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EFFECT OF HEAVY METAL CADMIUM ON *TRITICUM AESTIVUM*: DETERMINATION OF PEROXIDASE AS BIOMARKER

Jagdishchandra K. Monpara, Kiran S. Chudasama* and Vrinda S. Thaker

*Plant Biotechnology and Genetic Engineering Laboratory, UGC-CAS Department of Biosciences, Saurashtra University, Rajkot- 360005, (Gujarat), India

Abstract

The environment is constantly being polluted by the accumulation of heavy metal contaminants and it adversely affects the health of humans and animals. These contaminants also pose a major threat to agricultural sector. Cadmium (Cd) is extensively used in electronic and other industries as an important heavy metal. The present investigation was conducted to determine the phytotoxic effect of heavy metal cadmium on *Triticum aestivum* L. seedling. The different concentration of cadmium (25ppm; 50ppm; 75ppm and 100ppm) was used. Various physio-biochemical parameters *i.e.*, fresh weight, dry weight, water content and length chlorophyll content, protein, peroxidase enzyme and phenolic content were studied. Further peroxidase of shoot and root was characterized by Native-PAGE. Dose dependent decrease in Dry weight, water content, length and chlorophyll content was observed while peroxidase and phenol induction increased with Cd concentration.

Key words: Cadmium, Heavy metal, Native page, Peroxidase, Wheat

Introduction

Heavy metal toxicity is one of the major recent environmental health problems, and potentially dangerous due to bioaccumulation through the food chain and in plant products for human utilization (Schickler and Caspi, 1999). Therefore, heavy metal contaminations of soils and plants have become an increasing problem. The most potent sources of heavy metals in the environment are the anthropogenic activities, *i.e.* mining, smelting procedures, steel and iron industry, chemical industry, traffic, agriculture and domestic activities (Elbagermi *et al.,* 2013). Heavy metals inhibit physiological processes of plants *i.e.* respiration, photosynthesis, plant-water relationship, cell elongation, N-metabolism and mineral nutrition (Zornoza *et al.,* 2002). Cd is known to be one of the most phytotoxic heavy metals and enters in agricultural soils mainly from industrial processes, phosphatic fertilizers and atmospheric deposition, and is then tansferred to the food chain (Ederli *et al.,* 2004; Pilon-Smits, 2005). The Cd in the soil is mainly absorb by roots and then transported across plant tissues, and finally accumulated in roots, shoots, fruits and grain (Qian *et al.,* 2009). Excess Cd accumulation in plants generally causes various symptoms of phytotoxicity, results in inhibititon of plant growth and development (Milone *et al.,* 2003). These negative effects of Cd on plants may be associated with that Cd interferes with several metabolic processes (Liu *et al.,* 2005). These changes are reflected by an increase in activity of certain enzymes that play an imperative metabolic role under conditions of metal stress.

Peroxidase (POX) induction is a general response of higher plants in uptake of toxic amounts of metals (Wei-Ching and Ching, 2000). It has been observed in root and leaves of various species after treatment of various heavy metals (Tarvainem *et al.,* 1991). Plant peroxidase is widely distributed in all higher plants and this enzyme is involved in various physiological processes (Hiraga *et al.,* 2001). Plants produce a many type of secondary products that contain a phenol group a hydroxyl functional group on an aromatic ring. Phenolics are often produced and accumulated in the sub-epidermal layers of plant tissues exposed to stress and pathogen attack (Clé *et al.,* 2008). Phenolics compounds can be used as a potential biomarker of pollution because they participate in plant's response to the accumulation of heavy metals, acting as antioxidants able to scavenge free radicals

***Author for correspondence :** E-mail : kiranchudasama@gmail.com

formed by metal ions (Bia³ oñska *et al.,* 2007).

In the present study, wheat (*Triticum aestivum* L.) is selected as a model plant. It is the most important crop in India. Wheat at seed germination and seedling stages are very sensitive to environmental factor. The presence of heavy metals in the soil is also a considerable influencing seed germination and seedling growth. Considering to this in the present study, assessed the effect of different concentrations of heavy metal cadmium on *Triticum aestivum* L. seedlings and estimate the growth and different biochemical parameter.

Materials and Method

Test Plant:

Certified seeds of *Triticum aestivum* L. were purchased from the local market, Rajkot. Equal sized seeds were screened, washed with tap water for 2-3 times and soaked in distilled water for 2 h. seeds were surface sterilized with 0.1% $Hgcl_2$ to prevent any fungal contamination. Seeds were washed with five time's in double distilled water immediately before use.

Heavy metals and their different concentrations

The stock solutions of, Cd, was prepared at concentrations of 25ppm, 50ppm, 75ppm, and 100ppm. Distilled water was used as a control. The seeds were then allowed to germinate in sterilized Petri dishes on Whatmann filter-paper moistened with 5 ml of selected heavy metal test solution and kept in dark for 36 h. Each Petri dish was contained 25 seeds. The experiment was conducted in a growth room at 20 ± 25^{0}C for 7 days under whitelight, Lux 1680 (114240×1020 photons m^2s^{-1}), 12 h photoperiod. For each metal concentration and control groups 25 seedlings were used.

Growth analysis

Growths is measured in the terms of fresh weight, dry weight, water content and shoot and root length. The length of shoot and root were recorded by using a centimeter scale. For the measurement of fresh and dry weights, freshly harvested shoot and root were taken. Freshly separated shoot and root were weighed before and after oven drying to a constant weight at 65^{0}C for 72 hours. The Water content of each stage was determined by difference in fresh and dry weights. Data were taken in 5 replicate and the calculated with ± standard deviations.

Tolerence Index (TI)

Tolerance index (TI) was determined as suggested by Iqbal and Rahmati (1992) using the following formula: TI = RLs *100 / RLc

RLs=Average root length in stress, RLc= Average root length in control

Phytotoxicity

The phytotoxicity (%) for shoot and root of 7 day old seedlings were calculated by the formula given by (Chou and Lin ,1976).

$$\%\,Phytotoxiaty\,shoot = \frac{shoot\,lenght\,of\,control - shoot\,lenght\,of\,treatment}{shoot\,lenght\,of\,control} \times 100$$

$$\%\,Phytotoxicity\,root = \frac{Root\,lenght\,of\,control - Root\,lenght\,of\,treatment}{Root\,lenght\,of\,control} \times 100$$

Determination of Chlorophyll content

Chlorophyll content was determined according to Arnon (1949) by the spectrophotometer. The two wavelengths of absorbance at 645 and 663 nm were recorded.

Extraction of enzyme

Enzymes were extracted from wheat seedlings at 7th day after germination. The root and shoot separated from each other. The root and shoot were collected and powdered with liquid nitrogen. Five gram of crushed material was homogenized within 15 ml 0.1M Tris buffer pH 8.0 in a prechilled mortar and pestle. The homogenized was centrifuged at 10,000 g for 15 min at 4^{0}C and supernatant served as enzyme source. Protein content was determined according to the method of Lowry *et al.,* (1951).

Peroxidase assay (POX)

POX activity was measured spectrophotometrically using guaiacol as a substrate, according to Thaker (1998). The assay mixture 2ml contained 50mM Na-acetate buffer (pH 5.0), 25mM guaiacol and 25mM H_2O_2. By addition of 100µl crude enzyme extract, the reaction initiated, and enzyme kinetics was measured at 470 nm.

Native PAGE analysis and Activity staining for Peroxidase

Native-PAGE was performed according to the method of Laemmli (1970). Peroxidase isoforms were visualized by soaking the gels in staining solution containing 5.8mM o-dianisidine dissolved in 95% ethanol and 50mM Sodium acetate buffer (pH 6.0). POX staining was initiated by addition of 3% H_2O_2 The staining was then fixed in Na- acetate buffer (pH 5.0) and the gel was washed with distilled water (Stegemann *et al.,* 1983).

Extraction of phenolic compound from the seedlings

One gram of control and treated shoot and root was taken and to this 10ml of 80% methanol was added. The mixer was kept under dark for 48 h on shaking condition. Next day the extracts were centrifuged at 5000 g for 15 min. The supernatant was collected in Petri plate and

allowed for evaporation. The remaining dry residue was dissolved in sterile distilled water (5ml) and used as a source of phenols.

Quantification of monohydroxy, O-dihidroxy and total phenol content

The concentration of total, monohydroxy and o-dihydroxy phenol content was determined according to Pansuria *et al.*, (2006). Measurements were carried out in triplicate, total phenol, monohydroxy and o-dihydroxy phenolic content was calculated using a calibration curve of chlorogenic acid, hydroxybenzene and pyrocatechol, respectively. Phenol content was expressed as µg/ g fwt.

Statistical analysis

Correlation coefficient was worked out between growth parameters (*i.e.* Fwt, WC, Dwt, Chlorophyll, phytotoxicity, tolerance index and protein content by using the Excel 2007. P values significant at 0.1 or less than that were considered for the data interpretation.

Results and Discussion

In the present study, growth measured by the physical parameter of fresh and dry weight, water content and length measurements are shown in table 1. The fresh weight, Dry weight and water content were expressed as mg seedling root^{-1} or shoot^{-1}. Fresh weight in control shoot and root was recorded 93.86 mg and 78 mg respectively, and then decreased with increasing the concentration of Cd. Similarly, dry weight in control shoot and root was decreased with increasing the concentration of Cd treatment. Water content in control shoot was 83.38 mg and then decreased in treated plant up to 48.4 mg in 100ppm similar pattern was observed in root. Vajpayee *et al.,* (2001) was found that dry matter production affected by chromium concentration above 2.5 mgL^{-1} Ag in a nutrient medium. In our study, varied concentrations of Cd affected fresh weight of wheat was observed. The reduction in the growth of wheat seedling could be the suppression of the elongation growth rate of cells, because of an irreversible inhibition exerted by Cd on the proton pump responsible for the process (Rumana *et al.,* 2014). Amount of water per cell/tissue or organ play important role in the growth and development (Rabadia *et al.,* 1999). Direct correlation between Dwt accumulation and water content is observed in many studies (Gokani and Thaker, 2002; Chudasama and Thaker, 2007).

In this work shoot and root length decreased with increasing the concentrations of cadmium treatment. The average shoot length of seedling was between 11.3-7.3 cm, due to increased concentration of Cd. The average root length of wheat was decreased from 11.2-4.0 cm, due to increased concentration of, Cd, (table 1). Root length of wheat was significantly inhibited at all the treatments of heavy metals as compared to control. The main reason for high root sensitivity to Cd might be related to the fact that root is the first organ exposed to Cd and therefore, accumulates metal at much higher concentrations than the shoot (Tiryakioglu *et al.,* 2006). Cd is known to cause physiological drought by altering the plant water balance, nutrient uptake and permeability of plasma membrane which in turn affect cell enlargement and resulted in reduced growth (Hayat *et al.,* 2011).

The phytotoxicity of shoot and root are presented in table-1. Phytotoxicity of shoot and root was decreased at lower concentration (25 ppm) and increased at higher concentration (100ppm) thus showed dose dependent responsed. The phytotoxicity of shoot of wheat was

Table 1: Effect of different concentrations of cadmium on wheat seedling growth, Tolerance index and phytotoxicity.

Shoot						
Concentration ppm	**Fresh Weight (mg)**	**Dry Weight (mg)**	**Water Content (mg)**	**Length (cm)**	**Phytotoxicity (%)**	**Tolerance index**
Control	93.86±1.5	10.48±0.7	83.38±0.2	11.3±0.1	-	-
25	78.00	9.78±1.1	68.22±1.7	10.3±1.3	8.0	91.37
50	64.9±1.2	7.88±0.5	57.02±0.3	8.9±0.5	21.23	78.40
75	62.74±1.1	8.42±1.2	54.32±0.2	8.4±0.5	25.66	74.33
100	55.04±0.5	7.0±0.2	48.04±0.6	7.3±1.1	35.39	64.89
Root						
Control	78	10.62±0.2	67.38±0.5	11.2±0.5	-	-
25	73.82±0.4	5.84±1.1	67.98±0.8	6.3±0.2	44	56
50	65.58±0.1	6.06±0.3	59.52±0.7	5.7±0.2	49	51
75	60.78±1.0	5.46±0.2	55.30±0.1	4.9±0.4	56	44
100	39.48±0.5	4.06±0.9	35.42±1.1	4.0±0.1	64	35

Table 2: Correlation coefficient 'r' between seedling growth, phytoxocity, Tolerance index and Chlorophyll content.

Variable	FW shoot	FW root	DW shoot	DW root	WC shoot	WC root	Shoot length	Root length	Phytoxocity shoot	Phytoxocity root	Tolerance Index shoot	Tolerance index root	Total Chl
FW shoot	1												
FWroot	0.86***	1											
DWShoot	0.95***	0.90***	1										
DWroot	0.81***	0.99***	0.87***	1									
WC Shoot	0.99***	0.86***	0.95***	0.80***	1								
WCRoot	0.81***	0.99***	0.87***	1.000	0.80***	1							
Shoot length	0.98***	0.93***	0.97***	0.89***	0.97***	0.89***	1						
Root length	0.95***	0.762***	0.84***	0.68***	0.96***	0.68***	0.89***	1					
Phytoxocity shoot	-0.995	-0.939	-0.938	-0.945	-0.996	-0.945	-1.000	-0.960	1				
Phytoxocity root	-0.940	-0.966	-0.844	-0.967	-0.947	-0.967	-0.967	-0.999	0.96***	1			
Tolerance index shoot	0.99***	0.93***	0.94***	0.94***	0.99***	0.94***	1.000	0.95***	-1.000	-0.967	1		
Tolerance index root	0.92***	0.97***	0.83***	0.97***	0.93***	0.97***	0.95***	1.000	-0.960	-0.999	0.95***		
Total chl	**0.90*****	**0.994**	**0.92*****	**0.97*****	**0.90*****	**0.97*****	**0.96*****	**0.80*****	**-0.965**	**-0.984**	**0.96*****	**0.98*****	**1**

Note: * Significant at $P<0.05$, ** Significant at $P<0.01$, *** Significant at $P<0.001$

increased from 8.0-35.39 %, due to increased concentration of Cd. Similarly phytotoxicity of wheat root was increased from 44- 64 %, due to increased the concentration of Cd treatment. Tolerance index calculated on the basis ratio of root length ratio of treated to that of control shows gradual reduction of tolerance index during growth in all treatments. The table -1 shows indices of tolerance for shoot and root at different concentration treatments of Cd. The Tolerance index of shoot and root was decreased from 91.37 to 64.89 and 56 to 35, respectively due to treatments with increased concentrations of Cd (table-1). Higher rate of metal uptake by roots and its translocation to shoots might be the cause of the decrease in seedling growth and biomass production and therefore the potential of root growth have been proven to be an index of metal tolerance in plants (Subin and Francis, 2013).

The changes in chlorophyll a, chlorophyll b and total chlorophyll content are presented in figure-1. In this work the content of chlorophyll a, chlorophyll b and total chlorophyll was significantly decreased as the Cd concentrations increased. Control leaf shows 6.4 ± 0.5 and 2.55 ± 0.42 and 8.0±0.1 mg g^{-1} chlorophyll a, b and total contents respectively. The maximum decline in chlorophyll a was observed at 100 ppm 1.2±0.13 mg g^{-1} concentrations. The Chlorophyll-b content was equal in control, 25ppm and 50ppm then after decreased continuously up to 100ppm concentration. Total chlorophyll content was maximum observed in control (8.0 ± 0.1) then after gradually decline at 25-100ppm Cd concentrations. Chlorophyll content showed close correlation with fresh weigh and dry weight (table 2).

Similar results have been obtained by several workers working on various crops. Several report have shown that under Cd stress, decrease chlorophyll content in leaf garden grass, almond seedling, groundnut and black gram (Nada *et al.,* 2007; Nagajyoti *et al.,* 2008; Singh *et al.,* 2008; Gill *et al.,* 2012). Previously Rascio *et al.,* (1993) reported that cadmium is an effective inhibitor of plant metabolism, particularly photosynthetic processes and chloroplast development in higher plants. Cadmium damages the photosynthetic apparatus, in particular the light harvesting complex II and photosystems I and II (Krupa, 1988; Siedlecka and Krupa, 1996). In the present

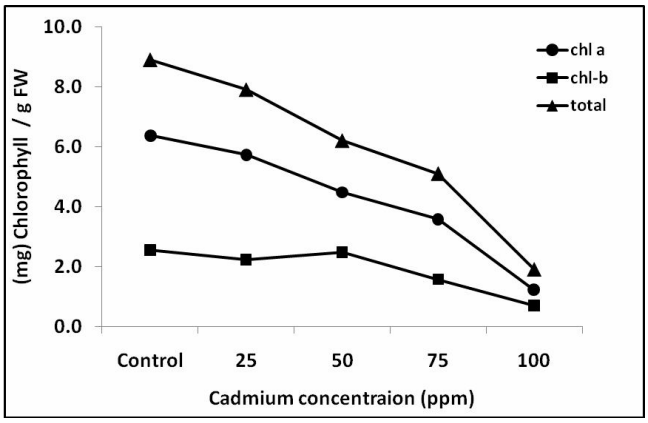

Fig. 1: Changes in Chlorophyll content of Chlorophyll a, Chlorophyll b and Total in control and treated wheat seedling exposed to cadmium

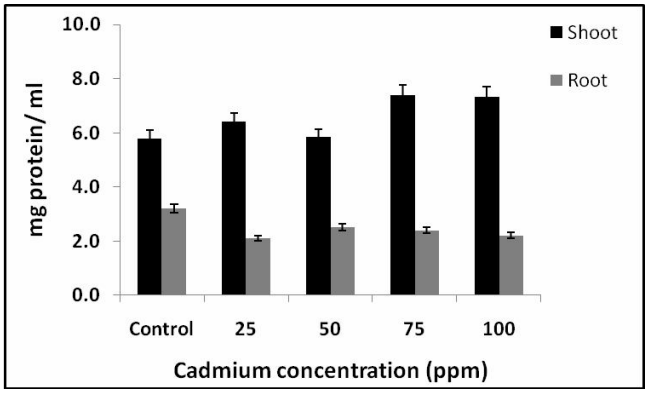

Fig. 2: Changes in protein content of Shoot and Root in control and treated wheat seedling exposed to cadmium

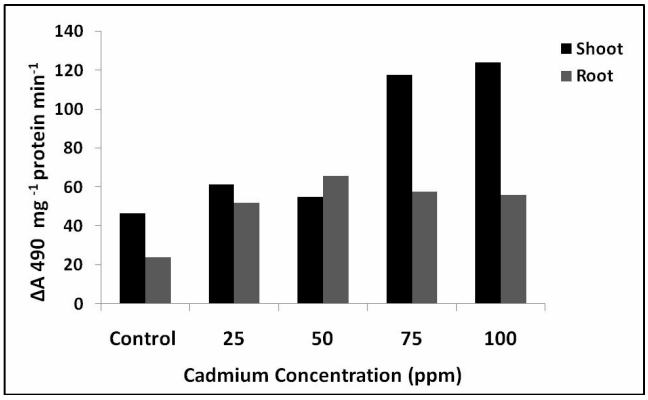

Fig. 3: Changes in Peroxidase activity Shoot and Root in control and treated wheat seedling exposed to cadmium

work the finding of a difference in chlorophyll content may be due to a change of chloroplast structure and thylakoid membrane composition under heavy metal stress conditions.

Protein concentration estimated from control and treated shoot and root are presented in fig. 2. The protein concentration in control and treated shoot was observed

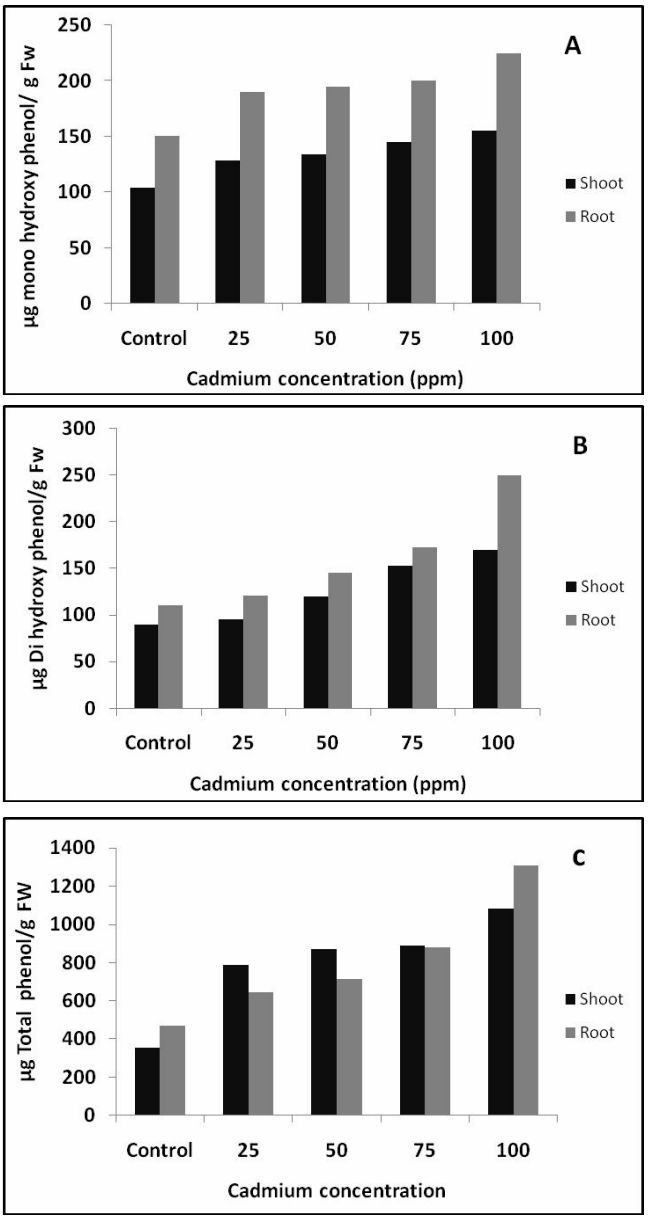

Fig. 4 : Changes in phenol content (A) Mono hydroxyl (B) Di hydroxy (C) Total in Shoot and root of control and treated wheat seedling exposed to cadmium

in the range of 5.8 -7.7 mg/ml. The protein content in root was observed between 3.2 - 2.2 mg/ml. In this work the total protein content of shoot was increased by raising the Cd concentration. Earlier (Heiss *et al.,* 2003) reported that protein accumulation in leaves was also seen in other plants treated with heavy metals.

The increased in total soluble protein content under heavy metal stress may be correlated to induce the synthesis of stress enzymes (Mishra *et al.,* 2006). Our results suggest that induction of total protein under Cd stress indicates that wheat shoot is adapted to produce specific proteins during metal stress conditions. In

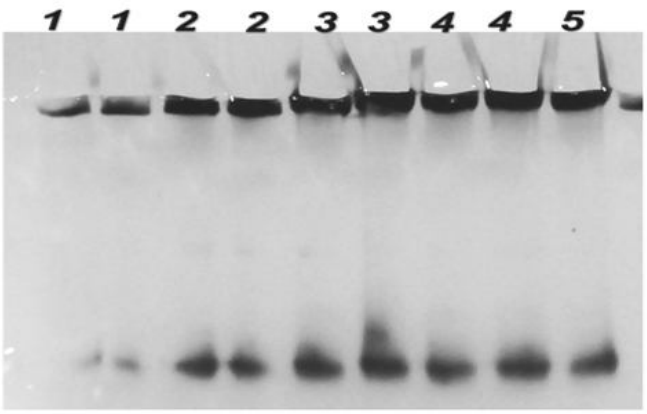

Fig. 5: Native profile of control and treated leaf peroxidase (1) Control (2) 25ppm (3) 50ppm (4)75ppm (5)100ppm

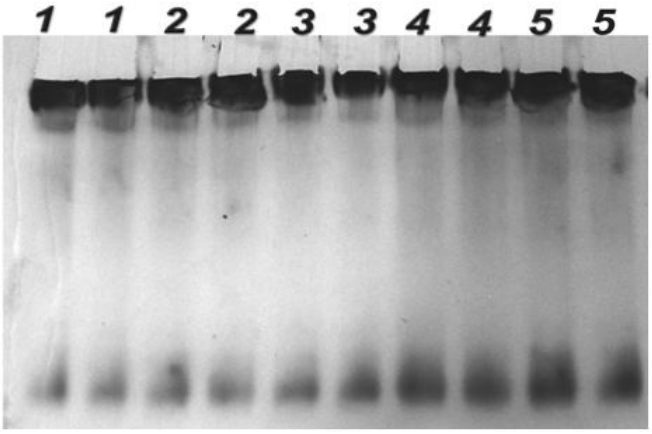

Fig. 6: Native profile of control and treated root peroxidase (1) Control (2) 25ppm (3) 50ppm (4) 75ppm (5)100ppm

contrast, under the Cd treatments, protein content in root was decreased steadily. Romero-Puertas *et al.,* (2007) reported that cadmium is able to decrease the protein content by inhibiting the uptake of Mg and K and promote posttranslational modification, decrease in synthesis or increase in protein degradation and the prevention of rubisco activity (Monteiro *et al.,* 2009).

Varying pattern of peroxidase activity was recorded in the control and treated seedlings**.** The changes of peroxidase activity in shoot and root with or without Cd treatment is shown in fig. 3. The shoot POX activity was gradually increased by raising the concentration of Cd. In root the activity was steadily increased up to 50 ppm and then declined at 75 and 100 ppm concentration. Peroxidase induction is a general response of higher plants after uptake of lethal quantities of metals (Assche and Clijsters, 1990). There are several reports that cadmium induced the activity of peroxidase (POX) in, *Cicer arietinum*, *Bacopa monniera,* and in the leaves of *Calamus tenuis* (Khan and Patra, 2007; Hayat *et al.,* 2007; Hasan *et al.,* 2007). In addition, POD participating in lignin biosynthesis can make a physical barrier against toxic heavy metals (Zhang *et al.,* 2007). This enzyme is suggested to be involved in lignifications of a cell wall, in the hypersensitive response and it was shown that increase in their activity protected plants against various stress factors.

In the present study, the protein samples of control and treated wheat root and shoot were analyzed by Native-PAGE for separation of POX isoforms. The native PAGE analysis of the shoot POX enzyme showed the two isoforms in control and treated leaf (fig. 5). But the intensity of isoform-bands were more in treated with Cd (25, 50, 75 and 100 ppm) compared to control. The native PAGE analysis of the root peroxidase enzyme showed the three isoforms in control and Cd treated root. But the intensity of isoform bands was more in treated root (fig. 6). The similar result of native PAGE profile was reported by (Rastgoo *et al.,* 2011), where they studied the differential expression of POX isoforms in *Aeluropus littorali* when treated with heavy metal.

The total phenols, monohydroxy and dihydroxy phenols from control and Cd treated shoot and root were expressed as µg phenol/g fwt (fig. 4). Phenolic content of plants treated with cadmium is significantly increased in comparison to the control. In this study increasing Cd toxicity, total, mono and Di phenol was also increased in dose-dependent manner. Enhancement of phenolic synthesis has been reported by environmental stresses. The effect of heavy metal stress on phenolics metabolism in plants has been studied extensively (Dýìaz *et al.,* 2001; Sgherri *et al.,* 2003). Synthesis of phenolic compounds under heavy metal stress is due to their high tendency to chelate metals, which is because to the presence of -OH and -COOH groups that bind to metal (Jung *et al.,* 2003). In addition, they expand their role in stress defense as radical scavengers, increase the activity of enzymes involved in phenolics synthesis (Michalak, 2006). Induction of phenolic accumulation observed in this study, because of their functions as intermediates in lignin biosynthesis and to protect the plant cells by building a physical a barrier.

Correlation coefficient was calculated for seedling growth showed fresh weight, root length, shoot length and tolerance index of seedlings were significantly and positively correlated with chlorophyll content. All interactions were highly significant ($P<0.001$) table 2. Phytotoxicy of shoot and root were negatively correlated with fresh weight, Dry weight and water content.

The study result clearly reveals Cd is inhibitory with respect to seed germination and early seedling growth in *Triticum aestivum*. The intensity of inhibition was directly

proportional to the concentration of Cd solutions employed. High concentrations of Cd treatment is found responsible for decreasing the percentage of tolerance indices in *T. aestivum* and that was clearly evident from the inhibition of shoot and root growth. Cd uptakes by the roots and their translocation to shoots at higher concentration might be the cause of drastic decline in seedling growth and biomass production. Increase in total protein in shoot with treatments suggests that there are some special proteins; they may have active roles in the tolerance of this plant to high concentrations of heavy metals. Cadmium also inhibits chlorophyll content, thus affecting photosynthetic capacity of the plant. The result of the present study also indicates that cadmium toxicity caused an enhancement in the production and activity of peroxidase enzyme and phenol content compared to healthy control seedlings. The induction of peroxidase and phenol may be a part of defense mechanism to protect the plant from heavy metal stress. Thus the present investigation showed that root length, chlorophyll, peroxidase and phenol can be used as biomarker for detection of metal stress on the plant. However, more study is required at the cellular and molecular levels for understand the mechanism of Cd toxicity.

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