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Response of Antioxidative Enzymes to Cadmium Stress in Leaves and Roots of Radish (*Raphanus sativus* L.)

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Abstract

Presented study has demonstrated that exposure of plants to toxic heavy metal Cd results a reduction in plant growth. Varied concentrations of $CdCl_2$, ranging from 0.0 to 50 ppm in the germinating media reduced leaf area of radish plant, chlorophyll and carotenoid contents. Greater loss of chlorophyll b content than chlorophyll a was observed especially under 50 ppm Cd exposure. With regards to the distribution of Cd in roots and leaves, the obtained data showed that the maximum accumulation of Cd occurred in roots followed by leaves. Generally, Fe, Zn, Mn and Cu declined in leaves compared to the roots. Furthermore, substantial increases were observed in antioxidant enzymes, such as catalase (CAT), glutathione S-transferase (GST) and peroxidase (POD), in Cd-stressed plants in comparison with control. The Cd stress also induced several changes in CAT and POD isozyme profiles and enhanced their activities. The results suggest that the reduction of leaf area and pigment content together with antioxidant enzymes and isozyme patterns can be used as indicators to Cd contamination.

Keywords: cadmium, radish, chlorophyll, catalase, peroxidase, glutathione S-transferase

Abbreviations: CAT: Catalase; POD: Peroxidase; SOD: Superoxide dismutase; GSH: Reduced glutathione; CDNB: 1-Chloro-2,4-dinitrobenzene; GST: Glutathione S-transferase; ROS: Reactive oxygen species; H_2O_2: Hydrogen peroxide; •OH: Hydroxyl radicals; $O2^{\cdot -}$: Superoxide radical; PAGE: Polyacrylamide gel electrophoresis; PAA: Polyacrylamide

Introduction

Cadmium (Cd) is one of most toxic pollutants found in air, water and soil and is non-essential for plants. Cd interacts with photosynthetic, respiratory and nitrogen metabolism on plants resulting in poor growth and low biomass accumulation growth inhibition, reduction of root length, leaf area and even plant death, although the mechanisms involved in its toxicity are still not completely understood (Sanitá di Toppi and Gabbrielli, 1999; Snadalio *et al.,* 2001). Cd produces oxidative stress possibly by generating free radicals and reactive oxygen species (ROS) as superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH) (Hendry *et al.,* 1992; Halliwell and Gutteridge, 1989). These toxic species react with lipids, proteins, pigments, and nucleic acids and cause lipid peroxidation, membrane damage, inactivation of enzymes, thus affecting cell viability for their protection. In order to deleterious effect of ROS, plant cells are equipped with several antioxidant enzymes and antioxidant compounds as sited by Dixit *et al.* (2001). Cd accumulates immediately in roots, later in the stem and finally in the leaves (Dixit *et al.*, 2001). Photosynthesis is also sensitive to Cd, chlorophyll being one of the targets as well as enzymes involved in CO_2 fixation (Somashekaraiah *et al.,* 1992). Cadmium toxicity is also correlated with disturbances in the uptake and distribution of macro- and micro-nutrients in plants (Gussarson *et al.*, 1996). The accumulation of oxygen free radicals mediated oxidative damage is prevented in the cell by peroxidase (POD), particularly in the cell wall, or by catalase (CAT) in the peroxisome (Azevedo *et al.,* 1998; Polidoros and Scandalios, 1999). Glutathione is also a precursor of phytochelations. The conjugation of glutathione to a variety of hydrophobic, electrophilic and cytotoxic substrates is accomplished by multifunctional enzymes glutathione S-transferases (GSTs). These enzymes play a regulatory role in heavy metal-induced oxidative stress (Dixit *et al.*, 2001). Cd toxicity can also modulate the expression of isozymes in plant tissue. For example, Van Assche and Clijsters (1986) detected two isoperoxidases in both roots and leaves which were absent in the control *phaseolus* plant. The aim of the present study was to explore the possibility of using some biochemical and molecular methods for the prediction of Cd toxicity on radish plant.

Materials and methods

Plant material and stress conditions

The healthy, homogenous seeds of radish (*Raphanus Sativus* L.*)* were subjected to surface sterilization with 0.1% sodium hypochlorite solution for 10 min and then rinsed with double distilled water. After 24 h imbibitions of seeds in water, seedlings were raised in sand cultures in

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plastic pots saturated with either Hoagland nutrient solution (Hogland and Arnon, 1950), which served as control or nutrient solutions supplemented with $CdCl_2$ to achieve concentrations of 1, 2.5, 5, 10, 25 or 50 ppm Cd^{2+} which served as treatment solution. Pots were maintained at field saturation capacity and received control and respective treatment solutions when needed to saturate the sand and the plants were watered as needed. Pots were kept for growth of seedlings at 28±1°C under 80% relative humidity and 12 h photoperiod with 40-50 $\mu mol^{-2}\ s^{-1}$ irradiance. All treatments were tested in four replications. For further study, the plant samples were collected at 40 day from pot culture and the biochemical parameters were analyzed.

Leaf area

Leaf area was estimated by leaf area-meter model LI-3000.

Determination of Cd content

To determine the amount of absorbed cadmium in the seedling, fresh root/shoot samples were surface sterilized with 1 M HCl and then with 1 mM Na_2EDTA to resolve excess surface bound Pb and then dried in oven at 70°C for 3-4 days. Dried samples were ground to a fine powder in a mortar and pestle and digested using the $HNO_3/HClO_4$ digestion method. Digested samples were dissolved in deionized distilled water and lead content was estimated using atomic absorption spectrometer (Unicam Sp 1900 model). Pb content of roots and shot was calculated in mg $g\ DW^{-1}$, where DW stands for dry weight.

Pigments contents

Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoids were extracted and estimated according to the method of Lichtenthaler (1987). About 100 mg of leaves from each Pb treatment was cut into tiny segments and kept in 10 ml of chilled 80% acetone in a capped glass tube. After 48 h extraction in dark at 4°C, the leaf segments were well-extracted for residual pigments. The contents of Chl *a*, Chl *b* and carotenoids were measured at 666 nm, 653 nm and 470 nm, respectively. Pigment contents were calculated in mg $g\ FW^{-1}$.

Preparation of crude enzyme extracts

The method described by Vitória *et al.* (2001) was used to prepare the crude enzymes extracts. In this method, roots' and leaves' tissues were homogenized in a chilled pastel and mortar with 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DL-dithiothreitol and 5% (w/v) insoluble polyvinyl pyrrolidone. The homogenates were centrifuged at 10.000 g for 30 min and then the supernatants were kept stored in separate aliquots at -20°C until the analysis.

Protein determination

Soluble protein was estimated by using the Coomassie Brillent Blue G-250 reagent according to the method of Bradford (1976) with bovine serum albumin as standard.

Antioxidant enzyme activities

Peroxidase (POD) activity was assayed according to the method of Hemeda and Klein (1990). A 100 ml of reaction mixture contained 10 ml of 1% guaiacol (v/v), 10 ml of 0.3% H_2O_2 and 80 ml of 50mM phosphate buffer (pH 6.6). Enzyme extract (75 µl) was added to the reaction mixture in a final volume of 3 ml. The increase in absorbance due to oxidation of guaiacol (extinction coefficient 26.6 $mM^{-1}\ cm^{-1}$) was monitored at 470 nm. Enzyme activity was expressed as units $min^{-1}\ mg^{-1}$ protein.

For measurement of the catalase (CAT) activity the method of Aebi (1983) was used. The 3 ml reaction mixture comprised of 50 mM sodium phosphate buffer (pH 7.0), 20 mM H_2O_2 and a suitable aliquot of enzyme. Decrease in the absorbance was taken at 240 nm (molar extinction coefficient of H_2O_2 was 0.04 $mM^{-1}\ cm^{-1}$). Enzyme activity was expressed as units $min^{-1}\ mg^{-1}$ protein.

Glutathione S-transferase (GST) was determined using the method of Habig and Jacoby (1981). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 6.5), 1 mM EDTA, 1 mM 1-chloro 2,4-dinitrobenzene (CDNB), 1 mM reduced glutathione and enzyme extract. The enzyme activity was measured at wave length of 340 nm. The activity of the enzyme was calculated using the extinction coefficient of the conjugate 9.6 $mM^{-1}\ cm^{-1}$ and expressed as units $min^{-1}\ mg^{-1}$ protein.

Native gel electrophoresis and isoenzyme staining

PAGE for peroxidase POD and catalase CAT isoenzymes assay was performed with 7% (w/v) polyacrylamide gel as described by Laemmli (1970). POD isoenzymes were detected by the Ros Barcelo method (Ros Barcelo, 1987). The gels were rinsed in water and the gel was stained in a solution containing 0.06% (v/v) H_2O_2, 0.1% (w/v) benzidine and 0.1% (v/v) acetic acid at room temperature till it reached the brown colour. CAT isoenzymes were detected by the Woodbury method (Woodbury *et al.* 1971). Gels were incubated in 0.01% H_2O_2 for 10 min and developed in a 2% (m/v) $FeCl_3$ and 2% $K_3Fe(CN)_6$ (m/v) solution for 10 min until the colourless bands appeared.

Statistical analysis

Statistical analysis were done using SPSS (version 10) program. Mean and standard error were descriptive measures of quantitative data using the analysis of variance test (ANOVA) for independent samples. $P \leq 0.05$ were considered significant. Values reported here are means of three replicates.

Results and discussion

Leaf area

Results pertaining to the effects of various concentrations of Cd on the average of leaf area of radish at harvest time under prevalent treatments are represented on Fig. 1. Data showed that the leaf areas of radish plants reflect the toxicity effects of cadmium. In low concentrations of Cd (1 and 2.5 ppm), slightly effects were noticed. In these treatments, the reduction of leaf areas reached 62.66 and 56.36 cm^2, respectively. Moderately inhibitory effects were seen in other Cd treatments (5, 10 and 25 ppm). At these concentrations, the values of leaf areas reached 51.13, 46.36 and 41.56 cm^2 with Cd treatments, respectively. At high concentration of Cd (50 ppm) very strong inhibitory effects were observed and reached the maximum value (31.87 cm^2). The reduction of leaf area could be attributed to toxic Cd levels, which induce negative effects on some key metabolic processes coupled to growth in radish (Van Assche *et al.,* 1984). The impact of Cd uptake by living cells has been shown to be drastic, normally leading to cell death depending on metal dose and time-length of exposure (Vitória *et al.,* 2001).

Cd accumulation and micronutrient contents

Regarding to the data of cadmium accumulation in Tab. 1, gradually increases in Cd accumulation in roots and leaves could be noticed,while Cd content in growth media was increased. The Cd concentration was ranged between 1.3 and 112 ppm in leaves and 5 to 206 ppm in roots. It means a progressive accumulation of Cd in roots which was translocated to leaves. Values in Tab. 1 also indicated that the concentration of Fe, Zn, Mn and Cu were decreased by increasing Cd concentration in the growth media. The lowest concentrations of micronutrients were found at 50 ppm Cd in both leaves and roots. Nevertheless this increase is not toxic to the plant and may be rendering to continuous addition of nutrient solution but its translocation from the roots to the leaves differ from one element to other. These results were in harmony with those obtained by several authors in many plants including radish (Vitória *et al.,* 2001; Mohamed *et al.,* 2009), peas (Dixit *et al.,* 2001; Sandalio *et al.,* 2001), maize and pea plants (Lozano-Rodriguez *et al*., 1997), groundnuts (Stefanov *et al.,* 1995) and beans (Wouter *et al*., 2002). They found an accumulation of cadmium in plants with increasing cadmium concentration in the growth media.

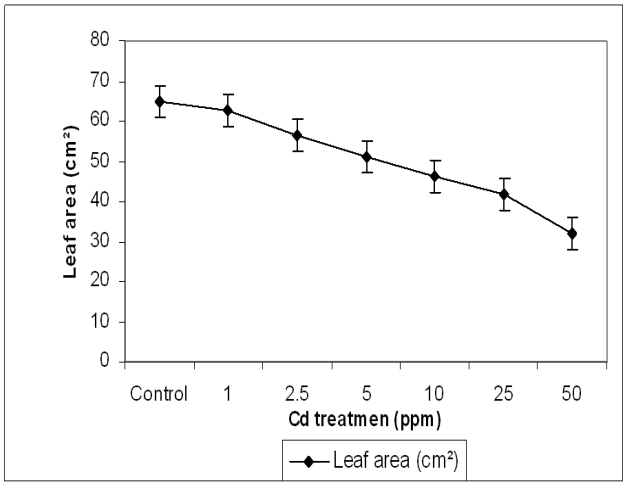

Fig. 1. Effect of various concentrations of Cd (as $CdCl_2$) on the average of leaf area (cm^2) of radish plants grown in sand culture at the harvest time (40 days)

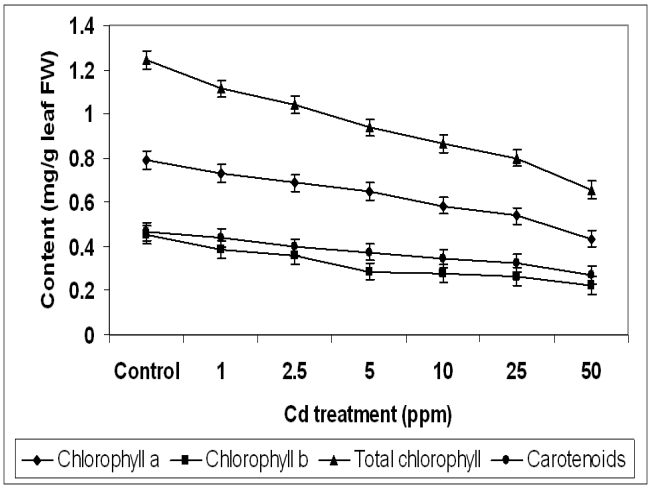

Fig. 2. Levels of chlorophyll and carotenoid contents (mg g^{-1} leaf FW) of Cd treated radish plants grown in sand culture at the harvest time (40 days). FW: fresh weight

Tab. 1. Cd accumulation and micronutrient contents in leaves and roots of radish plants at harvest time (40 days of growth)

Treatment (ppm)	Cd		Fe		Zn		Mn		Cu	
	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
0	1.3±0.1^{f}	5±0.5^{g}	817±40^{a}	948±23^{a}	36.3±1.2^{a}	18±1.5^{c}	327±15^{a}	302±7.1^{a}	39±1.5^{a}	77±4.6^{a}
1	12±0.5^{e}	30±4.0^{f}	755±39^{a}	711±18^{b}	33.3±0.6^{a}	21±1.0^{abc}	270±36^{b}	281±4.0^{b}	32±1.7^{b}	66±3.5^{b}
2.5	15±0.6^{e}	50±5.0^{e}	657±40^{b}	700±8.0^{b}	32.6±2.1^{a}	20±1.7^{bc}	183±14^{c}	257±6.7^{c}	28±1.0^{c}	59±3.6^{c}
5	23±0.6^{d}	91±4.2^{d}	617±16^{bc}	641±13^{c}	22.0±2.7^{b}	21±1.8^{abc}	145±8.1^{d}	220±11^{d}	24±0.6^{d}	51±2.0^{d}
10	41±2.1^{c}	112±5.0^{c}	555±30^{cd}	566±11^{d}	20.6±1.2^{b}	24±1.1^{a}	136±5.1^{de}	143±7.5^{e}	19±1.1^{e}	40±1.5^{e}
25	60±4.5^{b}	148±9.0^{b}	517±42^{d}	462±8.0^{e}	16.6±3.5^{c}	22±1.6^{ab}	109±8.1^{ef}	121±3.1^{f}	16±1.5^{f}	35±1.2^{f}
50	112±1.5^{a}	206±2.0^{a}	400±66^{e}	310±7.0^{f}	13.2±3.0^{c}	18±1.3^{c}	98±7.8^{f}	105±7.0^{g}	12±1.1^{g}	30±0.6^{g}
LSD	3.490	8.601	72.831	24.047	3.855	2.795	29.12	12.13	2.228	4.87

$^{a, b, c}$, Means within column different litter differ significantly (P> 0.5) from each other

Tab. 2. Specific activities of antioxidant enzymes in leaves and roots of radish plants

Treatment (ppm)	CAT unit mg protein^{-1}		GST unit mg protein^{-1}		POD unit mg protein^{-1}	
	Leaves	Roots	Leaves	Roots	Leaves	Roots
0	5.72±0.76^{e}	3.68±0.64^{e}	0.029±0.0008^{e}	0.025±0.0007^{e}	4.9±0.05^{g}	10.4±0.12^{g}
1	7.18±0.96^{e}	9.97±1.42^{d}	0.035±0.0012^{de}	0.042±0.0014^{d}	6.0±0.08^{f}	36.5±0.24^{f}
2.5	19.4±0.58^{d}	15.4±1.21^{c}	0.037±0.0008^{d}	0.047±0.0011^{d}	8.0±0.27^{e}	46.2±2.52^{e}
5	20.4±1.26^{d}	15.7±0.97^{c}	0.055±0.0028^{c}	0.048±0.0033^{d}	12.2±0.14^{d}	57.3±0.54^{d}
10	33.1±0.84^{c}	31.9±1.25^{b}	0.059±0.0030^{c}	0.113±0.0044^{c}	14.3±0.37^{c}	126±2.7^{c}
25	58.8±1.24^{a}	49.8±1.00^{a}	0.103±0.0051^{b}	0.142±0.0054^{b}	24.9±0.79^{b}	132±1.39^{b}
50	46.6±2.74^{b}	30.2±1.14^{b}	0.133±0.0054^{a}	0.189±0.0139^{a}	32.6±0.58^{a}	145±3.61^{a}
LSD	2.403	1.945	0.0057	0.0102	0.724	3.395

$^{a, b, c}$, Means within column different litter differ significantly (P> 0.5) from each other

Chlorophyll and carotenoid contents

Data of chlorophylls and carotenoids are shown in Fig. 2. The control leaves recorded the highest contents of chlorophylls and carotenoids while the lowest values were found in plants treated with the highest concentrations of Cd.

Chlorophyll *a* content at highest Cd concentration (50 ppm) was reached 0.436 mg g^{-1} leaf fresh weight compared to control (0.793 mg g^{-1} leaf fresh weight). A significant reduction was noticed in chlorophyll *b* content with different Cd treatments. The level of chlorophyll *b* in 50 ppm of Cd treatment reached to 0.223 mg g^{-1} leaf fresh weight compared to control (0.454 mg g^{-1} leaf fresh weight). The data showed that chlorophyll *b* was more reduced at the Cd concentrations than chlorophyll a. Furthermore, gradual decrease of carotenoid content has been noticed according to Cd concentration increase. With Cd treatments (1, 2.5, 5, 10, 25 and 50 ppm) of carotenoid contents recorded 0.437, 0.396, 0.375, 0.345, 0.324 and 0.271 mg g^{-1} leaf fresh weight, respectively. Generally, the reduction of chlorophyll and carotenoids content may be due to Cd induced inhibition of δ-aminolaevulinic acid hydratase (δ-ALA). This enzyme converts δ-ALA into porphobilinogen in the synthesis of chlorophyll (Wouter *et al.,* 2002). Also Cd inhibits the protochlorophyllide reductase by SH-interaction between heavy metals and SH group on the enzyme (Van Assche and Clijsters, 1990). In addition, heavy metals can cause substitution of Mg atom in chlorophyll molecules and led to breakdown in photosynthesis process as stated by Kupper *et al.* (1998) and Helene *et al.* (1998). The increases of Cd concentrations in growth media decreased chlorophyll and carotenoid contents (Wouter *et al.,* 2002).

Catalase specific activity

The increase in CAT activity demonstrated in Tab. 2 is therefore, circumstantial evidence to support hypothesis that Cd treatment causes the formation of ROS. The specific activity of catalase enzyme was increased by increasing the Cd concentration, and reached its maximum value with 25 ppm of Cd in leaves (58.8 unit mg protein^{-1}) and in roots (49.8 unit mg protein^{-1}). In contrast at highest concentration of Cd (50 ppm), the activity of CAT has been decreased relative to previous concentration of Cd (25 ppm) in both tissues (leaves and roots). The present data proved that Cd, like other heavy metals, induces specific responses from the plant antioxidant defense system including the increased activity of CAT (Arleta *et al.,* 2001). The reduction of CAT specific activity in highest concentration of Cd may be due to the long-term stress exposure (Chaoui *et al.,* 1997). The reasons for the increase in catalase activity after Cd treatments may be due to the scavenging role of CAT to H_2O_2, which could be quenched by the induction of specific enzymes like catalase (Elstner *et al.,* 1988). Generally, catalase activity increased as heavy metals concentration increased and decreased at higher concentration for long-term exposure. The present results are in agreement with those reported by Arleta *et al.* (2001); Vitória *et al.* (2001); Dixit *et al.* (2001); El-Beltagi *et al.* (2008); Salama *et al.* (2009); Shehab *et al.* (2010).

Glutathione S-transferase specific activity

Cd treatment caused a significant increase in GST specific activity in both roots and leaves (Tab. 2). GST activity at highest concentration of Cd (50 ppm) reached 459% in leaves and 756% in roots of control plants. Excess concentrations of Cd are known to cause cellular oxidative damage and lipid peroxidation. GST binds to Pb, Zn, Cd and Cu metal ions (Tommey *et al.,* 1991). So the Cd-induced increase in GST activity in the present study as a result to a detoxification response (Edwards, 1996). The induction of GST provides additional defense against metal toxicity and keeps the metabolic activities in roots and leaves functional. GST is known to be responsive to both biotic and abiotic stresses. This enzyme has not been characterized with respect to its antioxidative roles in plants (Dixit *et al.*, 2001).

Peroxidase specific activity

Peroxidase activity was increased with the increase of Cd treatments. The results showed that peroxidase specific activity increased by increasing Cd concentration and reached its maximum at highest concentration. The value of enzyme activity was 32.61 and 145 units mg protein^{-1}

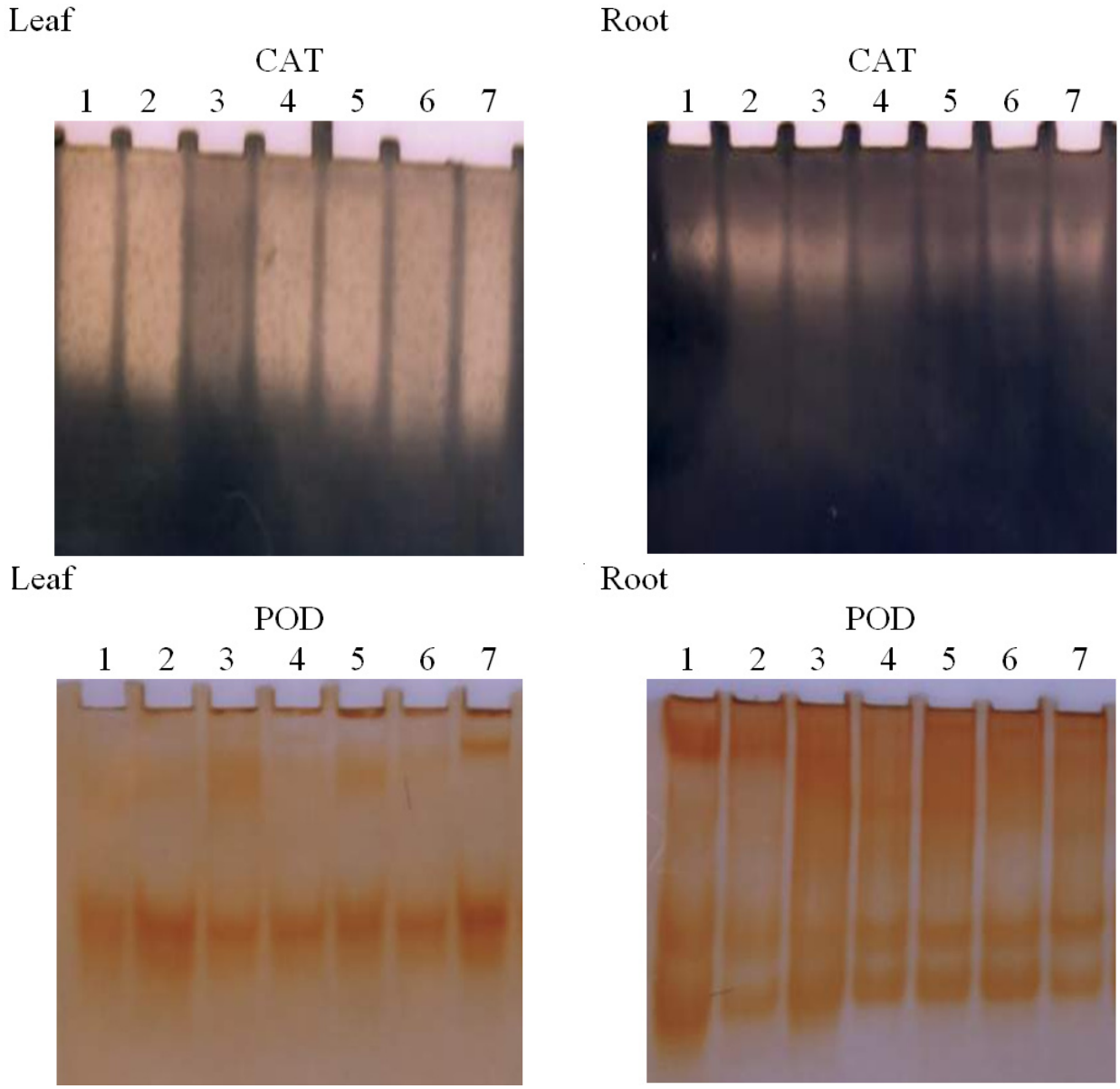

Fig. 3. Catalase and peroxidase isoenzyme patterns of radish tissues (leaves and roots) at harvest time (40 days growth) in plants grown in Cd containing media.

Lanes:1: Cd 0.0 ppm (control); 2: Cd 1.0 ppm; 3: Cd 2.5 ppm; 4: Cd 5.0 ppm; 5: Cd 10 ppm; 6: Cd 25 ppm; 7: Cd 50 ppm.

for 50 ppm Cd for leaves and roots, respectively compared to control 4.9 and 10.42 units mg protein^{-1} for leaves and roots, respectively as shown in Tab. 2. Van Assche and Clijsters (1990) cited that peroxidase induction is a general response in roots and leaves of higher plants to uptake of toxic doses of Zn, Cd, Cu, Ni and Pb. Van Assche and Clijsters (1988) noted that POD induction is significantly correlated to the level of Zn, Cd, Pb and Cu in the tissues, and with the degree of growth inhibition, observed on these plants. During heavy metal stress, intermediary forms of oxygen, (H_2O_2, hydroxyl and superoxide radicals) are formed. These very reactive molecules could be quenched by the induction of specific enzymes like POD, SOD and CAT (Elstner *et al.,* 1988). Dixit *et al.* (2001) stated that Cd enhances the level of lipid peroxidation and increasing tissue concentration of H_2O_2 in both roots and leaves of pea plants and causes oxidative damage to plants. He found that increasing of POD activity in both roots and leaves show that it was functioning concurrently to remove H_2O_2.

Catalase and peroxidase isozymes

The induction of new isozymes and the change in the isozyme profile is considered to play an important role in the cellular defense against oxidative stress, caused by toxic metal exposure. Among this enzymatic system, CAT and POD isozymes in both leaves and roots subjected to Cd stress are presented to Fig. 3. When protein extracts were

separated by native electrophoresis, one CAT isoenzyme in varying density was observed in both leaves and roots. This result agreed with the results of (Vitória *et al.,* 2001; El-Beltagi *et al.,* 2008). This band is very similar in its mobility in control and Cd treated leaf tissues except its density was increased under Cd stress, and reached its maximum at highest concentration of Cd (50 ppm). This result agreed with that of Vitória *et al.* (2001). However, in root tissue, the density was lower after treatment by Cd concentration especially at 5 and 10 ppm and then increased at 25 and 50 ppm treatments compared to control. Results of POD isozyme showed that, a new slow band appeared in leaves Cd treated plants and reached its maximum density at concentration of Cd 50 ppm, where the control and low Cd concentration 1 and 2.5 ppm not expected any band. Also the major bands were increased in its density with Cd treatments and reached its maximum density at concentration of Cd 50 ppm this result agreed with that of Van Assche and Clijsters (1986). However in root sample, one slow band was disappeared under treatments 5, 10, 25 and 50 ppm Cd compared to control. Also one fast band was disappeared with Cd at concentration 50 ppm, and other slow and fast bands were much less dense at in the same treatment of Cd compared to control.

Conclusions

It can be concluded that Cd causes oxidative stress as evidenced by the decreases in the leaf area, chlorophyll and carotenoid and micronutrient contents. Moreover, the data demonstrated a significant increment in the activities of three major enzymes, which are involved in the detoxification of ROS (CAT, GST and POD). However, the magnitude of this activity's increase varies with both the enzyme and location, in leaves or roots. It remains to be seen whether these increases due to induced gene transcriptional and de novo synthesis of protein, or are due to posttranslational modification of existing protein.

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