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ROLE OF CHELATORS IN ALLEVIATING OXIDATIVE DAMAGE IN RADISH SHOOT SUBJECTED TO CADMIUM STRESS

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ABSTRACT

Radish (*Raphanus sativus* L.) plants were grown with 0, 100 and 150 mg/kg soil cadmium chloride and treated with 100 or 200 mg/l chitosan or humic acid as soil additives to study the responses of leaf area, metabolite accumulation, oxidative stress and enzymatic and non-enzymatic antioxidant physiological processes to cadmium or chelators and to show the influence of chelating application on the alleviation of cadmium chloride induced adverse effects. Results revealed that cadmium stress caused reduction in leaf area, parallel to increased hydrogen peroxide levels, lipid peroxidation and electrolyte leakage in shoot tissues which were significantly reversed by either humic acid or chitosan. The antioxygenic enzymes viz. catalase and peroxidase were significantly decreased due to increasing cadmium concentration. Either humic acid or chitosan induced enzymes activities in shoot tissues of cadmium stressed plants. Proline, ascorbic acid, soluble sugars and total phenol significantly increased due to cadmium chloride or chelators as well as their combinations.

Anatomically, either chitosan or humic acid increased the diameter of radish root due to an increase in the thickness of cortex and diameter of vascular cylinder as well as diameter of metaxylem vessel, the thickness of radish leaf blade due to the increase in the thickness of mesophyll tissue as well as thickness of both lower and upper epidermal cells. In addition, the thickness of leaf blade through midrib region was also increased, due to the increase in the midrib vascular bundle thickness, as well as the size of the medvien vascular bundle, area of xylem and phloem tissues. Cadmium stress decreased all anatomical characteristics of root and shoot. It appears clearly that both chelators application partially overcame the depression effect of high cadmium level on the radish root and leaf structure.

This finding suggests that both chelators, in particular, chitosan might be activating antioxygenic enzymes and elevating antioxidants thereby controlling free radical generation, hence preventing membrane peroxidation and denaturation of biomolecules resulting into improved leaf area of radish plant grown under cadmium stress.

INTRODUTION

Environmental pollution by metals became extensive as mining and industrial activities increased in the late 19th and early 20th century. Cadmium salts are particularly dangerous environmental pollutants, due to their relatively high mobility in soils, large water solubility and extreme toxicity, even at low doses (Das *et al.* 1997). Cadmium (Cd) is a widespread heavy metal that occurs naturally in the soil in very small quantities. However, because of some anthropogenic or agricultural activities (Lux *et al.* 2011), its concentration has been increasing in soil and water. More seriously, Cd exposure will cause chromosome aberration, cancer and birth defects (Diels

et al. 2002). Excess Cd induces complex changes in plants at genetically, biochemical and physiological levels, leading to phytotoxicity. The most obvious symptoms are (1) depression of plant growth and even resulting in plant death by disturbing the uptake of nutrients (Farouk *et al.* 2011); (2) destruction of photosynthesis via degradation of chlorophyll and inactivation of enzymes involved in CO_2 fixation (Chamseddine *et al.* 2009), and (3) indirectly enhances the production of reactive oxygen species (ROS) resulting in oxidative damage to cellular constituents (Lux *et al.* 2011). Although cadmium is not a redox-active metal, such as copper and iron, and cannot catalyze Fenton-type reactions yielding ROS directly (Schu¨tzendu¨bel and Polle 2002), it can induce oxidative stress in an indirect manner, by disturbing cellular equilibria between the generation and the neutralization of ROS.

Under optimal growth conditions, ROS including H_2O_2, superoxide and hydroxyl radical are continuously produced at low levels mainly in chloroplasts, peroxisomes, and mitochondria of plant cells. The balance between production and removal of ROS are tightly controlled by the antioxidant systems (Apel and Hirt 2004). However, under severe environmental stress conditions, the reductive enzymatic pathway in plant tissues may be overwhelmed, resulting in oxidative damage. The ROS can cause lipid peroxidation, damage proteins, and DNA (Bao *et al.* 2011). Since ROS, as highly reactive molecules, can damage cell structure and function, plants possess effective scavenging mechanisms against cadmium stress: they are able to avoid metal toxicity through metal binding to the cell wall, by reducing transport across the cell membrane and by active efflux (Hall 2002). Apart from low molecular weight metabolites like ascorbic acid and reduced glutathione, important components of the ROS-scavenging system are antioxidative enzymes, such as peroxidases, catalase and superoxide dismutase (Khan *et al.* 2007). It was found that cadmium treatment could modify their activities, although the results were contradictory and depended on the type of the enzyme, cadmium concentration, the plant species and the environmental conditions. One of the common responses of many plant species exposed to different abiotic stresses is the accumulation of compatible organic solutes such as proline, glycine betaine, choline, and O-sulfate (Serraj and Sinclair 2002). Proline is an amino acid that is a highly soluble, non-toxic, and has a low molecular weight (Ashraf and Fooland 2007). It has been suggested that proline protect plants by functioning as a cellular osmotic regulator between cytoplasm and vacuole, and by detoxifying of ROS, thus protecting membrane integrity and stabilizing antioxidant enzymes (Bohnert and Jensen 1996). Ascorbic acid is the major primary antioxidant reacting directly with ROS. It also acts as a secondary antioxidant preventing membrane damage (Demirevska-Kepova *et al.* 2006).

Several strategies are required to remediate agricultural soils that have moderate and widespread metal-contamination to make food produced on these soils safe for human consumption. The use of metal-accumulating plants to remove toxic metals, including Cd from soil and aqueous streams has been proposed as a possible solution to this problem. This process of using plants for environmental restoration is termed "phytoremediation".

Phytoremediation has been viewed as a promising technique to remediate metal-contaminated soils because it offers advantages of being in situ, cost effective, and nondestructive (McGrath *et al.* 1995). However, it is limited by the fact that plants need time, nutrient supply and, the limited metal uptake capacity. The second approach recommends profitable use of synthetic chelators such as EDTA which have shown positive effects in enhancing heavy metal extraction through phytoremediation, but they have also revealed a vast number of negative side-effects on the soil. It is a non-selective agent which could extract various cations, including, calcium and magnesium, which are necessary for plant growth (Barona *et al.* 2001). As an alternative to these synthetic chelators, widespread natural sources of synthetic chelators, such as humic substances and chitosan, could be used for counteracting the harmful effect of cadmium stress. It has long been recognized that humic acid (HA) has many beneficial effects on plant growth and crop productivity (Farouk *et al.* 2008, 2011). For example, they influence nutrient uptake, nitrogen metabolism, enzymes activities, and membrane permeability in plants (Tan 1998). Recently, humic acid can work as antioxidant substances through increasing catalase, ascorbate peroxidase and polyphenol oxidase activities (Kesba and El-Belatgi 2012). Despite the abundance of descriptive effects, little is known about the chemical mechanism(s) by which HA influence these biological activities.

Chitosan (CHI), has an inherent property of being environmentally friendly and easily degradable. With high affinity and non-toxic, it does no harm to human being and livestock. Chitosan is reported to influence the production of substances related to stress response and to influence peroxidase activity (Kowalski *et al.* 2005). Chitosan treatments have also shown plant growth promoting effects, resulting in improved yields and plant health in numerous crops (Farouk *et al.* 2008, 2011). The data on antioxidant activity of chitosan and its derivatives are scanty and contradictory and were primarily obtained on in vitro models. Chitosan can scavenge OH and O_2^- radicals and has been shown to have DNA-protective properties (Harish Prashanth *et al.* 2007). In addition, treatment of *H. verticillata* with chitosan has been shown to increase the activity of superoxide dismutase (SOD) and to decrease malonaldehyde (MDA) concentrations (Xu *et al.* 2007). To our knowledge, there is currently no information available about the possible beneficial effects of either HA or CHI on the antioxidative system and stress markers in the performance of plants grown with Cd toxicity. Therefore, this study was undertaken to investigate the impacts of HA or CHI addition to the cadmium affected soil on: a) leaf area; b) changes in the activities of antioxidative enzymes including CAT, and POD; c) Endogenous proline, ascorbic acid, soluble carbohydrates, phenol, carotenoids, H_2O_2, MDA concentrations, and percentage of cellular membrane permeability in radish plants. It was hoped that this study would provide a basis for developing strategies for reducing the risks associated with Cd contamination in soils and maintaining sustainable vegetable plant production.

MATERIALS AND METHODS

Two pot experiments were carried out during the two successive seasons (2007 and 2008) in the greenhouse of Plant Pathology Department, Mansoura University. Closed plastic pots (30 cm in diameter) were filled with 8 kg air dried soil, its chemical analysis was recorded in Table (1). Then the pots were divided into three sets and contaminated with cadmium chloride at concentrations of 0, 100 and 150 mg kg^{-1} dry soil, Cd doses were added dissolving in the first irrigation water. In each set the pots were divided into 5 groups and treated with either humic acid or chitosan, both at 100 and 200 mg kg^{-1} soil or left untreated as a control. Twenty uniform seeds of radish (*Raphanus sativus*, L. var. sativus) were sown on 10th April in the previous pots and irrigated with tap water when ever required. The pots were arranged in a complete randomized block design with three replications.

Table (1): Physiochemical analysis of soil used in three experiments

Particle size distribution (%)		Physical properties		Soluble cations (meq L^{-1})		Soluble anions (meq L^{-1})		Available nutrients and cadmium (mg Kg^{-1})	
Sand	19	Bulk density (g cm^{-3})	1.24	Ca^{2+}	5.36	CO_3^{2-}	0	Nitrogen	43
Silt	29	Field capacity(%)	33	Mg^{2+}	3.23	HCO_3^-	4.21	Phosphorus	14
Clay	52	EC (dSm^{-1})	1.43	Na^+	5.28	Cl^-	6.74	Potassium	289
Soil texture	Clay	pH (Soil paste)	7.6	K^+	0.28	SO_4^-	3.20	cadmium	1.50
		Calcium carbonate (%)	3.7						
		Organic matter(%)	1.65						

Three weeks after sowing, plants were thinned to leave 5 uniform young plants per pot. At harvest (45 days from sowing), leaf area, were recorded in addition to the following stress-indicative biochemical parameters.

Oxidative damage and stress injury

Hydrogen peroxide (H_2O_2), lipid peroxidation (MDA) and membrane permeability (EC%) in shoot samples were measured to assess the oxidative damage and stress injuries. Lipid peroxidation was estimated as thiobarbituric acid reactive substances (TBARS). Malondialdehyde content "MDA" was determined and calculated as mM/g of fresh weight by the method of Shao *et al.* (2005). The amount of MDA present was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1}. Hydrogen peroxide content was estimated by forming a titanium-hydro peroxide complex via methods outlined by (Rao *et al.* 1997). Electrolyte leakage percentage measurement (ELP) was used to assess membrane permeability according to Goncalves *et al.* (2007), using an Electrical Conductivity Meter (Hanna, UK).

Assay for ROS scavenging (enzymatic and non-enzymatic)

Catalase (CAT) (EC 1.11.1.6) activity was assayed by measuring the rate of disappearance of H_2O_2 using the method of Barber (1980). Peroxidase (EC 1.11.1.7) activity was assayed by the method of Reuveni and Reuveni (1995). Ascorbic acid was extracted from plant material and titrated using 2.6-dichlorophenol indophenole as described by Sadasivam and Manickam (1996). Total phenolic compounds were determined according to the method

of Singleton and Rossi (1965) using Folin-Ciocalteau reagent. Proline content was assessed using the procedure decribed in Arbona *et al.* (2003). Total soluble sugars extracted by ethanol and then determined by phenol-sulphoric acid methods as described by Sadasivam and Manickam (1996).

Statistical analysis: The data were analyzed following Analysis of Variance (ANOVA) and mean separations were adjusted by the Multiple Comparison test using the statistical computer programme MSTAT-C v.1.2

RESULTS

Effect of chelating agents on Cd-induced oxidative damage in radish plant:

As shown in Table (2) and illustrated in Figure (1), increasing Cd concentration in the soil solution had an inhibitory effect on leaf area. However, pretreatment with chelating, in particular, 200 mg/kg soil chitosan, improved the leaf area which gave the highest values in this respect. Concerning the interaction effects, the data illustrated in Figure (1) proved that application of either chitosan or humic acid concentrations under cadmium levels alleviated the harmful effect of cadmium on leaf area comparing with untreated plants under such levels.

Table 2a. Leaf area (cm^2/plant), hydrogen peroxide (µM/g FW), membrane permeability (%) and lipid peroxidation (mM/g FW) in radish plants as affected by cadmium levels in the two growing seasons

Cadmium chloride (mg/g Soil DW)	Leaf area per plant		Hydrogen peroxide		Membrane permeability		Lipid peroxidation	
	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
0	287.601	282.917	15.210	15.361	71.396	71.428	17.631	17.913
100	231.009	229.265	17.543	17.592	80.759	81.134	24.472	24.423
150	203.367	200.665	18.854	19.108	86.317	86.692	27.228	27.357
LSD at 0.05	5.346	6.430	0.5143	0.4710	1.1280	1.4951	0.5181	0.4412

Table 2b. Leaf area per plant, hydrogen peroxide (µM/g FW), membrane permeability (%) and lipid peroxidation (mM/g FW) in radish plants as affected by chelator levels in the two growing seasons

Chelators (mg/Kg soil)	Leaf area per plant		Hydrogen peroxide		Membrane permeability		Lipid peroxidation	
	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
0	203.434	201.360	20.338	20.645	86.073	87.015	27.750	27.626
CHI100	226.287	219.969	16.893	16.901	81.533	80.996	24.401	24.691
CHI200	288.224	281.894	15.782	16.123	73.467	73.850	18.748	18.985
HA100	232.874	231.289	16.575	16.556	78.736	79.501	23.218	23.318
HA200	252.473	253.567	16.423	16.541	77.044	77.394	21.434	21.536
LSD at 0.05	7.355	8.961	0.6639	0.6079	1.4564	1.9306	0.6688	0.5698

As expected, cadmium stress significantly increased the H_2O_2 concentration in the shoot of radish plants in both growing seasons (Table 2a). The highest value 18.854 and 19.108 µM/g FW were obtained under high cadmium concentration in the first and second seasons as compared with control plants (18.854 and 19.108 µmol/g FW). Addition of CHI or HA to the soil significantly reduced the H_2O_2 concentration in both cadmium stressed and non-stressed shoots (Table 2b and Figure 2). The data illustrated in figure (2) proved that addition of chelators alleviated the harmful effect of cadmium stress on hydrogen peroxide concentration, whereas decreased the endogenous H_2O_2 under such cadmium concentration.

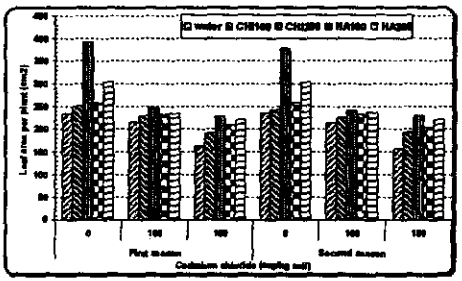

Figure (1): leaf area per radish plant as affected by the interaction between cadmium levels and chelators in the two growing seasons

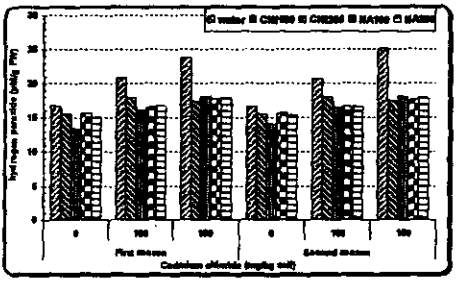

Figure (2): hydrogen peroxide concentration in radish shoot as affected by the interaction between cadmium levels and chelators in the two growing seasons

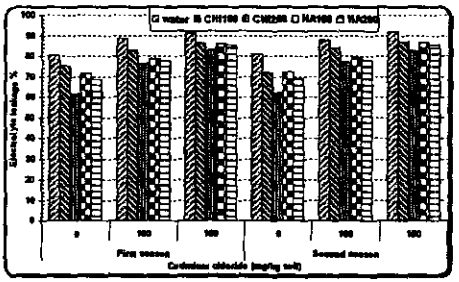

Figure (3): Electrolyte leakage percentage in radish leaf as affected by the interaction between cadmium levels and chelators in the two growing seasons

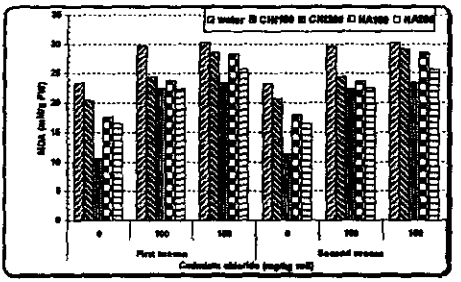

Figure (4): MDA concentration in radish shoot as affected by the interaction between cadmium levels and chelators in the two growing seasons

Cadmium chloride impaired cell membrane permeability by increasing electrolyte leakage (EL) compared to control (Table 2a). While the highest EL was observed under high cadmium concentration without chelator application. Either CHI or HA supplementation (in particular, chitosan at 200 mg/kg dry soil), under the assay condition significantly lowered electrolyte

leakage (Table 2b). Application of either chitosan or humic acid under all cadmium concentration significantly decreased electrolyte leakage percentage as compared with untreated plants under such cadmium levels (Figure 3). Lipid peroxidation levels of samples, measured as the concentration of MDA, are given in table (Table 2) and Figure (4). The results showed that MDA concentration increased with excessive oxidative stress when compared to control. The highest MDA content (27.228 and 27.357 mM/g FW) was obtained under high level of cadmium in the soil during the two growing seasons. However, significant decreases in MDA content of samples treated with either chitosan or humic acid, the lowest content of MDA was obtained due to addition of 200 mg/kg soil chitosan (Table 2b). Figure (4) proved that we can used both chelatore to alleviated the harmful effect of cadmium on MDA production in plant tissue. The magnitude of reduction was much more pronounced by applying 200 mglkg soil chitosan under such cadmium levels.

Antioxidant enzyme activities

Activities of catalase (CAT) and peroxidase (POD) enzymes in radish plants under the effect of cadmium or chelators as well as their combinations are given in Table (3) and Figures (5,6). It is evident that treatment with cadmium significantly inhibited the activities of CAT and POD enzymes of radish plants (Table 3a) and addition of chelators increased its activities (Table 3b) as compared to control plants, suggesting that the enzymes are sensitive to cadmium exposure. The data presented in figures (5, 6) indicated that the presence of chelators especially 200 mg/kg soil chitosan alleviated the adverse effect of cadmium on the activities of CAT or POD which increased it under such cadmium concentration

Table 3a. Catalase and peroxidase activities (unit/g FW) in radish plants as affected by cadmium levels in the two growing seasons

Cadmium chloride (mg/g Soil DW)	Catalase		Peroxidase	
	1st season	2nd season	1st season	2nd season
0	51.373	51.219	28.203	28.090
100	45.125	45.350	22.766	22.522
150	40.755	40.776	18.643	18.575
LSD at 0.05	0.5547	0.4970	0.4554	0.6269

Table 3b. Catalase and peroxidase activities (unit/g FW) in radish plants as affected by chelators levels in the two growing seasons

Chelators (mg/Kg soil)	Catalase		Peroxidase	
	1st season	2nd season	1st season	2nd season
0	39.085	39.518	17.168	16.872
CHI100	44.768	44.804	22.742	22.473
CHI200	50.224	49.946	26.599	26.466
HA100	46.494	46.422	24.104	24.187
HA200	48.184	48.220	25.408	25.314
LSD at 0.05	0.7165	0.6407	0.5879	0.8095

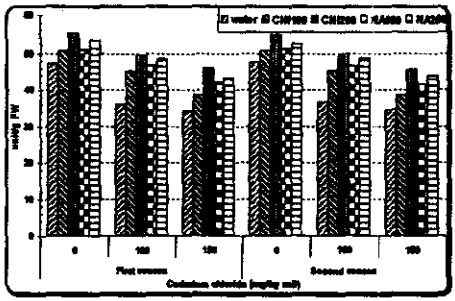

Figure (5): Catalase activity (unit/g FW) in radish plants as affected by the interaction between cadmium levels and chelators in the two growing seasons

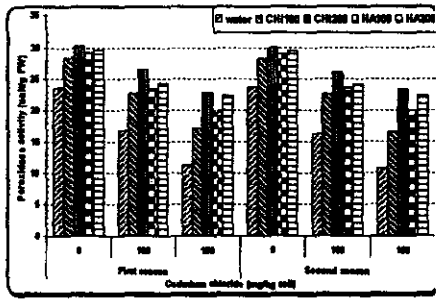

Figure (6): Peroxidase activity (unit/g FW) in radish plants as affected by the interaction between cadmium levels and chelators in the two growing seasons

Antioxidants compounds

Table (4) and Figures (7-10) show the time courses of non-enzymatic antioxidant in radish plants treated with or without chelators under normal or cadmium chloride condition. In general, the proline, ascorbic acid, soluble sugars and total soluble phenol were characterized by a gradual induction with increasing cadmium chloride levels up to 150 mg/kg dry soil or due to addition of chelators used compared to untreated plants.

Table 4a. Proline (mg/g FW), ascorbic acid (mg/g FW), soluble sugars (mg/g DW), soluble phenole (mg/g FW as gallic acid) and carotenoids (mg/g FW) concentration in radish plants as affected by cadmium levels in the two growing seasons

Cadmium chloride mg/g Soil DW)	Proline		Ascorbic acid		Soluble sugars		Phenol	
	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
0	14.248	13.992	12.911	13.010	41.814	40.656	16.272	15.928
100	16.922	16.844	16.911	16.867	53.853	53.743	22.325	22.328
150	18.821	18.170	18.311	18.333	58.696	58.413	23.876	23.950
LSD at 0.05	0.2726	0.3685	0.3176	0.3482	0.6382	0.6385	0.3820	0.3783

Table 4b. Proline (mg/g FW), ascorbic acid (mg/g FW), soluble sugars (mg/g DW), soluble phenole (mg/g FW as gallic acid) and carotenoids (mg/g FW) concentration in radish plants as affected by chelators levels in the two growing seasons

Chelators (mg/Kg soil)	Proline		Ascorbic acid		Soluble sugars		Phenol	
	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
0	13.842	13.732	13.740	13.944	43.956	43.802	18.485	18.496
CHI100	16.491	16.219	15.500	15.406	50.457	49.543	19.457	19.315
CHI200	18.519	17.763	17.444	17.463	56.135	55.642	23.162	23.263
HA100	17.065	16.806	16.463	16.406	52.243	51.730	20.846	20.650
HA200	17.401	17.156	17.074	17.132	54.482	53.968	22.176	21.951
LSD at 0.05	0.352	0.4756	0.410	0.4496	0.8239	0.8245	0.4932	0.4883

Exogenous application of chelators, in particular, chitosan at 200 mg/kg soil, counteracted the harmful effects of cadmium toxicity on non-enzymatic scavenging systems (Figures 7-10). Adding chelators to the contaimenated or noncontaimenated soils stimulated the accumulation of proline, ascorbic acid, soluble sugars and total soluble phenol in radish plant compared to untreated plants under the corresponding cadmium chloride levels.

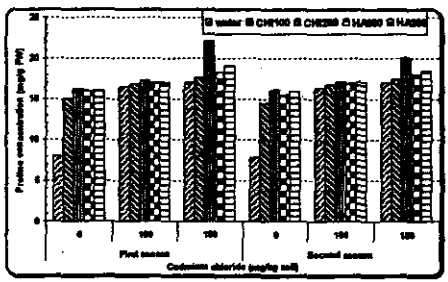

Figure (7): Proline concentration in radish shoots as affected by the interaction between cadmium levels and chelators in the two growing seasons

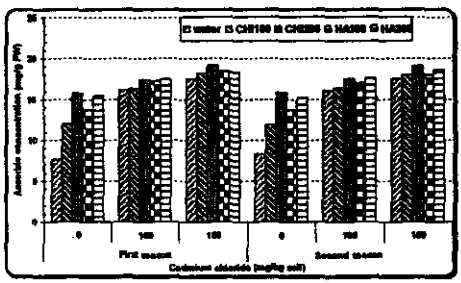

Figure (8): Ascorbic acid concentration in radish shoots as affected by the interaction between cadmium levels and chelators in the two growing seasons

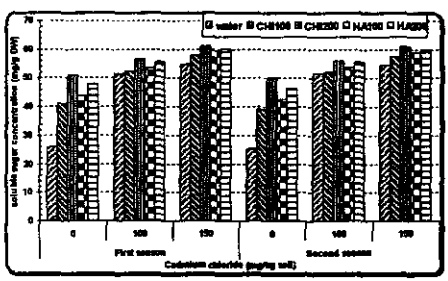

Figure (9): soluble sugars concentration in radish shoots as affected by the interaction between cadmium levels and chelators in the two growing seasons

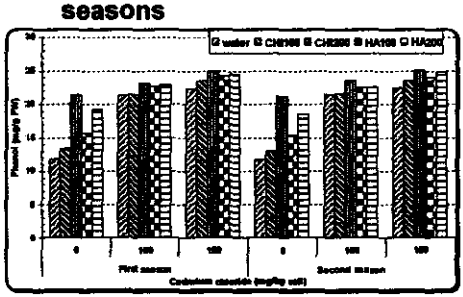

Figure (10): Phenol concentration in radish shoots as affected by the interaction between cadmium levels and chelators in the two growing seasons

Root structure

The most remarkable anatomical feature in radish root is early formation of secondary roots, which often woody and massive in size (Figure 11). Table (5) and Figure (11) reveal that either chitosan or humic acid increased the diameter of radish root due to an increase in the thickness of cortex and diameter of vascular cylinder as well as diameter of metaxylem vessel leading to enhancing the absorption of solutes and water from the soil , which undoubtedly reflects on growth and yield. With regard to the effect of

cadmium stress on anatomical characters of roots, Table (5) and Figure (11) show that cadmium stress decreased diameter of root cross section as a result of decreasing cortex tissue thickness and diameter of vascular cylinder. Metaxylem vessels diameter was also decreased.

Table 5. Anatomical characters (μ) of radish root as affected by the cadmium, chelators and their interactions in the second growing season

Treatments (mg/kg dry soil)		Root diameter	Cortex thickness	Vascular cylinder diameter	Metaxylem vessels diameter
Cadmium	Chelators				
Cd 0	0	2046	538	1508	48
	CHI100	2186	511	1675	49
	CHI200	2215	501	1714	57
	HA100	2190	491	1699	52
	HA200	2205	504	1701	52
Cd 100	0	1457	238	1219	34
	CHI100	1850	416	1434	42
	CHI200	2120	465	1655	49
	HA100	2021	538	1483	45
	HA200	2093	446	1647	48
Cd 150	0	1200	204	996	36
	CHI100	1590	298	1292	37
	CHI200	1969	501	1468	44
	HA100	1713	321	1392	40
	HA200	1831	405	1426	47

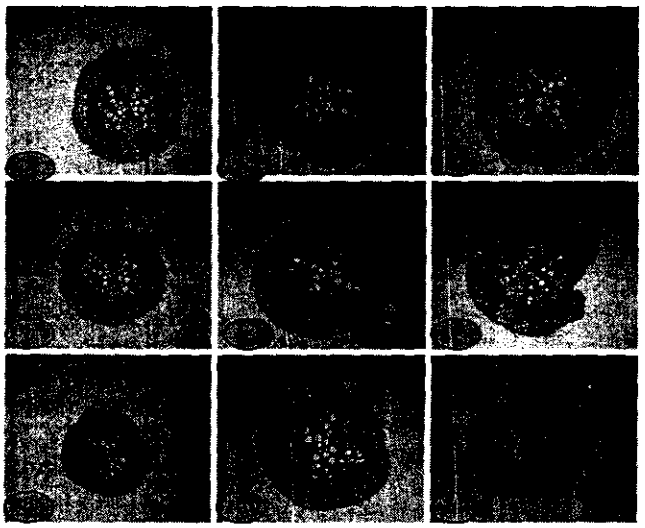

Figure (11): Cross section of radish main root as affected by cadmium stress and chelators as well as their interactions in the second growing season (obj 4x, oc. 10x) (A, control ; B, CHI 200 mg/kg soil ; C, HA 200 mg/kg soil ; D, 100 mg CdCl/kg soil; E, 100 mg CdCl/kg soil+ CHI 200 mg/kg soil ; F, 100 mg CdCl/kg soil+ HA 200 mg/kg soil ; G, 200 mg CdCl/kg soil; H, 200 mg CdCl/kg soil+ CHI 200 mg/kg soil ; I, 200 mg CdCl/kg soil+ HA 200 mg/kg soil)

The results due to high cadmium concentration may be correlated with inhibition the procambial activity leading to retardation in the differentiation of the root conductive tissues. It appears clearly that both chelators application partially overcame the depression effect of high cadmium level on the root structure. Chitosan at 200 mg/kg soil is the most effective than humic acid in increasing all anatomical characters compared with control plants and untreated plant under such levels.

Leaf structure

The leaf blade internal structure of radish plant as seen in figure consists of upper and lower epidermis and mesophyll tissue. Mesophyll tissue consists of a single or two layered palisade parenchyma cells and the spongy parenchymatous cells are loosely arranged with numerous intercellular spaces. The medvien vascular bundle is open collateral bundle having a narrow cambial zone. Cross section of radish leaves showed that there were significant changes in leaf anatomical characteristics induced by both chelators application. Application of either chitosan or humic acid at both concentrations increased the thickness of radish leaf blade respectively, due to the increase in the thickness of mesophyll tissue as well as thickness of both lower and upper epidermal cells. In addition, the thickness of leaf blade through midrib region was also increased, due to the increase in the midrib vascular bundle thickness, as well as the size of the medvien vascular bundle. Chelators resulted in increasing the area of xylem and phloem tissues, due to the stimulation of pro-cambium activity in the midrib bundle during their differentiation. Chitosan at 200 mg/kg soil was more effective in increasing all anatomical features of leaf (Table, 6 and Figure, 12).

Table 6. Anatomical characters (µ) of radish leaf as affected by the cadmium, chelators and their interactions in the second growing season

Treatments (mg/kg dry soil)		Leaf thickness in the midrib region	Thickness of leaf blade	Palisade parenchyma thickness	Spongy parenchyma thickness	Main vascular bundle thickness	Xylem tissue thickness	Phloem Tissue thickness
Cadmium	Chelators							
Cd 0	0	224	56	28	28	44	26	18
	CHI100	240	56	32	24	48	26	22
	CHI200	264	60	40	20	60	42	18
	HA100	196	72	40	32	52	26	26
	HA200	248	40	24	16	52	34	18
Cd 100	0	124	32	24	8	20	12	8
	CHI100	168	64	28	36	40	22	18
	CHI200	228	60	48	12	48	32	16
	HA100	146	88	48	40	44	28	16
	HA200	224	56	36	20	48	22	26
Cd 150	0	116	28	24	8	16	11	9
	CHI100	132	56	36	20	24	16	8
	CHI200	188	44	24	20	40	22	18
	HA100	132	36	20	16	32	14	18
	HA200	144	68	44	24	32	18	16

Regarding the effect of cadmium stress on radish leaf structure, the thickness of leaf blade through the midrib region as well as the mesophyll tissue thickness was decreased under cadmium chloride levels. In addition,

the thickness of midrib vascular bundle and big metaxylem vessels, and thickness of xylem and phloem were also decreased. The decrease in mesophyll tissue, xylem and phloem leads to a slow rate on the translocation of photoassimilates towards the developing tissues. Furthermore, the decrease in the diameter of metaxylem vessels in the leaf blade resulted in lowering the accumulation of necessary water required for photosynthesis (Table, 6 and Figure, 12). Concerning the interaction between cadmium and chelators, the interactions increased all leaf anatomical characters grown under low cadmium chloride level compared with control plants. On the other hand, chelators used partially overcame the depression effect of high cadmium chloride levels on the thickness of the midrib region and mesophyll tissue

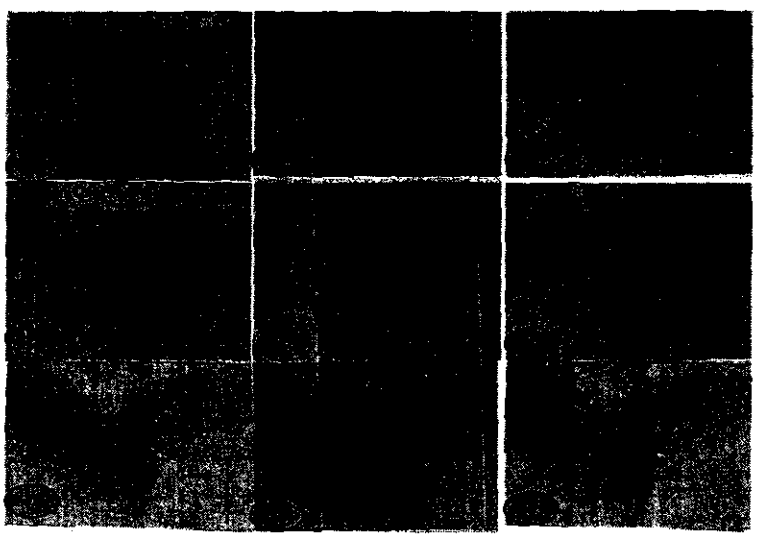

Figure (12). Anatomical characters (µ) of radish leaf as affected by the cadmium, chelators and their interactions in the second growing season (obj 4x, oc. 10x) (A, control ; B, CHI 200 mg/kg soil ; C, HA 200 mg/kg soil ; D, 100 mg CdCl/kg soil; E, 100 mg CdCl/kg soil+ CHI 200 mg/kg soil ; F, 100 mg CdCl/kg soil+ HA 200 mg/kg soil ; G, 200 mg CdCl/kg soil; H, 200 mg CdCl/kg soil+ CHI 200 mg/kg soil ; I, 200 mg CdCl/kg soil+ HA 200 mg/kg soil)

DISCUSSION

Cadmium (Cd) is considered to be among the most environmentally toxic pollutants. In plants, it interferes with several physiological and metabolic processes and can also produce oxidative stress and modify the activity of various antioxidant enzymes depending on the plant species and environmental conditions. The present study revealed that presence of Cd in

the soil solution significantly decreased leaf area per plant. The inhibitory effect of cadmium on leaf area is mediated through altered cell growth. Cd in cell gets associated with cell walls and middle lamella and increases the cross-liking between the cell wall components, resulting in the inhibition of cell expansion (Poschenrieder *et al.* 1989). Moreover, Cd also alters the water relation in plants, causing a physiological drought (Farouk *et al.* 2011) and cause metabolic dysfunctions such as production of reactive oxygen species "ROS", photosynthesis and the uptake, transport and use of several macro-elements (Chaugh and Sawhney 1999). However, application of either chitosan or humic acid increased leaf area not only in control but also in stressed plants (Table 2 and Figure 1). The plant growth enhancement by chelators might be due to inducing changes in the biochemical or physiological processes including cell division, cell differentiation and morphogenesis as indicated from the anatomical studies in this investigation.

In higher plants, Cd induces oxidative stress by generating ROS (Jouili and Ferjani 2004), which can rapidly damage biological molecules (DNA, RNA and protein) and membrane by including lipid peroxidation resulted in irreparable metabolic dysfunction and cell death (Weckx and Clijsters 1996). The present study's results and other investigations showed that there were very high increases in electrolyte leakage (Table 2), and MDA and H_2O_2 concentration (Table 2, Unyayar *et al.* 2010) in response to cadmium treatment, indicating that cadmium stress could damage the integrity of the cellular membrane, as well as cellular components, such as lipids and proteins. Malondialdehyde (MDA) has been used extensively as an indicator for free radical production and membrane injury under various a biotic stress conditions and is considered a biomarker of metal-induced oxidative stress (Ferrat *et al.* 2003). The results of the present investigation indicated that the membrane permeability, hydrogen peroxide accumulation and MDA concentration in the treatments containing chelators were lower than those in the control, which indicated that the addition of chelators alleviated the lipid peroxidation. Maintaining integrity of the cellular membranes under stress condition is considered as an integral part of the stress tolerance mechanism (Stevens *et al.* 2006). The results of the present study are concordant with Tan (1998), who reported that application of chelators, like HA facilitated the maintenance of membrane functions. This could be attributed to the production of the antioxidant compounds and enzymes that protect the plant from the oxidative damage by stress (El-Tayeb 2005). Decreased MDA indicates that there may be some antioxidative response alleviating or preventing lipid perxoidation (Nimptsch and Pflugmacher 2007). The mechanism by which chelators reduces the lipid peroxidation may be explained on the basis that chelators can promote plant growth and development and thus dilute the concentration of Cd, thus reducing their toxicity, or may be that chelator changed the DNA in plants. These results are in a good harmony with those obtained by Kesba and El-Beltagi (2012) who reported that, exogenous application of HA decreased the oxidative damage by reducing the malondialdehyde content and hydrogen peroxide in response to different stresses. In recent years, a growing attention has been directed towards the antioxidant activity of chitosan (Sun

et al 2008). The antioxidant properties of chitosan are primarily attributable to its abundant active hydroxyl and amino groups (Sun *et al.* 2008), which can react with ROS to form stable and relatively nontoxic macromolecular radicals and has been shown to have DNA-protective properties (Harish Prashanth *et al.* 2007). In the current study, it could be concluded that the antioxidant properties of chitosan can also enhance resistance to oxidative stress in radish plants subjected to cadmium stress. The present investigation show that chitosan played a key role in increasing cell membrane stability during cadmium stress. Taken together, the data suggest that chitosan may alleviate the adverse reactions of ROS towards membranes and reduce the level of superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide etc., possibly through activation of ROS scavenging enzymes.

To scavenge ROS, plants possess a well-organized antioxidative defense system comprising enzymatic and non-enzymatic antioxidants. The cooperative function of these antioxidants plays an important role in scavenging ROS and maintaining the physiological redox status of organisms (Cho and Seo 2005). In the present study, activities of antioxidative enzymes (CAT and POD) were generally depressed at highly toxic Cd level in shoot of radish plants. These results suggest that antioxidative systems are disrupted by Cd itself, while ROS is indirectly induced by Cd, engendering oxidative damage to the cells (Hegedus *et al.* 2001), might be the reason for the accumulation of hydrogen peroxide. The decrease may be associated with degradation caused by induced peroxisomal proteases or may be due to photoinactivation of enzyme (Sandalio *et al.* 2001) or/and changes in the assembling of CAT subunits and enzyme inactivation or proteolytic degradation by peroxisomal protease (Cakmak 2000). In fact, the increased CAT activity as found herein, which can be associated with hydrogen peroxide scavenging, was also observed due to application of HA (Kesba and EL-Beltagi 2012). This increase suggests a compensatory mechanism of defense against oxidative stress caused by toxic metal concentrations and can be explained by increase in its substrate to maintain the level of hydrogen peroxide as adaptive mechanism of the plants (Cargnelutti et. al. 2006). POD activity, a H_2O_2-scavenger that belongs to the ascorbate-glutathione cycle, was inhibited at all Cd concentrations tested. Such a decrease has also been reported in some Cd-treated plants (Gomes-Junior *et al.* 2006). Meanwhile, POD activities were enhanced with chelators. Increased total peroxidase activities in response to chelators were reported by Kowalski *et al.* (2005).

It is widely accepted that detoxification of metal ions within plant tissue usually depends on chelation by appropriate ligands. Antioxidants like proline, ascorbic acid, phenol, soluble sugars and nonprotein thiole play an important role in detoxification of toxic metal ions (Liu *et al.* 2007, Unyayar *et al.* 2010). Radish ascorbic 'AsA' concentration was enhanced with cadmium concentration or application of chelating agent and their combinations compared with untreated control plants, indicating that AsA is involved in antioxidants response to Cd toxicity. AsA is an important antioxidant that plays an important role to scavenge the free radicals like superoxide and hydroxyl anions as well as the lipid hydroperoxidases (Reddy *et al.* 2005). It

is well known that proline is another important component of the defence system of the plants to counter the environmental stress like cadmium. In the present study, an accumulation of proline in radish shoot in response to cadmium or chelators as well as their combinations was detected. Thus, the increased proline concentration indicated that proline tended to protect them from the Cd induced toxicity by hydroxyl radicals scavenging, contributing to alleviate the adverse effects of Cd-injury. In line with the present results, several studies have already described a positive correlation between the severity of stress and increase in proline content (Bao *et al.* 2011). There are many suggestions regarding mechanism(s) by which proline might reduce heavy metal stress; (1) proline has been proposed to act as a metal chelator, binding the metal ions, resulting in the formation of a non-toxic Cd-proline complex (Sharma *et al.* 1998), (2) act as antioxidant (Xu *et al.* 2009), (3) helping in stabilization of proteins and protein complexes in the chloroplast and cytosol, protection of the phoytosynthestic apparatus and enzymes involved in detoxification of ROS (Szabados and Savoure 2009). Proline accumulation in plant tissue has been suggested to result from (1) a decrease in proline degradation enzymes, (2) an increase in proline biosynthesis, (3) a decrease in protein synthesis or protein utilization and (4) hydrolyze of proteins (Charest and Phan 1990).

Result indicated that increasing levels of cadmium treatment markedly increased the phenolic concentration in radish. In this concern, an increase of phenolics correlated to the increase in activity of enzymes involved in phenolic compounds metabolism was reported (Michalak 2006), suggesting synthesis of phenolics under heavy metal stress. The phenolics are generally thought to prevent oxidative damage by scavenging active oxygen species and by breaking the radical chain reactions during lipid peroxidation, these antioxidative effects require the reduced form of phenolics, in the oxidized form act as prooxidants (Sakihama and Yamasaki 2002). The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Sakihama *et al.* 2002). It has been reported that the antioxidative properties of phenolic is due to their ability tochelating transition metal ion, the inhibition of superoxide-driven Fenton reaction, and membranes stability by decreasing membrane fluidity (Blokhina *et al.* 2003).

The results obtained in the present study clearly indicated the ameliorative effect of chelators on Cd toxicity stress. The stress alleviation effect of chelators was associated with enhanced levels of proline. Chelator strongly protects radish plant from Cd induced oxidative stress by minimizing the impact of reactive oxygen species by increasing antioxidant enzyme activity, which may represent a secondary defensive mechanism against oxidative stresses. The finding further indicated the ability of chelators to protect the membrane integrity as observed in the case of radish plant challenged with Cd stress. Hence, it can be concluded that the supplementation of chelators proved to be beneficial for the plant system in combating metal toxicity.

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دور المواد المخلبية في التغلب علي أضرار الأكسدة علي المجموع الخضري لنبات الفجل المعرض لإجهاد الكادميوم

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تم زراعة نباتات الفجل تحت مستويات من كلوريد الكادميوم وهي ٠، ١٠٠ و١٥٠ ملليجرام/كجم تربة مضافاً إليها اي من الكيتوزان أو حامض الهيوميك بتركيزات ١٠٠ و ٢٠٠ ملليجرام/كجم تربة كإضافات أرضية وذلك لدراسة إستجابة المساحة الورقية، تراكم نواتج التمثيل، أضرار الأكسدة والمواد المضادة للأكسدة سواء انزيمية أو غير إنزيمية لتلك المعاملات بالإضافة لدراسة دور المواد المخلبية في التغلب علي الآثار الضارة لكلوريد الكادميوم. أظهرت النتائج أن المعاملة بالكادميوم تقلل المساحة الورقية متبوعة بزيادة مستوي فوق اكسيد الهيدروجين، زيادة نواتج أكسدة الدهون مع زيادة نفاذية أغشية المجموع الخضري والتي يمكن التغلب عليه بإستخدام الكيتوزان وحامض الهيوميك. ينخفض معنوياً نشاط كل من انزيم الكتاليز والبيروكسيديز بزيادة تركيز الكادميوم، بينما إضافة الكيتوزان أو حامض الهيوميك يشجعا ويحفزا نشاط تلك الإنزيمات. إزدادمعنويا محتوي المجموع الخضري من البرولين، حامض الأسكوربيك، السكريات الذائبة والفينول إستجابة لكلوريد الكادميوم أو إستخدام المواد المخلبية كل علي حدة او نتيجة للتداخل بينهما.

تشريحيا، إستخدام الكيتوزان أو حامض الهيوميك يُزيد قطر الجذر نتيجة لزيادة سمك القشرة وقطر الإسطوانة الوعائية بالإضافة لزيادة قطر وعاء الخشب التالي. كما تعمل تلك المواد علي زيادة سمك نصل الورقة في منطقة العرق الوسطي متبوعا بزيادة سمك النسيج المتوسط وسمك البشرة العليا والسفلي وسمك الحزمة الوعائية في منطقة العرق الوسطي وسمك كل من نسيج الخشب ونسيج اللحاء. أما الكادميوم فهو يعمل علي إنخفاض جميع الصفات التشريحية لكل من الجذر والساق. ويمكن القول عموما بأن إستخدام المواد المخلبية تتغلب نوعا علي التأثير المثبط للكادميوم علي تشريح الجذر والاوراق في نبات الفجل.

نخلص من الدراسة أن إستخدام المواد المخلبية وبصفة خاصة الكيتوزان تنشط الإنزيمات المسئولة عن الأكسدة والإختزال بالتالي التحكم في مستوي الشوارد الاوكسيجينية الحرة، بالتالي تمنع من أكسدة الأغشية وتلفها بالتالي تتحسن وتزداد المساحة الورقية للنبات تحت تأثير كلوريد الكادميوم.

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