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Evaluation of post-germinative lipid peroxidation and enzymatic antioxidant potential in lead absorbing oat (*Avena sativa*) seedlings

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Abstract

The objective of the present research was to study the impact of lead (Pb) on growth, metal uptake and antioxidative potential of oat seeds under metal stress. To achieve these objectives, few experiments were conducted to assess the effect of this particular metal on various anti-oxidative enzymes, during initial metabolism after germination, in presence of lead. Pb is not an oxido-reducing metal, the oxidative stress induced by Pb in growing oat seedlings appears to be an indirect effect of Pb toxicity, leading to production of ROS with simultaneous decrease in tissue levels of superoxide dismutase and catalase. Content of free radical like superoxide anion and metabolite such as H_2O_2 were found to be more in plumule as compared to radical and endosperm of oat seedling. In response to various concentrations of lead ranging from 25-400 ppm, activities of peroxidase, glutathione peroxidase and ascorbate peroxidase were induced in plumule, radical and cotyledon on the 3rd, 6th and 9th days after germination. Growth parameters like length, fresh weight and dry weight were substantially affected in addition to reduced germination upto 49 % only. The results indicated that even at the lowest concentration tested, a low inhibition of growth was obtained.

Key words

Antioxidant, Enzymes, Lead, Peroxidation

Introduction

The growing urbanization and disposal of industrial effluents into soil and water is rapidly increasing the amount of phyto-toxic heavy metals in various habitats. Lead is one of the hazardous heavy metal pollutants in environment and its increasing levels in soil and water inhibit germination and exert a wide range of effects on growth and metabolism of plants (Gautam *et al.,* 2010). Biomagnification of metals leads to sustained accumulation in edible parts of plants thereby causing risk to plants and their consumers (Bhushan *et al*., 2013).

Seed germination is an initial phase of life cycle and is pre-requisite for establishment of healthy and vigorous seedlings. There is an increasing awareness on metal pollution through issues raised by environmentalists and scientific work on plant physiological responses to heavy metals. As an indicator of metal phytotoxicity, various authors have reported, from time to time, different bio-monitoring indices based on germination and seedling growth to indicate the effects of metal stress using different plant systems (Datta *et al*., 2009).

Heavy metals are known to cause disturbance to several physiological processes and their effects are manifested by various symptoms of injury. The cellular basis of this disturbance is oxidative damage to plants, either directly or indirectly by triggering an increased level of production of reactive oxygen species (ROS). These ROS are produced as by products during membrane linked electron transport activities as well as by a number of metabolic pathways and, in turn, cause damage to biomolecules such as membrane lipids, proteins, chloroplast, pigments, enzymes, nucleic acids etc. (Srivastava *et al*., 2011).

To combat oxidative damage, plants have an efficient system comprising of catalase (CAT), peroxidases (POD), superoxide dismutase (SOD) and the nonenzymic constituents

which remove, neutralize and scavenge the ROS. The enzymes of Halliwell-Asada pathway or ascorbate-glutathione cycle such as ascorbate peroxidase (APX), and glutathione peroxidase (GPX) also play a significant role in scavenging H_2O_2 mainly in chloroplasts and in maintaining the redox status of the cell (Nakano and Asada, 1981). The ROS are chemically aggressive species and attack of free radicals on polyunsaturated fatty acid components of membrane lipids initiates lipid peroxidation, an autocatalytic process that changes membrane structure and function.

Many workers (Bhushan and Gupta, 2008; Bhushan *et al*., 2013) have reported the toxic effects of heavy metals on various aspects of seed germination but biochemical studies pertaining to the effect of Pb on enzymatic antioxidative potential in germinating oat seeds are less. As Pb is one of the most abundant heavy metal pollutants in both aquatic as well as terrestrial environments and oat which is a particular crop that serves as feed for majority of the world animal population, the present study was undertaken to examine the uptake and distribution pattern of Pb in oat seedlings, to determine Pb^{2+}-induced possible induction of oxidative stress and likely alterations in behaviour of enzymes of antioxidant defense system in these plants.

Materials and Methods

The experiments were conducted on oat (*Avena sativa* L. cv HJ-8) seeds procured from Forage Section, Department of Plant Breeding, CCS Haryana Agricultural University, Hisar, India. The seeds of uniform size and colors were selected and surface sterilized by soaking in 0.5% NaCl for 15 min, then washed three times with sterile water and placed on Petri plates (15 cm diameter) lined with two layers of Whatman No. 1 filter paper for germination in a BOD incubator, under 16 hr light/8 hr, dark photoperiod and 60 ± 10% relative humidity. After germination, seeds were transferred to container having support and wicks for absorbing different treatment solutions prepared in half strength Hoagland solution. Seedlings were irrigated with distilled water (control) and aqueous solution of various concentrations (25, 50, 100, 200, 300 and 400 µg ml^{-1}) of lead (treated), kept for seedling growth at 25 ±1ºC.

Growth parameters : Seed germination was examined and calculated in different experiments with 20 seedlings in one petri plate per treatment and considered it as single replicate. Radical and plumule length were meaured starting from imbibitions towards post germination. Embyonic axis separated from cotyledon and their fresh weight was recorded at regular time interval. Samples of cotyledon, radical and plumule were withdrawn at three day interval from three replicates of control and treated germinated seedlings and thoroughly washed with distilled water in each experiment. The endosperm and embryonic axis were separated, dried at 70ºC until a constant weight was observed. The dried samples were ground to fine powder and stored in a dessicator over anhydrous $CaCl_2$. Total soluble proteins were extracted and estimated by modified method of Lowry *et al*. (1951), which involved malachite green in sodium maleate buffer, pH 6.0 and 1 mM EDTA.

Determination of cell viability : Loss of cell viability was determined using Evans blue staining method (Baker and Mock, 1994). Freshly harvested roots were stained with 0.25% (v/v) aqueous solution of Evans blue for 15 min. After washing with distilled water for 30 min, root tips of maximal size were excised and soaked with 500 µl of N, N-dimethylformamide for 1 hr at room temperature. Optical density of the released Evans blue was measured spectrophotometrically at 600 nm.

Antioxidant potential : Preliminary experiments were conducted to optimize the extraction conditions with respect to pH, molarity and type of buffer, concentration of stabilizing agent(s) and other constituents of extraction medium. Unless stated otherwise, all extraction procedures were carried out at 0-4ºC. Each experiment was repeated thrice and the estimations further made in duplicate. Finally, the standardized extraction medium for SOD and POX consisted of 0.1 M Tris-HCl buffer (pH 7.0) containing 3% (*w/v*) polyvinylpyrrolidone, 1mM EDTA and 1mM $CaCl_2$. The extraction medium for CAT, APX and GPX consisted of 0.1 M potassium phosphate buffer (pH 7.5) in place of Tris-HCl buffer. The enzymes were extracted by macerating 1 g tissue with 10 ml of ice cold extraction medium in a pre-chilled pestle and mortar using acid washed sand as abrasive. The homogenate was filtered through four layers of cheese cloth and the filtrate centrifuged at 15,000 rpm for 20 min in a refrigerated centrifuge (Remi, India) at 4ºC. The supernatant was carefully decanted and used as crude enzyme preparation.

Superoxide dismutase (SOD) : Superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) adopting the method of Beauchamp and Fridovich (1971). The reaction mixture (3.0 ml) contained 50 mM Tris-HCl (pH 7.0), 14 mM L-methionine, 60 µM NBT, 3 µM riboflavin, 0.1 mM EDTA and 0.1 ml of enzyme extract. Riboflavin was added at the end. The reaction mixture without enzyme extract developed maximum colour and its absorbance decreased with the addition of enzyme. The absorbance was recorded at 560 nm. One enzyme unit was defined as the amount of enzyme required to inhibit the photoreduction of one µmol of NBT.

Catalase (CAT) : Catalase activity was measured by slightly modified method of Sinha (1972). To 0.55 ml of 0.1 M potassium phosphate buffer (pH 7.5), 0.4 ml of 0.2 M H_2O_2 and 50 µl of enzyme extract was added and mixed thoroughly and incubated for 1 min. Then 3.0 ml of 5% potassium dichromate: acetic acid (1:3) solution was added to it. A control without enzyme extract was also run alongwith the samples. The tubes were kept in

boiling water bath for 10 min, cooled and absorbance was recorded at 570 nm using dichromate : acetate solution as blank. The absorbance of samples was subtracted from that of control and amount of H_2O_2 calculated from the standard curve ranging from 20 to 200 µmols of H_2O_2 using an equation of y = 0.0061x. One CAT unit was defined as amount of enzyme required to consume one µmol of H_2O_2 min^{-1}.

Guaiacol peroxidase (POD) : Peroxidase was assayed by determining the rate of guaiacol oxidation in the presence of H_2O_2 at 470 nm (Rao *et al*., 1996). Reaction mixture (3.0 ml) contained 2.15 ml of 0.1 M Tris HCl buffer (pH 7.0), 0.6 ml of 1% guaiacol, 0.1 ml of enzyme extract and 0.15 ml of 100 mM H_2O_2. H_2O_2 was added at the end, mixed and absorbance was recorded at 470 nm at 15 sec interval up to 3 min. The linear portion of O.D. change was taken to calculate enzyme activity using a molar extinction coefficient of 26.6 mM^{-1} cm^{-1} for guaiacol oxidation. One unit of peroxidase activity was defined as the amount of enzyme required to oxidize one nmol of guaiacol min^{-1} ml^{-1}.

Ascorbate peroxidase (APX) : Ascorbate peroxidase was assayed by the method of Nakano and Asada (1981). The reaction mixture (3.0 ml) contained 95 mM potassium phosphate buffer (pH 7.5), 0.5 mM L-ascorbate and 0.5 mM H_2O_2. The reaction was initiated by addition of 50 µl of enzyme extract. The decrease in absorbance at 290 nm was recorded spectrophotometrically for 2 min against reagent blank which corresponded to the oxidation of ascorbic acid. The enzyme activity was calculated using molar extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ for ascorbate. One enzyme unit was expressed as amount of enzyme required to oxidise 1 nmol of ascorbate min^{-1}.

Glututhione peroxidase (GPX) : Glututhione peroxidase was assayed by the method of Hopkins and Tudhope (1973). This assay is based on the GR catalyzed NADPH expensive continuous regeneration of oxidized glutathione produced by this enzyme. The reaction mixture (3.0 ml) contained 50 mM potassium phosphate buffer (pH 7.5) containing 200 µM EDTA, 150 mM reduced glutathione, 4.1 mM NADPH, 0.1 unit of commercial GR and 50 µl of enzyme extract. The reaction was initiated by addition of 2.2 mM t-butyl peroxide and decrease in absorbance was monitored at 340 nm against 3.0 ml potassium phosphate buffer (0.05 M, pH 7.0) as blank. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to calculate the amount of NADPH oxidized. One enzyme unit was defined as amount of enzyme required to oxidize one nmol of NADPH min^{-1}.

Lipid peroxidation and free radicals : The level of peroxidation in 12-day-old seedling and 3-day-old cotyledon was measured by estimating malondialdehyde using standardized methodology. The amount of lipid peroxidation in 12-day-old plumule sample was also monitored and expressed as Thiobarbituric acid reactive species (TBARS) according to the modified method of Yagi (1976). The adopted method for determining the level of superoxide anion is based on detection of NBT (nitroblue tetrazolium) reduction. NBT reducing activity was expressed as increase in absorbance per gram fresh weight of plumule. The level of hydrogen peroxide was calculated by coupled assay. The absorbance was expressed in nano moles per gram fresh weight.

Statistical analysis : The results presented are mean of three independent experimental values. Sample variability is given as the standard deviation of the mean.

Results and Discussion

Seedling vigour under heavy metal stress : Seed germination count was monitored through percent germination analysis as described in materials and methods. Percent seed germination reduced at all the concentrations of lead (25 ppm to 400 ppm) on all 4 days of water imbibition. After 4 days of water imbibition, percent seeds germination in control seeds was about 87 %, whereas at highest concentration (400 ppm) there was of 51 % reduction 36 % germination). Thus, it has shown that inhibition in germination count was concentration dependent. Other morphological parameters like length of embryonic axis was studied and their data were combined with germination count data to get germination , metal tolerance index and percent phyto-toxicity for better presentation of results (Table 1 a,b).

Yemeni (2001) reported that lead delayed germination and lowered the ability of seeds to germinate in dose dependent manner compared to control in *Vigna ambacensis*. Lead does not act by inhibing water uptake during imbibition and the coats highly permeable to lead are those with lower imbibition of water. Seed coats were impermeable to lead during the first period of imbibition when uptake of water was intense and later on water uptake was reduced and seed coats became more permeable to lead. Lead that penetrated into the embryos in the final stages of imbibition delayed germination (Gautam *et al.,* 2010).

Influence of lead on fresh and dry weight of endosperm and embryonic axis was recorded at 3 day interval upto 12 days after germination. With passage of time, there was a steady decline in fresh weight of endosperms of seed germinated in control as well as in presence of specified concentration of lead. The fresh weight of endosperm of seeds germinated in 400 ppm lead solution was 61.53 % greater than that of control seeds germinated in distilled water after 12 days. A converse effect was, however, registered for embryonic axis and its fresh weight was 11 % less with increasing level of treatment (Table 2a). From the results presented in Table 2b, it is clear that in control, the dry weight of endosperm decreased and that of embryonic axis increased progressively with passage of time. Similar trend was observed at all days of germination in dry mass of endosperms. It was also observed that 12 days after germination dry weight of endosperm and embryonic axis in plants treated with 400 ppm lead increased by 67 % and decreased by 38 % with respect to control.

Table 1 (a) : Impact of lead concentration on the germination index with respect to radical length

Treatment of Pb (ppm)	3 DAG	6DAG	9DAG	12 DAG
25	0.85±0.033	0.87±0.041	0.86±0.045	0.87±0.029
50	0.66±0.021	0.68±0.024	0.69±0.031	0.69±0.033
100	0.49±0.019	0.54±0.021	0.56±0.026	0.56±0.027
200	0.38±0.012	0.42±0.014	0.45±0.021	0.44±0.028
300	0.30±0.011	0.37±0.013	0.39±0.019	0.39±0.019

Table 1 (b) : Impact of lead concentration on the germination index with respect to plumule length

Treatment of Pb (ppm)	3 DAG	6DAG	9DAG	12 DAG
25	0.66±0.023	0.81±0.022	0.87±0.039	0.89±0.044
50	0.51±0.023	0.62±0.023	0.68±0.034	0.71±0.037
100	0.38±0.031	0.49±0.017	0.52±0.026	0.56±0.029
200	0.26±0.011	0.35±0.014	0.40±0.031	0.45±0.022
300	0.20±0.009	0.30±0.014	0.34±0.019	0.39±0.016
400	0.17±0.005	0.26±0.013	0.28±0.012	0.34±0.013

Germination Index (GI) = (G_sL_s) /(G_cL_c); G_s and L_s represents seed germination (%) and radical/plumule elongation (cm) for the treated samples respectively; G_c and Lc are the corresponding control values

Decrease in fresh and dry weight of endosperms with passage of time occurs largely due to mobilization and utilization of major seed reserves during germination stored in endosperm. The greater dry weight of endosperm in presence of increasing concentration of lead indicates progressive increase in inhibition of mobilization. One of the early important events during germination entails mobilization of reserve material stored in the endosperm. A part of these are respired and catabolized while others are translocated to growing embryonic axis, resulting in an overall decline in weight of endosperm. (Ramakrishna and Rao, 2005). The progressive decrease in fresh and dry weight of embryonic axis with increasing concentration of lead, as compared to control, were due to inhibition of seed reserve mobilization and nutrient's translocation (Bhushan and Gupta, 2008).

Influence of different concentrations of lead on growth was also monitored from elongation of embryonic axis. On specified days after germination, with increasing concentration of lead, impediment in the length of embryonic axis was observed (data not shown). This shows that lead affect the plumule length greater than the radical length (Table 3a,b). Lead retards the internal mobilization and transportation of reserve materials from endosperms so this could be one of the reasons for stunted growth of embryonic axis emerging from seeds germinated in presence of lead where embryo remain deprived of nutrients in the form of soluble hydrolysates derived from hydrolysis of endospermic reserves (Bhushan and Gupta, 2008; Bhushan *et al*., 2013; Gautam *et al*.,2010).

Deleterious effect of lead on embryonic axis growth appeared to be slightly more on elongation of plumule than that of radical (Table 4a,b). Few studies have, however, shown a slightly higher impairment in growth of radical as compared to plumule growth in *Vigna ambacensis* (Yemeni, 2001). Decreased seedling vigour in rice due to Pb could possibly be attributed to Pb interference with the metabolic and biochemical processes associated with normal growth and development of plant. This discrepancy between the results of comparative growth inhibitions of radical and plumule, found in present study and those reported in literature (Gautam *et al*., 2010; Ramakrishna and Rao, 2005), may be due to variation in physiological response of different plant species and difference in experimental conditions.

Evans blue uptake through root tips has been observed to be initiated after submerging them for one hour at 25 ppm lead nitrate solution and intensity of uptake also gradually increased upto 200 ppm (Fig.1). Beyond 200 ppm, no further increase in dye uptake was noted in root tips submerged in higher concentration of treatment. This suggests that transporter at root tips may be saturated with dye molecule ligand and displayed the sigmoidal nature of transporter kinetics with respect to this metal ligand. Siroka *et al*. (2004) showed that Pb was unevenly distributed in roots, where different root tissues acted as barriers to apoplastic and symplastic Pb transport and hence Pb transport to shoot got restricted.

With increase in metal concentration of supplementing nutrient solution to seedlings, high MDA, content with respect to control were recorded at different stages of seedling growth. After 9 days of germination, more than two fold increase in MDA content was observed at 400 ppm stage with respect to control. This suggests that lead enhanced lipid peroxidation content resulting in imposes oxidative stress during the germinative and post-germinative stages of life cycle. Surprisingly, after analyzing

pattern of thiobarbituric acid reactive species (TBARS) content of plumule at different stages under stress, it was concluded that depressing TBA level reactive species 31.71, 20.26 and 20.51 nmoles with respect to control was attributed to positively modulated enzyme antioxidant system at all stages except 12 DAG where no significant change was observed with respect to concentration and time (Fig. 2). Free radicals like superoxide anion and hydrogen peroxide were found to be five and two folds more in most of the treated samples as compared to control after 12 days of the germination (Fig. 3).

SOD is an essential component of anti oxidative defense system in plants and has been used as potential biomarker for stress occurrence. In the present investigation, Pb treatment

Table 2 a : Effect of lead on fresh weight of endosperm and embryonic axis of germinating oat seeds

Pb concen -tration (ppm)	Fresh weight (mg)/endosperm Days after germination				Fresh weight (mg)/embryonic axis Days after germination			
	3	6	9	12	3	6	9	12
0	37.66±2.08	26.00±2.00	23.00±3.00	13.00±1.00	70.05±4.58	121.61±2.08	248.33±8.32	419.33±2.51
25	40.66±3.05 (108)	27.00±2.00 (104)	24.00±3.00 (104)	14.66±1.52 (107)	62.33±3.21 (88)	116.00±2.64 (95)	240.00±3.00 (96)	406.33±7.76 (96)
50	42.00±3.60 (111)	28.00±2.00 (107)	24.66±4.50 (107)	16.00±1.73 (123)	60.00±3.60 (85)	112.00±3.60 (92)	237.00±3.60 (95)	402.66±7.63 (95)
100	42.66±5.50 (113)	29.00±2.00 (111)	25.66±4.50 (111)	17.00±1.73 (130)	55.66±3.05 (78)	108.00±4.58 (89)	232.66±3.78 (93)	392.33±3.21 (93)
200	44.33±4.50 (117)	30.00±2.00 (115)	26.66±4.50 (115)	18.33±1.15 (138)	49.66±3.05 (69)	102.33±4.16 (84)	228.33±5.50 (91)	387.66±4.00 (92)
300	45.00±4.00 (119)	31.00±2.00 (119)	27.66±4.50 (120)	19.33±1.15 (146)	47.00±4.00 (67)	99.00±4.35 (81)	225.00±4.58 (90)	381.66±2.51 (90)
400	45.33±3.51 (120)	32.00±2.00 (123)	28.33±4.04 (123)	21.00±1.00 (161)	42.66±4.72 (60)	98.33±4.16 (80)	218.33±7.09 (87)	373.33±1.52 (89)
		Endosperm					Embryonic axis	
	Treatment	2.503					3.683	
	Stages	1.891					2.784	
	Interaction	NS					NS	

Values in parenthesis denote fresh weight as per cent of respective controls

Table 2 b : Effect of lead on dry weight of endosperm and embryonic axis of germinating oat seeds

Pb Concen -tration (ppm)	Dry weight (mg)/endosperm Days after germination				Dry weight (mg)/embryonic axis Days after germination			
	3	6	9	12	3	6	9	12
0	16.00±1.00	14.00±1.00	11.66±0.57	7.33±0.57	11.00±1.00	21.00±1.00	26.00±1.00	34.00±1.00
25	17.00±1.00 (106)	15.00±1.00 (107)	12.33±0.57 (106)	8.67±0.57 (118)	7.00±1.00 (63)	16.00±1.00 (76)	21.00±1.00 (81)	30.67±0.57 (90)
50	18.00±1.00 (112)	15.66±0.57 (112)	13.33±0.56 (114)	10.33±0.57 (140)	6.67±1.15 (61)	15.00±1.00 (71)	20.00±1.00 (77)	28.64±1.52 (84)
100	19.00±1.00 (119)	16.66±0.57 (119)	14.33±0.57 (122)	11.00±0.50 (150)	6.33±0.57 (58)	14.00±1.00 (67)	19.00±1.73 (73)	27.32±0.57 (80)
200	20.00±1.00 (125)	17.67±0.57 (126)	15.00±0.00 (128)	11.33±0.57 (154)	5.67±0.57 (52)	13.33±1.15 (63)	17.67±1.52 (68)	24.66±0.57 (72)
300	20.66±0.56 (129)	18.00±0.00 (128)	16.00±0.00 (137)	12.00±1.00 (163)	4.66±0.57 (42)	12.33±1.56 (59)	16.00±1.00 (62)	22.64±1.52 (66)
400	22.00±1.00 (137)	19.33±0.11 (138)	17.33±0.57 (148)	12.34±0.57 (167)	3.67±0.57 (33)	11.33±1.52 (54)	14.37±1.52 (55)	21.00±2.64 (62)
		Endosperm					Embryonic axis	
	Treatment	0.550					1.001	
	Stages	0.415					0.757	
	Interaction	NS					2.004	

Dry weight of mature seed was 24mg; Values in parenthesis denote dry weight of endosperm and embryonic axis as per cent of respective controls

Table 3 (a) : Impact of lead on metal tolerance index (MTI) of radical (RAD)

Pb concentration (ppm)	3 DAG	6DAG	9DAG	12 DAG
25	92.3±8.23	94.0±8.46	93.4±5.89	94.3±6.17
50	85.8±7.12	89.5±7.79	90.3±4.34	90.1±5.91
100	76.4±7.13	83.5±7.43	87.1±4.51	86.4±4.88
200	70.8±6.65	79.1±7.02	84.2±4.22	81.7±4.87
300	59.4±4.34	73.3±6.67	78.0±3.69	77.5±4.34
400	48.1±3.78	66.8±4.78	70.7±3.55	74.8±4.41

Table 3 (b) : Impact of lead on metal tolerance index (MTI) of plumule (PLU)

Pb concentration (ppm)	3 DAG	6DAG	9DAG	12 DAG
25	71.4±3.45	86.8±4.43	94.1±5.11	95.7±6.13
50	66.6±3.31	81.5±4.02	88.5±4.72	92.8±5.43
100	58.5±3.11	76.3±3.56	80.5±3.86	86.7±4.22
200	49.0±2.98	65.7±3.33	74.0±4.11	83.9±3.21
300	40.9±3.01	58.6±3.13	66.9±3.45	77.8±3.11
400	36.2±2.46	53.4±3.22	58.9±3.59	69.6±4.02

Metal tolerance index (MTI) = radical or plumule length of cotyledon in test/ radical or plumule length of cotyledon in control X 100

caused suppression in SOD activity in oat seedlings. SOD activity decreased gradually during early and later days of seedling growth both under control as well as Pb treatments (Fig. 4). On 12th day of seedling growth 400 ppm of Pb treatment led to decrease in 28% SOD activity in shoots and about 80 % decrease in roots. Among antioxidant enzymes activity, of SOD is of more relevance in maintenance of the overall defense system of plants subjected to oxidative stress. Decrease in SOD activity could be due to its inactivation by ROS or decreased enzyme synthesis under stress conditions (Mathad and Pratima, 2009). However, unlike present investigation, heavy metal induced anti-oxidative enzyme activity has also been observed when plants were grown in nutrient medium containing $Pb(NO_3)_2$ (Madhava *et al*., 2000). Reduced SOD activity may partly results in enhanced production of free radicals.

Catalase is universally present as oxidoreductase that decomposes H_2O_2 to water and molecular oxygen and is one of the key enzymes involved in removal of toxic peroxides. In the present study catalase activity also decreased during early and late days of seedlings growth (Fig.5). With increasing levels of Pb treatment, a concomitant decrease in catalase activity was observed in roots while in shoots a higher Pb treatment level led to marked inhibition in enzyme activity. Seedling growing under

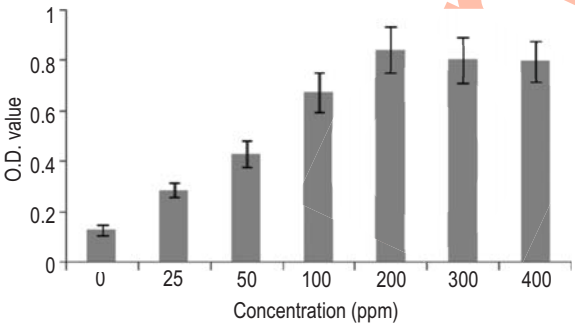

Fig. 1 : Evans Blue uptake through roots after short term exposure of lead treated oat seedlings

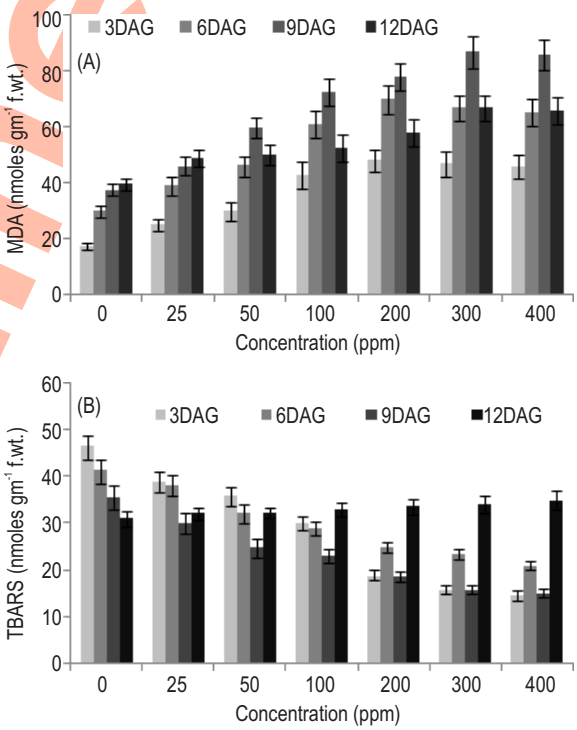

Fig. 2 : Stage bound level of lipid peroxidation by MDA content (a) and TBARS content (b) in plumule of lead treated oat seedlings

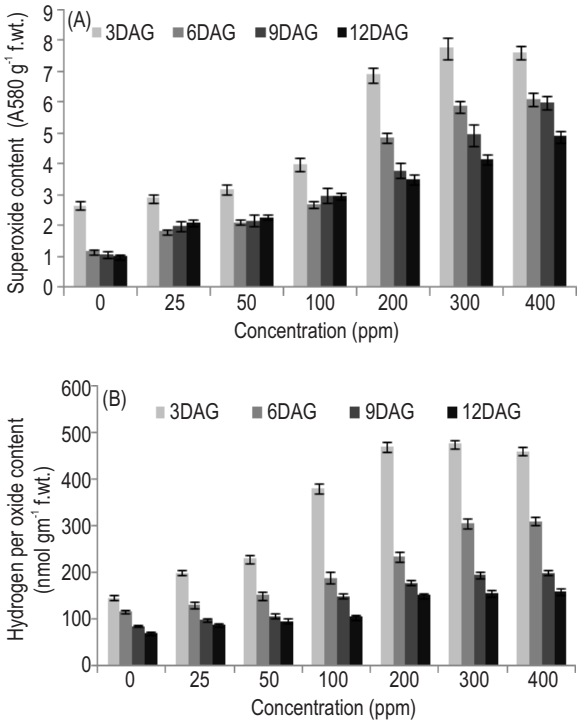

Fig. 3 : Stage bound production of superoxide anion (a) and hydrogen peroxide (b) in plumule of lead treated oat seedlings

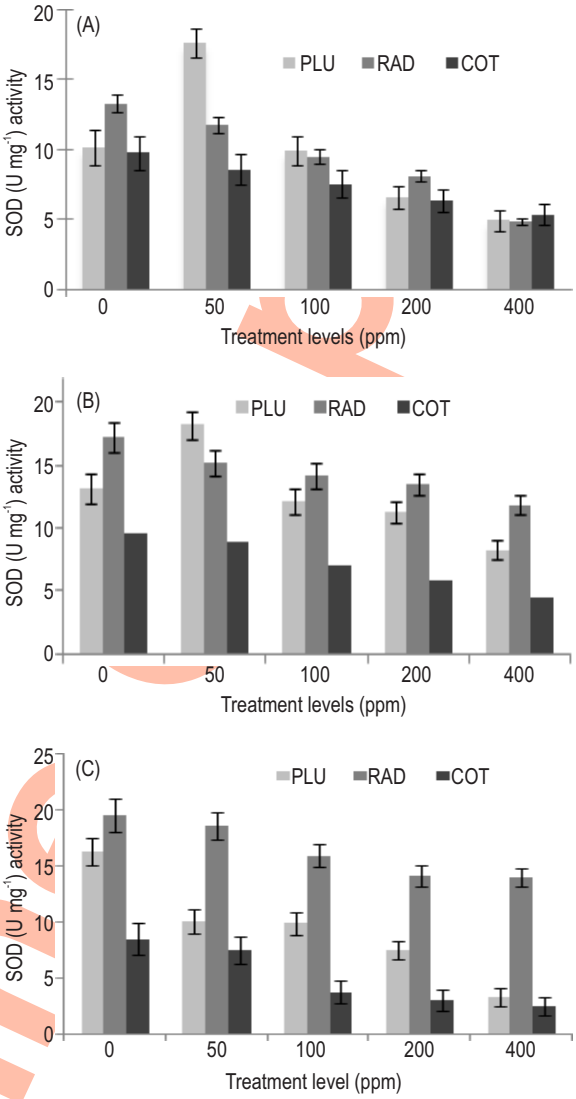

Fig. 4 : Activity profile of SOD in plumule, radical and cotyledon at (a) 3DAG, (b) 6DAG and (c) 9DAG

400ppm Pb showed about 36% decline in catalase activity in shoots and about 15% decline in roots after 12 days of growth. Compared to roots, a greater inhibition in catalase activity was observed in shoots with 400ppm Pb treatment. A decline in catalase activity under Pb toxicity, as observed in present study suggests a possible delay in removal of H_2O_2 and toxic peroxides by catalase and in turn, enhancement in free radical mediated lipid peroxidation under Pb toxicity. It was interesting to observe reduced SOD and POD activities and higher level of peroxides, it suggested that this could not have resulted from enzymatic dismutation of superoxide anions but from non enzymatic disproportionation of radicals or from other oxidative reactions (Gajewska and Sklodowska, 2007). Reduction in catalase activity under stressful conditions has been attributed to the inactivation of enzyme protein due to over production of ROS, decrease in enzyme synthesis or change in assembly of enzyme subunits. Similar decline in catalase activity was reported under various abiotic stresses. However, unlike these studies both increased and decreased catalase activities were observed when plants were grown in nutrient medium containing very low to very high concentration of heavy metals (Pandey and Pathak, 2006; Gopal and Nautiyal, 2013).

Peroxidases are widely accepted as 'stress enzymes'. Induction in peroxidase activity has been documented under a variety of stressful conditions, especially under toxic levels of heavy metals. Under sub-lethal salinity and metal toxicity conditions, level of peroxidase activity has been used as potential biomarker of stress. The present results indicate an enhancement in guaiacol peroxidase activity, suggesting that this enzyme serves as an intrinsic defense tool to resist Pb^{2+}-induced oxidative damage in plants (Srivastava and Dubey,2011; Sinha *et al*.,2012). Different parts of seedling maintained higher guaiacol peroxidase activity under Pb treatment than their control counterparts, after 3 days of germination. Germination in Pb, increase of 15 units in guaiacol peroxidase activity was found in 9-days-old plumule with respect to 3-day-old of oat cultivar (Fig. 6). As guaiacol

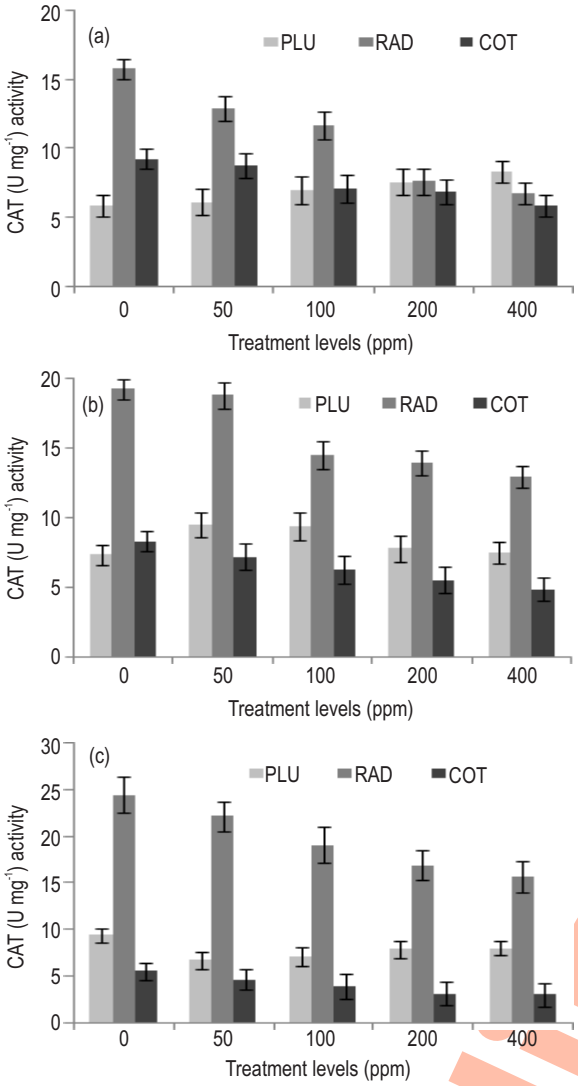

Fig. 5 : Activity profile of CAT in plumule, radical and cotyledon at (a) 3DAG, (b) 6DAG and (c) 9DAG

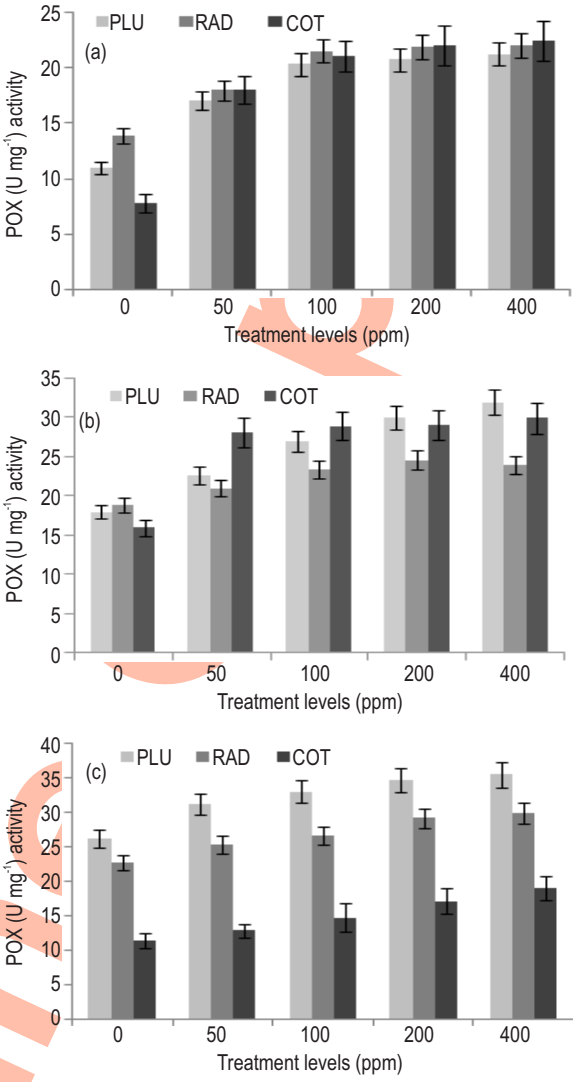

Fig. 6 : Activity profile of POD in plumule, radical and cotyledon at (a) 3DAG, (b) 6DAG and (c) 9DAG

Table 4 (a) : Impact of lead concentration on percent phytotoxicity of radical (RAD)

Treatment of Pb (ppm)	3 DAG	6DAG	9DAG	12 DAG
25	7.6±0.92	6.0±0.88	6.6±0.92	5.6±0.99
50	14.1±1.12	10.4±0.94	9.7±0.99	9.9±1.44
100	23.5±1.46	16.4±1.34	12.8±1.11	13.5±1.01
200	29.1±2.23	20.8±1.49	15.7±1.33	18.2±1.78
300	40.5±2.87	26.6±1.99	21.9±1.67	22.4±1.47

peroxidases are located in cytosol, cell wall, vacuole and in extracellular spaces, increased peroxidase activity in Pb stressed seedlings might be due to increased release of peroxidases localized in cell walls. Both APX and GPX utilizes the reducing powers to eliminate potentially harmful H_2O_2. Similar to guaiacol peroxidase ascorbate and glutathione peroxidase activity showed a concomitant increase in radical and plumule with increase in Pb treatment (Fig. 7, 8). This means that enzyme

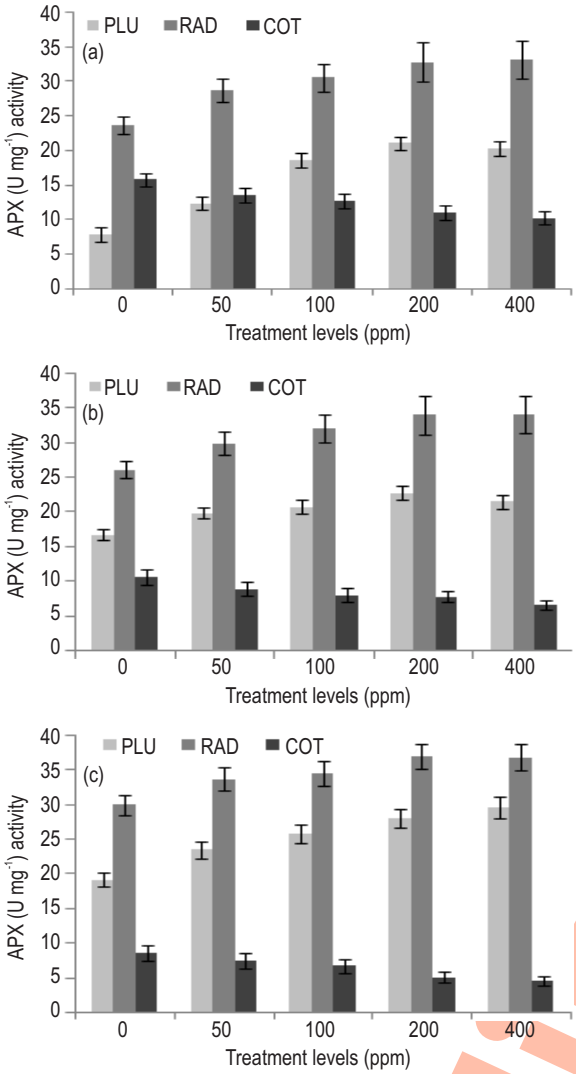

Fig. 7 : Activity profile of APX in plumule, radical and cotyledon at (a) 3DAG, (b) 6DAG and (c) 9DAG

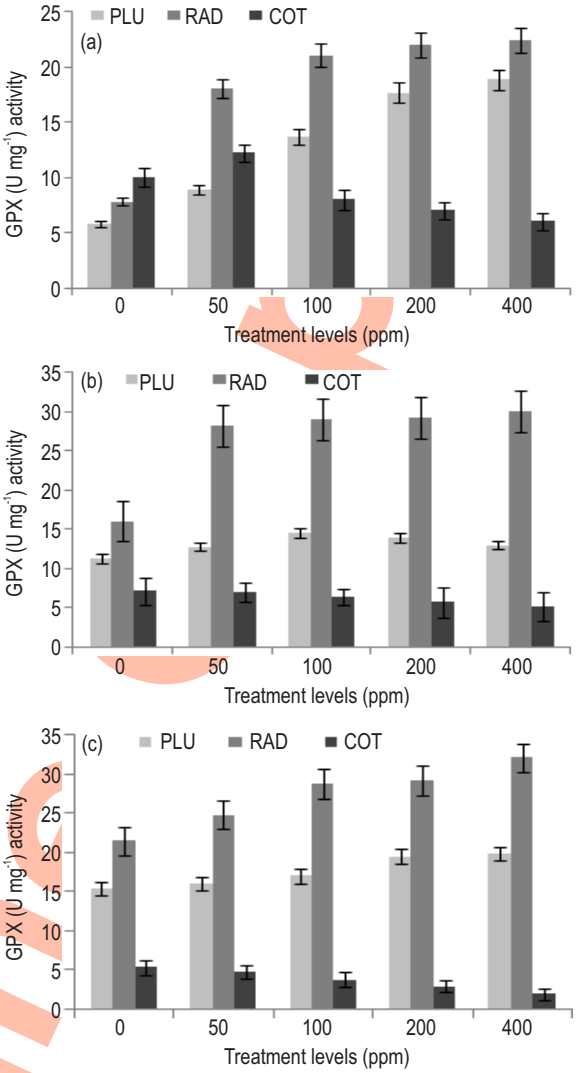

Fig. 8 : Activity profile of GPX in plumule, radical and cotyledon at (a) 3DAG, (b) 6DAG and (c) 9DAG

Table 4 (b) : Impact of lead concentration on percent phytotoxicity of plumule (PLU)

Treatment of Pb (ppm)	3 DAG	6DAG	9DAG	12 DAG
25	28.5±1.33	13.1±0.95	5.8±0.66	4.2±0.53
50	33.3±1.98	18.4±1.11	11.4±0.92	7.1±0.66
100	41.4±1.77	23.6±1.41	19.4±1.16	13.2±0.99
200	50.9±1.94	34.2±1.45	25.9±1.72	16.0±1.03
300	59.0±2.12	41.3±2.23	33.0±1.81	22.1±1.26
400	63.8±2.31	46.5±2.46	41.0±1.95	30.3±1.31

Percent phytotoxicity = radical or plumule length of control - radical or plumule length of test/ radical or plumule length of control X 100

activities are stress regulated. Whereas, under both control and Pb treatments, roots showed higher level of APX/GPX enzyme activity than shoots. For example 6 days after germination, 34.19 U of radical APX was recorded whereas COT and PLU - APX was 6.6 and 21.6 U respectively at 400 ppm Pb treatment. It is also important to observe increased enzymatic activities in radical and plumule with the reduced peroxide levels at 6DAG and 9 DAG. Enzyme induction have also been reported in response to mild

abiotic stresses (Srivastava and Dubey,2011; Sinha *et al*.,2012).

The present study suggests that lead treatment of oat seedlings has generated a tolerance response specifically in roots, where both APX and POD seem to be efficient in removal ofoxic peroxide in all germinating oat seedling stages. The level of free radicals and peroxides was diminished to a large extent in both activated enzyme system but their level was found to be highest under reduced activities of SOD and CAT at the initial stage of germination. So, from the present study it is concluded that functioning of complete anti-oxidative defence system is sufficient enough to reduce the level of free radicals. Variations in the level of free radicals and peroxides with their relevant enzymes in cotyledons has not attracted much attention.

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