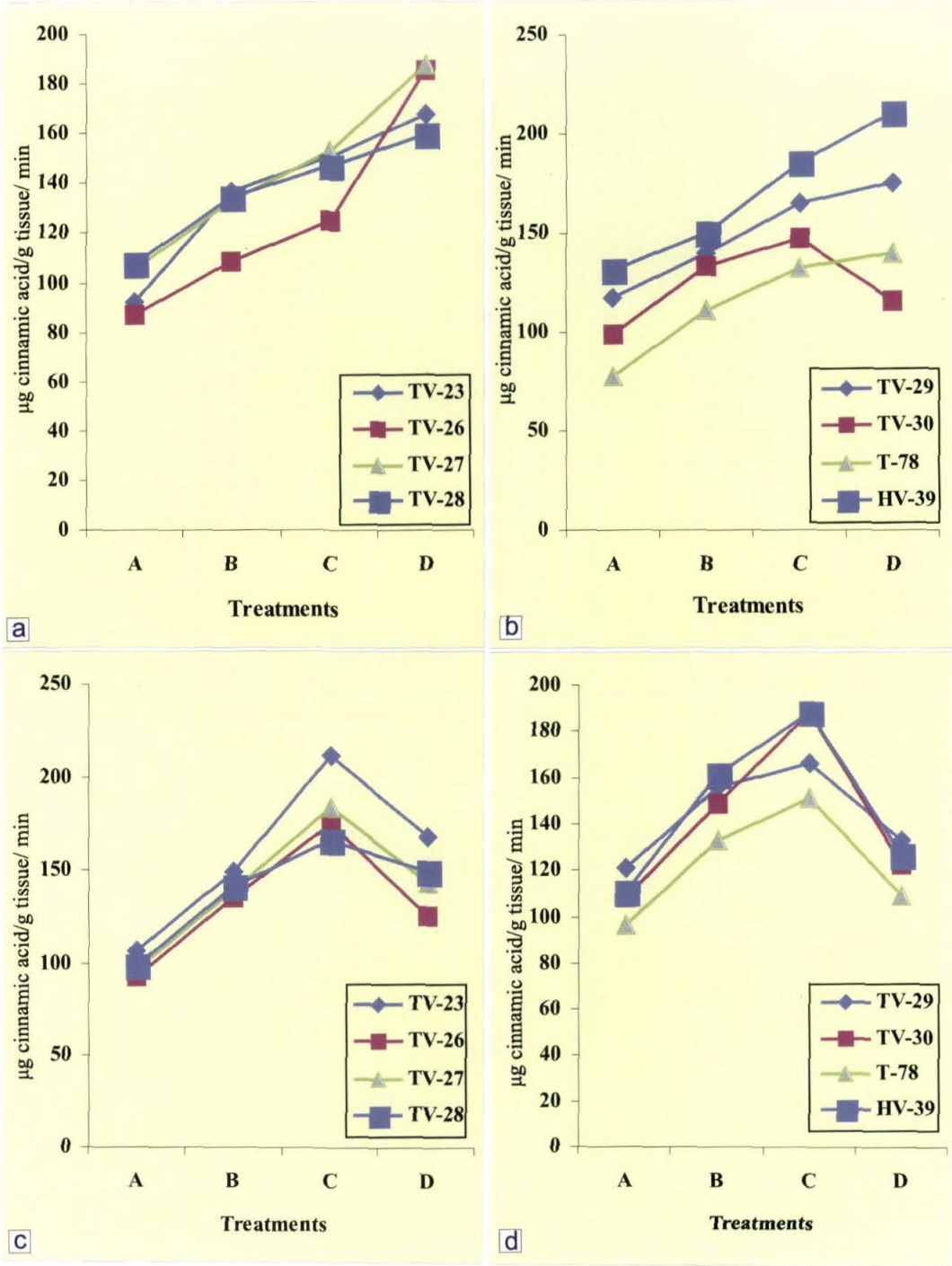


Fig.25: Changes in PAL activity of tea leaves of tea seedlings following 1st (a & b) and 2nd (c & d) application of Cu
A=Control, B= 100, C=500, D=1000 µg/ml

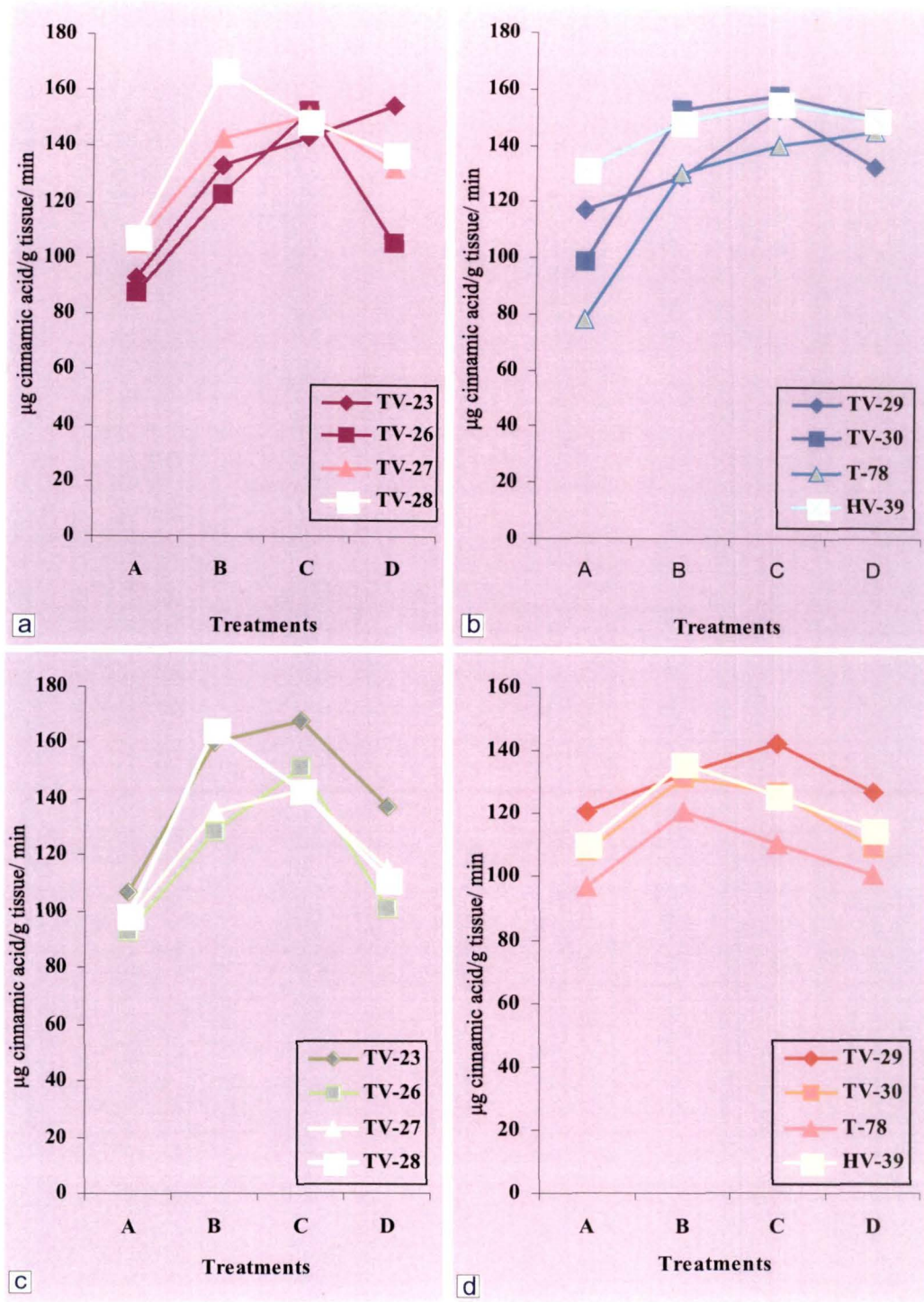


Fig.26: Effect of 1st (a & b) and 2nd (c & d), (d) application of Cd on PAL activity of tea leaves of potted tea seedlings.
A=Control, B= 100, C=500, D=1000 µg/ml

When seedlings were treated with insecticide or fungicide there were general decline of PAL activity after each spray (Fig 27a). When bushes were subjected to the spraying with both the chemicals PAL activity was reduced after each spray (Fig.28 a) . It was also evident that PAL was higher in bush than that of potted plants.

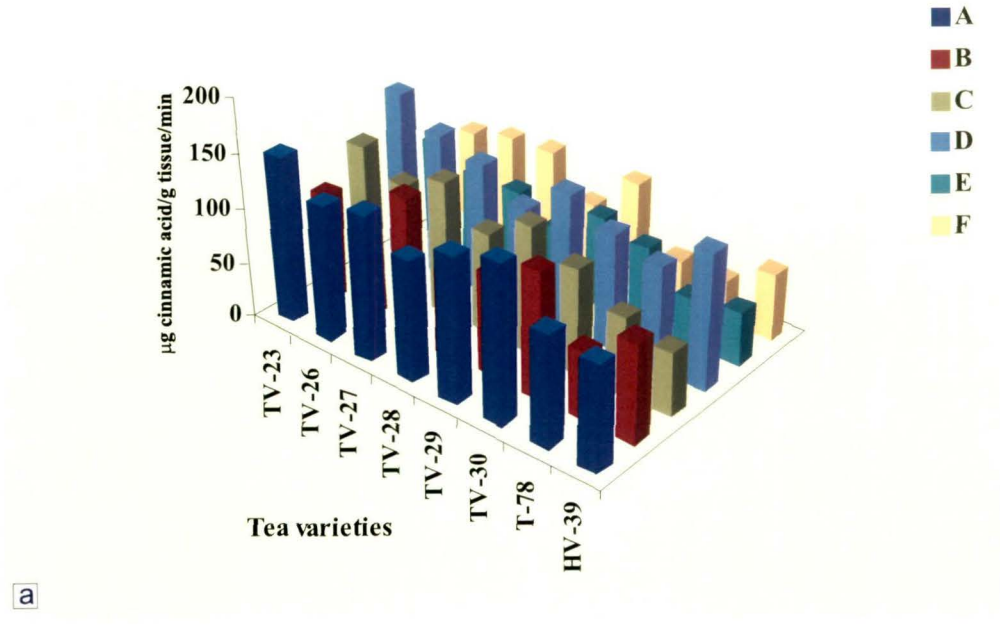
Results of all experiments relating to PAL activity has shown that in heavy metal treatment plant responded by increased enzyme activity, whereas in case of chemical spray the activity was declined after each spray. Constitutive PAL activity among the varieties also differed. Age of the plant was also an important factor for the PAL activity, as the older plant showed the greater activity than that of young seedlings.Thus, PAL activity in the plants was consecutively regulated by the varieties, age of the plant and different types of stresses.

4.6.2. Peroxidase

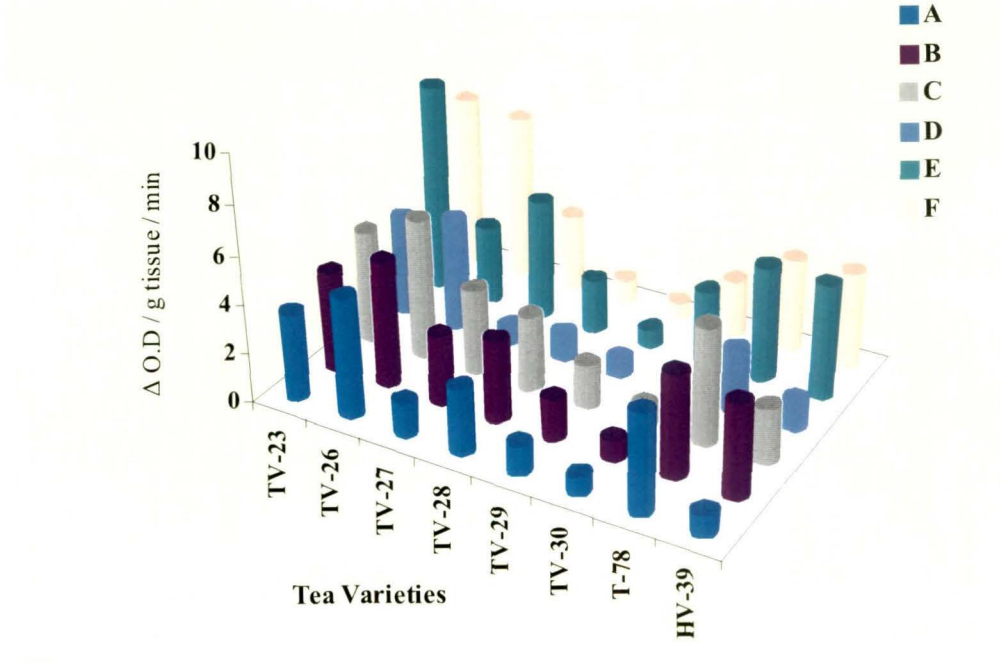
When the shoots were immersed in Cu solution increased activity of peroxidase was noted at 500µg/ml ; at the highest concentration the activity declined to a level even lower than control (Fig.29a) .In case of Cd heighest accumulation was noticed at 100 µg/ml ; in few varieties at 500µg/ml activity was enhanced ,though the activity was declined at 1000 µg/ml (Fig.29b).

In case of seedlings there was an increased activity after 1st application of Cu, but after 2nd application maximum accumulation was noted in most of the varieties at 500 µg/ml. TV-23, HV-39, TV-20 at 1000 µg/ml showed an increased activity but other varieties showed the declining trend (Fig. 30). Seedlings when treated with Cd after 1st application at the heighest concentration showed reduced enzyme activity and after 2nd treatment increased activity was noted at lower concentration but declined at the higher concentration (Fig 31).

When the seedlings, were subjected to fungicide or insecticide treatment increased enzyme activity was evident even after 2nd spray. The accumulation was much higher following 2nd spray. (Table 20).



a



b

Fig.27: Effect of inscticide/fungicide on PAL (a) and PPO (b) activity on leaves of Potted tea plants.
Legend: (A-C): 1st application and (D-F) 2nd application
A&D=Control;B&E=Insecticide;C&F=Fungicide treatments

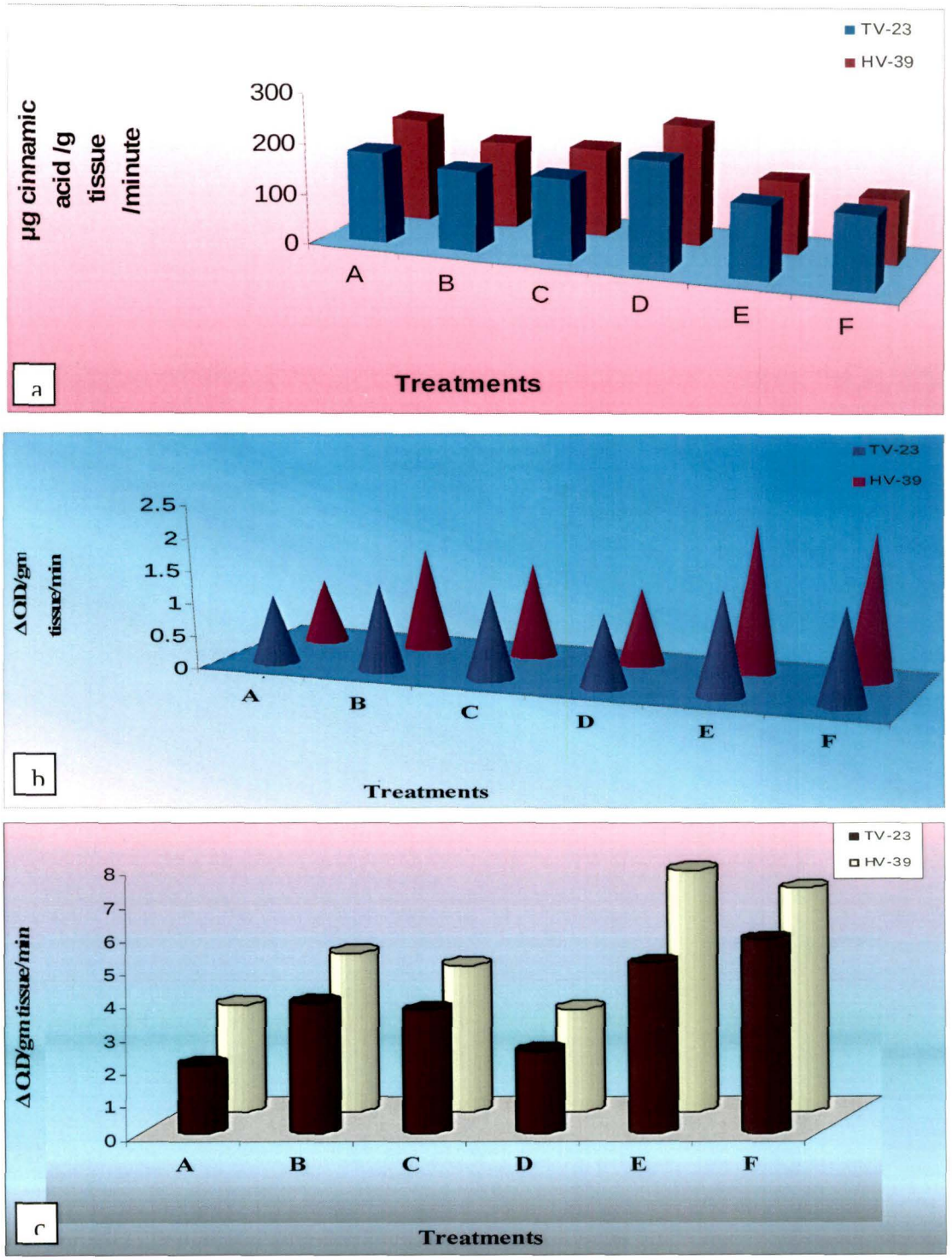


Fig.28: Changes in PAL (a); POX (b) and PPO (c) activity in Leaves of Tea bushes following insecticide/ fungicide application.
(A-C): First application and (D-F) second application
A & D = Control; B & E = Insecticide; C & F = Fungicide treatments

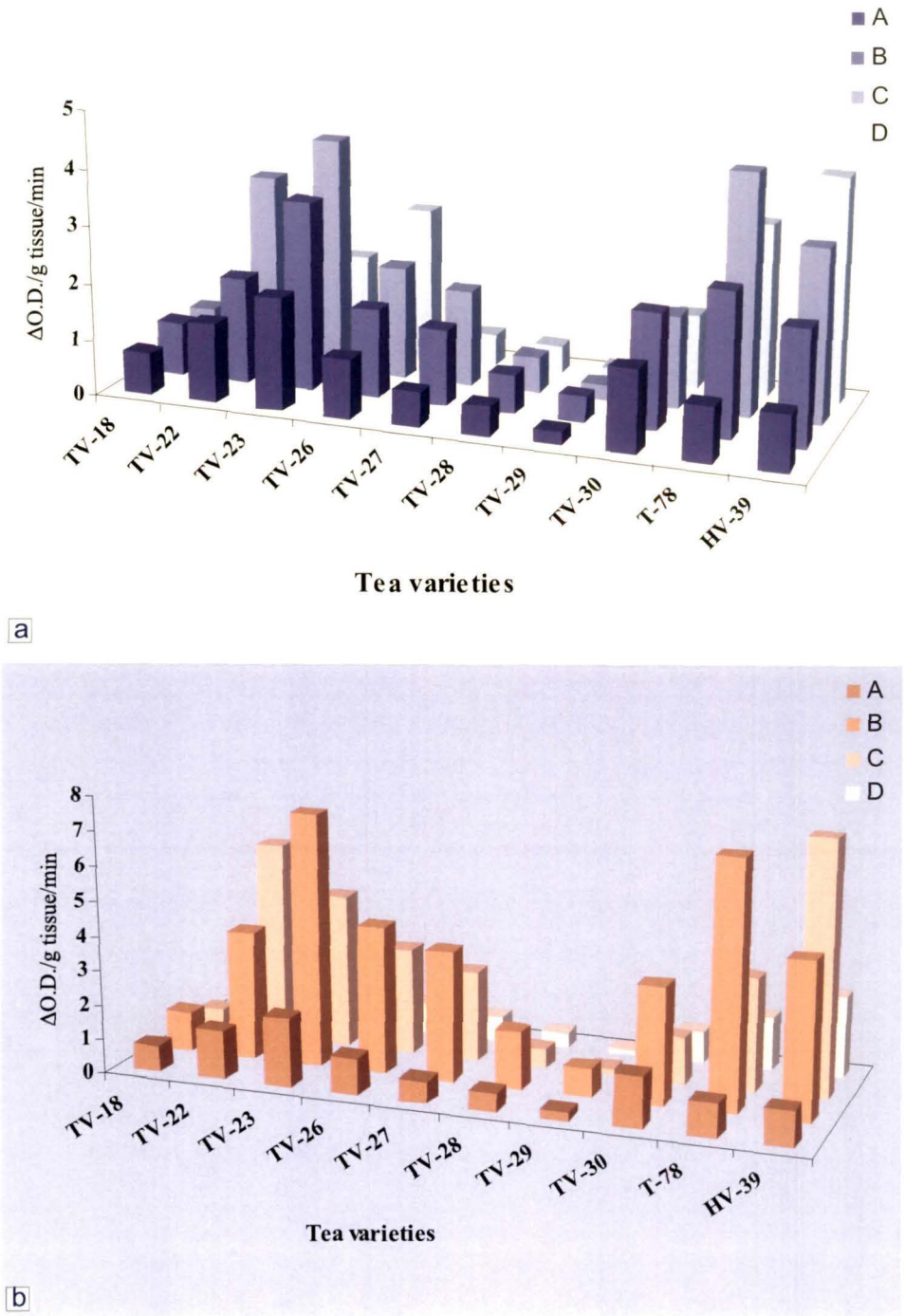


Fig.29: Alteration of POX activity induced by different concentrations of Cu (a) and Cd (b) in leaves of cut shoots of tea.
Legend: A=Control, B= 100, C= 500, D= 1000 μg/ml.

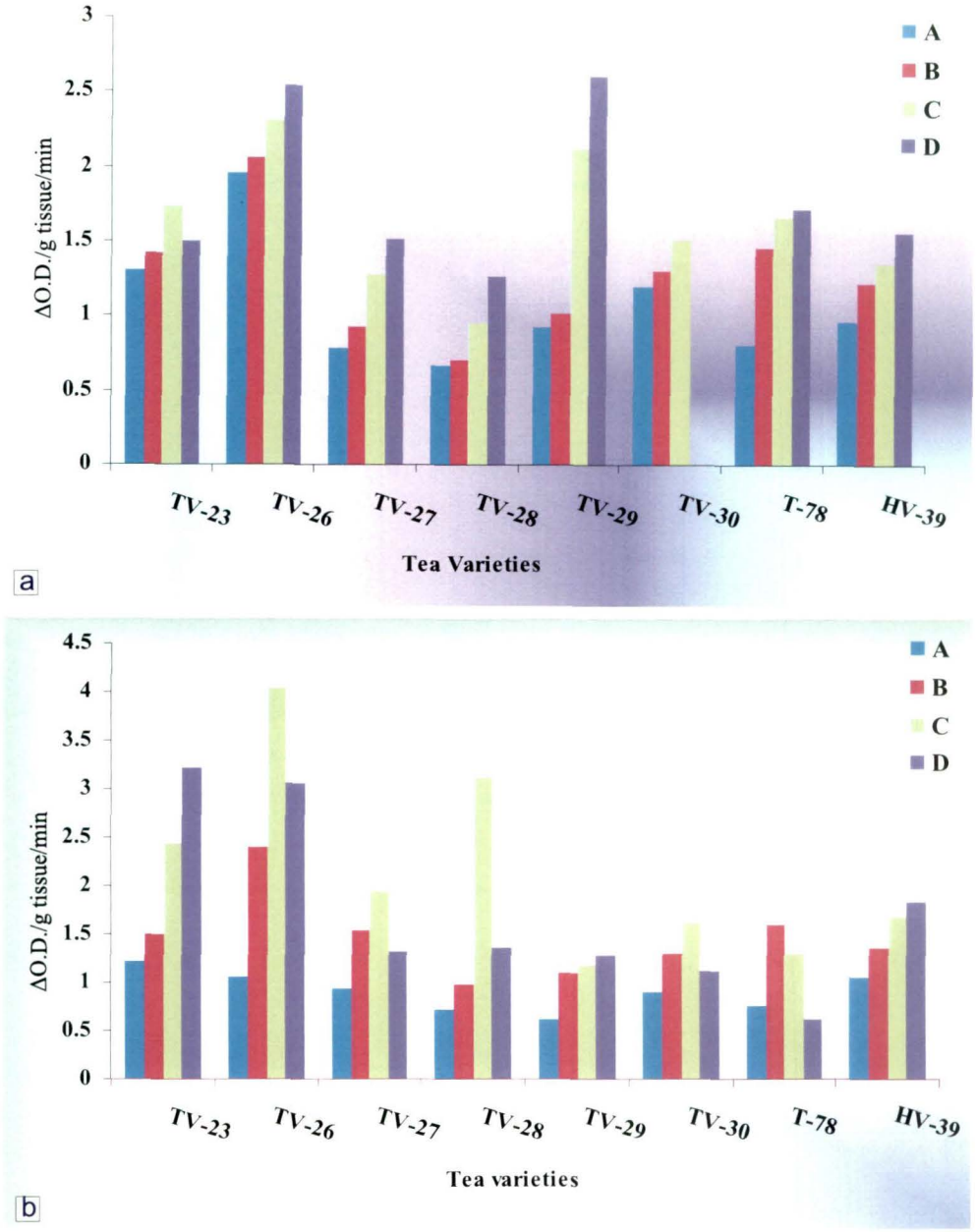


Fig.30: Changes in POX activity in leaves of potted tea plants after 1st (a) and 2nd (b) application of different concentrations of Cu
Legend: A=Control, B= 100, C= 500, D= 1000 μg/ml.

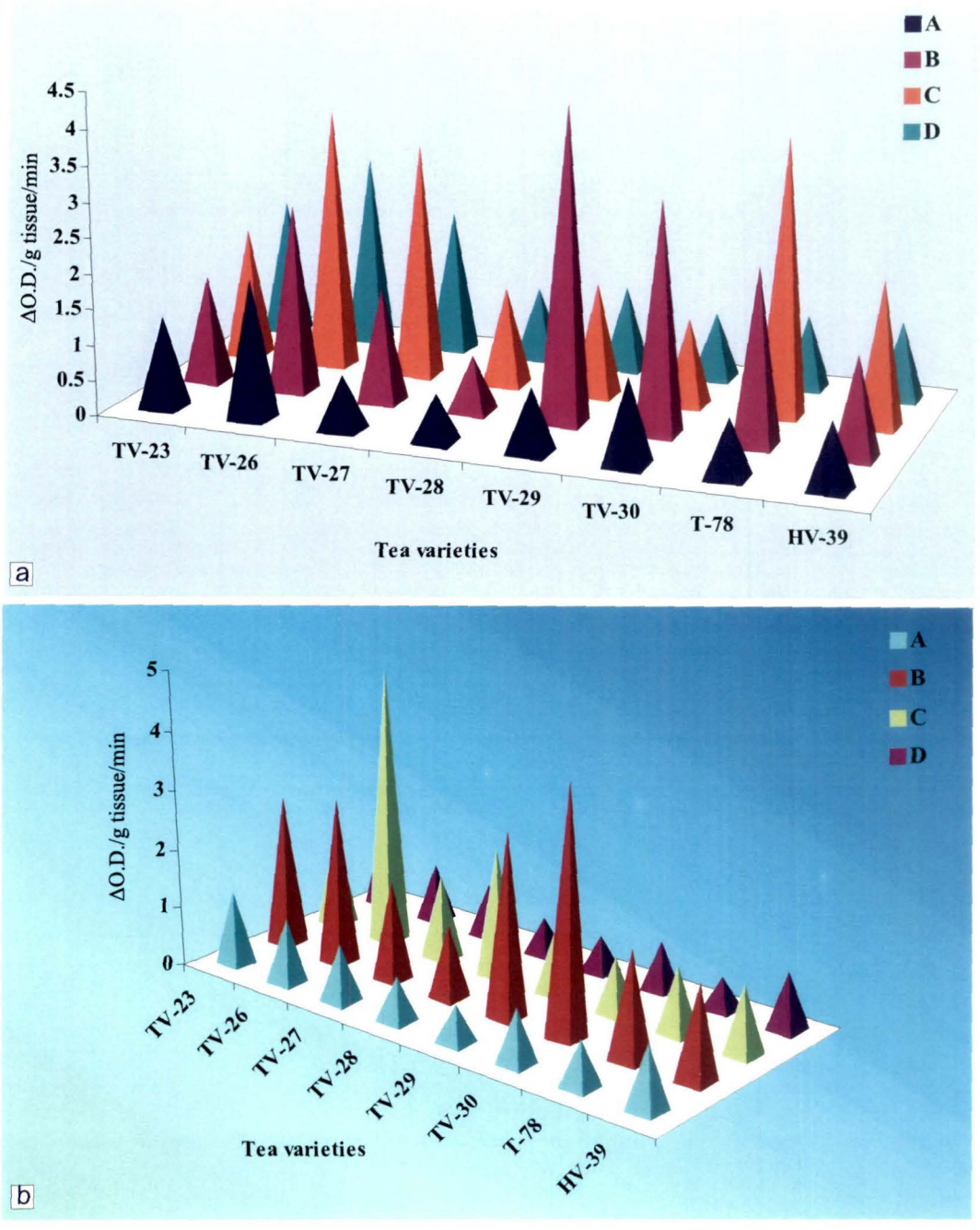


Fig.31: Changes in POX activity in leaves of potted tea plants after 1st (a) and 2nd (b) application of different concentrations of Cd
Legend: A=Control, B= 100, C= 500, D= 1000 µg/ml.

Table 20 : Changes in Peroxidase activity in seedlings following insecticide/ fungicide treatment.

Varieties	Peroxidase content (ΔO.D/g tissue/min)					
	1st treatment			2nd treatment		
	Control	Insecticide	Fungicide	Control	Insecticide	Fungicide
TV-23	1.92 ±0.07	3.22 ±0.02	2.60 ±0.04	1.75 ±0.15	4.72 ±0.10	3.60 ±0.11
TV-26	1.98 ±0.22	3.10 ±0.12	2.76 ±0.16	2.25 ±0.11	4.95 ±0.01	4.79 ±0.03
TV-27	2.40 ±0.75	2.75 ±0.50	2.90 ±0.86	2.60 ±0.15	5.75 ±0.07	4.55 ±0.12
TV-28	2.50 ±0.09	3.33 ±0.08	3.62 ±0.02	2.29 ±0.02	5.62 ±0.03	4.78 ±0.73
TV-29	1.31 ±0.83	1.73 ±0.62	2.14 ±0.84	2.41 ±0.15	4.84 ±0.11	7.60 ±0.15
TV-30	2.30 ±0.05	2.75 ±0.07	3.57 ±0.08	2.5 ±0.12	4.75 ±0.10	7.25 ±0.18
T-78	3.40 ±0.04	4.15 ±0.02	5.75 ±0.10	4 ±0.12	7.20 ±0.11	8.75 ±0.17
HV-39	2.32 ±0.14	6.62 ±0.12	5.25 ±0.13	1.96 ±0.08	9.45 ±0.09	7.40 ±0.13
CD Treatment (P=0.05)= 0.792547 CD Varieties (P=0.05) =1.294224				CD Treatment (P=0.05)= 1.282782 CD Varieties (P=0.05) =2.094775		

Values are mean of 3 replicates; ± = SEm

Table 20A: Analysis of variance of data presented in Table 20. (1st treatment)

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	8.3719	2	4.18595	7.663935	0.005648	3.738892
Columns	21.16632917	7	3.02376131	5.536117	0.003249	2.764199
Error	7.646633333	14	0.546188095			
Total	37.1848625	23				

Table 20B: Analysis of variance of data presented in Table 20. (2nd treatment)

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	66.58773333	2	33.29386667	23.2684	3.54E-05	3.738892
Columns	26.71833333	7	3.816904762	2.667556	0.056032	2.764199
Error	20.03206667	14	1.430861905			
Total	113.3381333	23				

Bushes also showed the similar trend i.e. the increased enzymatic activity after each spray (Fig 28b).

4.6.3. Polyphenol Oxidase

In case of young shoots treated with different conentartion of Cu enzyme activity was enhanced at the lower concentration, but decreased at higher concentration. Result is presented in Table 21.

When the young shoots were immersed in Cd solutions great differences in enzyme activity was noticed in different varieties. Highest enzyme activity was found in HV-39, TV-29. The results have been present below. (Table 22).

Table 21: PPO activity in tea leaves following Cu stress imposed on cut shoots of different varieties.

Varieties	**PPO content (ΔO.D/g tissue/min)**			
	Concentration of Cu (μg/ml)			
	0	**100**	**500**	**1000**
TV-18	2.10±0.02	2.52±0.23	3.08±0.02	1.09±0.08
TV-22	1.25±0.03	1.52±0.02	2.20±0.06	0.87±0.01
TV-23	1.32±0.11	2.37±0.05	3.06±0.02	0.73±0.19
TV-26	0.93±0.02	1.53±0.04	1.23±0.01	0.60±0.05
TV-27	0.46±0.05	0.54±0.11	0.61±0.07	0.30±0.09
TV-28	1.30±0.04	2.20±0.1	3.07±0.19	0.98±0.09
TV-29	0.97±0.01	2.60±0.07	1.15±0.02	0.60±0.05
TV-30	0.36±0.1	0.92±0.19	0.63±0.17	0.30±0.09
T-78	0.87±0.03	3.15±0.23	4.84±0.04	1.37±0.01
HV-39	0.78±0.15	3.08±0.1	2.07±0.19	1.11±0.09
CD Treatment (P=0.05)= 0.564549; CD Varieties (P=0.05) =0.892631				

Values are mean of 3 replicates; ± = SEm

Table 21A: Analysis of variance of data presented in Table 21.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	14.89577	3	4.965256667	13.1175	1.8E-05	2.960351
Columns	16.45306	9	1.828117778	4.829628	0.000676	2.250131
Error	10.22008	27	0.378521481			
Total	41.56891	39				

Table 22: PPO activity in tea leaves following Cd stress imposed on cut shoots of different varieties.

Varieties	**PPO content (ΔO.D/g tissue/min)**			
	Concentration of Cd (μg/ml)			
	0	**100**	**500**	**1000**
TV-18	2.10±0.23	2.22±0.20	0.95±0.15	0.85±0.09
TV-22	1.25±0.78	1.41±0.23	1.06±0.09	0.78±0.17
TV-23	1.32±0.10	1.69±0.14	1.57±0.9	0.56±0.11
TV-26	0.93±0.01	1.17±0.03	0.96±0.04	0.53±0.01
TV-27	0.46±0.03	1.51±0.07	1.43±0.05	0.93±0.09
TV-28	1.30±.02	3.41±0.04	2.12±0.14	0.87±0.05
TV-29	0.97±0.04	4.08±0.04	3.27±0.01	2.45±0.09
TV-30	0.36±0.03	2.57±0.05	1.46±0.09	0.48±0.04
T-78	0.87±0.02	2.07±0.05	1.10±0.07	0.92±0.11
HV-39	0.78±0.05	4.77±0.17	3.32±0.11	1.67±0.98
CD Treatment (P=0.05)= 0.59339; CD Varieties (P=0.05) =0.938232				

Table 22A: Analysis of variance of data presented in Table 22

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	14.77572	3	4.92524	11.77767	4.09E-05	2.960351
Columns	14.89474	9	1.654971111	3.957515	0.002594	2.250131
Error	11.29098	27	0.418184444			
Total	40.96144	39				

In the potted varieties in the seedlings treated with Cu general increment of PPO activity was noted after 1st application but, after 2nd application at the higher concentration activity was declined (Fig 32). Seedlings when treated with Cd solution PPO activity was changed. Lower concentration enhanced the enzyme activity whereas, higher concentration showed lower activity after each application (Fig 33).

When seedlings were treated with insecticide or fungicide the PPO activity was enhanced after each spraying. The activity was more pronounced after the 2nd treatment of the fungicide / insecticide treatment.The results have been presented in (Fig.27b)

Bushes, after the application of the chemicals, i.e. insecticide and fungicide showed the increment in enzyme activity .The greater activity of enzyme was found after 2nd treatment (Fig 28c). HV-39 showed the greater activity than that of TV-23.

4.7. Changes in isozyme profile of peroxidase and polyphenol oxidase following anthropogenic stress

4.7.1. Isozyme of peroxidase

Presence of multiple isozymes of peroxidase in tea was reported previously. Experiments were carried out to determine whether the different stresses induce the activity of isozymes or whether the activity of any isozyme were lost due to the same stressed environment. Analyses of isozyme were done on native PAGE as described earlier in materials and methods and the results are presented below.

4.7.1.1. Heavy metal

Peroxidase extracts from leaves subjected to different heavy metal stresses exhibited different types of isozyme pattern.

In TV-23, analysis of isozymes in leaves of cut shoots revealed a faint band of Rm value 0.22 in only Cd and Cu (1000µg /ml).One band of Rm. 0.77 was expressed in all the treated leaves (Table 23, Plate X, fig.A).

Peroxidase extracts from leaves of young shoots of HV-39 immeresed in Cu500, Cu1000, Cd500, Cd 1000 exhibited more or less similar isozyme pattern to that of control leaves i.e. there was no differences in number of bands between control and stressed leaves as visualized on the gel. However, when activity of peroxidase enzymes was assayed, significant changes were evident. (Table 23, Plate X, fig.B).

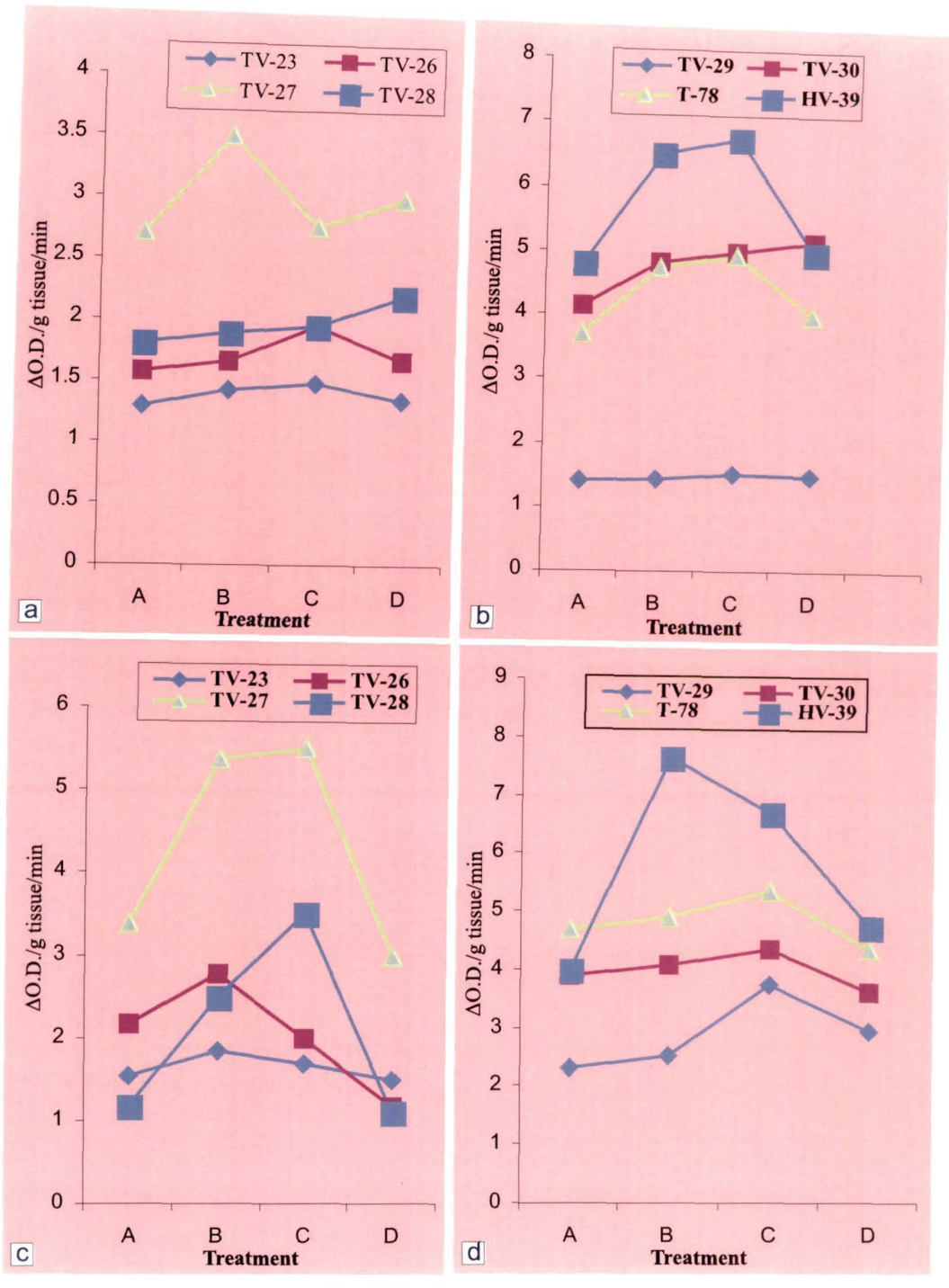


Fig.32: Changes in PPO activity in leaves of potted tea plants after 1st (a&b) and 2nd (c&d) application of different concentrations of Cu.
A=Control, B= 100, C= 500, D= 1000 µg/ml.

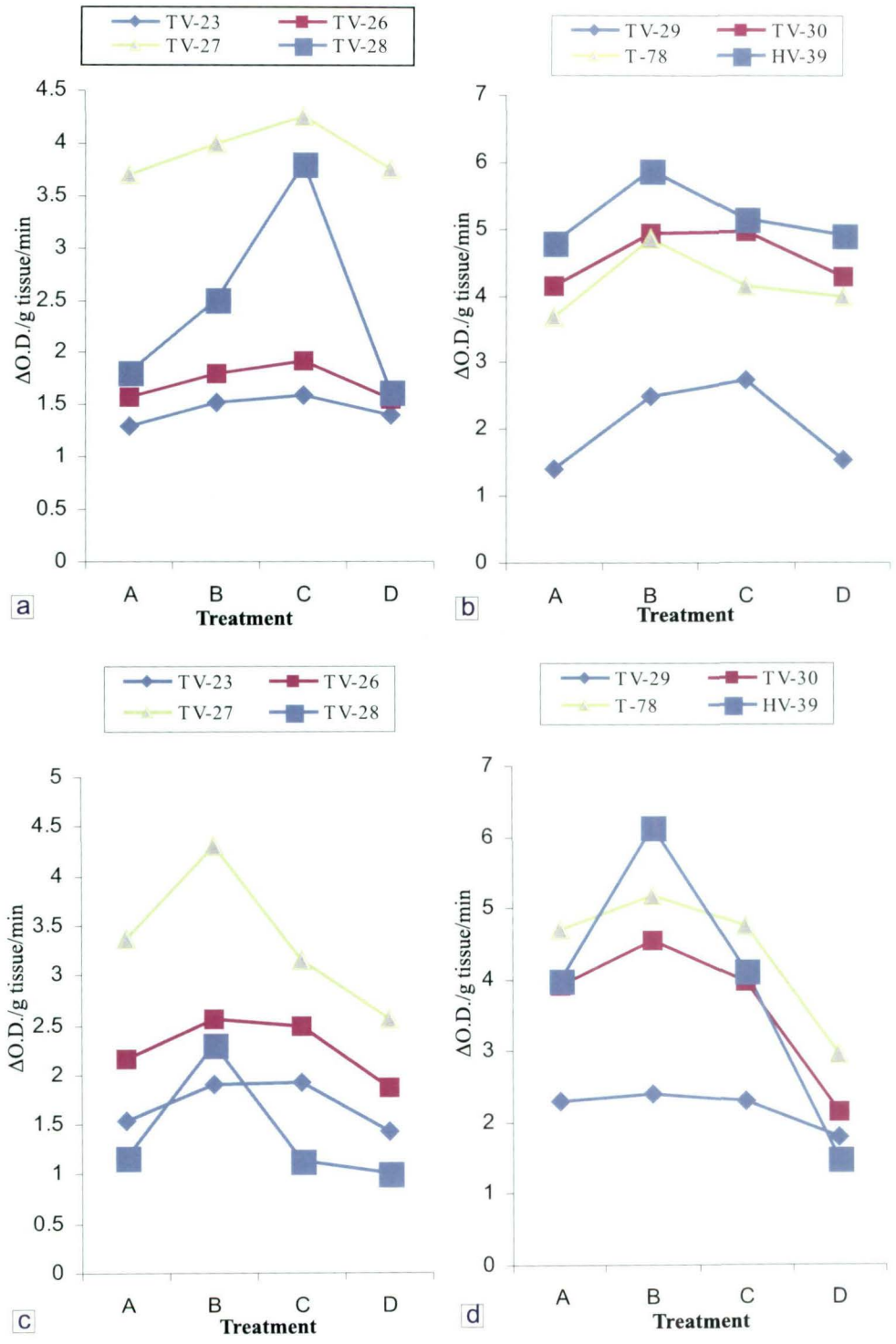


Fig.33: Changes in PPO activity in leaves of potted tea plants after 1st (a&b) and 2nd (c&d) application of Cd.
A=Control, B= 100, C= 500, D= 1000 µg/ml.

Peroxidase was extracted from leaves of control and young shoots of T-78 dipped in Cu100, Cu500, Cu1000, Cd100, Cd500 μg/ml solutiions . Different isozyme pattern was evident in most of the treatments. The upper most band of Rm. 0.07 was expressed only in Cu 1000. A band Rm. Value 0.59 was missing in Cu100 and Cd100. Another band of Rm. 0.66 was absent in Cu100 . The lowest band of Rm value of 0.73 was present only in Cu 1000 (Table 23, Plate X, fig. C).

In seedlings HV-39 treated with Cu1000 and Cd1000, isozyme pattern was noted. New isozymes of Rm 0.18 were expressed only in treated leaves (Table23, Plate XI, fig. A).

When TV-23 seedlings were treated with different concentrations of heavy metal solutions, changes in isozyme pattern was evident.In Cu500, Cu1000 and all the concentrations of Cd new band of Rm 0.09 was expressed. A new band of Rm. 0.17 was noticed in Cu1000 and Cd. Another new band of Rm 0.62 was expressed in all the treated leaves. A new band of 0.66 was visualized in Cu500, and all Cd concentrations. Two bands of Rmvalues 0.84 and 0.85 noticed in Cu1000 and Cd (Table 23, Plate XI, fig. B).

4.7.1.2. Insecticide / Fungicide

Seedlings were treated with insecticide /fungicide and peroxidase was extracted from control and treated plants and were analysed by PAGE after 2nd treatment. Significant changes were evident in treated leaves with control. In TV-29, seedlings when treated with fungicide/insecticide expression of new isozyme were more than that of control or insecticide treated leaves. Three distinct new bands of Rm 0.5, 0.55, 0.6 were visualized in fungicide treated leaves. (Table24 , Plate XII, fig. A).

In HV-39 seedlings, after 2nd application of fungicide significant changes in control and fungicide treated leaves was evident. Upper band of Rm value 0.04 was absent in treated leaves. Two new lower bands of Rm. Value 0.55 and 0.63 were found only in fungicide treated leaves. (Plate XII, fig. B).

In mature bush of HV39 there was no diiferences could be visualized in both after the 2nd application of insecticide and fungicide treated leaves. , though the changes

in peroxidase enzyme activity was noted after each treatment.(Table 24, PlateXII, fig.C).

Table 23. Relative mobility of peroxidase isozymes of tea plants subjected to heavy metal stress.

Variety	Isozyme No	Relative mobility	0	Cu 100	Cu 500	Cu 1000	Cd 100	Cd 500	Cd 1000
TV-23 (Cut shoot)	1	0.22	–	–	–	+	–	–	+
	2	0.28	+	+	+	+	+	+	+
	3	0.42	+	+	+	+	+	+	+
	4	0.48	+	+	+	+	+	+	+
	5	0.77	–	+	+	+	+	+	+
HV-39 (Cut shoot)	1	0.18	+	+	+	+	+	+	+
	2	0.25	+	+	+	+	+	+	+
	3	0.43	+	+	+	+	+	+	+
	4	0.49	+	+	+	+	+	+	+
	5	0.76	+	+	+	+	+	+	+
	6	0.86	+	+	+	+	+	+	+
T-78 (Cut shoot)	1	0.07	–	–	–	+	–	–	+
	2	0.24	+	+	+	+	+	+	+
	3	0.35	+	+	+	+	+	+	+
	4	0.41	+	+	+	+	+	+	+
	5	0.59	+	–	+	+	–	+	+
	6	0.66	+	–	+	+	+	+	+
	7	0.73	–	–	–	+	–	–	+
HV-39 (Pot)	1	0.18	–	–	+	+	–	+	+
	2	0.29	+	+	+	+	+	+	+
	3	0.34	–	–	+	+	–	+	+
	4	0.49	+	+	+	+	+	+	+
	5	0.76	+	+	+	+	+	+	+
	6	0.86	+	+	+	+	+	+	+
TV-23 (Pot)	1	0.09	–	–	+	+	+	+	+
	2	0.17	–	–	–	+	+	+	+
	3	0.31	+	+	+	+	+	+	+
	4	0.37	+	+	+	+	+	+	+
	5	0.54	+	+	+	+	+	+	+
	6	0.62	–	+	+	+	+	+	+
	7	0.66	–	–	+	–	+	+	+
	8	0.84	–	–	–	+	+	+	+
	9	0.85	–	–	–	+	+	+	+

+ = Band present; — = No band visualized

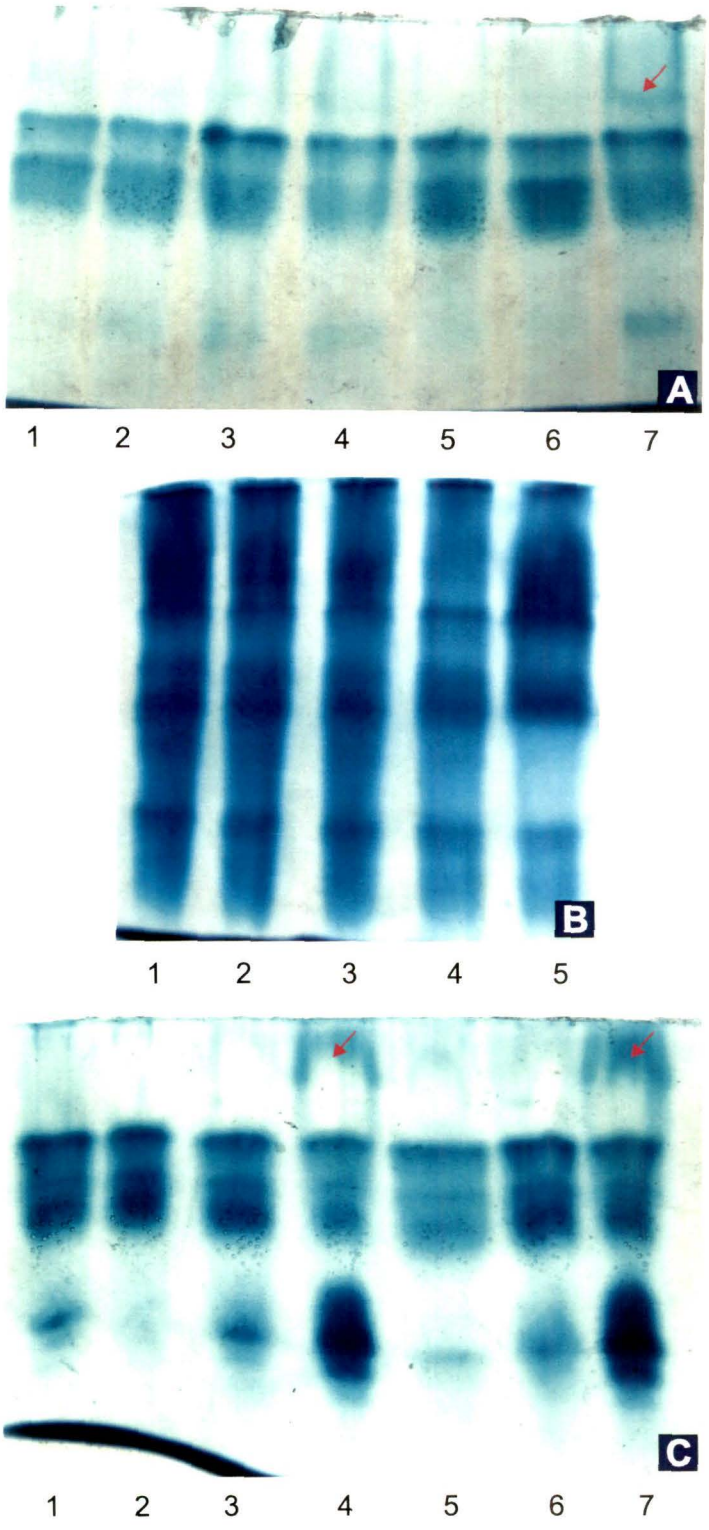


Plate X(A-C): Analysis of isozymes of peroxidase by PAGE in TV-23 (A), HV-39(B)&T-78 (C) following heavy metal treatments.

A-Lane 1: Control; Lanes 2-4: 100, 500 &1000 µg/ml of Cd, respectively; Lane 5-7: 100 ,500 & 1000 µg/ml of Cu respectively.

B-Lane 1: Cd 500 & 2:1000µg/ml; Lane 3: Cu 1000µg/ml; Lane 4: Control; Lane 5: Cu 500 µg/ml).

C: T-78 Lane 1: Control; 2-4: Cu 100, 500 &1000µg/ml, respectively; 5 & 6: Cd 100 &500 µg/ml; Lane 7: Cu 1000 µg/ml).

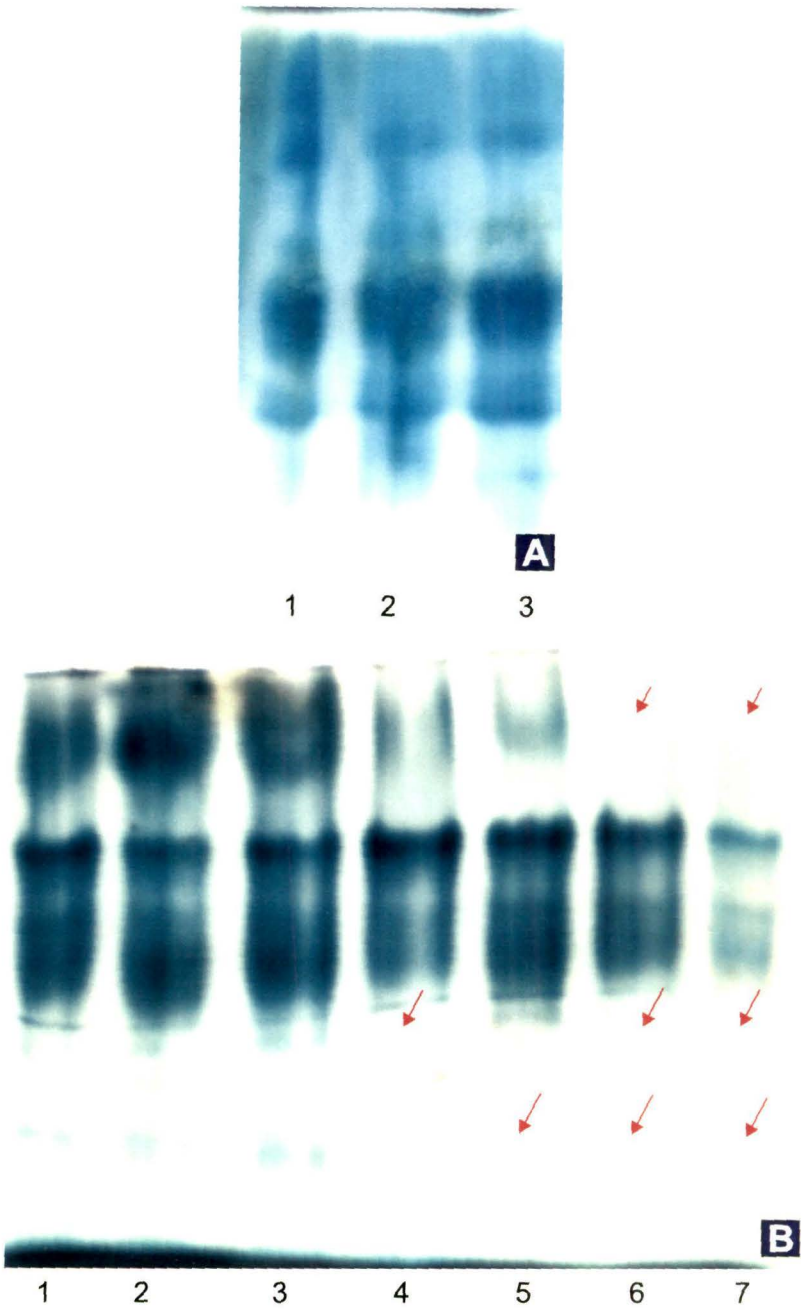


Plate XI (A-B): Analysis of isozymes of peroxidase in leaves of tea seedlingsby PAGE.

A: HV-39 Lane 1: Cd 1000µg/ml; Lane2: Cu 1000µg/ml; Lane 3: Control).

B: TV-23 Lane 1: Cd 100µg/ml; Lane 2: Cd 500µg/ml; Lane 3: Cd 1000µg/ml; Lane 4: Cu 1000 µg/ml; Lane 5: Cu 500 µg/ml; Lane 6: Cu 100 µg/ml; Lane 7: C2

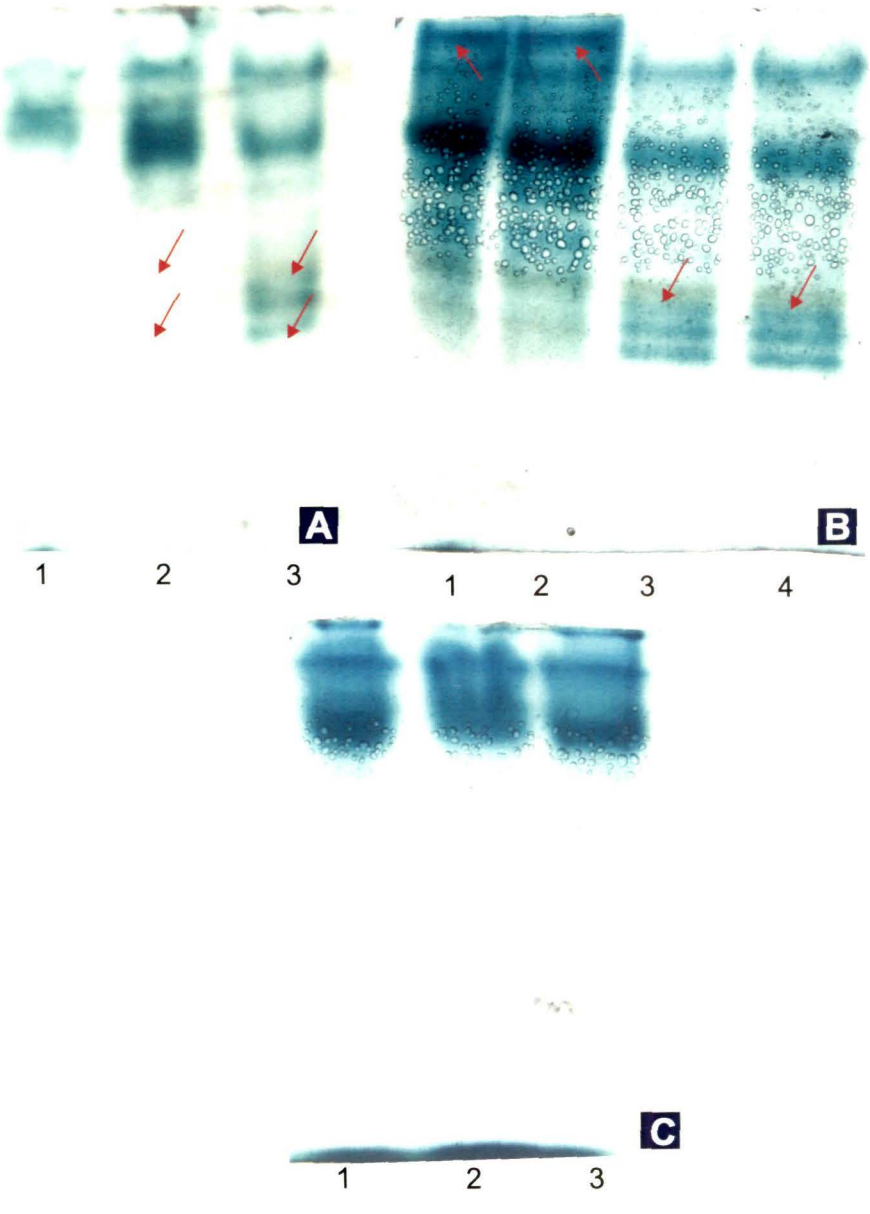


Plate XII(A-C): Peroxidase isozyme analysis of tea plants following fungicide/insecticide application.

A-Seedling of TV-29: Lane 1: Control; 2: Insecticide & 3: Fungicide.

B-Seedling of HV-39: Lane 1& 2: Control; Lane 3&4: Fungicide.

C-Bush of HV-39: Lane 1: Control; 2: Insecticide & 3: Fungicide.

Table 24. Relative mobility values of the isozymes of peroxidase from control, insecticide, fungicide treated tea leaves.

Variety	Isozyme No.	Relative mobility	Control	Insecticide	Fungicide
HV-39 (Bush)	1	0.08	+	+	+
	2	0.15	+	+	+
	3	0.21	+	+	+
	4	0.31	+	+	+
	5	0.38	+	+	+
	6	0.44	+	+	+
TV-29 (Pot)	1	0.12	+	+	+
	2	0.25	+	+	+
	3	0.5	–	–	+
	4	0.55	–	–	+
	5	0.6	–	–	+

+ = Band present; — = No band visualized

4.7.2. Isozyme of Polyphenol oxidase

Experiments were conducted to determine the changes of polyphenol oxidase isozyme pattern in tea leaves which are important in various anthropogenic stress responses.Young shoots of TV-23 when dipped in different concentrations of heavy metal solutions of Cu no changes in isozyme pattern was evident, but, a significant changed activity was evident (Table 25, Plate XIII, fig. A).

In seedlings of TV-23 treated with fungicide /insecticide no changes of isozyme pattern found (Plate XIII, fig. B).

Seedlings of TV-29 when treated with the different concentrations of tested metals new isozyme of Rm. Value 0.12 was noted in Cd treatments, but absent in all Cu concentrations and control leaves. (Table 25, Plate XIII, fig. C).

In bush TV-23 almost similar isozyme pattern was noted after insecticide /fungicide treatment .Lower band of Rm 0.44 was absent in control leaves. But the intensity of band was more in fungicide treated leaves. (Table- 26 Plate XIV, fig. A).

In HV-39 after 2nd treatment lower band of Rm. 0.55 was present in control leaves only which is absent in treated one. Whereas, a band of Rm. 0.85 was newly expressed in treated leaves (Plate XIV, fig. B). Analysis of isozyme patterns of the enzymes revealed that changes were more significant in POX than in PPO. Isozyme patterns differed with the varieties , type of plants as well as with the type of treatments.

Table 25. Relative mobility of polyphenol oxidase isozymes of tea plants subjected to heavy metal stress.

Variety	Isozyme No.	Relative mobility	0	Cu 100	Cu 500	Cu 1000	Cd 100	Cd 500	Cd 1000
TV-23 (cut-shoot)	1	0.29	+	+	+	+	+	+	+
	2	0.44	+	+	+	+	+	+	+
	3	0.48	+	+	+	+	+	+	+
TV-29 (Potted plants)	1	0.12	–	–	–	–	+	+	+
	2	0.3	+	+	+	+	+	+	+
	3	0.4	+	+	+	+	+	+	+
	4	0.46	–	+	+	+	+	+	+

Table 26. Relative mobility of polyphenol oxidase isozymes of control, insecticide, fungicide treated tea leaves.

Variety	IsozymeNo.	Relative mobility	Control	Fungicide	Insecticide
TV-23 (Potted plants)	1	0.1	+	+	+
	2	0.27	+	+	+
	3	0.41	–	+	+
	4	0.64	+	+	+
	5	0.87	+	+	+
TV-23 (Bush)	1	0.1	+	+	+
	2	0.14	+	+	+
	3	0.44	–	+	+
	4	0.61	+	+	+

+ = Band present; — = No band visualized

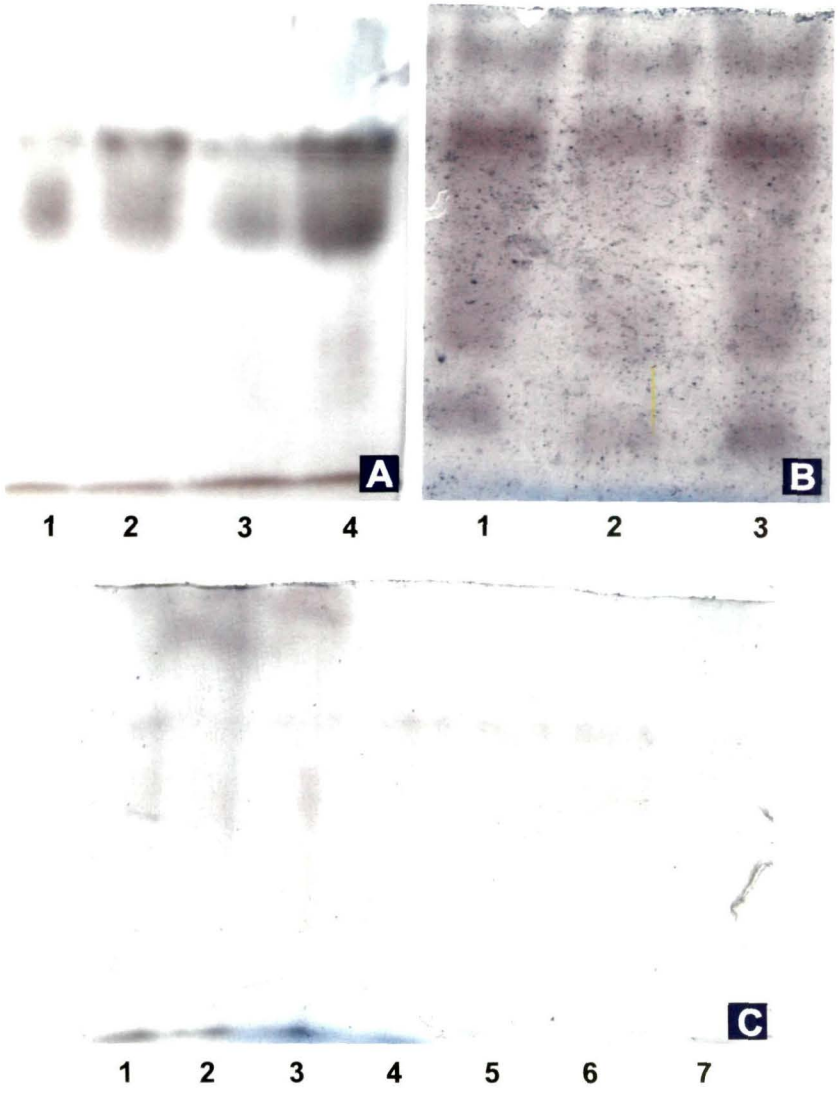


Plate XIII: Analysis of isozymes of polyphenol oxidase by PAGE.

A: TV-23- Lane 1: (Control; Lanes 2-4: Cu 100, 500 & 1000µg/ml treatments.

B: TV-23-Lane 1: Fungicide; Lane 2: Control; Lane 3:Insecticide.

C: TV: 29 - Lane 1: Cd 1000µg/ml; Lane2: Cd 500µg/ml; Lane 3: Cd 100µg/ml; Lane 4: Cu 1000µg/ml; Lane 5: Cu 500µg/ml; Lane 6: Cu 100µg/ml;Lane 7: Control).

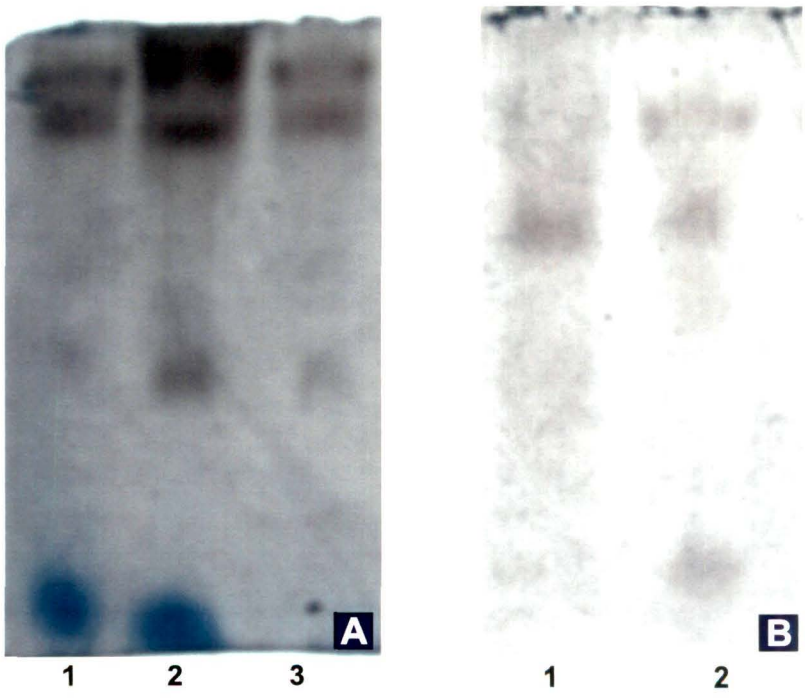


Plate XIV: Analysis of isozymes of polyphenol oxidase by PAGE.
A: TV-23- Lane 1: Fungicide; Lane 2: Control; Lane 3:Insecticide.
B: HV: 39- Lane 1:Control ; Lane 2: Fungicide;

4.8. HPLC analysis of catechins from tea leaves

Catechins were extracted from tea leaves and analysed by HPLC as mentioned in materials and methods. The peaks were compared with the authentic catechin isoforms i.e. gallo catechin (GC) , epi gallo catechin (EGC), epicatechin(EPC), epi gallo catechin gallate (EGCG) , gallo catechin gallate (GCG) and catechin gallate (CG). Results are presented below.

4.8.1. Heavy Metal

In this experiment catechins were extracted from leaves of potted two year old plants of three varieties (TV-23, HV-39 and T-78) treated with different concentrations of heavy metal solution as mentioned in materials and methods.Sampling was done after 48 hr of 2nd treatment.In all cases results revealed that , the treatment led to changes in the isomer patterns of the catechins. In TV-23 the peak of Cu 100µg/ml was more or less similar to that of control, while in Cu 500 µg/ml there was a suppression of many of the peaks. Interestingly, in Cu1000 µg/ml some of the peaks of epicatechin and catechin gallate was quite high (Table 27; Figs. 34A&B). In HV-39 also Cu at 1000 µg/ml treatment showed peak where absorbance was higher or similar to that of control (Table 28, Figs. 35A-C). In T-78 on the other hand Cu1000 µg/ml showed the reduction in peak height. Whereas, Cu 500 µg/ml was almost similar to that of control. (Table 29, Figs. 36A- C).

In case of Cd both 500 µg/ml and 1000 µg/ml treatments of T-78 enhanced the catechin peaks. Similar results were also obtained Cd1000 µg/ml in HV-39 and TV-23.

4.8.2. Fungicide /Insecticide

The isomer profile of catechins in tea bushes was different than those of potted plants. Spraying with insecticide/ fungicide suppress the peak heights of catechins in TV-23 (Table 30 ; Fig. 37). In HV-39 insecticide spray resulted in decrease in height as compared to control (Table 31; Fig. 38). In TV-23 insecticide and fungicide spray led to a decrease in number of isomers as well as the peak height of the isomers.

Table 27 (A-F): Peak results of HPLC analysis of catechin extracts of heavy metal stressed tea leaves (TV-23) of potted plants. A: Control; B: Cu 100; C: Cu 500; D: Cu 1000; E: Cd 500 and F: Cd 1000 µg/ml.

Table 27 A

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.930	957.0284	75.543	0.706	2.297
2	6.040	1565.1920	70.567	1.154	2.146
3	12.270	26544.6880	759.222	19.573	23.086
4	14.180	2219.7841	61.155	1.637	1.860
5	14.910	934.1855	49.010	0.689	1.490
6	15.830	3645.6313	78.702	2.688	2.393
7	16.860	44713.0697	992.241	32.970	30.171
8	18.440	48545.3398	994.342	35.795	30.235

Table 27 B

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.910	1216.2018	108.108	0.848	2.326
2	11.870	15107.5358	493.618	10.535	10.621
3	13.970	3725.3966	77.301	2.598	1.663
4	14.760	1104.0471	56.183	0.770	1.209
5	15.470	4401.3492	83.137	3.069	1.789
6	16.140	14818.3600	997.344	10.333	21.459
7	17.190	33309.5698	797.416	23.228	17.157
8	18.440	32660.8028	996.092	22.776	21.432
9	19.560	29049.8054	795.292	20.258	17.111

Table 27 C

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.950	1636.4141	139.875	2.786	6.869
2	5.210	784.0123	46.391	1.335	2.278
3	12.790	2506.2991	73.513	4.268	3.610
4	15.850	2492.5351	50.812	4.244	2.495
5	16.380	15200.3107	345.598	25.882	16.971
6	18.010	2167.0871	103.487	3.690	5.082
7	18.470	13965.0594	613.925	23.779	30.147
8	19.260	2055.7787	123.053	3.500	6.043
9	19.600	14071.2426	410.028	23.960	20.133

Table 27 D

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.940	2233.9957	191.710	1.180	5.231
2	12.510	28190.3956	906.862	14.887	24.746
3	14.290	5101.7134	104.709	2.694	2.857
4	15.040	1377.3574	77.367	0.727	2.111
5	15.650	6249.7139	137.578	3.300	3.754
6	16.980	59467.5406	991.815	31.404	27.064
7	18.540	77474.1445	990.769	40.913	27.037

Table 27 E

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.910	694.3665	57.292	1.471	2.640
2	11.830	10905.6392	330.295	23.108	15.218
3	16.140	3745.4065	237.453	7.936	10.941
4	16.750	3486.5386	198.884	7.388	9.164
5	17.620	1282.4181	66.450	2.717	3.062
6	17.990	885.8358	62.507	1.877	2.880
7	18.390	4178.9865	274.143	8.855	12.631
8	18.710	2589.9945	144.105	5.488	6.640
9	19.200	1453.4166	66.088	3.080	3.045
10	19.550	1530.5908	105.741	3.243	4.872
11	19.840	3497.4973	66.340	7.411	3.057

Table 27 F

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.900	1267.0149	111.411	0.766	2.145
2	5.920	2256.5007	70.244	1.364	1.352
3	8.370	2357.6462	66.273	1.425	1.276
4	11.760	34936.3635	1002.036	21.115	19.292
5	13.340	1773.3626	46.210	1.072	0.890
6	13.950	3373.2219	88.917	2.039	1.712
7	14.770	1468.4978	69.731	0.888	1.343
8	15.150	1870.3387	86.198	1.130	1.660
9	16.130	16434.2477	903.438	9.933	17.394
10	16.800	38840.8142	1000.239	23.475	19.257
11	18.410	31581.9933	999.636	19.088	19.246
13	19.560	23714.0114	533.961	14.332	10.277

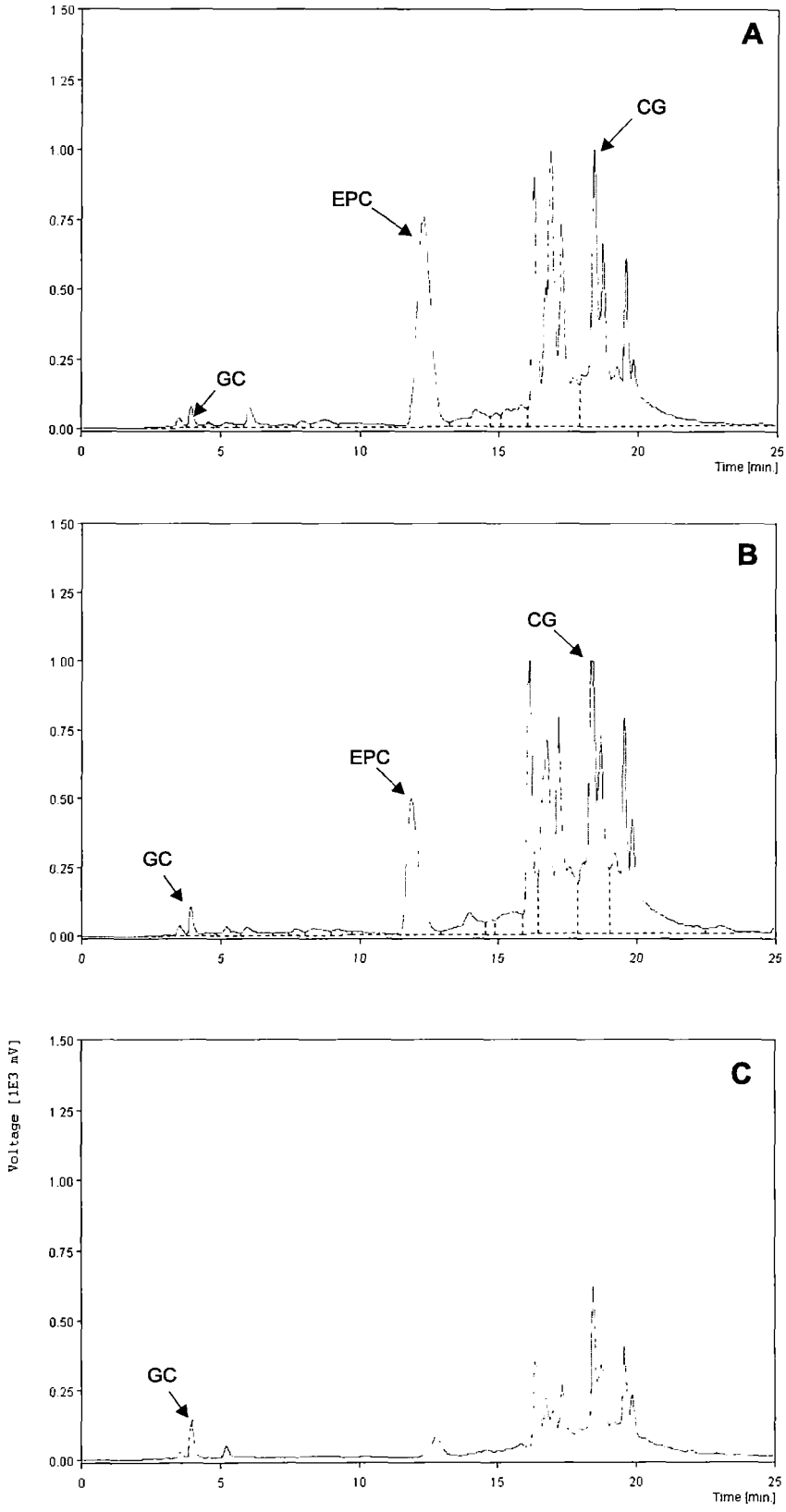


Fig. 34A: Changes in HPLC profile of catechin of tea leaves following heavy metal stresses in TV-23. A. Control; B. Cu 100; C. Cu 500.

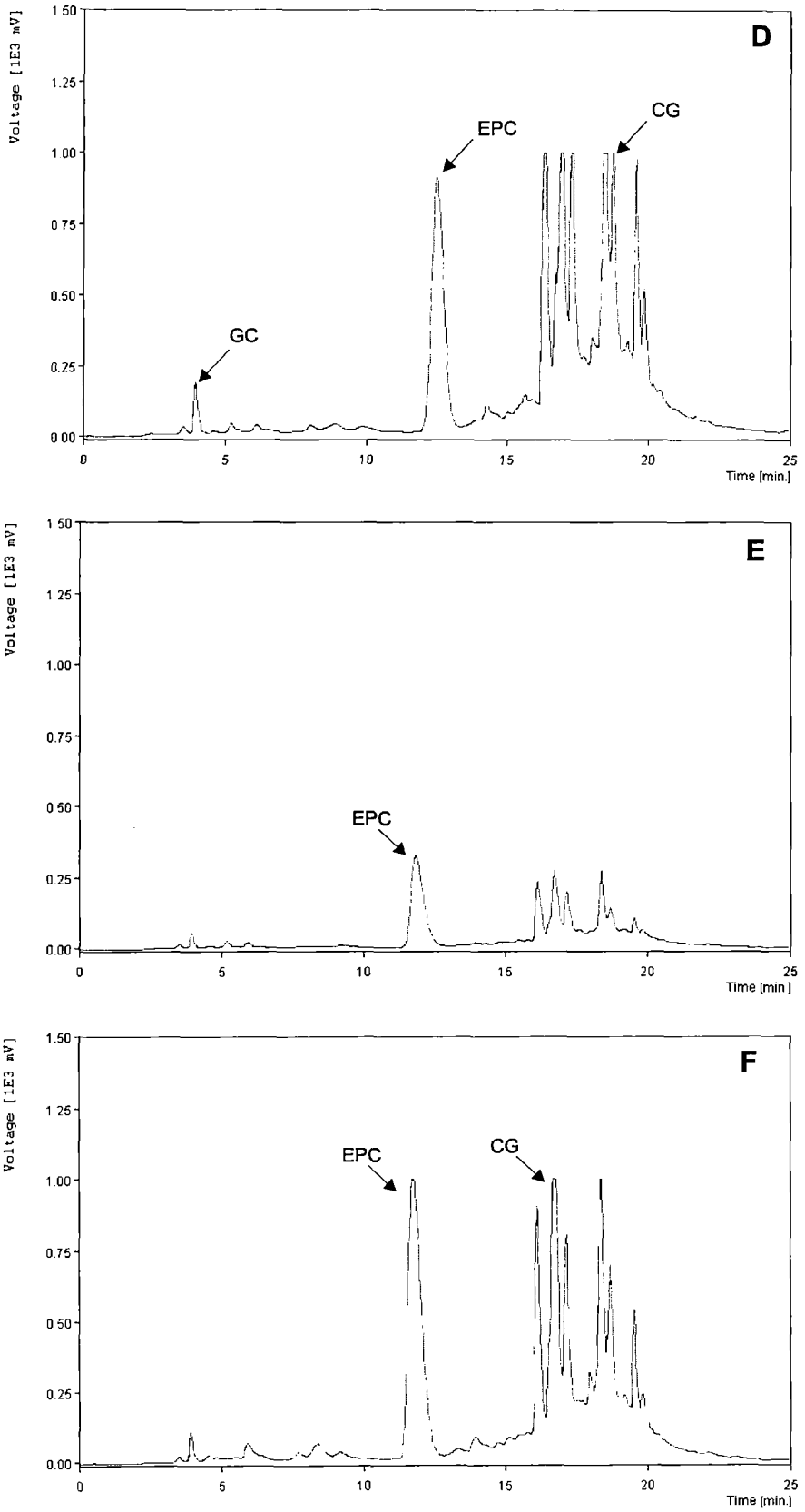


Fig. 34B: Changes in HPLC profile of catechin of tea leaves following heavy metal stresses in TV-23. D. Cu 1000; E. Cd 500; F. Cd 1000.

Table 28 (A-G): Peak results of HPLC analysis of catechin extracts of heavy metal stressed tea leaves (HV-39). A: Control; B: Cu 100; C: Cu 500; D: Cu 1000; E: Cd 100; F: Cd 500 and G: Cd 1000 µg/ml.

Table 28 A

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	4.020	15545.3665	1006.767	19.491	38.589
2	6.040	1662.5516	53.496	2.085	2.050
3	12.320	9209.0847	229.610	11.546	8.801
4	14.200	2348.1852	39.260	2.944	1.505
5	15.870	3096.0418	68.419	3.882	2.622
6	16.310	11458.2447	348.876	14.366	13.372
7	17.300	7521.2748	281.261	9.430	10.781
8	18.470	22631.6545	374.949	28.376	14.372

Table 28 B

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.940	5607.8877	521.872	11.695	33.224
2	5.220	1669.6835	107.442	3.482	6.840
3	12.590	3387.5449	92.238	7.064	5.872
4	15.940	2558.7652	46.315	5.336	2.949
5	16.380	3232.1707	181.802	6.740	11.574
6	17.350	7895.6685	152.214	16.465	9.690
7	18.510	16402.2946	269.988	34.205	17.188
8	21.340	1738.6711	54.210	3.626	3.451

Table 28 C

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	12.280	1550.8857	59.284	4.160	3.893
2	16.760	4894.5825	162.777	13.128	10.688
3	18.730	7582.4449	90.525	20.338	5.944

Table 28 D

PeakNo.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.950	10863.8926	1006.662	13.160	28.875
2	6.090	1391.8111	50.125	1.686	1.438
3	8.750	2308.5856	56.896	2.797	1.632
4	12.360	15710.3968	467.439	19.031	13.408
5	14.280	2201.0946	65.864	2.666	1.889
6	15.670	1870.3895	61.811	2.266	1.773
7	15.950	1309.0627	65.350	1.586	1.875
8	16.360	5416.4869	342.049	6.561	9.811
9	16.920	7904.3100	446.538	9.575	12.809
10	17.340	7696.6822	306.113	9.323	8.781
11	18.500	19635.0238	395.720	23.785	11.351

Table 28 E

PeakNo.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.940	7942.6311	765.228	6.299	14.485
2	6.110	1586.4782	54.417	1.258	1.030
3	8.730	3013.2599	81.250	2.390	1.538
4	12.280	31949.5733	1003.369	25.339	18.993
5	13.630	1735.8031	51.797	1.377	0.980
6	14.220	3230.6426	100.820	2.562	1.908
7	14.990	1012.2420	49.903	0.803	0.945
8	15.920	4045.7908	90.542	3.209	1.714
9	16.310	10653.0279	734.739	8.449	13.908
10	16.890	13000.9017	822.901	10.311	15.577
11	17.320	12764.2734	623.208	10.123	11.797
13	18.490	30536.2486	749.583	24.218	14.189

Table 28 F

PeakNo.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	4.110	22030.3178	1006.126	23.086	22.540
2	4.590	1388.5329	61.752	1.455	1.383
3	6.100	4111.3175	217.027	4.308	4.862
4	12.220	13382.8998	404.872	14.024	9.070
5	15.310	1538.7710	66.580	1.612	1.492
6	15.660	1000.6838	67.239	1.049	1.506
7	15.990	1160.4331	67.081	1.216	1.503
8	16.380	2526.8800	138.696	2.648	3.107
9	16.760	9360.2276	594.868	9.809	13.326
10	17.320	3490.7944	166.209	3.658	3.723
11	17.610	1489.7420	116.184	1.561	2.603
12	17.840	1320.4654	120.372	1.384	2.697
13	18.090	2966.0033	171.541	3.108	3.843
14	18.510	3337.6669	199.577	3.498	4.471
15	18.740	4957.3729	245.012	5.195	5.489
16	19.310	2898.5727	131.648	3.037	2.949
17	19.650	1814.8224	116.220	1.902	2.604

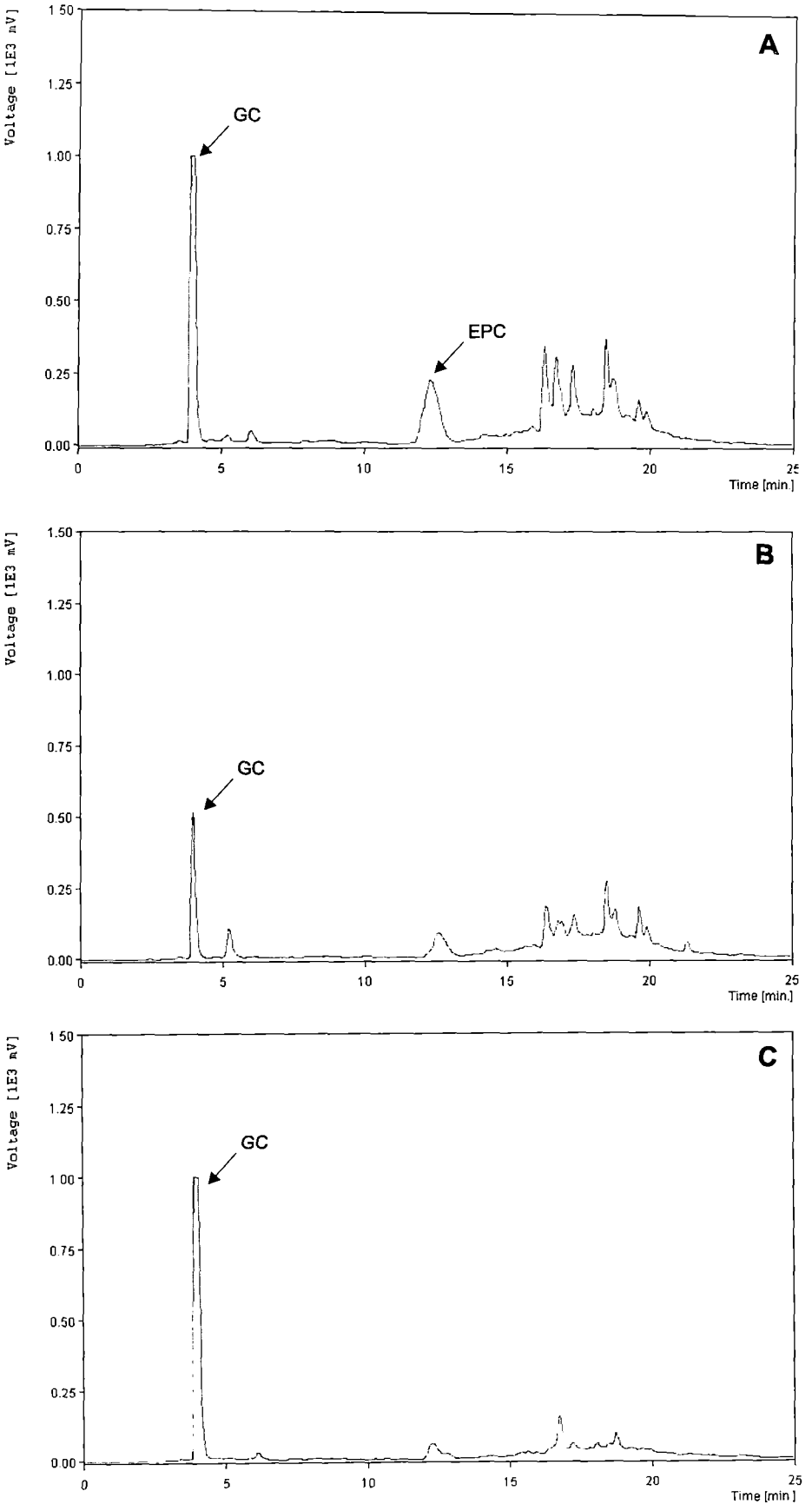


Fig. 35A: Changes in HPLC profile of catechin of tea leaves following heavy metal stresses in HV-39. A. Control; B. Cu 100; C. Cu 500.

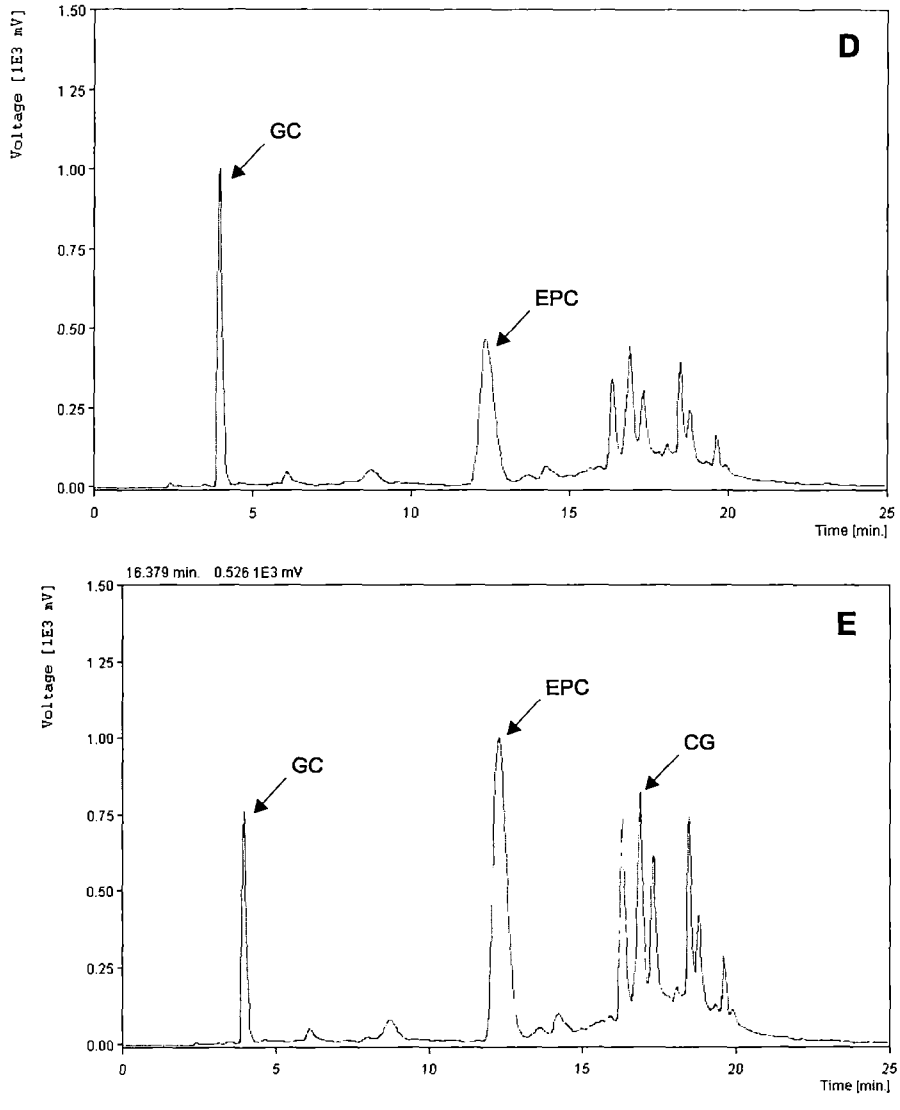


Fig. 35B: Changes in HPLC profile of catechin of tea leaves following heavy metal stresses in HV-39. D.Cu 1000; E. Cd 100.

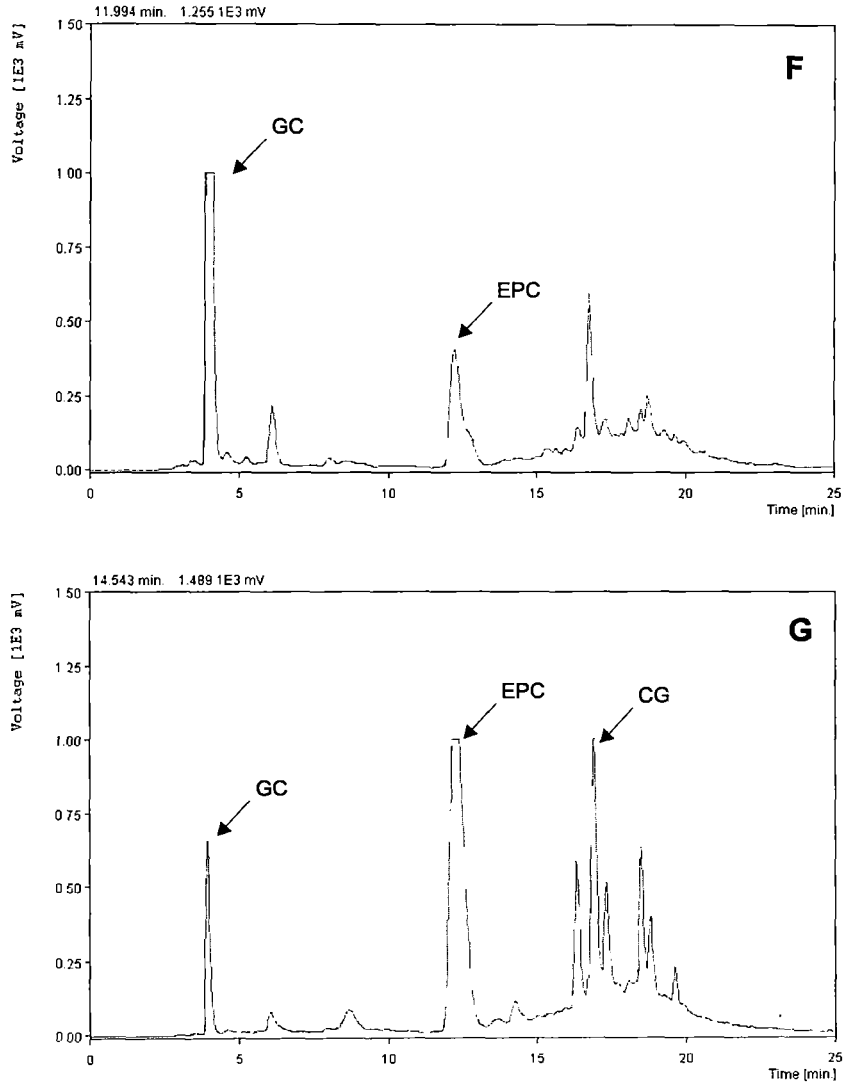


Fig. 35C: Changes in HPLC profile of catechin of tea leaves following heavy metal stresses in HV-39.F.Cd 500; G. Cd 1000.

Table 28 G

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.930	6832.6273	662.74	5.207	14.735
2	6.050	2120.4324	79.358	1.616	1.764
3	8.670	3145.9856	84.621	2.397	1.881
4	12.350	36998.0212	1001.771	28.195	22.272
5	13.670	1794.2462	51.921	1.367	1.154
6	14.260	3332.9382	111.482	2.540	2.479
6	15.030	1322.5064	59.726	1.008	1.328
8	15.680	2278.6312	73.945	1.736	1.644
9	16.330	10721.5090	584.165	8.170	12.988
10	16.920	27225.4264	999.341	20.748	22.218
11	18.500	31188.3929	630.541	23.768	14.019

Table 29 (A-G): Peak results of HPLC analysis of catechin extracts of heavy metal stressed tea leaves (T-78). A: Control; B: Cu 100; C: Cu 500; D: Cu 1000; E: Cd 100; F: Cd 500 and G: Cd 1000 µg/ml.

Table 29 A

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.910	2223.4766	202.960	0.926	3.132
2	5.930	4380.7468	160.685	1.825	2.480
3	7.730	2064.8181	68.130	0.860	1.051
4	8.400	7745.8334	268.931	3.227	4.150
5	9.220	3687.6600	85.945	1.536	1.326
6	12.050	62240.5756	995.669	25.928	15.365
7	13.260	4927.9597	127.278	2.053	1.964
8	13.960	5267.4963	161.035	2.194	2.485
9	14.780	2154.4939	109.722	0.898	1.693
10	15.150	3936.9885	164.406	1.640	2.537
11	15.620	3722.9726	177.788	1.551	2.744
12	16.150	13858.8309	735.914	5.773	11.357
13	16.870	41216.7337	992.380	17.170	15.315
14	18.000	15751.3490	521.013	6.562	8.040
15	18.450	35206.5346	991.291	14.666	15.298
16	19.550	22531.2376	436.495	9.386	6.736
17	21.990	4477.5892	79.041	1.864	1.223

Table 29 B

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.900	3182.4721	275.951	1.998	5.734
2	5.920	1328.4245	64.455	0.834	1.339
3	8.410	2798.1006	79.239	1.757	1.646
4	11.890	40923.8205	993.755	25.690	20.648
5	13.290	2119.3571	50.603	1.330	1.051
6	13.960	3308.0890	94.617	2.077	1.966
7	14.800	3013.3302	112.732	1.892	2.342
8	15.510	4141.2244	120.555	2.600	2.505
9	16.170	10593.1239	523.875	6.650	10.885
10	16.810	18731.5826	989.266	11.759	20.555
11	17.190	9768.6810	532.470	6.132	11.063
12	18.430	45060.2999	589.043	28.287	12.239
13	21.300	6204.7302	109.719	3.895	2.282

Table 29 C

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.900	3004.0636	270.653	1.405	3.423
2	5.920	2790.9143	139.151	1.305	1.760
3	7.670	1813.3160	49.000	0.848	0.620
4	8.340	4846.7669	157.366	2.266	1.990
5	9.140	1569.9904	71.085	0.734	0.899
6	9.390	1821.4719	67.164	0.852	0.850
7	11.960	57841.0927	995.397	27.045	12.590
8	13.220	3400.3363	84.870	1.590	1.073
9	13.920	4729.2472	139.544	2.211	1.765
10	14.760	2775.8026	119.333	1.298	1.509
11	15.130	3217.4127	137.824	1.504	1.743
12	15.630	2731.9664	143.598	1.277	1.816
13	15.880	2492.2462	171.028	1.165	2.163
14	16.150	10315.8064	630.295	4.823	7.972
15	16.850	23939.0221	992.124	11.193	12.549
16	17.170	13840.3242	719.529	6.471	9.101
17	17.610	6815.9127	312.810	3.187	3.956
18	18.030	9747.7939	442.752	4.558	5.600
19	18.430	12064.1632	835.689	5.641	10.570
20	18.750	15490.9091	550.638	7.243	6.965
21	19.580	13656.0307	328.601	6.385	4.156
22	20.610	2519.6862	108.067	1.178	1.367
23	21.090	3168.0561	110.112	1.481	1.393
24	22.020	2069.4561	62.089	0.968	0.785

Table 29 D

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.900	1814.2743	160.623	2.097	4.493
2	11.700	16565.2792	532.341	19.146	14.891
3	13.890	1278.2192	50.199	1.477	1.404
4	14.760	2326.8428	78.581	2.689	2.198
5	15.850	3153.8737	75.853	3.645	2.122
6	16.130	4238.7788	237.852	4.899	6.653
7	16.730	11479.6493	729.231	13.268	20.399
8	17.160	4852.1726	250.600	5.608	7.010
9	17.590	3038.5544	137.890	3.512	3.857
10	18.010	3144.8388	175.205	3.635	4.901
11	18.420	6428.7243	377.331	7.430	10.555
12	18.740	7618.1432	253.521	8.805	7.092
13	19.580	9115.6721	153.040	10.536	4.281

Table 29 E

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.890	3726.4786	331.828	2.875	7.630
2	11.550	22506.9826	712.808	17.362	16.390
3	13.790	2886.8182	60.095	2.227	1.382
4	14.710	3711.4613	106.210	2.863	2.442
5	15.400	3518.7788	110.704	2.714	2.546
6	16.060	10518.2128	542.637	8.114	12.477
7	16.670	12844.9733	759.858	9.909	17.472
8	17.110	13919.3494	503.72	10.738	11.583
9	18.000	5246.6965	276.799	4.047	6.365
10	18.380	40520.0016	646.944	31.259	14.874

Table 29 F

PeakNo.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.880	2433.9602	219.010	1.385	3.369
8	5.790	3432.1805	128.048	1.952	1.970
3	6.720	738.3710	26.650	0.420	0.410
4	7.530	1377.6901	46.291	0.784	0.712
5	8.080	6012.5668	198.692	3.420	3.057
6	8.790	2586.6770	62.409	1.471	0.960
7	11.490	49524.9813	996.703	28.171	15.334
8	12.870	3827.4584	91.643	2.177	1.410
9	13.680	4100.1045	108.809	2.332	1.674
10	14.560	2686.9898	97.639	1.528	1.502
11	15.140	2693.6967	112.767	1.532	1.735
12	15.380	3144.0017	145.425	1.788	2.237
13	16.020	7907.4339	326.604	4.498	5.025
14	16.710	22198.9284	993.753	12.627	15.289
15	17.060	9142.4620	491.540	5.200	7.562
16	17.500	3130.1612	236.917	1.781	3.645
17	17.710	2549.1407	245.233	1.450	3.773
18	17.970	7657.8848	372.674	4.356	5.734
19	18.370	8201.2695	536.514	4.665	8.254
20	18.690	8951.8294	369.160	5.092	5.680
21	19.230	2923.3518	190.898	1.663	2.937
22	19.510	13555.1326	220.076	7.711	3.386

Table 29 G

PeakNo.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.880	2404.2392	212.573	1.526	3.555
2	5.800	3046.4196	112.302	1.934	1.878
3	8.110	4187.1619	131.263	2.658	2.195
4	8.820	947.0720	50.073	0.601	0.837
5	9.200	2012.4085	59.871	1.278	1.001
6	11.510	44746.3157	997.333	28.410	16.677
7	12.920	3009.5127	69.309	1.911	1.159
8	13.700	3812.5281	100.598	2.421	1.682
9	14.550	2424.0242	89.097	1.539	1.490
10	15.010	1236.2251	92.852	0.785	1.553
11	15.180	1081.5598	97.096	0.687	1.624
12	15.400	2697.0645	119.994	1.712	2.007
13	16.030	9349.3102	454.567	5.936	7.601
14	16.700	20315.6695	994.850	12.898	16.636
15	17.090	8929.0129	524.673	5.669	8.774
16	17.510	4795.3975	209.189	3.045	3.498
17	17.990	5952.2665	280.279	3.779	4.687
18	18.360	7548.2068	526.186	4.792	8.799
19	18.700	10886.0190	396.986	6.912	6.638
20	19.540	10083.4937	170.499	6.402	2.851

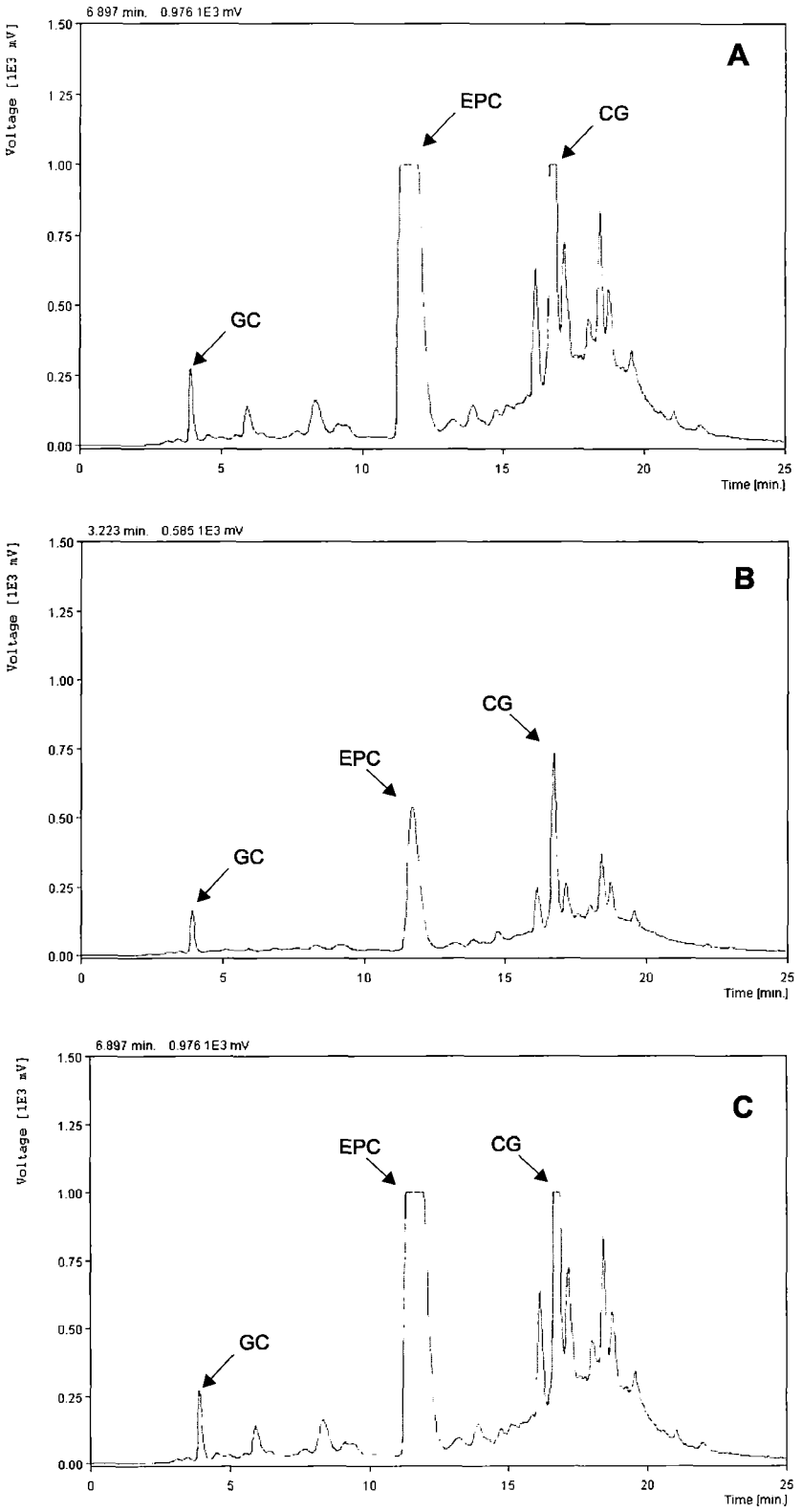


Fig. 36A: Changes in HPLC profile of catechin of tea leaves following heavy metal stresses in T-78. A.Control; B.Cu 100; C. Cu 500.

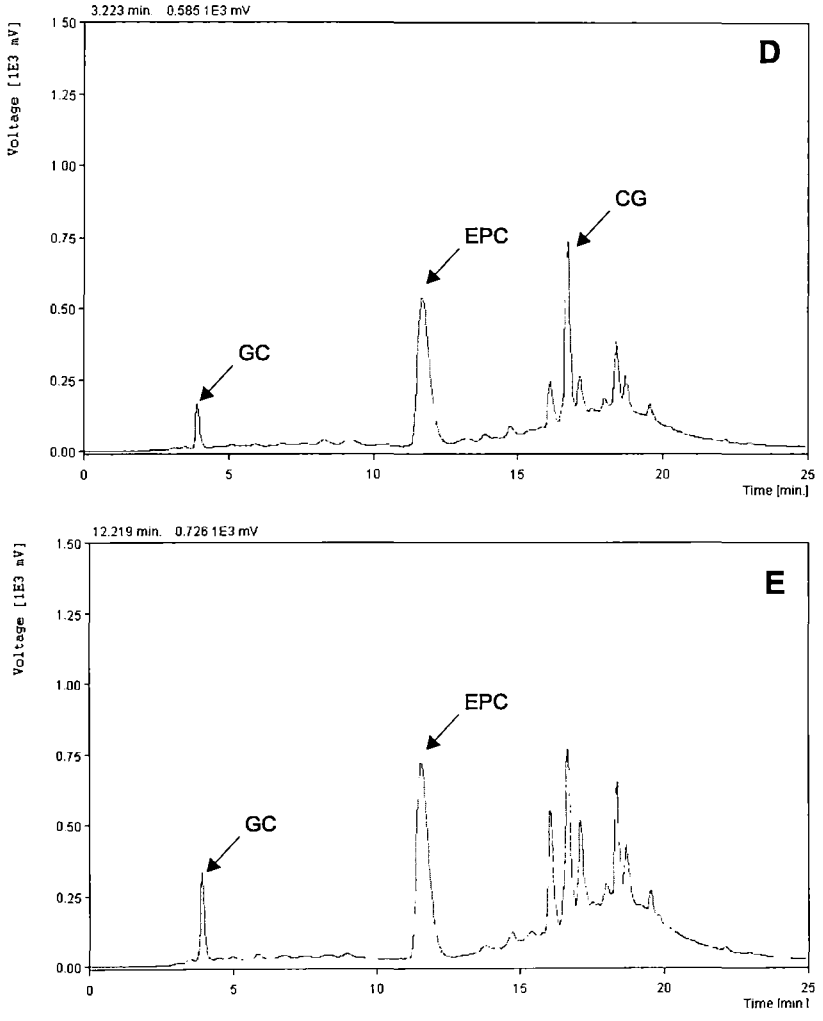


Fig. 36B: Changes in HPLC profile of catechin of tea leaves following heavy metal stresses in T-78. D.Cu-1000; E. Cd 100.

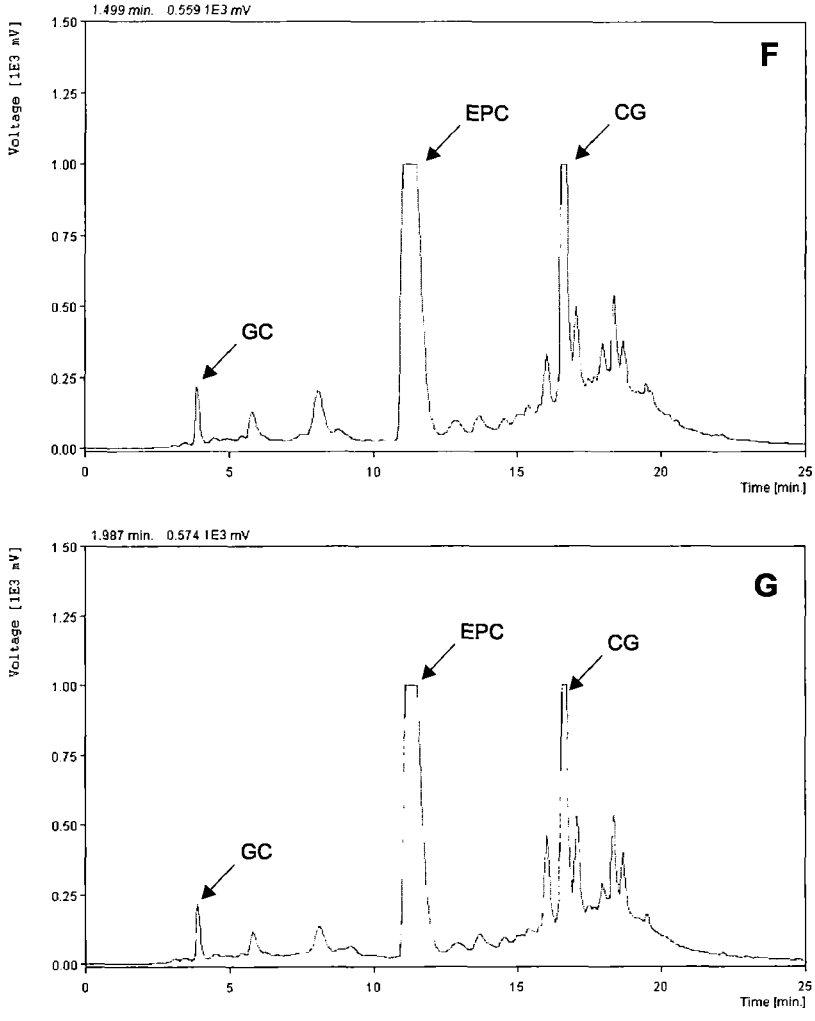


Fig. 36C: Changes in HPLC profile of catechin of tea leaves following heavy metal stresses in T-78. F. Cd 500; G. Cd 1000.

Table 30 (A-C): Peak results of HPLC analysis of catechin extracts of tea leaves (TV-23) bush following fungicide/insecticide application. A: Control; B: Fungicide; C: Insecticide.

Table 30 A

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	6.210	10840.3033	270.919	4.011	4.231
2	6.750	18426.3206	995.423	6.817	15.545
3	7.240	29955.9228	955.054	11.083	14.915
4	8.390	55159.3084	995.222	20.408	15.542
5	9.250	9264.1726	272.575	3.428	4.257
6	10.140	7410.9784	212.602	2.742	3.320
7	11.430	71761.2879	994.662	26.550	15.533
8	12.930	4785.0871	145.196	1.770	2.267
9	13.910	14000.6638	525.382	5.180	8.205
10	15.230	12314.1143	282.660	4.556	4.414
11	16.370	6396.4400	239.898	2.367	3.746
12	17.310	26859.1623	434.372	9.937	6.783
13	20.340	2465.6255	45.213	0.912	0.706

Table 30 B

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.510	536.1743	65.380	0.351	1.199
2	3.950	1059.1350	127.964	0.694	2.347
3	5.720	3229.3572	245.542	2.116	4.504
4	7.500	2145.6954	120.544	1.406	2.211
5	8.130	1240.1862	58.130	0.813	1.066
6	10.880	27174.3332	721.375	17.807	13.231
7	11.180	23902.6475	734.674	15.663	13.475
8	13.140	2247.5405	89.843	1.473	1.648
9	13.800	3396.3356	89.094	2.226	1.634
10	14.550	2042.3002	95.488	1.338	1.751
11	15.170	1690.6049	97.640	1.108	1.791
12	15.420	2646.0353	147.800	1.734	2.711
13	16.150	6330.5819	210.277	4.148	3.857
14	16.600	32801.3973	781.818	21.494	14.340
15	17.650	5847.2793	263.645	3.832	4.836
16	18.010	3632.9237	318.830	2.381	5.848
17	18.230	18529.2666	743.856	12.142	13.644
18	19.210	10181.6223	290.879	6.672	5.335
19	21.300	826.0229	69.541	0.541	1.275

Table 30 C

PeakNo.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	5.340	2779.3257	88.140	0.929	1.221
2	6.410	24331.4271	651.975	8.129	9.028
3	7.300	25494.8759	599.170	8.518	8.297
4	8.410	52329.0589	995.668	17.482	13.788
5	9.050	9556.5405	319.474	3.193	4.424
6	9.910	10381.4821	232.391	3.468	3.218
7	10.670	8495.9753	230.313	2.838	3.189
8	11.320	12810.2025	760.064	4.280	10.525
9	12.110	58047.0370	994.496	19.393	13.772
10	13.970	5797.5221	117.618	1.937	1.629
11	15.320	6981.3211	252.750	2.332	3.500
12	15.890	28346.6120	920.167	9.470	12.742
13	17.530	49365.7157	952.217	16.492	13.186
14	20.510	3056.8285	69.139	1.021	0.957

Table 31 (A-C): Peak results of HPLC analysis of catechin extracts of tea leaves (HV-39) bush following fungicide/insecticide application. A: Control; B: Fungicide; C: Insecticide

Table A.

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.450	897.4373	58.282	0.240	0.631
2	3.870	5319.4546	549.829	1.424	5.955
3	4.440	3310.0187	192.227	0.886	2.082
4	5.500	835.0794	44.728	0.223	0.484
5	5.820	4472.5534	259.086	1.197	2.806
6	6.340	983.2594	68.392	0.263	0.741
7	6.640	1470.1099	49.620	0.393	0.537
8	7.620	4217.9694	208.247	1.129	2.255
9	8.250	4609.8822	170.802	1.234	1.850
10	8.960	2863.3873	110.483	0.766	1.197
11	9.440	1238.4655	52.425	0.331	0.568
12	11.710	65346.4324	989.793	17.489	10.720
13	13.140	2682.6167	87.474	0.718	0.947
14	13.760	27818.7746	987.748	7.445	10.698
15	14.710	4373.8208	170.359	1.171	1.845
16	15.170	4505.0671	198.190	1.206	2.147
17	16.110	34809.7979	985.410	9.316	10.673
18	17.200	68802.6960	984.374	18.414	10.661
19	18.190	20827.4988	983.367	5.574	10.651
20	18.830	40308.7311	982.770	10.788	10.644
21	19.720	71487.4182	981.851	19.134	10.635

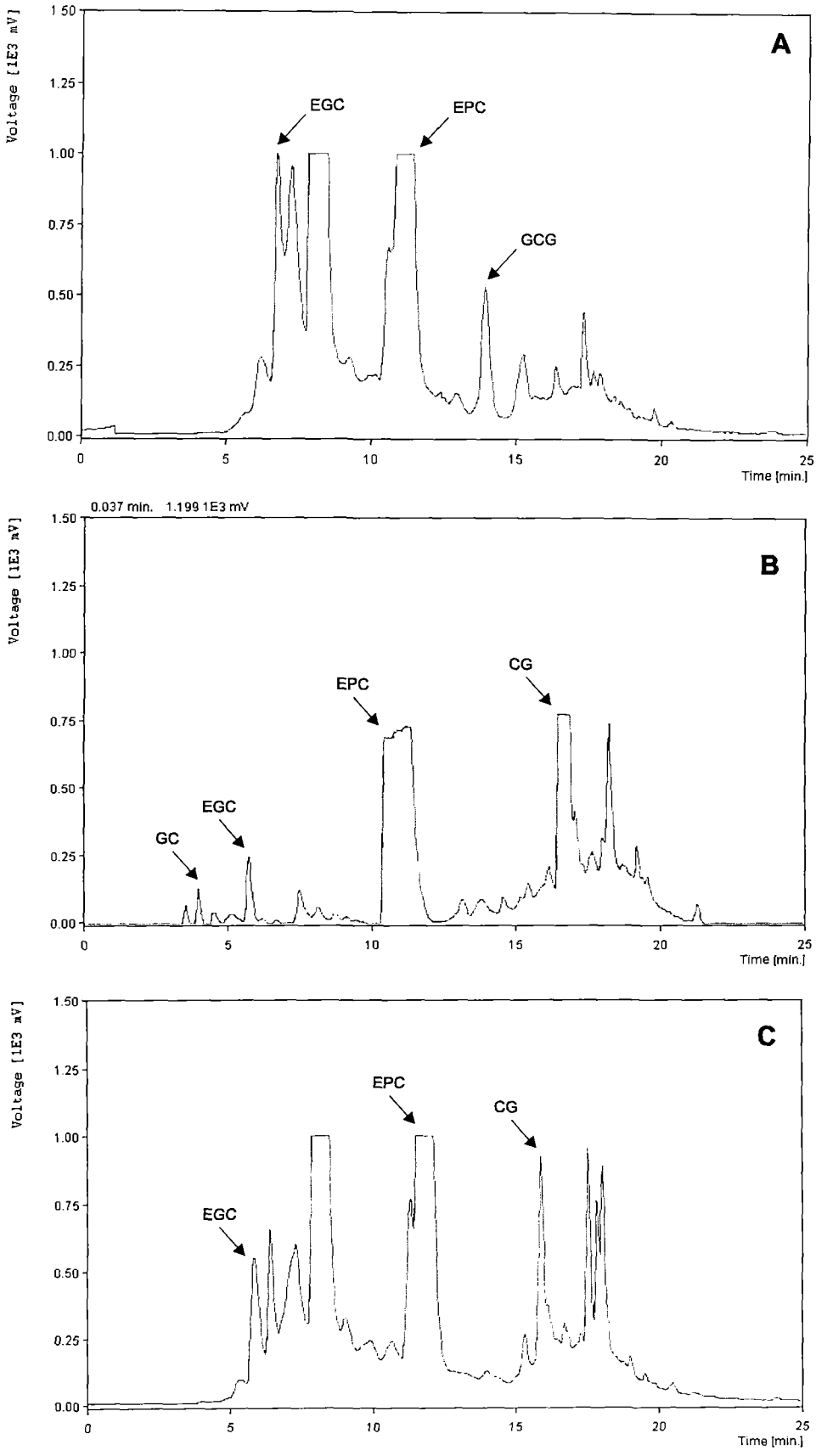


Fig. 37: Changes in HPLC profile of catechin of tea leaves in bush TV-23 following hexaconazole/acephate application.

A: C ; B: Fungicide ; C: Insecticide

Table 31 B

Peak No.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	3.490	734.9684	87.049	0.328	1.279
2	3.920	2316.4461	270.142	1.035	3.968
3	4.490	1837.2196	137.979	0.821	2.027
4	5.060	1702.6081	48.847	0.761	0.718
5	5.680	5690.4421	391.594	2.543	5.752
6	6.130	1177.9482	68.340	0.526	1.004
7	7.400	3274.1003	205.317	1.463	3.016
8	8.010	3088.5949	115.614	1.380	1.698
9	8.490	2040.5217	112.973	0.912	1.659
10	8.900	1474.5404	78.781	0.659	1.157
11	9.400	1521.6123	64.857	0.680	0.953
12	10.980	63262.8715	826.685	28.274	12.144
13	12.840	2853.4577	90.854	1.275	1.335
14	13.320	5758.8242	176.221	2.574	2.589
15	14.070	1408.2309	90.159	0.629	1.324
16	14.360	4395.7897	157.457	1.965	2.313
17	15.000	3238.1889	168.325	1.447	2.473
18	15.350	3970.7277	304.878	1.775	4.478
19	15.530	12871.9692	732.903	5.753	10.766
20	15.980	4305.5921	277.266	1.924	4.073
21	16.450	36521.4822	758.444	16.323	11.141
22	17.640	9195.7622	377.751	4.110	5.549
23	18.160	33682.3797	759.739	15.054	11.160
24	19.190	14403.7621	315.169	6.438	4.630
25	21.300	860.1313	56.483	0.384	0.830

Table 31 C

PeakNo.	Ret time	Peak area (mV.s)	Peak Height mV	Area (%)	Height (%)
1	4.120	1646.4646	91.311	0.742	1.771
2	4.970	1440.7661	80.415	0.650	1.560
3	5.630	3092.3486	84.227	1.394	1.634
4	6.370	1038.0778	56.756	0.468	1.101
5	6.720	5726.0551	263.508	2.582	5.111
6	7.880	1340.8469	53.731	0.605	1.042
7	9.110	3589.6531	127.100	1.619	2.465
8	10.570	3673.9864	62.671	1.657	1.216
9	11.160	802.5401	35.836	0.362	0.695
10	11.910	3870.6770	123.393	1.745	2.394
11	12.670	722.6269	28.957	0.326	0.562
12	13.510	17464.4675	993.845	7.875	19.278
13	14.180	41205.1337	993.178	18.581	19.265
14	15.200	13094.5640	319.354	5.905	6.195
15	16.070	5949.9700	259.801	2.683	5.039
16	16.610	12917.0392	442.164	5.825	8.577
17	17.590	100081.6418	989.557	45.130	19.195
18	21.940	2293.4738	91.429	1.034	1.773

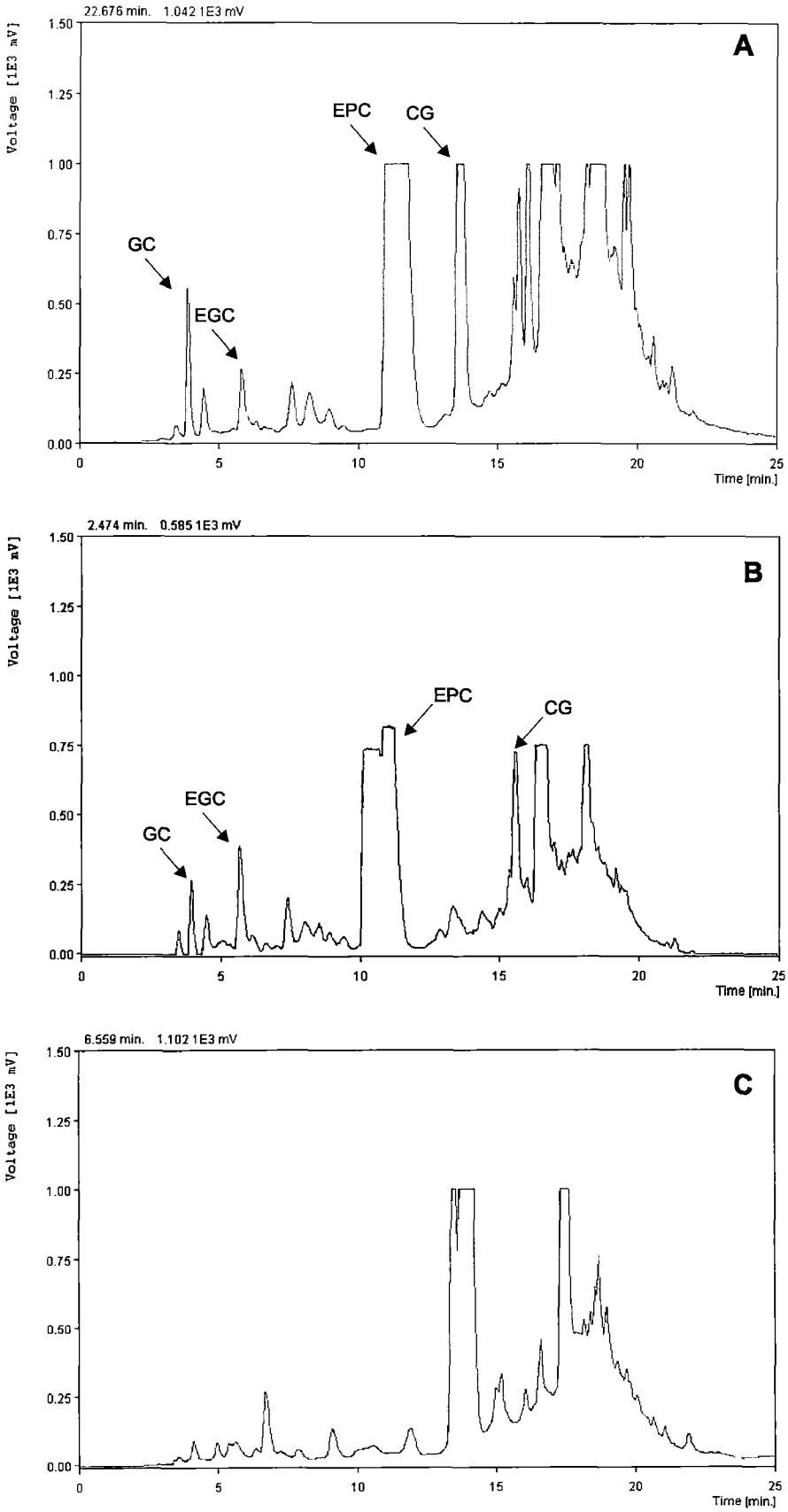


Fig. 38: Changes in HPLC profile of catechin of tea leaves in bush HV-39 following hexaconazole/acephate application.

A: C ; B: Fungicide ; C: Insecticide

4.9. Determination of heavy metal content of tea leaves

Accumulation the heavy metals in the tissues of plants treated with heavy metal solution were determined. Two varieties of tea seedlings i.e. TV-23 and HV-39 were taken for the study. Heavy metal solutions were applied to the soil as described in materials and methods.Analysis were performed after the 1st and 2nd application of the metal solutions to the soil. In case of control only watering was done. Traces of heavy metals Cu, and Cd were found in untreated control of all the varieties. Differences in heavy metal accumulation were evident among the different varieties. Accumulation of Cu was much higher than Cd after every application of the said metals.The result is presented in Table.32.

Table 32. Cu and Cd of tea seedlings treated with the respective heavy metals

Variety	Treatment	Cd content (ppm)					
		1st treatment			2nd treatment		
		0	100 µg/ml	1000 µg/ml	0	100 µg/ml	1000 µg/ml
TV-23	$Cd(NO_3)$, $4H_2O$	0.036	0.049	0.099	0.036	0.080	0.122
HV-39		0.034	0.050	0.070	0.034	0.076	0.081
Cu content (ppm)							
TV-23	$Cu(SO_4)$, $5H_2O$	0.222	0.241	0.261	0.222	0.304	0.394
HV-39		0.182	0.231	0.274	0.182	0.279	0.369

Discussion

Plants, being immobile are constantly subjected to adverse environmental conditions such as extreme temperatures, water scarcity, flooding, heavy metals, excessive salts, high intensity irradiation and infection by pathogenic agents. Besides, various activities of human beings also impose different stresses on plants. Plants react to such stresses by various adjustments to their metabolic processes leading either to avoidance strategies, tolerance or in extreme cases they are victims of the stress. Not all metabolic responses are deleterious or injurious to the plants and most changes represent the adaptations of the plants, to withstand the particular stress. Hence, genetic programme in normal plant is altered by the stress stimuli to activate the biochemical pathway that ensure survival.

Tea (*Camellia sinensis* L.) is grown in rain fed eco-systems in specific regions of India in the hilly regions of Darjeeling and Nilgiri, Terai and Dooars of West Bengal, Brahamputra valley in Assam and certain other areas. Since the plant is grown in non-irrigated eco-systems they are subjected to the different environmental stresses which modify morphology and rate of development, limit yield and quality and reproduction (Leinhos and Bergman, 1995). Huge amounts of pesticides, fungicides and chemical fertilizers are used in tea plantations to ensure the production of disease free leaves for the planters. Soil pollution is caused as a run out of these chemicals used which also contribute to the anthropogenic stress on tea. Activities such as mining and smelting as well as agriculture have contaminated extensive areas of world mostly by heavy metals such as Cd , Cu, Zn, Pb, Cr, Ni (Smith *et al.* 1996; Zantopolus 1999; Herawati *et al* . 2000). Inorganic and organic fertilizers are most important sources of heavy metals to agricultural soil which include liming, sewadge/ sludge, irrigation water and pesticides (Sharma and Agarwal,2005).

In the present study investigations have been done on the biochemical changes induced in the tea leaves by an insecticide (Acephate) and a fungicide (Hexaconazole) which are commonly used in the plantations. Besides, effects of two heavy metals i.e. Cu (taken as $CuSO_4$) and a Cd (taken as $CdNO_3$) were also studied on the various biochemicals process in the leaves. Changes in the biochemical components of tea leaves could alter the flavour components in tea which in turn would affect the quality of made tea. It was observed that in general, plats sprayed with insecticide or fungicide

were healthier due to less infestation by pests. However, the biochemical changes induced by the treatements which were considered as anthropogenic stress were determined. At the onset the effect of these chemicals on accumulation of phenol content in tea leaves of different varieties was investigated. Phenols are major components of tea leaves and some of the phenols are responsible for the flavour of tea. An increase in total phenols as well as o-phenol was observed in all treatments in cut shoots, potted plants as well as bushes. In higher concentration of heavy metals leaves from cut shoot showed a decline in phenol content. Phenols are considered to be involved in plant's defense to various stresses (Leinhos and Bergman, 1995). In case of tea, polyphenols are major components and their biosynthesis seems to be well regulated to help the plant to overcome various stresses. Kotasthane and Vyas, (1992) reported quantitative changes in phenol content in mustard following application of synthetic fungicide. Alteration of phenol metabolism following infection has been observed in many diseases and phenolics have been implicated in defense reaction in several instances (Zaichuk *et al.* 1988; Nicholson and Hammerschimidt, 1992; Chakraborty *et al.* 1996). The activation of defense reaction in plants is associated with increased expression of a number of genes that encode enzymes involved in the biosynthetic pathway of phenolic compound. It has been suggested that, certain fungicides used to mitigate or prevent pathogen attack may be involved in certain defense responses in plants (Gracia *et al.* 2003). Increased accumulation of polyphenols to heavy metal stresses has also been reported by several previous workers. Tripathy and Tripathy (1999) reported that phenol contents in *Albizzia lebek* increased with concentration of Ni, Hg and Cr . On the other hand Basak *et al* .(2001) also reported increased accumulation of phenols in tea leaves following Cu treatment but decreased accumulation due to Hg and Ni treatments. Phenolics have been also been implicated in diverse functional roles such as antioxidants and metal chelators as UV-light screens and signaling agents both above and below ground (Cooper *et al*: 1998) . Thus , results of the present study as well as those of previous workers suggest that, phenolics accumulate in plants as a response to various kinds of stresses and they play important roles in the resistance or tolerance of plants to various stresses.

Proline content in all tea varieties increased following insecticide /fungicide or heavy metal treatments. Proline is an important amino acid which has gained prominence over the years due to its phenomenal accumulation in plants subjected to different stressed conditions. Most of the early reports where proline has increased related to water stress.Proline being an osmoregulator may accumulate due to different stresses which ultimately create osmotic stress in the cell. Schat *et al.*(1997) reported that massive accumulation of proline occurs in leaves of *Silene vulgaris* in response to Cu, Cd and Zn . Increased accumulation proline due to heavy metal stress has also been reported by other workers (Tripathy and Tripathy 1999). Barman *et al.* (2002), and Panda and Khan,(2003), also reported that high concentration Zn and Cr induced accumulation of proline. Siddiqui and Ahmed, (2002) also reported increased accumulation of proline in varieties of *Triticum aestivum* following application of synthetic fungicides. In tea plants, Basak *et al* .(2001) reported increased accumulation of proline following Cu, Hg and Ni treatments while Chakraborty *et al.* (2002), also observed increased accumulation of proline following Cu stress . In a study involving heavy metal stress of okra, Chakraborty and Lahon (2007) reported increased accumulation of proline. However, in their study it was observed that the degree of enhancement varies with both varieties and the types of heavy metals. Different types of stresses which ultimately lead to osmotic stresses in the cells induce proline accumulation. At least 3 possible mechanisms has been proposed by various authors i.e. stimulation of enzymes of proline synthesis such as glutamic dehydrogenase, the inhibition of enzymes of proline catabolism and inhibition of protein synthesis. According to Yoshiba *et al.* (1997) synthesis of proline from L-glutamic acid via pyroline 5-carboxylate is mainly regulated by two enzymes pyrolline carboxylate synthese and pyrroline carboxylate reductase . Proline is metabolized to L-glutamate by two enzyme i.e. proline dehydrogenase and pyrroline carboxylate dehydrogenase and such metabolism of proline is inhibited when dehydration occurs. During dehydration the gene for pyrroline carboxylate synthase is strongly induced while the expression of the gene proline dehydrogenase is inhibited. Therefore, they suggested that the levels of proline are regulated in the transcriptional level of these two genes during dehydration and re-hydration.

Proteins, being major biochemical components of plants, influence of the heavy metals and pesticides on protein contents were investigated. Spraying with insecticide and fungicide led to an initial increase in protein content in some of the varieties. However, further application of the chemicals resulted in decrease in the protein content. Similarly , in case of heavy metals though the first treatment induced the accumulation of protein to a certain extent further treatment led to decline.Dewan and Dhinghra (2004), reported that Cd treatment in general did not affect the seed protein appreciably in two parent cultivars of pea and their hybrid. However, high doses of Cd decreased protein content in one of the cultivar HFP4. Reports of induction of phytochelatin synthesis by Cd are numerous (Raineri *et al.*2005; Mishra *et al* . 2006). Giannaza *et al.* (2007) reported inhibition of storage protein catabolism and plant protein anabolism in *Lipidum sativum* plantlets exposed to Cd stress. Besides, they also reported that the appearance of two proteins may be related to cellular stress and another two which may be involved in embryogenesis. Siddiqui *et al.* (2002) reported that , application of systemic fungicide to susceptible and resistant variety of wheat caused a significant decrease in total protein content which was maximum in susceptible cultivar.

Chlorophyll plays important role in plant metabolism as it controls photosynthetic activity of a plant and thereby is one of the determinants of the productivity of the plant. In case of tea, chlorophylls also contribute to the "blackness" of made tea that is considered to be one of the important criteria in the commercial evaluation of tea (Liyanage and Penyasiri,1993). Differences in chlorophyll content among different tea clones have been reported previously. In a study by Desilva and Shivapalan (1982), it was reported that, chlorophyll content of different Srilankan clones varied and varieties were observed with climatic changes. Increase in chlorophyll content was correlated with rainfall by Wikramasingha and Perara (1996). The present study was undertaken to determine the effect of the various anthropogenic stresses on chlorophyll content of tea leaves. Results showed that, while low concentration of Cu increased chlorophyll content to certain extent, higher concentration of Cu inhibited chlorophyll. The effect of Cd was more pronounced as even lower concentration of Cd inhibited chlorophyll accumulation. Chlorophyll content was also observed to decrease

in tea cultivars following insecticide / fungicide spray. In a similar study, Upadhyay and Pandey (2004), reported that treatment with a pesticide showed decrease in chlorophyll content of seedlings. There are several reports on the effect of Cd influencing chlorophyll content of plants. Keshan and Mukherjee (1992) reported that chlorophyll content of *Vigna radiata* leaves declined at all concentrations of Cd and the reduction in chlorophyll b was higher than that of chlorophyll a. However, in the present study, the reduction in the chlorophyll b was greater than chlorophyll a leading to higher a/b ratio. The results are in confirmity with those of Bhattercharjee and Mukkherjee (2003) , who also reported that , $CdCl_2$ treatment of *Amaranthus lividus* seedlings induce slightly higher chlorophyll a/b ratio and the loss of chlorophyll b was greater than that of chlorophyll a . Other heavy metals like Ni, Mn and Zn have also been reported to decrease chlorophyll content of plants (Prathiva and Rathore ,2002 ; Sinha *et al.* 2002; Singh *et al.* 2005 ; Hou *et al* .2007). The impairing of chlorophyll development by heavy metal may be due to interference with the synthesis of proteins which are structural component of chloroplasts. On the otherhand reduction in chlorophyll content might be due to stimulation of enzymes like chlorophyllase which degrade chlorophyll (Keshan and Mukherjee (1992); Kaur and Deshmukh , 1980) . Janave (1997) suggested that, chlorophyll degradation during senescence in cavandish bananas was a result of two types of catabolic pathways-chlorophyllase pathway and chlorophyll bleaching pathway. Variation in chlorophyll content due to a number of factors including variety, nature of leaf, increase in rainfall, shade , rainy weather, low elevation and different seasons has been previously reported (Bera *et al.* 1997). In the present study it was also observed that carotenoids showed an initial increase due to heavy metal treatment but, with further treatment or increasing concentration of heavy metal the carotenoid content decreased. However, spraying with insecticide/ fungicide enhanced carotenoid content to certain degree. In many of the previous studies it has been reported that , heavy metals decrease carotenoid content of leaves. Mathur *et al.*(2006) reported that Cd induce decline in carotenoid pigment in moth bean cultivars. However, carotenoids being antioxidants it might be possible that plants respond to stresses initially by enhancement of antioxidants which led to certain tolerance. The increase in carotenoid content in the study may be explained by the above. Chlorophyll

biosynthesis was inhibited by Cu stress in *Thalaspi ocrolucum* (Ouzounibou, 1992) and *Phaseolus vulgaris* (Gadallah, 1995). In general, it has been reported that chlorophyll accumulation is highly sensitive to heavy metal toxicity (Gupta and Chandra,1996).

Since carbohydrates form important biochemical constituents of any plants, in the present study, the influence of the various stresses on total soluble sugar and reducing sugar were determined. Total soluble sugars showed an increase in initial treatment but with increase in concentration the total sugar decreased. Reducing sugars also showed an increased accumulation at lower concentration which however declined with higher concentrations. Spraying with insecticides / fungicides led to an decrease in total and reducing sugar content. 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 concentrations of arsenate induce a decrease in total soluble sugar in *Vigna radiata* seedlings (Debnath and Srivastava ,2003).

Phenols being the most abundant and one of the important biochemical constituent of tea leaves enzymes involved in their metabolism i.e. phenylalanine amminia lyase (PAL) , polyphenol oxidase (PPO)and peroxidase (PO) also play important role in tea plant's metabolism. Hence, in the present study activities of these 3 enzymes under controlled and stressed conditions were analysed. Results revealed that the activity of the constitutive enzymes in different tea varieties also varied. PAL activity increased in 100and 500 µg /ml treatments of Cu and Cd but at 1000 µg /ml there was a decline. A decrease in PAL activity was noticed following spray with both fungicide/ insecticide. Matsumato *et al.* (1994) reported that Japaneese green tea cultivars belonging to variety 'sinensis' could be divided into 3 groups on the basis of their PAL c DNA cloning. They confirmed the exsistance of many kinds of PAL gene, the expression of which varied depending on the varieties.An elevation in the level of activity of PAL has been frequently demonstrated to be one of the earliest responses of plant to biotic stress (Southerton and Deverall ,1990; Chakraborty *et al* . 1993) or to other environmental stresses (Eckey-Kaltenbach *et al.* 1997). Yan *et al.* (2008) reported that Ni toxicity induced PAL activity which had a positive correlation to Ni concentrations.Gracia *et al.*(2001) determined the effect of application of different concentration of carbendazim on the metabolism of the phenolic compounds in tobacco

plants not suffering biotic or abiotic damage. Their results indicated that, the application of carbendazim increased PAL activity as well as accumulation of phenolics. PAL plays a key role in linking primary metabolism to phenyl propanoid metabolism by converting L-phenylalanine to trans cinnamic acid. This reaction provides an entry point for the biosynthesis of large number of defense related functional products and PAL is considered a part of defense mechanism. The regulation of PAL activity in plants is made more complex in many species due to the exixtance of multiple PAL encoding genes some of which are expressed only in specific tissues or only under certain environmental conditions (Macdonald and D'cunda , 2007) . It has also been shown previously that PAL is generally stimulated in plant tissues exposed to heavy metal stresses (Santiago *et al* .2002). PPO activity was also found to be enhanced in the lower concentration of heavy metals and also due to spraying of in secticide/fungicide. Heighest concentration of heavy metals reduced PPO activity . Increased activity of PPO was demonstrated in cucumber leaf caused by some foliar pathogen or by phosphate application (Avdinshko *et al*, 1993). More over, polyphenol oxidase could be induced by abiotic inducer such as jasmonic acid (Constabel and Ryan, 1998). Increased accumulation of polyphenol oxidase induced by Cd was reported in *Arabidopsis thaliana* by Saffar *et al.* (2009). In the present study the isozyme patterns of PPO were also found to be affected by various heavy metal treatments as also treatment with insecticide / fungicide . However, changes in isozymes could not be correlated with overall change in activity.

Peroxidase is one of the most worked out enzymes either in connection with its role in defense or as an antioxidative enzymes. Peroxidase is a metallo enzyme containing porphyrin bound iron. The enzyme acts on a wide range of substrate including phenols, aromatic amines, amino acids and inorganic compounds. These are ubiquitous to plants and are characterized by a large number of isozymes. The activity of peroxidase is markedly influenced by various naturally occurring synthetic substances, growth regulators and environmental factors. It was observed in the present investigation that peroxidase activity was greatly increased by anthropogenic stresses. Decline in activity was observed at high concentration of Cu or Cd. Chen *et al.*(2002) reported that, treatment of radish seedling with $CuSO_4$ solution increased peroxidase

activity which was concentration dependent. Activities of both anionic and cationic peroxidases were found to be enhanced but differed in the time of enhancement. Mourato *et a.l* (2009) also reported that peroxidase and polyphenol oxidase activity increased in *Lupinus luteus* plants grown in hydroponic solution for 15 days under different Cu concentrations. Cd was also reported to enhance activity of peroxidase in *Arabidopsis thaliana* (Saffar *et al* .2009).

Isozyme analysis of the peroxidase in the present study revealed changes in isozyme pattern induced by chemical treatments. The existence of multiple molecular form of peroxidase in tea have been reported by previous workers (Takeo and Kato 1971; Gunashekhar *et al.* 1996) . Yan *et al* . (2008) detected atleast 5 peroxidase isoforms in *Jatropa curcas* cotyledons with different patterns. The staining intensity of 3 isoform bands were stimulated with increasing Ni concentrations. In the present study also staining intensity of certain bands increased in some treatments while in others there was an inhibition. Peroxidases are commonly found as several isozymes in plants because of its multiple functions. The pattern of expression of isoforms varies in the different tissues of healthy plants and is regulated at different time and places by various kinds of biotic and abiotic stress inducers (Passardi *et al.* 2005) .

Catechins are phenol flavonoid flavour components of tea and as such it was considered worthwhile to analyse the changes in catechins in tea leaves following the different stresses. HPLC analysis revealed that, some of the isoforms of catechins was suppressesd by the stresses but at the lower concentration , the pattern was similar to that of control. Some of the peaks showed quantitative differences in the treatments. Accumulations of various heavy metals in different tissues in organs of plants which are taken up by the plant are the important consideration for human beings. Dietery exposure to heavy metals mainly Cd, Pb, Zn and Cu has been identified as a risk to human through consumption of vegetable crops (Kachenko and Singh,2006). Accumulation of heavy metals in tea leaves is particular by important because these heavy metals are taken by the plant and translocated to the leaves and which may remain in the leaves used as beverage. Hence, in the present study the actual content of heavy metal in leaves of two varieties of tea plants subjected to heavy metal stress were determined. It was observed that significant accumulation of both Cu and Cd occurred

in the leaves though, Cu accumulation was greater than Cd leaves of untreated plants were also found to contain certain amount of Cd and Cu specially more of Cu.

In conclusion, it can be stated that, either heavy metal treatment or spraying with insecticide/ fungicide induced definite metabolic changes in the tea plants. While the lower concentration of heavy metals or initial application of insecticide/ fungicide led to an initial increase in the metabolic products, higher concentration or prolonged treatments led to decline in metabolism. The plant's ability to withstand the stress was evident by enhanced antioxidative and other such responses of the different tea varieties.It is clear that tea plant which is a perennial develops mechanisms to overcome various environmental stresses including anthropogenic stresses. This would finally be regulated by the genetic make-up of the individual variety. Several genes are known to respond to different stresses commonly encountered in agriculture. The genetic manipulation of these genes holds considerable promise as a first step towards increasing stress tolerance (Hare *et al* .1996).

Summary

1. A review of literature relating to investigation on changes in different biochemical parameters as a result of commonly encountered environmental stresses in plants i.e. heavy metals, insecticide and fungicide application has been presented.

2. The materials and methods used in this study have been discussed in detail.

3. Tea plants, seedlings, young shoots and full grown tea bushes of different varieties i.e. TV-27, TV-23, TV-26, TV-30, TV-29, TV-28, TV-22, TV-18, HV-39 and T-78 were subjected to different types of anthropogenic stresses i.e. heavy metal, spraying with fungicide and insectide.The two heavy metals cadmium nitrate 4-hydrate [Cd $(NO_3)_2$, 4 H_2O], Copper sulphate 5 hydrate [$CuSO_4$, 5 H_20] used in the present study were applied in the form of their respective salts at 100, 500 and 1000 µg /ml concentration. Among the fungicide hexaconazole and insecticide acephate were selected for the study were applied at 0.1% and 1:400 concentrations respectively.

4. The result of quantification of total phenol as well as O-dihydroxy phenol content of leaves immersed at different contentration of heavy metal solutions revealed significant differences among the varieties. An increase in phenol content was evident only at lower concentration but decrease at the higher concentration of the tested metals. When seedlings were treated with the heavy metal solutions phenol content was increased after each application though reduction was noted at the highest concentration after 2^{nd} application. An increase in phenol content was evident following 1^{st} and 2^{nd} spray with both fungicide and insecticide application.

5. An overall increased accumulation of proline content was noted in tea leaves subjected to heavy metal as well as fungicide / insecticide treatment.

6. An increase in protein content was evident due to heavy metal treatment, although at higher concentration accumulation was decreased. Protein content was overall reduced following the application of insecticide /fungicide to the seedlings and bushes.

7. There was a little alteration in protein band pattern revealed in SDS gel electrophoresis after treatment with heavy metal solutions or insecticide / fungicide treatment. In lower concentration of heavy metal new band was expressed, wherease, at highest concentration band disappearance was noted.

8. Chlorophyll content of the leaves showed an overall decline in all treatments. Carotenoid contents also decreased at the higher concentrations of heavy metals. Spraying with insecticide /fungicide in seedlings and in mature bushes revealed increased carotenoid content after each application.

9. Treatment with the heavy metal solutions induced accumulation of total or reducing sugar content only at lower concentration but at the higher concentration or prolonged treatement sugar content declined. In case of insecticide / fungicide treatment total sugar as well as reducing sugar content was decreased.

10. Phenyl alanine ammonia lyase enzyme activity showed general increment after heavy metal treatment both *in vitro* and *in vivo*. However, enzyme activity was declined significantly after spraying with insecticide and fungicide in relation to control.

11. Peroxidase activity increased at the lower concentration of the heavy metals , whereas activity decreased with increase in concentration. A significant increase in peroxidase activity was noticed after spraying with insecticide and fungicide application. The accumulation was much higher following 2^{nd} spray .

12. Polyphenol oxidase showed greater activity at the lower concentration of the tested metals but activity declined at the higher concentration or prolonged application of the heavy metal solutions. There was an increase in activity of the enzyme in the leaves following spraying with both insecticide and fungicide.

13. Different peroxidase isozyme pattern was evident in most of the heavy metal treatments. The disappaerence or expressions of new bands were noticed after heavy metal treatments. Significant changes of band pattern were observed in tea leaves treated with the insecticide/fungicide. Expression of new band was noted in the treated leaves.

14. The polyphenol oxidase isozyme pattern of leaves treated with heavy metals or insecticide/ fungicide revealed little alteration in band pattern.

15. HPLC analysis of catechins revealed changes in the isomer pattern after treatment with the two heavy metals. Significant variation was noted in peak heights and number in the different varieties. Chemical spraying led to a decrease in number and the height of isomers.

16. Heavy metal accumulation in tea leaves was determined after treatment with heavy metal solutions. Traces of heavy metals were observed in untreated control also. Accumulation of Cu was much higher than Cd after every application of the said metals.

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Corrigendum A

Page No.8. **Gorlach *et al*** should be **Gorlach and Gambus**.

Page No. 10 (Not 2 as given by examiner) **Baruah and Bharatnath** should be **Baruah and Nath** and page 202 **Bharathnath** should be **Nath B** .

Page 204 **Chung** should be **Chugh**.

Page No. 16. **Toppi *et al*** should be **Toppi and Gabrielli**

Page No.17.**Cher** should be **Cher and Kao**.

Page No. 21.**Pratibha and Rathore** should be **Rathore** and page no. 215 **Pratibha Rathore VS** . should be **Rathore VSP**.

Rearrangement of references of V series - Page No. 221 & 222 as follows

Upadhay H , Panda SK.(2004) is followed by **Wagner GJ.(1993).** It should be as follows-

Upadhyay H, Panda SK **(2004) , Van Assche F.,** Clijsters H **(1988), Vangronsveld J,** Clijsters H **(1994), Viard B,** Pihan F, Promeyrat S and Pihan JC**(2004), Videa JRP**, Rosa G dela,Gonalez,Torresdey JLG **(2004), Vitoria AP,** Da Cunha M and Azevedo MA **(2005) Wagner GJ (1993)**

This is to be followed by the references of W, X, Y and Z in given order

Corrigendum B

Page no. 1.and 217. **Schlicker** should be **Schlickler**

Page no. 3 – Reference of **Weast RC (1984)** – which is given in **Page 221**, will be shifted to **Pg.222** due to rearrangement of V series; **pp will be 2386**

Page No. 201. Alla MM, Hassan NM. (2005). ..... **lives** should be ..... **lines**.

Page. No.210 **Mahadavan** A , Sridhar R.(1982). Methods in physiological plant pathology, 2nd edition, Sivakami publication, India should be - **Mahadevan** A , Sridhar R.(1982). *Methods in physiological plant pathology*, 2nd edition, Sivakami publication, India **pp 316**

Pg.No. 216 Salt DE..... *Biotechnology*. Should be *Biotechnology*

Page No. 216 Sambrook..... Molecular cloning- a laboratory manual ..... should be *Molecular cloning- a laboratory manual*.....

Gargi Saha.

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