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Full Length Research Paper

Chromium stress on peroxidase, ascorbate peroxidase and acid invertase in pea (*Pisum sativum* L.) seedling

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In this investigation, chromium (Cr) toxicity on enzymes like peroxidase, ascorbate peroxidase and acid invertase was studied in pea seedling to evaluate the relative tolerance of the different pea varieties. Effect of Cr (0, 1.0, 2.0, 3.0 mM) on different parts of the seedling that is, cotyledon, plumule and radicle were studied separately to have a comparative study of the enzyme activity in these parts of the plants and its varieties. Acid invertase activity in cotyledons of Arkel was not affected significantly by chromium but increased in Rachna and HFP-8712. Chromium decreased acid invertase activity in radicle and plumule. Chromium treatment increased peroxidase activity in all component of seedling and found to be highest in untreated cotyledons of Arkel. Ascorbate peroxidase activity in the radicle and plumule was higher in Rachna. Enzyme activity decreased significantly with the increasing chromium treatment in cotyledons of Arkel while converse was true with regard to HFP-8712. These results indicate that Rachna is more tolerant followed by HFP-8712 and Arkel.

Key words: Chromium toxicity, peroxidase, ascorbate peroxidise, acid invertase, *Pisum sativum*.

INTRODUCTION

Industrialization and technological advancements have led to increase of heavy metals like cadmium (Cd), lead (Pb), mercury (Hg), nickel (Ni), copper (Cu), aluminum (Al), chromium (Cr) and silver (Ag) into the soil environment. Some heavy metals have accumulative effect and some show external toxic effects. The level of protein, nucleic acid and carbohydrates are affected by environmental stresses in growing plant parts due to alteration in the activities of synthetic and hydrolytic enzyme (Dubey, 1997).

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Abbreviations: ROS, Reactive oxygen species; **MTS,** metallothioneins; **POX,** peroxidase; **CAT,** catalase; **GPX,** guaiacol peroxidise; **GR,** glutathione reductase; **APX,** ascorbate peroxidise; **SOD,** superoxide dismutase; **Mn-SOD,** Mn-superoxide dismutase.

At the time of seed germination, the proteins are hydrolyzed and the products are transported to the growing parts of the plant for synthesis of new proteins. Cellular influences like temperature stress (for example, heat shock protein), water supply (for example, desiccation protein), pathogen attack (for example, pathogennesis related (PR) protein), and heavy metal stress (for example, heavy metal stress induced protein) change the cellular protein patterns.

A developmentally and environmentally induced change in the cellular proteins is manifested at qualitative and quantitative levels, for example, increase or decrease in enzymatic activity occurs due to changes in the amount of that particular protein. Chromium (Cr) is the seventh most abundant metal in the earth's crust and an important environmental contaminant released into the environment due to its huge industrial use. Chromium is mainly present in the environment as insoluble Cr (III) and Cr (VI) compounds. These forms differ markedly in their mechanism of crossing the biological membranes. Cr (VI) as chromate, readily permeates through a general

ion transport system, whereas Cr (III) complexes cannot diffuse through the membrane. The rate of absorption of Cr (VI) in the intestinal track is high as compared to Cr (III) and hence, the hexavalent form of this metal is biologically more toxic (Dubey, 1997). Chronic exposure to the chromium has been reported to cause liver and kidney damage, which are coupled with the appearance of perforations in the nasal septum. It also produces mutagenic and carcinogenic effect on biological systems. Chromium is toxic even at moderately low level. The adverse effect of chromium on plant growth, first noted by Koening (1910), has been documented by numerous workers. It is known to effect seed germination, plant metabolism, minerals nutrition, depress protein and chlorophyll content and to interfere with photosynthetic reaction. These alteration lead to decreased growth and ultimately crop yield coupled with the deterioration in quality of the seeds. Chromium also induces phytotoxic symptoms in plants like morphological changes, proline accumulation and alterations in antioxidant metabolism (Panda et al., 2003).

Heavy metals can induce the generation of reactive oxygen species (ROS) such as super oxide radical (O^{-}_{2}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^{-}), etc., which cause severe oxidation of the biomolecules like lipids, proteins and nucleic acids (Chaoui et al., 1997; Sumitra and Nayna, 2003). High concentration of these ROS can disrupt the normal physiological and cellular functions. The presence of heavy metal in toxic concentration can result in the formation of ROS, which can be initiated directly or indirectly by heavy metals. To counter the deleterious effects plants have evolved various enzymic and nonenzymic antioxidant systems which can protect the plant from the toxic action of various ROS. These antioxidant activities are induced as a response to adverse and abiotic stresses. ROS formation affects the antioxidant metabolism. Chromium, copper and zinc can induce the activity of various anti-oxidant enzymes like catalase (EC1.11.1.6), super-oxide dismutase (EC1.15.1.1) and glutathione reductase [EC1.6.4.2) (GR)] and also non-enzymes like ascorbate and glutathione (Panda, 2003; Choudhury and Panda, 2004).

Pulses constitute an important source of dietary proteins in human and animal nutrition. Among the pulses, pea has been shown to be a sensitive crop to heavy metal toxicity (Rodriguez et al., 1997). There is a great degree of variability available in pea germplasm which differ in tolerance to stresses, diseases and pest. Varieties used as green vegetables like Arkel, Hisar-Harit etc., are tall, leafy, early flowering and sensitive to water logging and powdery mildews diseases. Those used as pulses like HFP-4, HFP-8712 etc., are dwarf, completely tendriller except the stipules, late flowering and resistant to aforementioned stresses. However, no systematic work has been conducted to decipher the tolerance limit of various cultivars of pea for chromium. So in the light of the foregoing points, the present study was conducted to understand the effect of chromium on peroxidase, ascorbate peroxidase and acid invertase enzymes in pea seedling.

MATERIALS AND METHODS

Seeds

Seeds of uniform size were selected from three different varieties of pea viz: Arkel, Rachna and HFP-8712 differing in their relative tolerance to chromium from Pulses Department, College of Agriculture, Chaudhary Charan Singh Harayana Agricultural University, Hisar, India.

Surface sterilization

Seeds were surface sterilized by treating them with 20% sodium hypochlorite solution for five minutes to which one or two drops of teepol (detergent) also added. The seeds were shaken regularly. After surface sterilization, seeds were washed thoroughly with distilled water.

Germination of seeds and chromium treatment

Surface sterilized seeds were soaked in water. After 48 h of soaking when radicle emerged and acquired a length of approximately 2 mm, these were transferred to Petri plates lined with filter paper these contained 10 ml of a range of $K_2Cr_2O_7$ solution (0, 1.0, 2.0, 3.0 mM Cr (VI) ions). These were than kept in dark at 25±2°C in an incubator. Cotyledons, radicles and plumules were separated after the fifth day of chromium treatment and employed for estimation of different metabolites and associated enzymes.

Extraction and estimation of enzymes

150 mg of fresh weight that is, cotyledons, radicle and plumule were taken separately and washed in chilled distilled water and homogenized separately with a chilled pestle mortar in 5 ml of enzyme extraction buffer. Extraction medium contained 0.1 M phosphate buffer, pH 7.0; 0.25 mM ethylene diamine tetra-acetic acid (EDTA); 2.5 mM cysteine HCl and 2.5% polyvinyl pyrollidone (PVP). The extract was then centrifuged at 10,000 g at 4°C for 20 min. The supernatant was then used for the estimation of enzymes.

Acid invertase

Acivity of acid invertase was measured by estimation of total reducing sugars (glucose and fructose) by dinitrosalicylic acid (DNSA) using the method of Sumner (1935).

1 g of DNSA was dissolved in 20 ml of 2 N NaOH. Added was 50 ml of distilled water and 30 g of sodium potassium tartrate and the volume was made up to to 100 ml by adding distilled water. To a clean test tube, 0.4 ml of 0.2 M acetate buffer (pH 4.8), 0.25 ml of 0.4 M sucrose and 0.35 ml of approximately diluted enzyme extract were added to make a final volume of 1.0 ml. In the control tube, sucrose solution was added only when enzyme had been inactivated by boiling for about 5 min. After 30 min of incubation at 30°C, 1 ml of DNSA reagent was added. The test tubes were kept in a boiling water bath for 10 min and then final volume was made to 5.0 ml and absorbance was recorded at 560 nm. Standard curve was prepared using graded concentration of D-glucose (10- to100 µg/ml) with DNSA reagent. Enzyme activity was expressed in terms

of µg reducing sugars produced mg^{-1} protein.

Peroxidase

$$\text{Guaiacol} + H_2O_2 \xrightarrow{\text{Peroxidase}} \text{Oxidised guaiacol} + 2H_2O$$

The rate of formation of guaiacol dehydrogenation product is a measure of peroxidase activity and was measured spectrophotometrically at 436 nm.

3 ml (0.1 M) phosphate buffer was pipetted out in test tube and 0.05 ml guaiacol solution added followed by 0.1 ml of enzyme extract. Before assay the temperature was maintained at 25°C. Reaction was started by adding 0.1 ml H_2O_2, mixed well, placed the cuvette in the spectrophotometer, waited until the absorbance increased by 0.05, stop watch was started and the time required in minutes noted (Δt) to increase the absorbance by 0.1. The enzyme activity was expressed as unit min^{-1} mg^{-1} protein. One unit can be defined as the amount of enzyme which catalyses the conversion of one micromole of hydrogen peroxide per minute at 25°C.

Ascorbate peroxidise

Ascorbate peroxidase activity was measured (Nakano and Asada, 1981) by a modified spectrophotometric method based on the rate of decrease in absorbance of ascorbate at 265 nm during ascorbate oxidation. The assay was performed in 3 ml quartz cuvette containing 0.5 mM ascorbate, 0.1 M phosphate buffer (pH 7.0), 1 mM H_2O_2. Blank did not contain H_2O_2. To 0.3 ml of enzyme extract, 0.7 ml of 0.5 mM ascorbic acid was added and reaction was started by adding 0.1 ml H_2O_2. Decrease in absorbance was recorded at 10 upto 30 sec at 265 nm. The enzyme activity was expressed as unit min^{-1} mg^{-1} protein. One unit can be defined as one micromole ascorbate oxidized min^{-1} (mg of total protein)$^{-1}$.

Statistical analysis

Data analysed as factorial completely randomized design (CRD) using the software “OPSTAT was developed for comparison of treatment (http://hau.ernet.in/link/spas.htm). Critical difference (CD) was calculated at 5% level of significance. One way t-test was applied for significance of the results.

RESULTS

Peroxidase

Maximum peroxidase activity was observed in the untreated cotyledon of Arkel followed by Rachna and HFP-8712 (Figure 1). It increased significantly with increase in chromium treatment in all the tested cultivars. Increase in peroxidase activity was higher in Rachna followed by HFP-8712. Whereas peroxidase activity in untreated radicle was maximum in Rachna followed by Arkel and HFP-8712 (Figure 1). The activity was increased with the increasing dose of chromium treatment.

The maximum increase was observed in Rachna and least in Arkel. The same activity in untreated plumule was found to be comparable in all the tested cultivars (Figure 1). With the increasing dose of chromium treatment, same results were obtained as in case of plumule.

Ascorbate peroxidise

Arkel cotyledons which were not exposed to Cr (VI) showed the maximum activity of ascorbate peroxidase (Figure 2) and the value was significantly less by nearly 250% in HFP-8712. Low chromium treatment (1 mM) enhanced the activity of ascorbate peroxidase except in Rachna where it remained unaffected. Enzyme activity decreased consistently, in Arkel with the increasing dose of treatment, while converse was true with regard to HFP-8712. An increase in enzyme activity was evident by 2.0 mM Cr (VI) treatment in Rachna.

Activity of ascorbate peroxidase enzyme in untreated radicle was found to be maximized in Rachna (Figure 2) while, the activity was nearly identical in other two varieties. Activity of ascorbate peroxidase remained unaffected at lower concentration of 1.0 mM chromium except in Rachna which registered a significant increase at 1.0 and 2.0 mM Cr (VI). Chromium did not affect enzyme activity significantly in other two cultivars.

Plumular ascorbate peroxidase activity of untreated seedling was higher in Rachna followed by HFP-8712 and least in Arkel. The effect of chromium treatment was found to be statistically significant at high concentration (3.0 mM) in comparison to control in all cultivars.

Acid invertase

Activity of acid invertase in the untreated cotyledons was found to be maximam in Arkel followed by Rachna and least in HFP-8712 (Figure 3). Chromium treatment did not affect acid invertase activity significantly in Arkel but increased in Rachna and HFP-8712. The increase in enzyme activity was gradual in Rachna with the increase in substrate Cr (VI) and this gradual increase was evident up to 2.0 mM Cr (VI). However, there was sharp increase (that is, 250%) in activity by 3 mM Cr (VI) as compared to 2 mM treatment in HFP-8712.

Untreated radicle of Rachna showed the maximum activity of acid invertase (Figure 3). This was followed by Arkel and HFP-8712 showed the least activity. The enzyme activity decreased with the increasing dose of chromium treatment in Rachna while, activity remained unaffected by Cr (VI) treatment in Arkel and HFP-8712.

The activity of acid invertase was least in the untreated plumule in Arkel and maximum in Rachna (Figure 3). The activity of this enzyme, in general, decreased with the increasing concentration of chromium except Arkel. Activity of the enzyme remained unaffected by Cr (VI) treatment up to 2.0 mM in HFP-8712 and got enhanced in Arkel at 1.00 mM chromium. One way t- test analysis of results indicates that enzymes activities are significantly correlated with chromium concentrations in almost all the cases (Table 1).

DISCUSSION

Activity of peroxidase in general increased with the

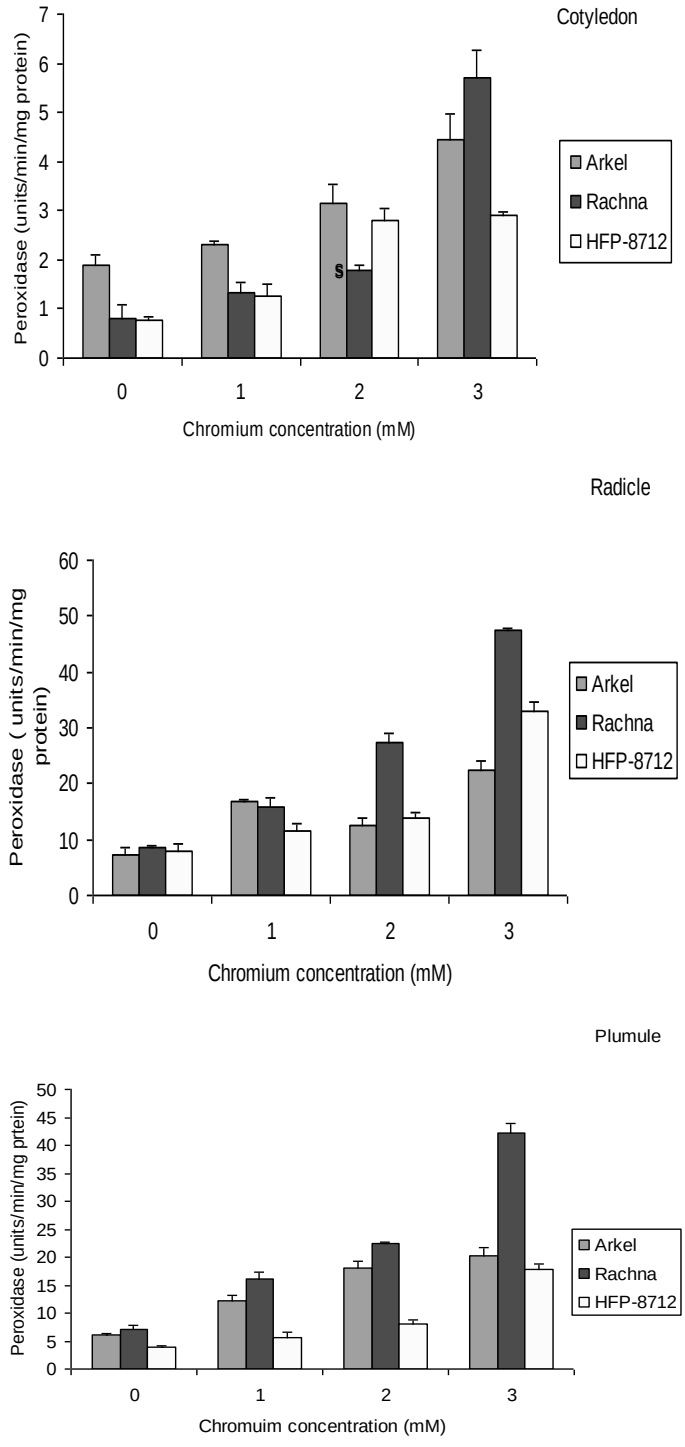

Figure 1. Effect of chromium Cr (VI) on activity of peroxidase in seedling components of pea after five days (Bars on top indicate standard error).

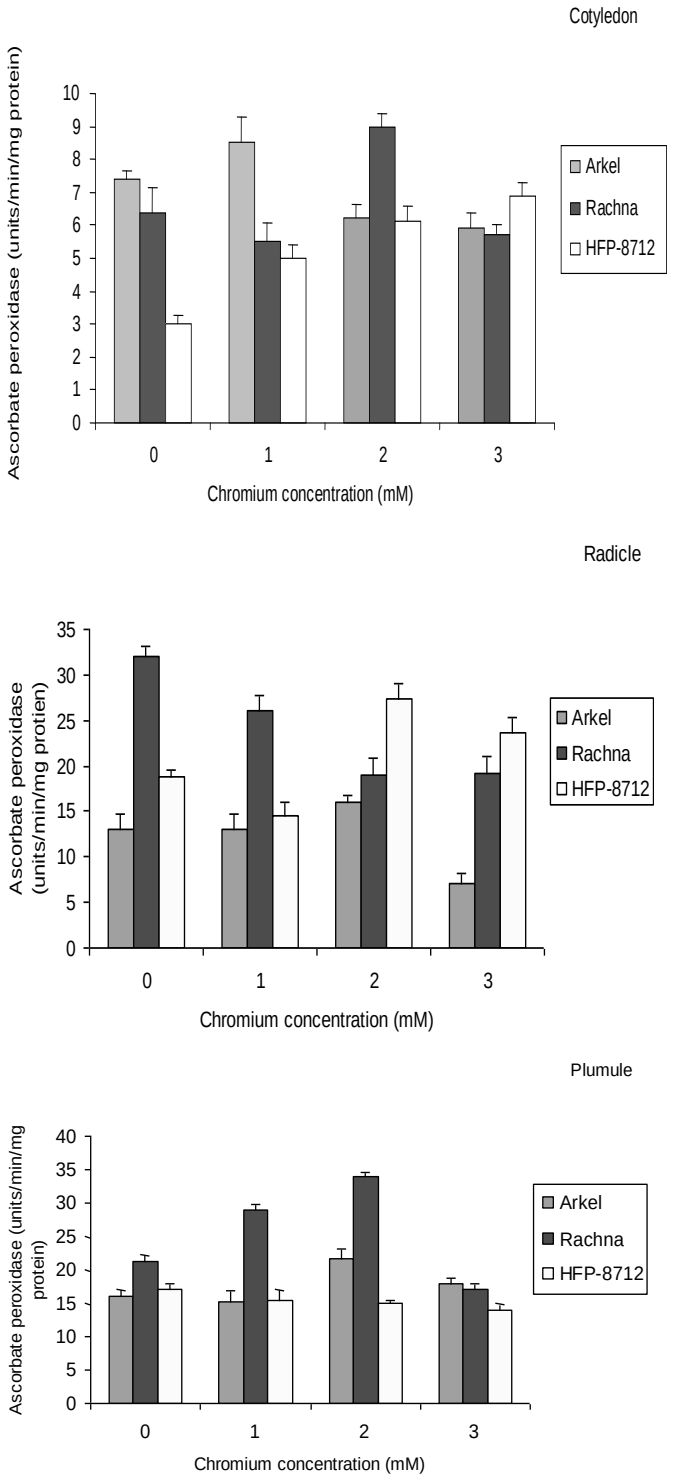

Figure 2. Effect of chromium Cr (VI) on activity of ascorbate peroxidase in seedling components of pea after five days of treatment (Bar on top indicate standard error).

increasing treatment of chromium in cotyledons, radicle and plumule in all the tested varieties. Similar results were reported in cadmium treated pea seedling (Divya, 1999). Pb (II) and Hg (II) treated rice seedling (Mishra and Choudhari, 1996) and chromium treated radish (Jayakumar et al., 2007). Conversely, Bhattacharjee (1998) reported a gradual decline in activity of peroxidase over untreated control in *Amaranthus*. Seedling under

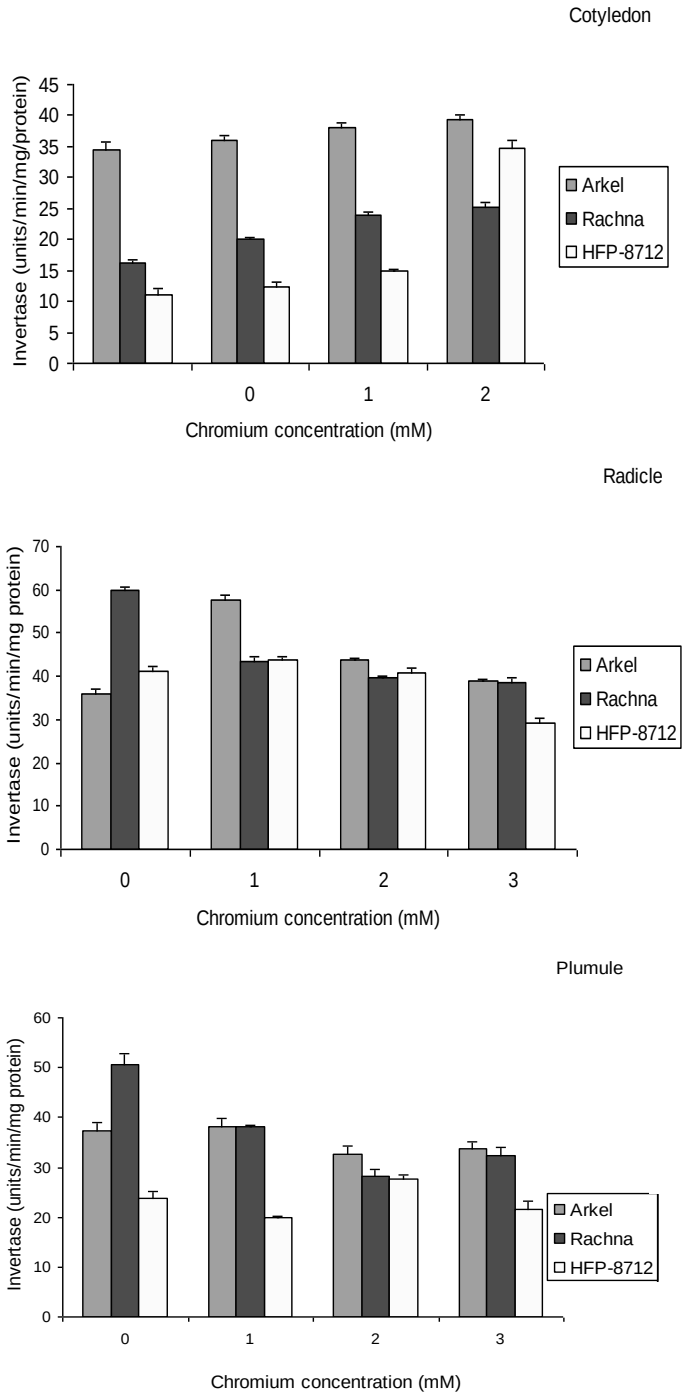

Figure 3. Effect of chromium Cr (VI) on activity of invertase in seedling components of pea after five days of treatment (Bar on top indicate standard error).

hypocotyls elongation in *Phaseolus vulagaris* and showed an increase in the peroxidase activity at both the chromium concentrations (Sumitra and Nayana, 2003).

Panda and Choudhury (2005) found that like copper and iron, chromium is also a redox metal and its redox behaviour exceeds that of other metals like Co, Fe, Zn, Ni etc. The redox behaviour of a metal has a direct involvement in inducing oxidative stress in plants. Chromium is a redox metal and its redox behaviour exceeds other metals like Co, Fe, Zn, Ni etc. (Sharma and Sharma, 1996) Chromium affects antioxidant metabolism in plants. Antioxidant enzymes like peroxidase (POX), catalase (CAT), guaiacol peroxidase (GPX) glutathione reductase (GR), ascorbate peroxidase (APX) and superoxide dismutase (SOD) are found to be susceptible to chromium resulting in a decline in their catalytic activities. Cell wall-bound peroxidases and Mn-superoxide dismutase (Mn-SOD) coupled together are known to generate hydroxyl radical (OH^-) from H_2O_2 which may in turn be derived from hydroxycinamic acid in the cell wall (Kukavica et al., 2009).

Peroxidase activity in radish leaves increased with increasing concentration of cobalt from 50 to 250 mg kg^{-1} soil. The peroxidase activity was minimum at 15 mg Co kg^{-1} of soil and highest at 50 mg Co kg^{-1} (Jayakumar et al., 2007). Sharma and Sharma (1996) found that the application of 0.05, 0.5 mM Cr in wheat cultivar cv. UP2003 decreased the activities of both enzyme catalase and peroxidise. Intracellular soluble peroxidases were found to be stimulated by Ni (II) toxicity more in the shoots at a lower concentration than in the roots (Pandolfini et al., 2006). Activity of ascorbate peroxidase increased in the cotyledons of Arkel and HFP-8712 at low level of chromium treatment but it remained unaffected in Rachna. Higher dose of chromium inhibited the activity of ascorbate peroxidase significantly in Arkel. On the other hand, an increase in ascorbate peroxidase activity was evident at 2.0 mM Cr (VI) in Rachna and HFP-8712. Higher dose of chromium (3.0 mM) inhibited the activity of enzyme. Radicle and plumule of Rachna showed the maximum activity of ascorbate peroxidase. Enzyme activity remained unaffected in response to various chromium treatments in plumule of Arkel, but showed a significant decrease in Rachna at 1.0 mM chromium and with a further rise in chromium levels, the activity remained unaffected in Rachna, Plumule of HFP-8712 also showed a decrease in enzyme activity at 1.0 mM chromium than control and thereafter remained unaffected by higher dose of chromium. Activity of asorbate peroxidase remained unaffected at low dose of chromium in case of radicle of Arkel and HFP-8712 and showed an increase in Rachna. But at the highest dose of chromium, radicle of all the tested varieties showed a decrease in enzyme activity. Similar results were reported by Sairam et al. (1998) and they found that abiotic stresses like water stress also increased the antioxidant enzyme like ascorbate peroxidase. Mn^{2+} toxicity resulted in increased activity of ascorbate peroxidase and this increase was more in susceptible genotype than tolerant genotype of bean. However, Divya (1999) reported a decrease in the activity of ascorbate peroxidase with increase in cadmium concentration in radicle and plumule of pea seedling and decrease was prominent in susceptible than the tolerant variety in roots than in shoots. The decline in activity of

Table 1. t-Test statistics of effect of chromium on the activities of the enzymes.

Enzyme	Pea cultivar	Seedling part	Chromium concentration			
			0	1	2	3
Invertase	Arkel	Cotyledon	2.15**	2.37*	2.60*	2.66*
	Rachna	Radicle	2.74*	2.97*	3.19*	3.24*
	HFP-8712	Plumule	2.59*	2.74*	3.25*	3.20*
Peroxidase	Arkel	Cotyledon	2.23**	5.19*	7.57*	6.02*
	Rachna	Radicle	2.97*	3.12*	2.68*	2.83*
	HFP-8712	Plumule	2.73*	2.58*	2.72*	2.58*
Ascorbate peroxidase	Arkel	Cotyledon	2.49*	3.23*	4.04*	6.45*
	Rachna	Radicle	2.40*	2.67*	3.04*	2.70*
	HFP-8712	Plumule	2.88*	2.66*	2.71*	3.78*

**Significant at 1% level of significance; *Significant at 5% level of significance.

this oxidative enzyme has been ascribed to inhibition of enzyme biosynthesis and the denaturation of enzyme proteins (Mohapatra, 1995).

Invertase is an important enzyme of carbohydrate metabolism and hydrolyses non reducing sucrose to reducing sugars like glucose and fructose. Chromium treatment did not affect acid invertase activity significantly in cotyledons of Arkel but increased it in Rachna and HFP-8712. On the other hand, radicle of Rachna showed the maximum activity of acid invertase followed by Arkel while HFP-8712 showed the least activity. The enzyme activity in general, decreased with increasing chromium concentration except in HFP-8712, where it showed an increase at 2.0 mM chromium. Acid invertase activity was least in untreated plumule of Arkel and maximum in Rachna which decreased with increasing concentration of chromium treatment. Dua and Sawhney (1991) also reported a decrease in the invertase activity with the increasing dose of chromium treatment. They found that Cr (VI) ions upto 1.0 mM in the assay mixture did not affect *in vitro* activity of invertase. The metal may be interfering with synthesis of this enzyme. Lowering of the activity of invertase would impair the capacity of cotyledon to generate monosaccharides from sucrose. More importantly, sucrose happens to be the principal form in which sugar are exported. Owing to the deleterious effects of chromium, reduced activity if invertase would restrict the ability of embryonic axis to hydrolyse the incoming sucrose and thus impair its growth. Jha and Dubey (2005) also reported an increase in acid invertase activity in endosperm as well as in embryonic axis under arsenic treatment (25 and 50 µM As_2O_3) in rice seedlings.

Stress condition in general lead to an increased production of toxic reactive oxygen species which is generated in plant cells during metabolic functions especially in chloroplast during photosynthesis. To counteract the hazardous effects of oxygen radical, all aerobic organisms have evolved a complex antioxidative defence mechanism comprising enzymatic constituents as well as free radical scavengers such as ascorbate, glutathione and tocopherol. Karkonen and Fry (2006) reported that ascorbate scavenged H_2O_2 in the culture medium of lignin producing *Picea* cells in spent and boiled spent medium Oxygen toxicity emerges when the production of reactive oxygen species exceeds the quenching capacity of natural protective systems due to adverse environmental conditions, such as chilling (Pinhero et al., 1998), drought, flooding and other stresses. Therefore, the antioxidant mechanism plays an important role not only in plant metal tolerance but also works in any kind of the abiotic stress (Jayakumar et al., 2007).

Conclusion

Acid invertase as well as peroxidase activity increased with the increase in concentration of chromium in the radicle and plumule of the tested pea cultivars except Arkel where it was not affected. The increase in peroxidase activity in Rachna was maximum. Activity of ascorbate peroxidase decreased in the cotyledons of Arkel, while the converse was true regarding HFP-8712. An increase in enzyme activity by 2.00 mM Cr (VI) in the radicle of Rachna was evident. These results indicate that Rachna is more tolerant followed by HFP-8712 and Arkel. The analyses of the results indicate that enzyme activities are significantly correlated with chromium concentrations in almost all the cases.

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