Research Article

Lead induced oxidative stress and development change on *Coriandrum Sativum*

Summary

Lead (Pb) is ubiquitous pollutant in environment which causes many toxic effects, at high and low dose, Pb make disruptions at all plants by reduction its growth and development of aerial parts and roots. In this study, assessing the capacity of lead to induce a dysfunctioning in development of roots and aerial parts at differents concentrations after 21-day and 40 days, biochemical parameters of oxidative stress and morphological change on Coriandrum sativum was studied in the present investigation. The seeds were sprayed with a solution containing lead to different concentrations for 40 days with six Pb levels (0 as control, 500, 1000, 1500, 2000, 2500 and 3000 mg.l-1). The soil used was characterized; the results show that there is no change in its properties. Parameters such as growth, oxidative damage markers (lipid peroxidation, prolin, chlorophyll and hydrogen peroxide contents) were investigated. Roots, sheets and stalks indicate that chlorophyll and carotenoid concentration were significantly increased at 3000 mg.l-1 Pb than control. Lipid peroxidation and H2O2 levels were increased at the dose of 2500 and 3000 mg l-1 compared to control treatment; no difference was noted between 500mg.l-1 and control in all part in plant. Morphological studies show that the group exposed to 3000 mg.l-1 of Pb shows a very important development of the tissues of roots and stems compared to control and to the group exposed to 500 mg.l-1 of Pb. changes in morphological structure and non-enzymatic antioxidants have shown that Pb exposure causes a significant perturbation on Coriandrum sativum Linn seedlings affecting biochemical and physiological processes.

Key words: Coriandrum sativum, chlorophyll, Proline, lead, lipid peroxidation.

Introduction

The contamination of soil by heavy metals is one of the most serious environmental problems and has significant implications for human health. Some industrial activities and some modern agricultural practice increase the disponibility of heavy metals in soil, plants and local environmental. Pb is a non-essential element for plant growth, being highly toxic metallic pollutant of soil, inhibits root and shoot growth and yield production, affects nutrient uptake and homeostasis, and the accumulation of lead is the point to enters the food chain with a significant potential to impair animal and human health, Coriander (Coriandrum sativum L.), which belongs to the family Apiaceae (Umbelliferae) is mainly cultivated from its seed throughout year (Mehndi et al., 2011), is widely used in the human diet, changing their composition or properties would cause deleterious effects to their consumers.

Engineering industrial techniques used to clean up contaminated soils are expensive and suitable only for small polluted areas (Lutts et al., 2004). Furthermore, these technologies are not only costly, but they also cause soil disturbances and they are not readily accepted by the general public (Perchet, 2008; Saifullah et al., 2009). Phytoremediation has been highlighted as an alternative technique to traditional methodologies, for the removal of heavy metals from soil. Two approaches have been generally proposed for the phytoremediation of heavy metals. The first one is using of natural hyperaccumulator plants with high metal accumulating capacity. However, the research must be pursued at the level of the increase of the assimilation of heavy metals by the vegetation we taking as example the lead.

Quantities of lead absorbed by the roots depend on the concentration of metal in the soil, but also of its migration capabilities from the ground to the roots and the amount of lead present in the various part of plants depends on the transport of outside of roots toward the inside, and then of its translocation to leaves (Patra et al., 2004).

Lead was recognized as causing oxidative stress in plants, so coriander may have a strong resistance to Pb, but little is known about the effects of Pb on the physiological processes and the biochemical changes at short and long term of exposed to lead of coriander seedlings. The objective of the present investigation is to study the effects of different concentrations of Pb on leaf, roots and tiges of coriander including growth, physiological and biochemical processes such as the different pigments, soluble proteins, lipid peroxidation and hydrogen peroxide contents; and description of morphological changes. The possible mechanisms of Coriander seedlings tolerance of Pb stress are briefly discussed in the present study.

Materials and Methods

Plant material

Coriander (Coriandrum sativum L.) seeds were used in our experimentation, in the first we have tempered of seeds overnight before sow this for accelerated germination and we conducted the experiment in 14 pots clean plastic, which have a diameter greater than maximum of 20 cm and a diameter of less than 10 cm, perforated to allow the water to drain out. Filled with compost added to the sterilized sand (2V of sand / 1V of compost) then we have sowed our seeds (70 seeds) to approximately 2.5 cm in depth and we have carried out regular watering during 40 days with solutions of lead to different concentrations (500,1000,1500 , 2000, 2500, 3000 $mg.l^{-1}$) and a witness sprayed with distilled water. Our seedlings were then placed in a greenhouse in Oran 1 University in controlled conditions as it is shown on Table 1.

Chlorophyll and carotenoid determination

Fresh biomass (leaves) was homogenized in 80% icecold acetone in the dark and then centrifuged at 10000g for 10 min at 4°C and the supernatant was used for the immediate determination of pigments. Absorbance of the solution was determined spectrophotometrically at 663, 645 and 480 nM the contents of chlorophyll a, b, and carotenoid, respectively; with the following equations help of Arnon's formulae (Arnon, 1949), for quantification of the total chlorophyll, chlorophyll a and chlorophyll b content in an 80% acetone extract:

Total chlorophyll = 20.2 (A645) + 8.02 (A663)
Chlorophyll a = 12.7 (A663) - 2.69 (A645)
Chlorophyll b = 22.9 (A645) - 4.68 (A663)
And Carotenoids= (1000A480 - 3.27[chl a] - 104[chl b])/227
Chlorophyll and carotenoid concentrations were expressed as mg g-1fresh weight.

Estimation of lipid peroxidation

The level of peroxidation was measured in terms of malondialdehyde (MDA) (a product of lipid peroxidation) content determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Frozen shoot was homogenized in 5 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 8000g for 20 min and 4.0 ml of 20% TCA containing 0.5% TBA was added. The mixture was heated at 95°C for 30 min and then quickly cooled on ice bath. The contents were centrifuged at 8000 g for 20 min and the absorbance of the supernatant was measured at 532 nM and the value for the non-specific absorption at 600 nM was subtracted. The concentration of MDA was calculated using coefficient of absorbance of 155 mM^{-1} cm^{-1}. MDA content expressed as nM g^{-1} fresh weight.

Determination of hydrogen peroxide

The H_2O_2 concentration was determined according to Loreto and Velikova (2001). Approximately 0.1g of shoots was homogenized at 4ºC in 2 ml of 0.1% trichloroacetic acid (TCA) (w:v). The homogenate was centrifuged at 12000 g for 15 min at 4ºC. Then, 0.5 ml of the supernatant was added to 0.5 ml of 10 mM K-phosphate buffer (pH 7.0) and 1 ml of 1M KI. The H_2O_2 concentration of the supernatant was evaluated by comparing its absorbance at 390 nM with a standard calibration curve. Hydrogen peroxide concentration was expressed as $\mu M.g^{-1}$ fresh weight.

Extraction and assays of soluble proteins

The proteins have been doses according to the method **(Bradford, 1976)**, briefly 1 g of fresh tissue, was crushed in the presence of sand, in 10 ml of medium of extraction with following composition: phosphate buffer (0.1M, pH 7); K_2HPO_4 to 0.1M; Triton x 100 to 0.1 % ; EDTA and centrifuged at 3000 rpm for 10 min, the supernatant (protein extract) is recovered for the assay of protein and reaction was read to 595 nm and express by mg /g MF (fresh material).

Proline assay

400 Mg of plant material is put into a mortar and chopped with 5 ml of ethanol at 95% followed by three flushing and washes with 5ml of ethanol at 70 %. The final solution is collected in a test tube so that it is decanted during 60 min. two phase are distinguished (a higher phase of light green color and a lower phase of dark green color) **(Nguyen and Paquin , 1971).** 5 mL of the upper phase are collected, to which are added 2 ml

of chloroform and 3 ml of distilled water. After agitation, the solution is maintained at rest for 24 hours in the cold for a good separation. After assay the optical density is read using a colorimeter, the wavelength is 515 nm.

Data analysis

All data were analyzed in three replications and the obtained data were evaluated statistically using Student's test, and least significant difference (LSD) was calculated at $p < 0.05$.

Results

Growth and fresh weight

The results after exposure of seeds to different concentration of lead (500, 1000, 1500, 2000, 2500, 3000 $mg.l^{-1}$) for 20 days, a significant reduction in leaves numbers, by 40%, 58%, 70 ; 74%, 84% and 92% respectively, was obtained compared to control, and the weight of total sheets, rods and roots decrease significantly after poisoned in all groups compared to control by 90%, 87%, 81%, respectively. After 40th days of exposure to different concentration of lead, show a net increase in weight of rods and sheets compared to the control. The numbers of sheets was significantly decreases with increasing the concentration from 500 to 3000, respectively compared to control (Table. 2 and 3). After testing the contaminated soil and the rest of the lead solution was used to perform analysis

Chlorophyll and carotenoid

After 20^{th} days, our results shown a significant decrease ($p<0,05$) in the rate of chlorophyll (a) in all groups exposed to lead (from 500 $mg.l^{-1}$ to 3000 $mg.l^{-1}$) compared to control group by 10.41 %, 24.94 %, 43.27 %, 47.50 % ,65.5% and 76.46 %. Chlorophyll (b) have significantly lower levels compared to control groups by 23.95 %, 55.98 %, 51.39%, 70.89%, 72.56% and 84.95%, respectively. Increased lead exposure causes a significant reduction in carotene levels compared to control from 49.08 % to 94.56 % respectively (Table. 4).

After 40^{th} days, the rate of carotenoid and total chlorophyll decreased significantly at the dose of 2500 $mg.l^{-1}$ and 3000 $mg.l^{-1}$ compared to the control (-27%, -41% and -24%, -34%, respectively) and no difference was noted in the other concentrations.

Lipid peroxidation and hydrogen peroxide

At 20^{th} days of exposed to increased levels of lead (500, 1000, 1500, 2000, 2500 and 3000 $mg.l^{-1}$), we noted a significant increase level of hydrogen peroxide in sheets compared to control. In rods, the level of hydrogen peroxide was higher by 2 to 4 times in all groups exposed to lead compared to control. According our results (fig. 1), we noted that the hydrogen peroxide content was very lower in roots of control, however in contrast the level of hydrogen peroxide was 60 times more important ($p<0,05$) in stems at 3000 $mg.l^{-1}$ compared to control. After 40 days, the level of hydrogen peroxide is significantly higher (400 times) in the sheets of plant exposed to 3000 $mg.l^{-1}$ of lead compared to control.

Fig. 1, shows a significantly increased content of lipid peroxidation by 1.5 to 2.5 compared to control in sheets after 20 day of plant exposed to 2000 $mg.l^{-1}$ to 3000 $mg.l^{-1}$, in rods the level of lipids peroxidation was increased from 1.8 to 5 times in all groups exposed to different concentration of lead compared to control. It has been noted that the rate of TBARS increases each time that the concentration in lead increases, however in roots, after 20 day, we noted that the level of lipids peroxidation was increased by 87.5%, 150%, 250 %, 275% and 300% respectively to a concentration in lead of 1000mg/l, 1500 mg/l, 2000 mg/l, 2500 mg/l and 3000mg/l. after 40 days of exposed to lead, we obtain that the rods and roots indicate a significant increased of level of lipids paroxidation in all groups by 1.2 and 2.7 times compared to control; in sheets, we noted the rate of lipid peroxidation decreases significantly with increasing the rate of lead in the ground, from 1.3 to 3.8 times less.

Soluble protein and Proline levels

The results obtained in Table 5. indicated that the level of soluble protein in leaves was significantly increased by 35% and 60% in groups receive 2000 $mg.l^{-1}$ and 3000 $mg.l^{-1}$ respectively compared to control, at 500, 1000, 1500 mg/l dose respectively, we noted a significant decrease 12.5% to 50 %. In rods, the rate of soluble protein was 14.6 and 13.6 times more important in plant receive 2500 $mg.l^{-1}$ and 3000 $mg.l^{-1}$ compared to control; in roots, soluble protein was 2 to 4 times more increased in groups receive 1000, 1500, 2000, 2500, 3000 $mg.l^{-1}$ compared to control. In the Table. 6, the soluble protein was significantly higher ($p<0.05$) from 28% to 63% in sheets, rods and roots after irrigation of plant with water contain 3000 $mg.l^{-1}$ of lead.

At level of leaves, rods and roots (Table. 5 and 6), it was observed that the level of proline increase gradually with increase of lead concentration from 10 to 16 times more compared to control. After 40 days of exposition to lead at different concentration, level of proline was significantly higher from 2 to 8 times more than control, in sheets, rods and roots.

Discussion

Lead is an element not very mobile and has a very high persistence in soil (Saddler and Berthelin, 1998). The plants have many systems of detoxification to limit interaction of these ions with biological molecules; lead induce a range of deleterious effects for the agencies, he disrupts the membrane structures and parietal, modified the statute of waterborne, disrupted the absorption and/or the translocation of essential minerals elements (calcium, manganese, zinc, iron etc….) or further reduced photosynthesis (Seregin and Ivanov, 2001; Sharma and Dubey, 2005). Depending to environment conditions, plants can absorb a part of the lead present in the soil and Pb2+ ions broadcast in the root, but are blocked by the physical barrier like endoderme. At the macroscopic scale, the plants exposured to lead induces a reduction in growth of biomass, yields and when stress is too severe, led to the emergence of resprouts necrosis and foliar, chlorotic, or even to the death of the plant (Sharma and Dubey, 2005). It also presents a high affinity for proteins which possess thiol groups or of metal cofactors (metallo-enzymes). However, despite the toxicity certain of these two types of interactions, it is not enough to explain the large variety of deleterious effects observed in plants treated with lead and particularly the genotoxicity induced by this metal (pourrut et al., 2008).

In our results indicate that the treatment with lead induces an increase in production of H_2O_2 and $O_2^{\bullet -}$, we can explain this by fact that it is possible to estimate indirectly the level of ERO production and the generation of oxidative stress, by measuring the activity of antioxidant enzymes, or the rate of lipid peroxidation, which are biomarkers of oxidative stress (Sharma and Dubey, 2005; Chen et al., 2007; Wang and al., 2007). An increase in lipid peroxidation content in coriander grown under Pb stress was observed (Fig. 1). It is possible that increase of MDA concentration in Coriander may be due to a increase in polyunsaturated fatty acid concentration relative to saturated fatty acids, which has also been reported in some plants under stressful conditions (Rucinska and Gwozdz, 2005; Grappa et al., 2007).

The increased of H_2O_2 may be essentially due to reduction of catalase activity, the case of this enzyme is complex, since half of publications reported an inhibition of activity by lead (Verma and Dubey, 2003; Choudhury and Panda, 2004; Seregin and al., 2004; Chen and al., 2007; Dey and al., 2007; Hu and al., 2007; Qureshi and al., 2007; Gopal and Rizvi, 2008), the other half shows an increase of his activity (Reddy and al., 2005; Mishra and al., 2006). This inhibition seems to be due to the species treated, to duration or to intensity of the treatment. The inhibition of catalase activity has been observed during different stress, for all the organisms studied, this inhibition is not a general rule; the origin of this phenomenon is still widely debated. In addition, when the CAT is inhibited, this mechanism is dose-dependent and the CAT is a metallo-enzyme, its inhibition could be due to a direct interaction with the lead (Landberg and Greger, 2002).

Some molecules non-loaded are also able to spread through the plant walls and can thus cause of multiple damage as oxidation of DNA (Britt, 1997), or even the oxidation of proteins including at the level of the cysteines and methionines (Rinalducci et al. 2008; Bartoli et al., 2004), these are the two amino acids that are most sensitive and since they are quite often involved in the fixing of metals or in the catalytic properties of many enzymes and proteins; reactive oxygen species inactivate enzymes and damage important cellular components. ROS are responsible for protein, lipid and nucleic acid modification and are thought to play a major role in ageing and cell death (Jacobson et al., 1996).

The lead exposure leads to a strong inhibition of photosynthesis, the photosynthetic yield, and to a limitation of the rate of assimilation of CO_2.This inhibition is explained by the decrease of the levels of chlorophylls and carotenoids generally found (Kosobrukhov and al., 2004; Gopal and Rizvi, 2008). The chlorophyll b seems more sensitive than chlorophyll a (Kacabova and Natr, 1986 ; Wozny and al., 1995; Vodnik and al., 1999). Singlet oxygen is the first excited electronic state of O2. Insufficient energy dissipation during photosynthesis can lead to formation of chlorophyll (Chl) triplet state. And the Chl triplet state can react with $3O_2$ to give up very reactive singlet oxygen. It has been proved that singlet oxygen formation during photosynthesis can have damaging effect on photosystem I (PSI) and a photosystem II (PSII) and on whole machinery of photosynthesis.

Lead interacts at this level in two ways:

- In a direct manner, by substituting divalent ions related to the metallo-enzymes. This is particularly the case with the δ-aminolevulinate deshydratase (ALAD) which is at the basis of the synthesis of the chlorophylls and whose ion Zn2+ is replaced by Pb2+;

- In an indirect way, by inducing a deficiency in these divalent ions.

Proline, α-amino acid is an antioxidant and potential inhibitor of programmed cell death and is considered as an indicator of stress; in several plant, the accumulation of proline has been observed as a response to biotic and abiotic stress ((Boguszewska et al. 2010; Torres 2010; Khatamipour and al., 2011). It has been suggested that free proline act as osmoprotectant, a protein stabilizer, a metal chelator, an inhibitor of lipid peroxidation and OH• and $^1O_2$ scavenger. Increased proline accumulation appears especially during salt, drought and metal stresses (Trovato et al., 2008). Therefore proline is not only an important signaling molecule, but also an effective ROS quencher. It has been found that the important role of proline is in potentiating pentose-phosphatase pathway activity as important component of antioxidative defense mechanism (Hare & Cress 1997).

Conclusion

Urban activities, industrial and agricultural are responsible for a growing contamination of soils and water by heavy metal, particularly lead. Overall the effect of lead toxicity on the cellular metabolism of plants depends on the concentration in Pb^{2+} and effectiveness of the protection systems of plant. In recent years, the development of the effective techniques to decontaminate polluted sites has become indispensable. Phytoremediation, which leverages the properties of certain plants to accumulate large amounts of heavy metals.

Our results have allowed us to see the disturbances in metered parameters, an increase in the content of proline, lipid peroxidation and hydrogen peroxide each time there is an increase in the concentration of lead, and a decrease in the pigment content chlorophyllien. Following our results, we can see that this plant can be regarded as being a good plant remediation activity itself because it has the ability to push in high concentrations of lead; that indicate that this plant may be a good accumulatrice of lead.

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Table 1: General condition of culture in the greenhouse.

Parameters	Brightness L	Temperature °C	Air W/M2	SUNSHINE W/m2	Wind km/h	Humidity %	Side
Day 1	10000	23.9	474	468	0	100	Right
Day 2	9915	15.6	50	51	K/h	100	Left
Day 3	9911	15	50	48	K/h	100	Left
Day 4	9663	15	49	46	K/h	100	Left
Day 5	9410	14.8	50	44	K/h	100	Left
Day 6	9174	14.6	50	43	K/h	100	Left
Day 7	10000	24	474	466	0	100	Right
Day 8	10000	24.9	475	470	0	100	Right
Day 9	8914	14.7	50	43	K/h	100	Left
Day 10	8876	14.3	47	41	K/h	100	Left

Table 2: Plant morphology modification after 20 days to lead-exposed at different level

Concentration mg/l	Length (cm)	Number of Sheets	Weight (g)		
			Sheets	Rods	Roots
Control	11.5	113	1.641	1.315	0.563
500	7.04	67	0.458	1.115	0.347
1000	4.02	47	0.337	0.675	0.331
1500	3.07	33	0.284	0.259	0.102
2000	2.6	29	0.155	0.164	0.84
2500	1.5	17	0.122	0.077	0.75
3000	1.01	9	0.117	0.36	0.34

Table 3: plant morphology modification after 40 days to lead-exposed at different level

Concentration Mg/l	Length Cm	Number of sheets			Weight (g)		
		Green	Clear Green	Yellow	Sheets	Rods	Roots
0	23	595	0	0	17.961	5.726	2.828
500	19	567	158	0	15.460	5.222	3.443
1000	17.5	487	182	28	15.470	5.594	4.671
1500	18	334	298	131	17.635	5.377	3.847
2000	18.5	266	184	141	18.814	5.433	4.635
2500	19	158	398	157	19.323	5.245	4.213
3000	17.8	165	390	149	17.524	5.317	5.423

Table 4: Effect of lead on different pigments in *Coriandrum sativum* L.

	20th Day				40th Day			
	Chlorophyll mg,g^{-1} Fresh tissue weight				Chlorophyll mg,g^{-1} Fresh tissue weight			
	a	b	Total	Total Carotenoid	a	b	Total	Total Carotenoid
Control	9,22±1.12	7,18±1.22	16,4±2.55	5,99±2.85	7,47±0.99	3,6±0.44	11,07±1.13	2,95±0.75
500	8,26±0.73	5,46±2.43	13,72±2.10	3,05±1.25	7,23±1.57	3,4±0.97	10,63±1.55	2,71±0.61
1000	6,92±1.43	3,16±0.91	10,08±1.98*	1,73±1.97	7,13±1.62	3,23±0.34	10,36±1.92	2,69±0.54
1500	5,23±1.29*	3,49±1.09*	8,72±1.21*	1,73±2.44	6,81±2.55	3,16±1.07	9,96±2.40	2,6±0.16
2000	4,84±0.81*	2,09±1.46*	6,93±2.45*	1,35±1.11*	6,26±2.09	2,93±0.81	9,19±1.41	2,42±0.45
2500	3,18±1.32*	1,97±1.66*	5,15±1.68*	0,48±0.22*	5,7±1.84	2,63±0.71	8,33±1.34	2,13±0.72
3000	2,17±0.75*	1,08±0.99*	3,25±2.59*	0,32±0.14*	5,01±1.24*	2,21±0.42*	7,23±1.75*	1,73±0.64

(*) the average of three replication are significantly different compared to control at $p<0.05$.

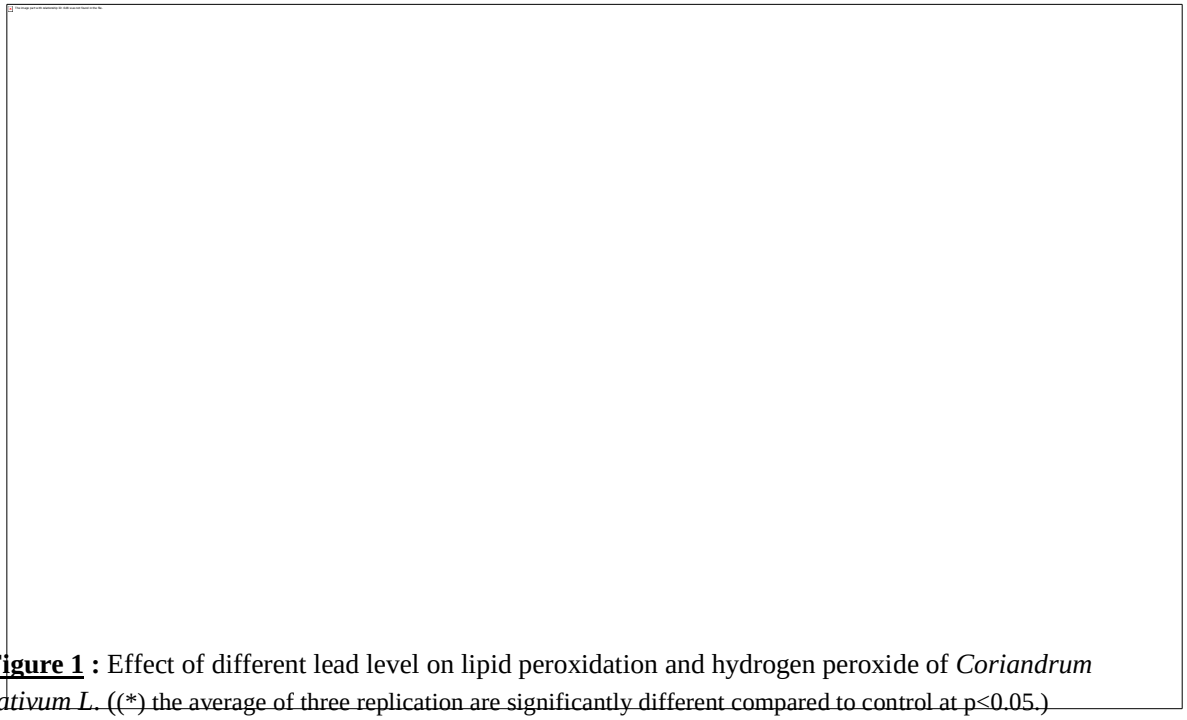

Figure 1 : Effect of different lead level on lipid peroxidation and hydrogen peroxide of *Coriandrum sativum L.* ((*) the average of three replication are significantly different compared to control at $p<0.05$.)

Table 5: Effect of lead on soluble proteins (µg/g fresh tissue weight) and proline ($mM.g^{-1}$) in *Coriandrum sativum* L. after 20 days

	Sheets		Rods		Roots	
	Soluble Protein	**Proline**	**Soluble Protein**	**Proline**	**Soluble Protein**	**Proline**
Control	114,8±6.4	1,78±0.45	8,4±1.14	1,11±0.61	103,6±10.2	0,56±0.42
500	58,8±4.8*	2,28±0.65	16,8±1.01*	2,94±0.35*	98,0±9.5	1,33±0.54
1000	67,2±5.7*	3,28±0.49*	47,6±3.21*	3,83±0.73*	224,0±20.2*	1,94±0.96
1500	98,0±4.9*	4,67±0.46*	22,4±2.22*	4,94±1.04*	302,4±34.3*	4,72±1.40*
2000	151,2±15.5*	6,33±1.07*	25,2±2.71*	5,89±0.89*	355,6±40.1*	9,67±2.20*
2500	112,0±16.4	10,94±2.22*	123,2±15.51*	6,94±1.55*	291,2±35.5*	12,56±2.75*
3000	179,2±25.5*	12,78±3.33*	114,8±13.43*	9,00±2.22*	431,3±55.5*	16,89±4.61*

(*) the average of three replication are significantly different compared to control at $p<0.05$.

Table 6: Effect of lead on soluble proteins (µg/g fresh tissue weight) and proline ($mM.g^{-1}$) in *Coriandrum sativum* L. after 40 days

	Sheets		Rods		Roots	
	Soluble Protein	**Proline**	**Soluble Protein**	**Proline**	**Soluble Protein**	**Proline**
Control	30,8±4.5	0,009±0.001	28,0±2.22	1,01±0.29	19,6±3.12	1,1±0.44
500	61,6±6.6*	0,014±0.004	22,4±1.75*	1,72±0.42	19,6±4.42	2,3±0.82
1000	58,8±3.7*	0,019±0.003*	14,0±0.95*	1,90±0.72	14,0±2.71	2,7±0.42*
1500	50,4±3.1*	0,021±0.007*	8,4±1.40*	3,51±1.21*	14,0±3.65	3,0±0.75*
2000	75,4±7.4*	0,021±0.005*	11,2±0.75*	4,85±1.53*	11,2±1.71*	4,0±1.09*
2500	58,8±5.2*	0,022±0.006*	25,2±2.33	6,50±1.95*	19,6±3.10	5,3±1.17*
3000	81,2±8.8*	0,027±0.004*	30,8±4.41	8,31±2.12*	25,2±2.73	7,4±1.54*

(*) the average of three replication are significantly different compared to control at $p<0.05$.