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Assessment of oxidative stress and genotoxicity in *Allium cepa* exposed to soils from rice fields

Rajneet Kour Soodan[1], Jatinder Kaur Katnoria[1], Renu Bhardwaj[1], Yogesh B. Pakade[2] and Avinash Nagpal[1]*

[1]*Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India*
[2]*CSIR-Institute of Himalayan Bioresource Technology (IHBT), Palampur*
[3]*Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India*

ABSTRACT

The present study evaluates the genotoxic potential of different soil samples collected from rice fields employing Allium cepa root chromosomal aberration assay using in situ and root dip modes of treatment. The study also involves assessment of oxidative stress by analyzing responses of certain antioxidative enzymes viz. catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione-S-transferase (GST), superoxide dismutase (SOD), guaicol peroxidase (POD) and dehydroascorbate reductase (DHAR) in A.cepa bulbs exposed to soils. Besides these, estimation of various physico-chemical parameters including metals was also carried out. All the parameters were shown to vary among different soil samples studied. Exposure of onion bulbs to soil samples resulted in induction of different types chromosomal aberrations (physiological and clastogenic). The activities of different antioxidative enzymes in A. cepa bulbs exposed to different soil samples was found to be higher for CAT, APX, GST, DHAR, SOD and lower for POD and GR than those observed for control bulbs.

Key words: Agricultural soils; Metals; *Allium cepa*; Antioxidative enzymes; Genotoxicity

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INTRODUCTION

Soil has a complex nature that depends on its physical, chemical and biological composition, representing a dynamic interface between atmosphere, hydrosphere and biosphere. In densely populated regions, soil may be contaminated as a result of discharges from industries, by domestic sewage, from metallurgical activities, by vehicular emissions, and application of fertilizers and pesticides. Once contaminants enter the soil, they may stay there for a long period or may slowly percolate to other compartments *viz.*, ground water as leachates, surface water bodies as run offs, and the atmosphere as dust particles. Metals have attained great attention in this respect as some may accumulate in agricultural soils and via consumption of contaminated food crops can enter human beings [1]. For ecotoxicological assessments, plant bioassays have gained attention due to the fact that plants are readily available and easier to handle than animals [2]. Among different plant bioassays, the *A. cepa* root chromosomal aberration assay is routinely used to evaluate the genotoxic potential of chemicals in the environment [3]. Enzymes involved in detoxification, in particular antioxidative enzymes may serve as markers of environmental pollution [4]. It has been shown in several studies that some metals may cause oxidative stress by intervening with the activities of antioxidative enzymes [5-7]. Stress induced by some metals may lead to changes in the activities of SOD, GR, CAT, APX and DHAR [8].

The present study was planned to investigate the agricultural soils of Amritsar, Punjab (India) under rice cultivation for genotoxicity and antioxidative defense response in the *A. cepa* test system. The study also comprised estimation of various physico-chemical parameters *viz*., pH, alkalinity, soil texture, bulk density, and concentrations of nitrates, phosphates, potassium and metals *viz*. iron (Fe), manganese (Mn), zinc (Zn), nickel (Ni), copper (Cu), lead (Pb), chromium (Cr) and cadmium (Cd).

EXPERIMENTAL SECTION

1.1. Study Area

The study area of the present investigation is the district of Amritsar, located in the northwestern part of the Punjab (India), between $31^0$28'30" to $32^0$03'15" north latitude and $74^0$29'30" to $75^0$24'15" east longitude, with a population of 2,490,700 and 778 villages [9].

1.2. Collection of samples

The soil samples were collected from 8 agricultural fields under rice cultivation during September 2009 (Figure 1). The samples were collected from 4-5 sites of each agricultural field randomly by digging soil to depth of 15-20 cm (10×10×20 cm^3 approximately) and were pooled to make single sample of that site. All the samples were brought to the laboratory, dried at room temperature for 72 h, and ground to fine powder with the help of mortar and pestle. The samples were coded and 4-5 Kg of powdered soil per sample was packed in poly bags until further investigation.

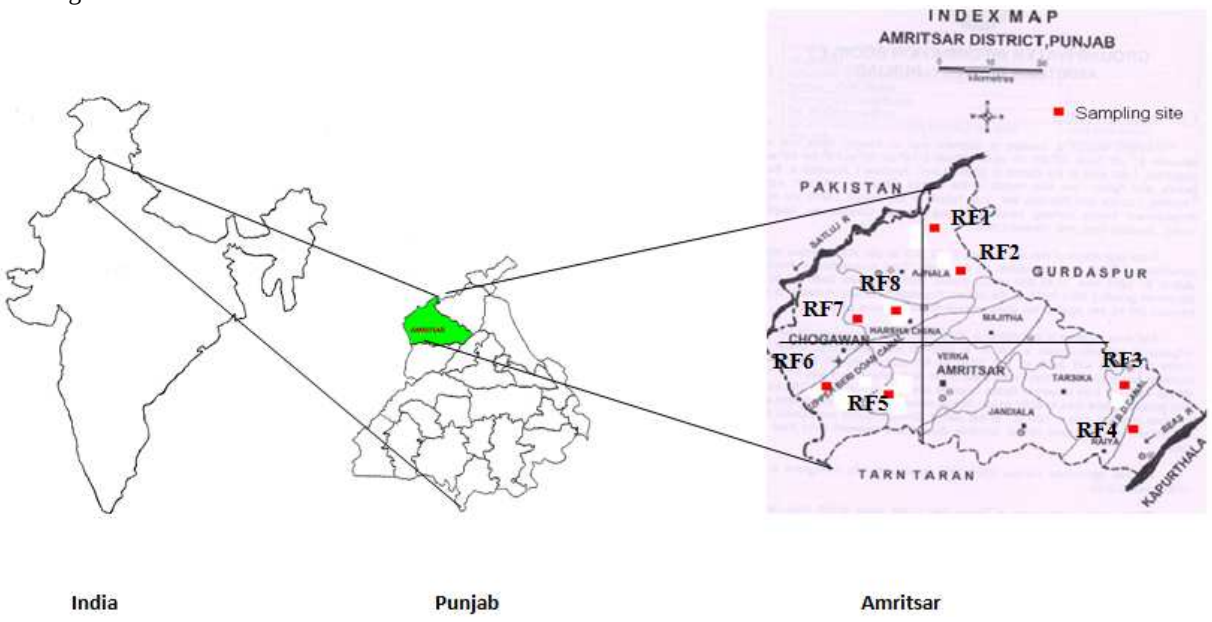


Fig.1. Location of different regions of study

Source: CGWB/NWR (S. K. Singh) Do.No.219/2008

1.3. Chemicals

The chemicals for physico-chemical analyses were obtained from Sisco Research Laboratories (Mumbai, India), Loba Chemie (Mumbai, India), and Spectrochem (Mumbai, India). The chemicals for genotoxicity study were obtained from Spectrochem, Thomas and Baker Chemicals (Mumbai, India), and Qualigen Fine Chemicals (Mumbai, India). For the enzyme analyses, substrates and reagents were obtained from Himedia (Mumbai, India). Bulbs of *A. cepa* were purchased from a local market.

1.4. Estimation of physico-chemical parameters

The physico-chemical properties *viz.*, pH, calcium, alkalinity, nitrates, phosphates, sodium and potassium were determined following standard protocols [10]. Soil texture classification, based on fractions of soil separates (sand, silt and clay) present in a soil, was done using sieving method. 20 g of soil sample was taken and passed through a series of sieves of decreasing pore size. The fractions were weighed individually. Soil texture was determined by

calculating percentage of each fraction. The soil extract was prepared by suspending soil in distilled water in ratio of 1:5 (w/v) and shaken on mechanical shaker for 12 h at room temperature. pH was measured by pH meter (Systronics; model µ pH system 361). The parameters like alkalinity and calcium were determined using titration method. Nitrates (NO_3^-) and phosphates (PO_4^{3-}) were estimated using a UV-Visible spectrophotometer (Systronics; model GS5701A). For estimation of nitrates soil extract was taken in a crucible, heated till dryness and cooled. Phenol disulphonic acid was added in left residue and the mixture was then diluted to 50 ml with double distilled water followed by the addition of ammonium hydroxide (30%) and kept undisturbed for 15 min for development of yellow color. The optical density was recorded by using spectrophotometer at 410 nm and nitrate content was calculated using standard calibration curve. For estimation of phosphates the ammonium molybdate and stannous chloride were added to the soil extract and kept undisturbed for blue color development which showed the presence of phosphates and the optical density was recorded at 625 nm using calibrated standard graph curve. The contents of sodium and potassium were estimated using flame photometer (ELICO; model CL 26D) and concentrations were estimated using calibration curves.

1.5. Atomic absorption spectrophotometric method for metal analysis

1 g soil sample was digested in glass digestion tube of 250 ml with 15 ml of nitric acid (HNO_3) at 140°C for 2 h in digestion chamber. The content was evaporated to dryness. The dried sample was treated with 3 ml of perchloric acid ($HClO_4$) for further oxidation at 240°C for 1h. After digestion, the content was cooled; filtered and final volume was made up to 50 ml with double distilled water [11]. Metals were analyzed using atomic absorption spectrophotometer (Shimadzu model AA 6300, Tokyo, Japan).

2.6. Estimation of genotoxic potential

2.6.1. A. cepa root chromosomal aberration assay

Two modes of treatment were employed, *viz., in situ* and root dip. *In situ* conditions were simulated by allowing the denuded onion bulbs to root directly in soil samples contained in pots for 24 - 48 h. Sand was used as negative control. For root dip treatment the soil extracts were prepared by suspending soil in distilled water in ratio of 1:2 (w/v) on shaker for 12 h and then filtered [12]. The filtered extract was considered as 100 % and different concentrations (20, 40, 60, 80 and 100%) of soil extract were made. The denuded onion bulbs were placed on Couplin jars containing distilled water for rooting. After 24 - 48 h, the emerged roots of about 0.5 - 1.0 cm length were treated for 3 h by placing them on treatment jars containing different concentrations of each soil extract. Distilled water was used as negative control.

2.6.2. Cytological investigations

After treatment, the bulbs were thoroughly washed, root tips were plucked and fixed in Farmer's fluid (glacial acetic acid : ethanol: 1:3) for 24 h and preserved in 70 % ethanol till further use. The fixed root tips were hydrolyzed in 1N HCl with intermittent heating for 1 min at 60ºC and then transferred to a watch glass containing mixture of 1 N HCl and aceto-orcein (1:9). The root tips in watch glass were heated intermittently for 3 - 5 min using spirit lamp, covered and kept aside undisturbed for 15-20 min [13]. Then the root tips were squashed in a drop of 45% glacial acetic acid. The slides were observed for different types of chromosomal aberrations. About 900-950 dividing cells from 9 root tips (~ 100 cells/root tip) were scored. Photomicrographs were taken with the help of a digital camera (Olympus-E520) fixed on microscope (Magnus- MLxi) that was connected to a computer in order to transport images.

2.7. Estimation of antioxidative enzymes

CAT, APX, GR, SOD, POD and DHAR were assayed in onion bulbs exposed to agricultural soil samples for 72 h. The denuded onion bulbs were placed on soil samples contained in pots which were kept in a seed germinator in the darkness at about 25^{o}C. The protocol of Fatima and Ahmad (2005) was followed. *A. cepa* bulbs after 72 h of treatment were cut into small pieces with the help of sharp knife and 1 g of sample was homogenized with the chilled sodium phosphate buffer (50 mM , pH 7.0) in a pre-chilled mortar and pestle. The homogenate thus obtained was centrifuged at 8,000 rpm for 20 min at 4 ^{o}C. The onion bulb extract was further used for determination of antioxidant enzymes and proteins as described by Sharma et al [14].

2.7.1. Protein quantification

Total protein content of treated as well as untreated *A. cepa* bulbs was quantified by following the Lowry's method [15] using bovine serum albumin as a standard. The total protein content was calculated as protein mg/g fresh weight of the sample.

***2.7.2. Ascorbate peroxidase assay* (APOX, EC 1.11.1.11)**
The activity of ascorbate peroxidase was determined spectrophotometrically according to the protocol described by Nakano and Asada [16]. The enzymatic reaction mixture of 3.0 ml contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 1.0 mM H_2O_2 and 100 µl of onion bulb extract. The H_2O_2 dependent oxidation of ascorbate was followed by monitoring the decrease in absorbance at 290 nm for 3 minutes at 25° C and enzyme activity was calculated using the extinction coefficient 2.8 mM^{-1} cm^{-1}. One unit of APOX activity is defined as the amount of enzyme that can oxidize 1 µmol of ascorbate per minute.

***2.7.3. Catalase assay* (CAT, EC 1.11.1.6)**
The activity of catalase was measured in terms of initial rate of H_2O_2 disappearance or liberation of O_2 according to the protocol of Aebi [17]. The enzymatic reaction mixture of 3.0 ml contained 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 100 µl onion bulb extract. The decrease in hydrogen peroxide was followed and catalase activity was estimated as decrease in optical density at 240 nm for 30 s at 25 ^oC as a result of H_2O_2 decomposition . The enzyme activity was calculated using an extinction coefficient 39.4 mM^{-1} cm^{-1} for H_2O_2.

***2.7.4. Dehydroascorbate reductase assay* (DHAR, EC 1.8.5.1)**
The enzymatic activity of dehydroascorbate reductase was measured according to the protocol of Dalton et al [18]. The enzymatic reaction mixture of 3.0 ml contained 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM dehydroascorbate, 0.1 mM EDTA, 2.5 mM reduced glutathione (GSH) and 100 µl of onion bulb extract. The DHAR activity was measured by following the increase in absorbance at 265 nm due to formation of ascorbate and glutathione disulphide as their final product at 265 nm using extinction coefficient of 14 mM^{-1} cm^{-1}.

***2.7.5. Glutathione reductase assay* (GR, EC 1.6.4.2)**
The enzymatic activity of glutathione reductase was measured using oxidized glutathione (GSSG) as a substrate and decrease in NADPH was recorded according to the protocol by Carlberg and Mannervik [19]. The enzymatic reaction mixture of 3.0 ml contained 50 mM potassium phosphate buffer (pH 7.6), 1 mM oxidized glutathione (GSSG), 0.5 mM EDTA, 0.1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 100 µl onion bulb extract. The absorbance of GR activity was measured spectrophotometerically as oxidation of NADPH at 340 nm and enzyme activity was calculated using extinction coefficient of 6.22 mM^{-1} cm^{-1}.

***2.7.6. Guaiacol peroxidase assay* (POD, EC 1.11.1.7)**
The enzymatic activity of guaiacol peroxidase was measured using the protocol of Sanchez et al [20]. with slight modifications. The enzymatic reaction mixture of 3.0 ml contained 50 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 12.3 mM H_2O_2 and 100 µl onion bulb extract. The absorbance of POD activity was measured at 436 nm, using an extinction coefficient of 26.6 mM^{-1} cm^{-1}. One unit of POD activity represents the amount of enzyme catalyzing the oxidation of 1 µmol of guaiacol in 1 min.

***2.7.7. Superoxide dismutase assay* (SOD, EC 1.15.1.1)**
The enzymatic activity of superoxide dismutase was measured as the ability of the onion bulb extract to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) dye which is reduced to superoxide radicals produced from autooxidation of hydroxylamine [21]. The enzymatic reaction mixture of 3.0 ml contained 50 mM sodium carbonate (pH 10.2), 24 µM NBT, 0.1 mM EDTA, 1 mM hydroxylamine, 0.03% (v/v) Triton X-100 and 70 µl onion bulb extract. The activity was calculated by recording increase in absorbance at 560 nm for 2 min. One unit of SOD activity was defined as the amount of enzyme required that caused 50% of NBT reduction.

2.8. Statistical analysis
All experiments were conducted in triplicate, and data were analyzed by one-way analysis of variance (ANOVA). Pearson correlation coefficients (r) were calculated and a correlation matrix was produced between metal content, enzyme activities and genotoxicity. Significance was considered at four levels, i.e. $p < 0.05$, $p < 0.02$, $p < 0.01$, and $p < 0.001$).

RESULTS AND DISCUSSION

3.1.Physico-chemical analyses
Physico-chemical analysis constituted estimation of parameters like pH, alkalinity, contents of calcium, nitrates, phosphates, sodium, potassium and soil texture are summarized in Table 1.The pH of all agricultural soil samples

was found to be basic in nature and ranged from 7.4 (RF4) to 8.1 (RF8). pH is an important parameter which measures hydrogen ion concentration and depends largely on the relative amount of the absorbed hydrogen and hydroxyl ions. It indicates the chemical composition and acidic or alkaline nature of soil. Moreover availability of nutrients is also directly influenced by pH of the soil. Various anthropogenic activities result in change in pH of soil and also enhance toxicity of soil. In this study, the pH of all the agricultural soil samples was found to be basic in nature and the results were similar to previous report by [22]. who reported that pH of soil samples collected from sides of Al- Khums city ranged from 8.1 - 8.6. Similarly some other studies have also indicated basic nature of agricultural soils [23]. Alkalinity of soil measures the amount of carbonates, bicarbonates and hydroxides in soil and is important in determining the fertility of soil and its impact on plant growth. In the present study, the alkalinity of different soil samples. In the present study, alkalinity of different soil samples ranged from 5.3 meq/kg (RF4) to 33.0 meq/kg (RF2) and was similar to observations reported by earlier studies [23]. Calcium is an essential part of plant cell wall structure which regulates the transport and retention of other elements in the plants. It controls the effect of alkali salts and organic acids within the plants. In present study, the calcium content of different soil samples ranged from 48.1 g/kg (RF1) to 101.5 g/kg (RF6). Doi and Ranamukhaaracchchi [24] estimated the content of calcium of soil under paddy growth from different villages of Thailand. The study revealed the calcium content to range from 1.47 to 5.44 mg/g in all the samples studied. Nitrogen is one of the essential nutrients for healthy plant growth. It acts as an important component of different proteins, enzymes and metabolic processes of growing plants. It is available in the form of ammonia and nitrate ions to the plants. The content of nitrates was found to range from 0.30 (RF1) – 0.62 g/kg (RF3) which is very low as compared to other reports. Yao et al [25]. studied the influence of sewage irrigation on agricultural soils of China and found the mean content of nitrates to be 653.18 mg/kg and 514. 17 mg/kg for waste water and regular water irrigated fields respectively. Phosphorous is the second most important macronutrient after N and is essential for the plant growth. It plays an important role for the development of healthy roots and fruits. It provides disease resistance and is available to the plants in the form of phosphate ions. In the present study, phosphate content of different soil samples ranged from 0.54 mg/g (RF2) to 2.13 mg/g (RF8). Ashraf et al [26] also estimated the content of phosphates in soils collected from Yusmarg hill resort, Kashmir and found it to range from 12 µg/g to 36µg/g in different soil samples. They are responsible for the plant's ability to resist diseases and to survive in stress conditions. Sodium is involved in regeneration of phosphoenolpyruvate (an anion with high energy phosphate bond involved in biosynthesis of various aromatic compounds) in Crassulacean Acid Metabolism (CAM). It helps in cation-anion exchange processes in soils. In the present study, sample RF8 showed minimum content (38 mg/kg) of sodium, where as maximum content (135 mg/kg) was found in soil sample RF3. Ashraf et al [26] also found the content of sodium to range from 4.5 mg/100g - 11.7 mg/100g in soils of Kashmir, India. Potassium helps in the process of photosynthesis, improves plant growth and provides resistance against drought and crop diseases. The content (mg/kg) of potassium observed in the present study was found to range from 8mg/kg (RF5) to 103 mg/kg (RF5) and was less as compared to earlier reports [26]. Ashraf et al [26] reported the content of potassium in soils of Kashmir, India to range from 5.0 mg/100g - 9.35 mg 100g. Soil texture determines the relative proportion of different sized particles (sand, silt and clay) present in soils. In the present study, the analysis of textural composition of different soil samples revealed that content of sand in all soil samples was highest followed by clay and content of silt was very low (< 2%). The content of sand, silt and clay particles ranged from 56.8 – 65.7%, 0.1 - 2.0% and 30.1 – 41.5 % respectively in rice cultivated agricultural fields. Ashraf et al [26] estimated physico-chemical characteristics of the grassland soils of Yusmarg Hill resort (Kashmir, India) and found that major proportion comprised the sand fraction.

3.2. Metal content

Metals are ubiquitous in the environment and are of considerable concern. Although some are essential for normal plant growth and are constituent component of proteins including enzymes but elevation in their concentration can lead to interactions at cellular and molecular levels resulting in various toxicity symptoms. Some metals also stimulate the inhibition of plant growth, formation of free radicals and reactive oxygen species causing oxidative stress. Studies concerning geochemical baseline data on background values of different metals in Indian soils are limited [27]. In the present study metal concentrations showed significant variations between different agricultural soil samples of Amritsar. The mean concentration (mg/kg) of metals ranged from 2.4 (RF1) to 13.1 (RF6) for chromium, 14 (RF3) to 28.1 (RF8) for copper, 15.8 (RF1) to 34.5 (RF7) for nickel, 53.4 (RF1) to 82.2 (RF2) for zinc, 208 (RF5) to 458 (RF7) for manganese, 7 (RF5) to 17.3 (RF2) for lead and 14.8 (RF3) to 20.5 g/kg (RF7) for iron (Table 2). Cadmium was not detectable for most of the soil samples except RF3 (0.7 mg/kg) and RF8 (4.6 mg/kg).The order of metals on the basis of their content in different soil samples was found to be Fe > Mn > Zn > Ni > Cu > Pb > Cr > Cd. Our results are in line with study by Jalali and Hemati [28] who determined content of Cd, Cu, Fe, Mn, Ni, Pb and Zn in agricultural top soils of Isfahan province in central Iran and reported that Fe (1240.4

mg/kg) was the most abundant metal followed by Mn (95.7 mg/kg), Pb (51.6 mg/kg), Zn (23.8 mg/kg), Ni (13.4 mg kg^{-1}), Cu (7.0 mg/kg) and Cd (2.8 mg/kg). Concentrations of Cd and Zn however were found to be higher when compared with mean metal limits recommended by the UK Interdepartmental Committee for Restoration of Contaminated Land [29]. for Cd (1 mg/kg) and Zn (25 mg/kg) in soil used for agriculture and recreation. On the contrary, this study revealed that concentration of Pb (50 mg/kg) was within the limits given by ICRCL. Agricultural soils of Amritsar showed contamination of soils by one or the other metal when compared to soils of other places of India like Karnataka [30] and Delhi [31].

Table 1. Physiochemical parameters of agricultural soils of Amritsar

Physiochemical parameters N=3										
Sample code	**pH**	**Alkalinity (meq/kg)**	**Calcium (g/kg)**	**Nitrate (g/kg)**	**Phosphate (mg/kg)**	**Sodium (mg/kg)**	**Potassium (mg /kg)**	**Soil texture (%)**		
								Sand	**Silt**	**Clay**
RF1	7.6±0.00	31.6±0.33	48.1±0.00	0.30±0.02	1.27±0.03	65±0.00	76±0.00	59.8	0.1	30.1
RF2	8.0±0.00	33.0±0.58	80.1±0.00	0.49±0.06	0.54±0.03	81±0.00	73±0.00	61.0	2.0	36.9
RF3	7.8±0.00	15.3±0.00	80.1±0.00	0.62±0.11	1.00±0.02	135±0.00	103±0.00	56.8	1.7	41.5
RF4	7.4±0.00	5.3±.033	88.1±0.00	0.51±0.11	1.07±0.02	91±0.00	30±0.00	65.7	1.5	32.8
RF5	7.6±0.00	12.6±0.08	69.4±2.67	0.51±0.03	1.37±0.05	85±0.00	8±0.00	60.0	1.6	38.4
RF6	7.7±0.00	7.0±0.05	101.5±2.69	0.51±0.04	1.30±0.02	68±0.00	46±0.00	60.8	0.4	38.8
RF7	7.7±0.00	14.6±0.05	50.7±2.67	0.44±0.02	1.44±0.02	55±0.00	53±0.00	61.2	0.5	38.3
RF8	8.1±0.00	12.3±0.03	80.1±0.00	0.50±0.18	2.13±0.00	38±0.00	52±0.00	63.7	1.4	34.9

Table 2. Contents of some metals (mg kg^{-1}) of agricultural soils of Amritsar (Mean ± S.D, N=3)

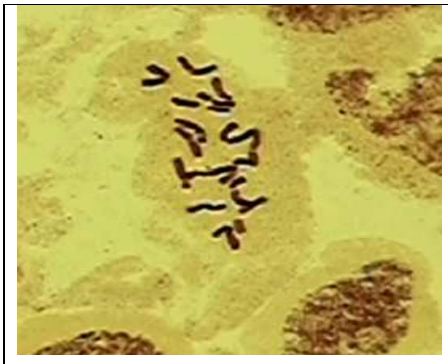
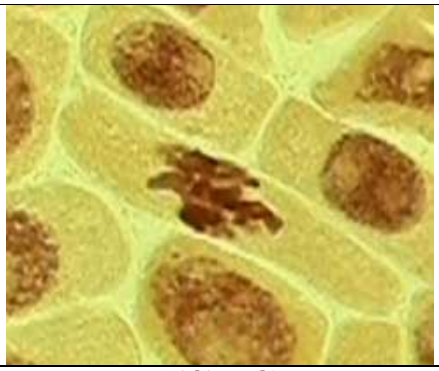
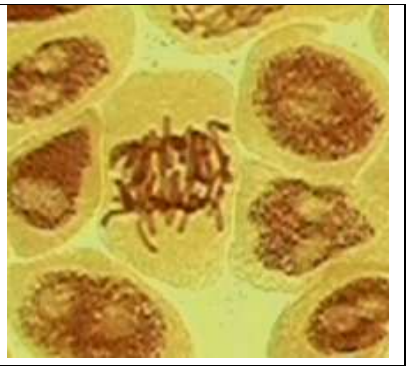
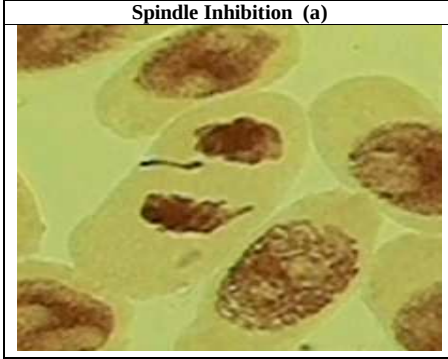
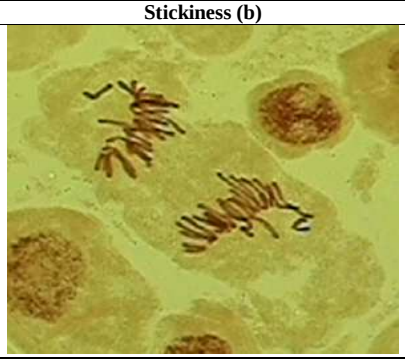
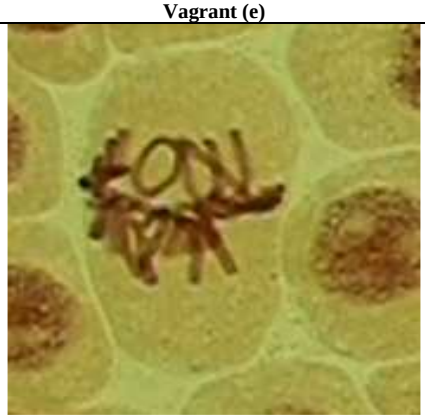
Sample code	**Chromium**	**Copper**	**Nickel**	**Zinc**	**Manganese**	**Lead**	**Iron**	**Cadmium**
RF1	2.4 ± 1.1	16.3 ± 0.3	15.8 ± 0.2	53.4± 0.2	329.7± 2.6	12.5± 1.3	17238.7 ± 61.4	-
RF2	8.5 ± 0.7	23.1± 0.3	24.8 ± 0.5	**82.2 ± 0.2**	359.1± 1.7	**17.3± 1.0**	20012.3 ± 56.1	-
RF3	8.2 ± 1.1	14.0 ± 0.2	18.0 ± 0.6	56.6 ± 0.2	251.2± 2.1	8.5± 0.7	14804.7± 59.5	0.7 ± 0.1
RF4	11.0 ± 1.1	24.9 ± 0.3	25.5 ± 0.1	74.7 ± 0.2	356.3± 1.7	11.4± 0.4	17824.0 ± 157.2	-
RF5	4.2 ± 1.5	14.8 ± 0.1	18.9 ± 0.5	55.8 ± 0.1	208.0± 1.4	7.0± 1.4	15677.9± 143.4	-
RF6	**13.1 ± 0.8**	24.1 ± 0.1	29.1 ± 0.4	70.1± 0.3	397.6± 3.0	14.2± 1.0	18358.0 ± 207.7	-
RF7	10.6 ± 1.2	28.1 ± 0.1	**34.5 ± 0.2**	76.8± 0.2	**458.0± 2.0**	12.7± 0.4	**20536.0± 44.1**	-
RF8	9.9 ± 0.7	**28.4± 0.4**	32.6± 0.40	68.7± 0.1	412.5± 2.1	11.8± 0.7	1999.5± 59.7	4.6 ± 0.1

All the data were significantly different at p≤0.05

3.3. Genotoxic potential

A. cepa root chromosomal aberration assay was used to estimate genotoxicity of 8 soil samples collected from agricultural fields of Amritsar under rice cultivation. Two modes of treatment *viz, in situ* and root dip were used to evaluate genotoxicity in terms of chromosomal aberrations in root tip cells of *A. cepa*. Both the treatments resulted in appearance of various types of physiological (c-mitosis, delayed anaphases, stickiness laggards, vagrants) as well as clastogenic (chromosomal breaks, chromatin bridge and ring chromosomes) aberrations were observed during both the modes of treatments. Fig.ure 2 represents the photomicrographs of representative physiological (a-e) and clastogenic (f-i) aberrations in *A. cepa* root tip cells treated with soil samples. Some aberrations which could not be included in any of above category were counted as abnormal metaphase (such as asteroid structures and deviation of alignment of chromosomes at metaphase) and abnormal anaphase (asteroid structures and deviation of chromosomes from the poles at anaphase). In *in situ treatment* frequencies of chromosomal aberrations induced by different soil samples were higher than negative control (sand) which was 4.14%. Among physiological aberrations, percentage of delayed anaphases was highest where as chromatin bridges dominated clastogenic aberrations. The percentage of chromosomal aberrations ranged from 7.71 (RF2) to 23.79% (RF5) (Figure 3). In *Root dip* mode of treatment, the squash preparations of root tips of control *A. cepa* bulbs showed 3.46% of cells with chromosomal aberrations. Among different soil samples, the sample RF6 showed maximum (25.95 %) percentage of aberrations while sample RF8 showed minimum (12.53%). Different types of aberrations observed in root tip cells during root dip mode of treatment with different agricultural soil sample extracts are given in Table 3.

Figure .2. Representative photomicrographs showing physiological (a-e) and clastogenic (f-i) aberrations in *Allium cepa* root tip cells treated with soil samples

		
Spindle Inhibition (a)	**Stickiness (b)**	**Delayed anaphase (c)**
		
Laggard (d)	**Vagrant (e)**	**Single chromatin bridge (f)**
		
Double chromatin bridge (g)	**Ring chromosome (h)**	**Chromosomal break (i)**

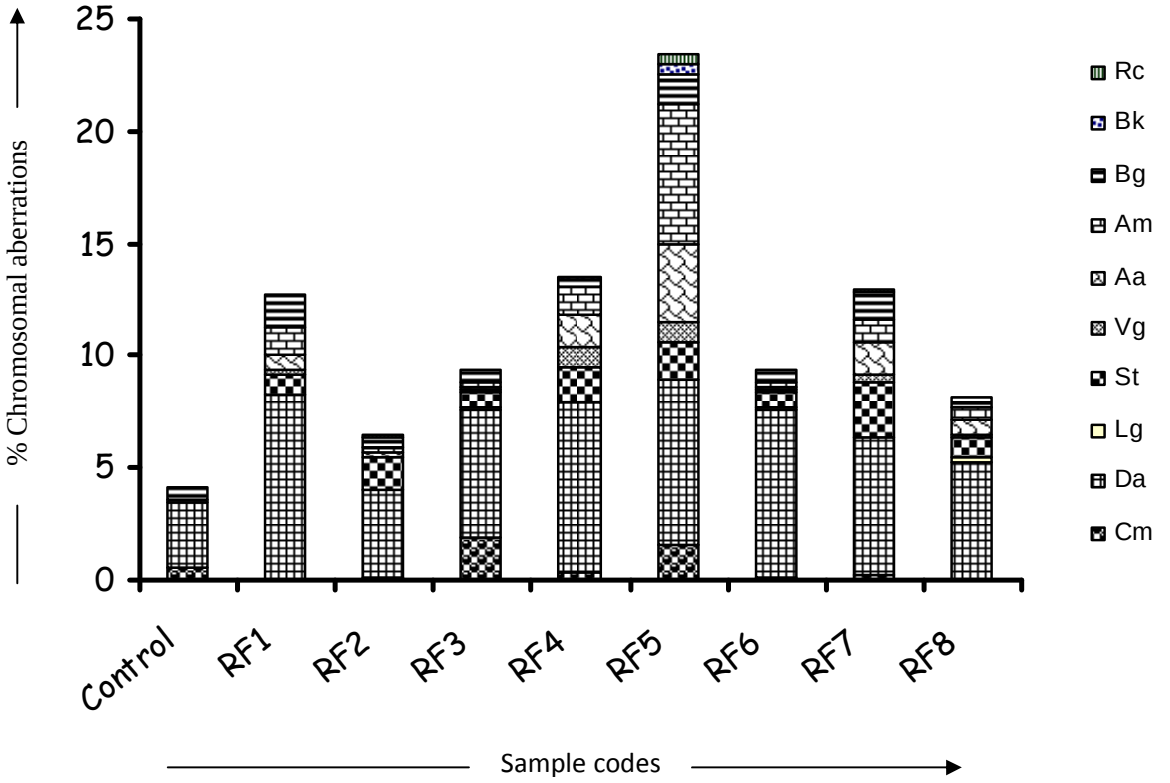


Fig.3 . Spectrum of different types of chromosomal aberrations induced in root tip cells of *Allium cepa* after treatment (*in* situ) with different agricultural soil samples under rice cultivation

Cm-C-mitosis; Da- Delayed anaphase; Lg- Laggard/s; St- Stickiness; Vg- Vagrant/s; Aa- Abnormal anaphases/s; Am- Abnormal metaphases; Bg- Chromatin Bridge/s; Bk- Chromosomal Break/s; Rc- Ring chromosome/s

All the data were significantly different at $p \leq 0.05$

The order of different samples with respect to percent aberrant cells observed at maximum concentration of soil extract tested from minimum to maximum was: RF7 (12.51%) < RF2 (16.17%) < RF6 (16.88) < RF1 (19.26) < RF3 (20.26%) < RF8 (22.70%) < RF4 (24.07%) < RF5 (25.95%). All the soil samples collected showed increase in chromosomal aberrations with increase in concentration of soil extract. Samples RF4, RF5 and RF8 had paper industry, sugar mill industry and distillery in their vicinity. High genotoxicity in terms of chromosomal abnormalities observed in the present study could be due to the contamination caused by use of agrochemicals and various industries present in the vicinity of agricultural fields from where the soil samples were collected. Chromosomal aberrations are considered as end result of genotoxic effects of various physical and chemical agents and are also estimates of exposure of various organisms to different physical and chemical agents that impair human health to which various organisms are exposed [1]. The present results on genotoxicity of soil samples are in conformity with some other earlier studies showing genotoxic potential of soil from different parts of the world [32, 33]. Masood and Malik [32]. reported the cytotoxic and genotoxic potential of soil from various toxic metal contaminated agricultural fields in the vicinity of industrial area of Jajmau, Kanpur (India) in terms of chromosomal aberrations which included c-mitosis, anaphase bridges, laggards, stickiness, broken and unequal distribution of chromosomes. Souza et al [33] investigated the clastogenic/aneugenic potential of land farming soils from a petroleum refinery before and after addition of sugar vinasse using the *A. cepa* root chromosomal aberration assay. The study revealed that sugar vinasse potentiates the clastogenicity of land farming soil from petroleum refineries. Main types of mitotic and chromosomal abnormalities observed in *A. cepa* included anaphase with chromosome loss and micronuclei, polyploidy and chromosomal adherence, multipolar anaphases, chromosomal breaks and bridges.

Table 3. Genotoxic potential of different agricultural soil samples of Amritsar in *Allium cepa* root chromosomal aberration assay (Root dip treatment) studies

Sample code@	Conc. of sample. (%)	No of dividing cells	No. of cells with aberrations											
			Physiological aberrations (PA)							Clastogenic aberrations (CA)			Total aberrant cells (TAC)	
			Cm	Da	Lg	St	Vg	Aa	Am	Bg	Bk	Rc	No.	%
Control		924	4	25	-	1	-	-	-	2	-	-	32	3.46
RF1	20	919	20	59	1	24	-	7	3	4	-	-	118	12.84
	40	919	2	68	-	45	5	14	9	11	3	-	157	17.08
	60	917	14	79	6	40	5	14	9	16	-	-	163	17.77
	80	924	10	87	12	40	1	1	-	13	2	-	166	17.96
	100	919	16	89	-	37	1	4	4	24	1	1	177	19.26
RF2	20	910	5	36	2	5	1	-	-	5	-	-	54	5.93
	40	920	10	59	2	7	1	-	-	1	-	-	80	8.69
	60	919	10	68	4	8	-	-	-	7	2	1	100	10.88
	80	917	12	85	3	15	1	-	-	16	3	-	135	14.72
	100	921	12	96	2	18	-	-	-	15	4	2	149	16.17
RF3	20	920	29	48	1	13	-	-	1	-	3	-	95	10.32
	40	916	21	52	4	39	1	3	4	-	6	-	130	14.19
	60	915	25	41	2	70	3	6	1	-	10	-	158	17.11
	80	923	27	56	8	54	4	8	7	4	10	-	178	19.28
	100	923	10	62	10	70	8	10	16	-	12	-	187	20.26
RF4	20	918	32	46	-	14	-	-	-	-	13		105	11.43
	40	920	37	53	1	19	1	-	-	-	15		126	13.69
	60	919	42	71	1	21	-	-	-	1	23		159	17.30
	80	912	39	86	-	32	2	-	-	3	24	1	183	20.06
	100	918	46	110	1	34	1	-	-	2	26	1	221	24.07
RF5	20	914	26	64	3	18	2	-	-	-	13	-	126	13.78
	40	919	31	88	2	21	1	-	-	2	16	1	162	17.62
	60	913	44	96	3	34	2	-	-	2	19	-	200	21.90
	80	911	46	113	2	41	3	-	-	-	25	-	230	25.24
	100	924	52	120	3	40	2	-	-	4	38	1	255	25.95
RF6	20	926	43	46	4	5	1	-	-	-	8	-	107	11.55
	40	923	43	59	-	8	-	-	-	-	10	-	124	13.43
	60	919	42	48	4	18	2	-	-	-	16	2	130	14.68
	80	920	42	56	1	14	4	-	-	3	18	-	138	15.00
	100	918	31	75	4	21	3	-	-	2	19	-	155	16.88
RF7	20	919	10	40	-	5	-	-	-	5	-	-	50	5.44
	40	910	9	49	1	4	-	-	-	3	-	-	66	7.25
	60	918	10	56	2	4	-	-	-	6	1	-	79	8.60
	80	921	13	65	4	8	-	-	-	4	-	-	94	10.20
	100	919	21	79	5	6	-	-	-	4	-	-	115	12.51
RF8	20	925	18	44	1	5	1	-	-	11	-	-	79	8.54
	40	921	16	79	1	12	1	-	-	26	-	-	135	14.65
	60	926	30	98	3	14	2	-	-	29	2	-	178	19.22
	80	922	35	95	2	21	2	1	-	31	2	-	190	20.60
	100	925	36	108	2	24	2	2	-	34	2	-	210	22.70

Cm-C-mitosis; Da- Delayed anaphase; Lg- Laggard/s; St- Stickiness; Vg- Vagrant/s; Aa- Abnormal anaphases/s; Am- Abnormal metaphases; Bg- Chromatin Bridge/s; Bk- Chromosomal Break/s; Rc-Ring chromosome

3.4. Antioxidative enzymes

Proteins are considered as the most important group of biomolecules and their types as well as quantity vary not only among different organisms but also in different parts of the same organism. In the present study, the protein content of onion bulbs (1.08 - 2.48 mg/g) treated with different soil samples was found to be higher as compared to that of negative control bulbs (0.98 mg/g) (Figure 4). This change in total content of protein clearly indicated that under stress, some of the genes responsible for overcoming stress become highly active and ultimately up regulate the total protein content. Although both the expression and functions of such proteins are unclear, yet it indicates that there is a relationship between some forms of plant adaptation and tolerance to stresses and expression of stress-induced proteins [8]. Our results are in conformity with those of Olorunfemi and Lolodi [34] who estimated total protein content in *A. cepa* treated with different concentrations (0%, 0.2%, 0.4%, 0.8% 1%, 2%, 3%, 4% and 5%) of fresh effluents from the cassava processing mills in Uselu Quarters, Benin City and found dose dependent increase in protein content up to four-fold at 1% as compared to control. After this concentration (1%), protein content was found to decrease. Sharma et al [14] also reported higher content of total soluble proteins in *Raphanus sativus* seedlings under nickel stress as compared to control.

Different types of environmental stresses not only enhance the protein content but also trigger the active defense mechanisms in plants resulting in expression of various detoxifying enzymes. The balance between ROS production and detoxification is maintained by enzymatic antioxidative system which involves different enzymes such as CAT,

SOD, GST, APX, DHAR, GR and POD. It is well established that reactive oxygen intermediates (ROI) and antioxidative enzymes play a crucial role in the establishment of normoxia in biological systems and in resistance to oxidative stress. The dual role of ROI as toxic and signalling molecules are ensured by complex and elaborate system controlling intracellular ROI levels. The ROI are detoxified by single or a series of antioxidative enzyme reactions. It is clear that the capacity and activity of the antioxidative defense system are important in limiting oxidative damage and in destroying active oxygen species that are produced in excess of those normally required for metabolism [14].

Catalase and other peroxidases are the major enzymes which are required for the detoxification of H_2O_2 produced during photorespiration and are present in peroxisomes and different cell compartments such as chloroplasts, cytosol, peroxisomes, and mitochondria [6]. APX activity was found to be higher in onion bulbs treated with soil samples as compared to control sample (Figure 5). The negative control showed APX activity of 0.36 mol UA/ mg fresh protein. Among all the soil samples collected from different fields under rice cultivation, the maximum APX activity (1.57 mol UA/ mg fresh protein) was shown by onion bulbs treated with sample RF5 and the minimum APX activity (0.42 mol UA/ mg fresh protein) was shown by onion bulbs treated with sample RF3. Control bulb showed the CAT activity of about 0.005 mol UA/ mg fresh protein. Among all the soil samples collected from different fields under rice cultivation, maximum catalase activity (0.012 mol UA/ mg fresh protein) was shown by the onion bulbs treated with sample RF7 and minimum (0.005 mol UA/ mg fresh protein) was shown by the onion bulbs treated with sample RF2 which was similar to that of control onion bulb (Figure 5). Bhardwaj et al [35] estimated effect of enhanced lead and cadmium in soil on physiological and biochemical attributes of *Phaseolus vulgaris* L**.** and found that activity of APX increased with increasing concentration of metal. Tepe and Aydemir [36] reported increase in activity of APX in barley plants under boron toxicity.

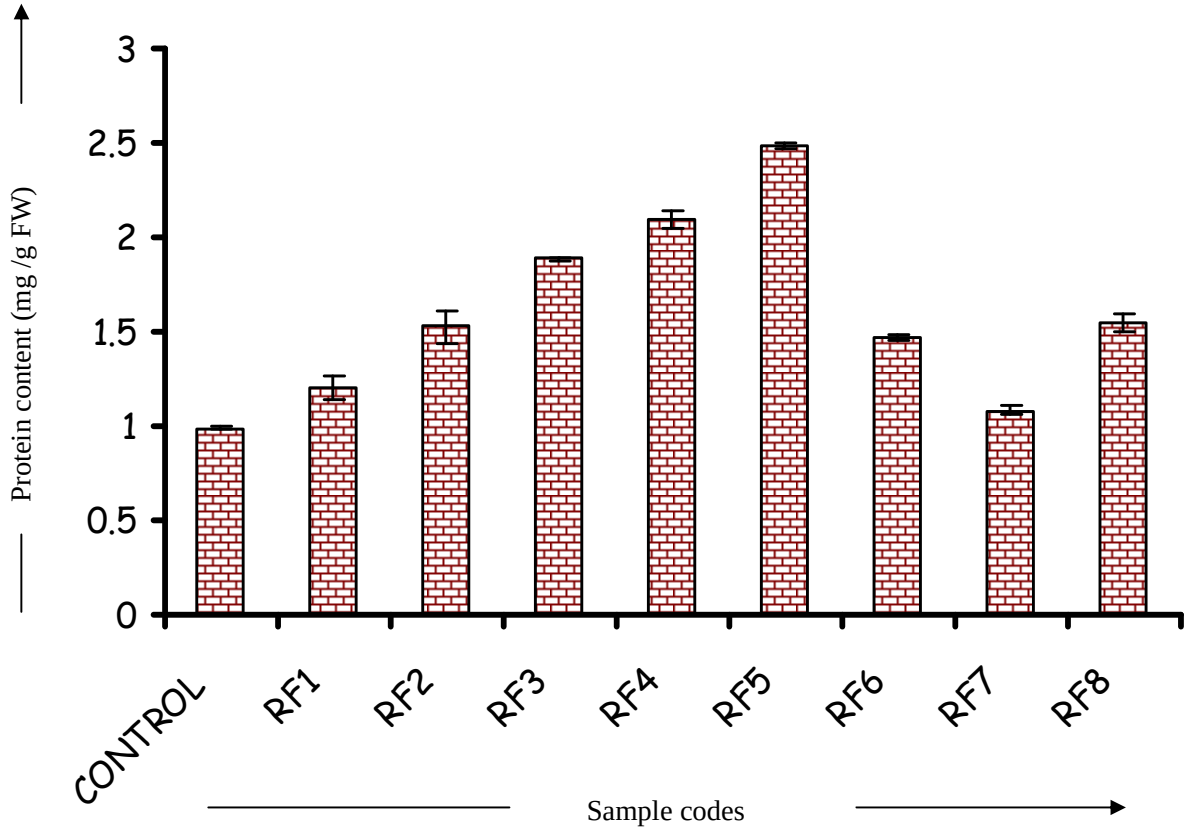


Fig. 4. Effect of different agricultural soils under rice cultivation s on protein content in *Allium cepa* bulbs
All the data were significantly different at p≤0.05

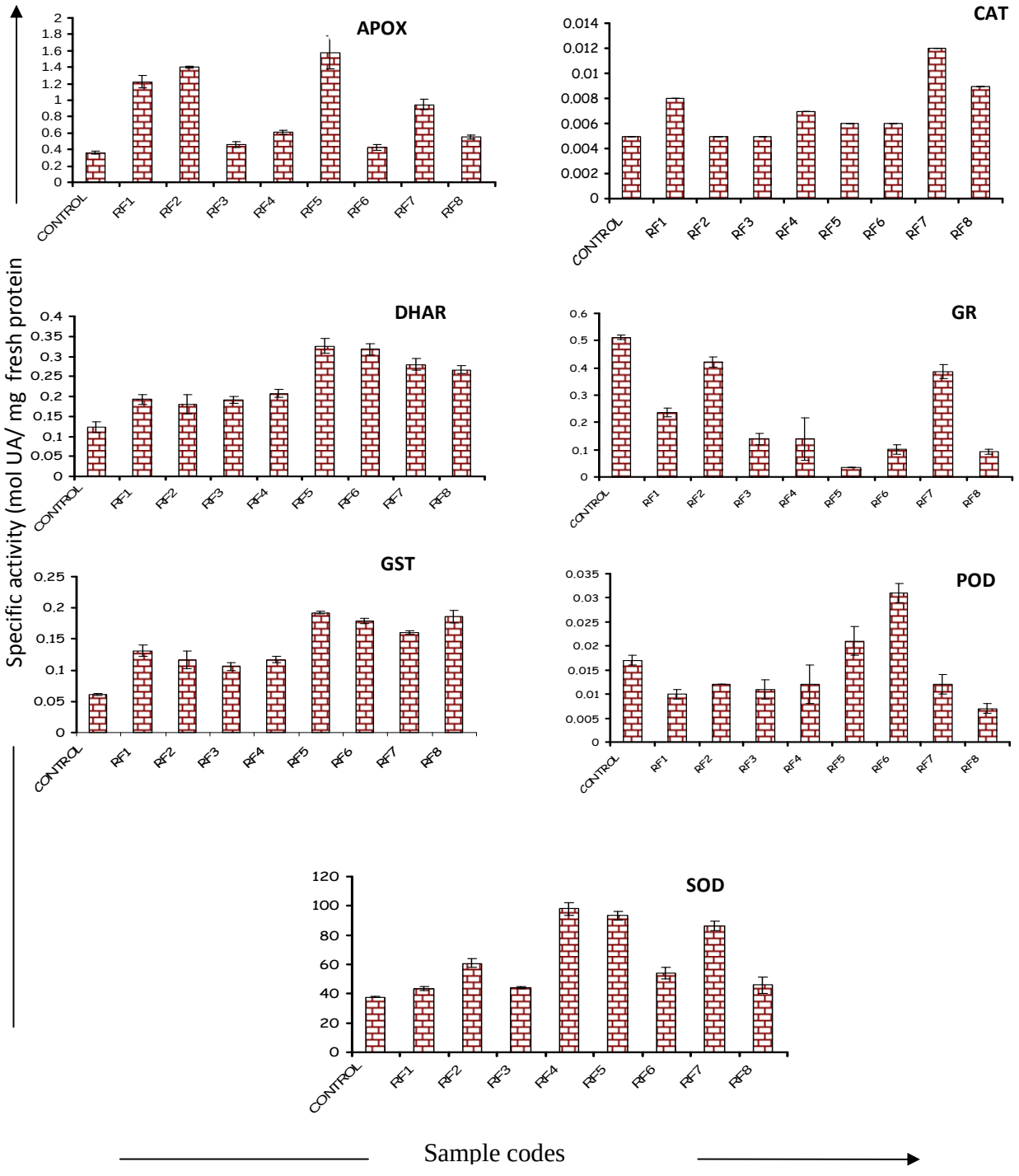


Fig.5. Effect of agricultural soils from different zones on protein content and antioxidative enzyme activities of ascorbate (APOX), catalase (CAT), glutathione- s- transferase (GST), dehydroascorbate reductase (DHAR), superoxide dismutase SOD), guaicol peroxidase (POD), glutathione reductase (GR) in *Allium cepa* bulbs.

All the data were significantly different at p≤0.05

Dehydroascorbate reductase (DHAR) plays an important role in signaling and maintains the cellular level of ascorbic acid by regulating its redox state thereby, affecting cell response and tolerance to oxidative stress [37,38].

The control onion bulbs showed DHAR activity of 0.12 mol UA/ mg fresh protein (Figure 5). Among all the soil samples studied, the onion bulbs treated with soil sample collected from RF5 showed maximum DHAR activity (0.32 mol UA/ mg fresh protein) while minimum activity (0.18 mol UA/ mg fresh protein) was shown by the onion bulbs treated with soil sample RF2. Arora et al. [39] studied antioxidative defense system of *Brassica juncea* L. subjected to cobalt ion toxicity and reported increase in activity of DHAR. Karuppanapandian and Kim [40] also investigated the effects cobalt induced oxidative stress, as well as the role of antioxidant systems on the leaves of hyperaccumulating plant *Brassica juncea* L and reported the increase in activity of DHAR. *Oryza sativa* L. seedlings also showed increase in activity of DHAR on exposure to slight salinity by NaCl.

Glutathione-S-transferases (GSTs) are a super family of enzymes, principally known for their role in detoxification reactions. The role of GST in detoxification is well documented by researchers throughout the world [5, 7]. Increase in GST activity was observed in onion bulbs treated with agricultural soil samples from different zones. The control onion bulb (negative control) showed GST activity of 0.061 mol UA/ mg fresh protein as shown in Figure 5. Among all the soil samples studied, the onion bulbs treated with soil sample RF5 showed maximum GST activity 0.192 mol UA/ mg fresh protein while minimum GST activity (0.106 mol UA/ mg fresh protein) was shown by onion bulbs treated with soil sample RF3. The activity of GST in *Allium* bulbs treated with all the soil samples was found to be slightly higher than control. Similar increase in activity of GST has been reported by Gupta and Ahmad [8]. when they studied the effects of Mathura Refinery waste water (MRWW) in *A. cepa* bulbs.

Glutathione reductase is a member of flavoenzyme family which catalyzes the NADPH dependent reduction of glutathione disulphide (GSSG) to glutathione (GSH) and it maintains glutathione in the reduced state which in turn reduces dehydroascorbate to ascorbate. The GR activity declined in onion bulbs treated with rice cultivated agricultural soil samples collected from different zones of Amritsar. It was found that control onion bulbs showed GR activity of 0.512 mol UA/ mg fresh protein as shown in Figure 5. Among all the soil samples studied, the onion bulbs treated with soil sample RF5 showed the minimum GR activity (0.034 mol UA/ mg fresh protein) while maximum GR activity was shown by RF2 (0.422 mol UA/ mg fresh protein). The GR activity in agricultural soil exposed *A. cepa* bulbs declined with respect to control. GR participates not only in H_2O_2 scavenging, but also favors a high GSH/GSSG ratio to maintain a proper cellular redox mechanism [14]. Decline in GR activity was also reported as a result of biotic and abiotic stress in earlier studies. Similar trend was observed in a study by Tabrez and Ahmad [41] who reported decrease in GR activity under the effect of trichloroethylene in *A. cepa*.

POD also plays an important role in carrying out different physiological functions which include biosynthesis of cell wall components like lignin and suberin, oxidation of toxic compounds and various developmental processes [14]. Onion bulbs grown in washed sand which was used as negative control showed total POD activity of 0.017 mol UA/ mg fresh protein as shown in Figure 5. Among all the soil samples collected from different fields under rice cultivation, maximum POD activity (0.031 mol UA/ mg fresh protein) was shown by onion bulbs treated with sample RF6 and minimum (0.007 mol UA/ mg fresh protein) was shown by onion bulbs treated with sample RF8. In the present study, POD activity in *Allium* bulbs also showed a decrease as compared to control except in bulbs treated with two samples RF7 and RF8 where activity was found to be ennahnced. The results were in consistence with other report where Kou et al [40] found a decrease in POD activity in Huang guan pears (*Pyrus purifolia* Nakai) that were treated with calcium chloride, chitosan and pullulan for prolonging the post harvest life of pears.

SOD is a family of metaloenzymes which is considered as the first line of defense against ROS generation because superoxide radical is considered as a precursor to several other ROS. Superoxide is considered as the central component of the signal transduction which activates the genes responsible for enzymes of defense system including SOD and could serve a very useful marker for metal stress. Superoxide radical generated by any means, is dismutated to H_2O_2 and oxygen by SOD, and this H_2O_2 generated is then removed by peroxidases. H_2O_2 is converted to water by APX in concert with oxidation of ascorbate and also by GPX with oxidation of GSH in the cytosol and chloroplasts [4, 6, 41]. A significant increase in SOD activity was found in onion bulbs treated with different soil samples as compared to control onion bulbs (negative control). Control bulb showed SOD activity of 37.82 mol UA/mg fresh protein (Figure 5). Among all the soil samples collected from rice fields, the onion bulbs treated with RF4 showed maximum SOD activity (97.91 mol UA/ mg fresh protein) while minimum activity (43.42 mol UA/ mg fresh protein) was shown by onion bulbs treated with soil sample RF1. Increase in SOD activity in the present study was consistent with few other studies. Fatima and Ahmad [4] also studied the utility of SOD in *A. cepa* as biomarker for detection of toxic metals in waste water collected from the industrial estate of Aligarh city, India. Significant enhancement of about 8 times in SOD activity was found with respect to control. Tepe and

Aydemir [36] also reported an increase in SOD activity of lentil and barley plants when exposed to boron stress. Sharma et al [14] also found a significant increase in activity of DHAR in *Raphanus sativus* L. with Ni treatment.
Pearson correlation analysis between all the variables was performed. Correlation matrix has been produced to determine relationship between metal content, anti-oxidative enzymes activities and percent chromosomal aberrations (Table 4). Heavy metals are generally closely associated with each other, strongest positive correlations were obtained within Cr, Cu, Ni, Zn and Mn; Pb showed positive correlation with Cr while Cd showed strong negative correlation with Fe. Significant correlations between these metals indicated that these metals and contaminants in the soil have similar source of origin. Moreover, absence of any geologic factor to yield high correlations could clearly indicate that these elements are of anthropogenic origin (could be excessive use of agrochemicals, existence of industrial activities as well as urban and vehicular emissions). A closer analysis of correlation between antioxidative enzymes and metals in present study revealed that among antioxidative enzymes, APX showed significant negative correlation with Cr, Cu and Ni. Significant negative correlation coefficients values were found between CAT and metals like Cu, Ni and Zn. GST and DHAR showed significant negative correlation with Mn whereas SOD was positively correlated with Cd. POD showed positive correlation with Pb. Furthermore, among different antioxidative enzymes, only DHAR showed positive correlation with GST and POD. Chromosomal aberrations showed significant negative correlation with Mn and GR and positive correlation with GST.

Table 4. Correlation coeffient (r) matrix for relationship between heavy metal content, enzyme activity and percent chromosomal aberrations

	Cr	**Cu**	**Ni**	**Zn**	**Mn**	**Pb**	**Fe**	**Cd**	**APX**	**CAT**	**GST**	**DHAR**	**SOD**	**POD**	**GR**	**% CA**
Cr	1															
Cu	0.723***	1														
Ni	0.775****	0.944****	1													
Zn	0.699***	0.808****	0.735***	1												
Mn	0.625***	0.916****	0.854****	0.702***	1											
Pb	0.538*	0.201	0.269	0.153	0.290	1										
Fe	-0.012	-0.177	-0.192	0.231	-0.027	0.190	1									
Cd	0.152	0.391	0.397	-0.005	0.259	-0.166	-0.964****	1								
APX	-0.809****	-0.713***	-0.740***	-0.406	0.742****	-0.408	0.118	-0.246	1							
CAT	-0.146	-0.512*	-0.525*	-0.543*	-0.451	-0.258	-0.167	0.038	-0.05	1						
GST	0.0315	-0.43	-0.398	-0.359	-0.595**	0.308	0.142	-0.345	0.091	0.309	1					
DHAR	0.131	-0.407	-0.253	-0.390	-0.551*	0.440	0.040	-0.225	0.015	0.232	0.925****	1				
SOD	-0.156	-0.265	-0.107	-0.365	-0.487	-0.236	-0.723***	0.618**	0.317	0.160	0.140	0.325	1			
POD	0.029	-0.228	0.094	-0.290	-0.139	0.812****	0.147	-0.232	0.054	0.293	0.423	0.593**	0.033	1		
GR	-0.144	-0.266	-0.315	0.091	-0.114	-0.232	0.154	-0.079	0.332	0.290	-0.447	-0.459	-0.002	-0.328	1	
% CA *Insitu*	-0.482	-0.419	-0.352	-0.421	-0.562*	-0.268	0.183	-0.355	0.415	-0.099	0.546*	0.483	0.137	0.162	-0.608**	1

** represents significance at p≤ 0.05; ** represents significance at p≤ 0.02; *** represents significance at p≤ 0.01; **** represents significance at p≤ 0.001.*
***Cr**- Chromium; **Cu**-Copper, **Ni**- Nickel; Zn- Zinc; **Mn**- Manganese; **Pb**- Lead; **Fe**- Iron; **Cd**- Cadmium; **APX**-ascorbate peroxidase; **CAT**- catalase; **GST**- glutathione-S-transferase; **DHAR**- dehydroascorbate reductase; **SOD**- superoxide dismutase; **POD** - guaicol peroxidase; **GR**- glutathione reductase; **% CA**- percent chromosomal aberrations*

CONCLUSION

Results from the present study aligned with previous studies, recommend using combination of bioassays and physicochemical analysis for monitoring of soils of a particular area. *A. cepa* root chromosomal aberration assay is a simple and sensitive method for investigating genotoxic potential of soil. In fact *A. cepa* root aberration assay is a cost effective assay routinely used in genotoxicity assessment of environmental chemicals and mixtures as well as air, soil and water. Present study also suggests that variation in antioxidative enzymes of *A. cepa* can serve as a suitable biomarker for studying contamination of soils of a particular area.

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