ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 3, Issue, 6, pp.018-023 June, 2011

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

INVESTIGATION OF CADMIUM AND LEAD INDUCED PHYTOTOXICITY ON SEED GERMINATION, SEEDLING GROWTH AND ANTIOXIDANT METABOLISM OF CHICK PEA (*Cicer arietinum* L.)

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ARTICLE INFO

Article History:
Received 9th March, 2011
Received in revised form
11th April, 2011
Accepted 27th May, 2011
Published online 2nd June 2011

Key words:
Heavy metals,
Lipid peroxidaion,
Antioxidants,
Radical,
Hypocotyls,
Germination,
Reactive Oxygen Species (ROS)

ABSTRACT

Heavy metals accumulation in soil has become a worldwide problem, leading to loss of agricultural productivity. Lead and cadmium are the toxic elements of primary importance. A study was conducted to determine the effect of different concentrations of cadmium and lead on seed germination, seedling growth, oxidative stress and antioxidant response of Chick pea (*Cicer arietinum* L.). Seeds were grown under laboratory conditions at 0.25, 0.5, 1, 2 and 4 mM $l^{-1}$of metal ions. All of these metals were added as single metal solution, i.e. one experimental set contained a single metal in particular concentration. A slight stimulatory effect on seedling growth was observed, especially at low metal concentrations (0.25-0.5 mM l^{-1}). Both lead and cadmium treatments showed toxic effects on various growth indices of chick pea at high metal concentrations (1-4 mM l^{-1}). The level of lipid peroxidation of germinated seeds was measured as Malondialdehyde (MDA) contents and were significantly enhanced by a high Cd and Pb concentration. The activity levels of some antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GPx, EC 1.11.1.9), did not change much at low metal concentrations, but fluctuated drastically at high Cd and Pb concentrations. These results suggest that Cd and Pb toxicity causes oxidative stress in plants and the antioxidative enzymes SOD, CAT and GPx could play a crucial role against oxidative injury.

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INTRODUCTION

Heavy metal pollution of air and agricultural soils is one of the most important ecological problems on world scale. Lead and cadmium are the toxic elements of primary importance (Breckle and Kahile, 1992). Contaminant metals can often accumulate in considerable amounts in the plant tissue and exceed the levels that are toxic to man or animal before they produce visible phytotoxic effects. More cadmium is being found in soil due to the amendment of chemical fertilizers, sludge and sewage irrigation and atmospheric deposition (Adams *et al.,* 2004 and Ranieri *et al.,* 2005). This is a growing concern because Cd in soil can be transferred to plants, resulting in phytotoxicity and threats to animal and human health through the food chain (Wagner, 1993). Various researchers have reviewed the toxic effects of Cd on biological systems; some general symptoms of Cd toxicity in plants include growth inhibition, chlorosis, and alterations of anatomical, morphological, physiological, or biochemical properties in different tissues (Watanabe and Suzuki, 2002; Maksymiec and Krupa, 2006).

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Although Cd is generally considered to be a highly toxic element and its negative effects on plant development and growth have been frequently observed in previous studies (Rubio *et al.,* 1994; Watanabe and Suzuki 2002; Maksymiec and Krupa 2006), a positive effect on plant growth at low concentration has also been reported in plants such as rice (Liu *et al.,* 2003; Aina *et al.,* 2007), soybean (Sobkowiak and Deckert 2003), barley (Wu *et al.,* 2003), and miscanthus (Arduini *et al.,* 2004) in hydroponics experiments. Lead is also one of the best known heavy trace elements, with a long history of toxicity. Its exposure is becoming a great concern because of its toxic nature, wide spread occurrence and long life in biological system. The major sources of lead in soil are usually derived from weathered bedrock, parents material from lead mine, smelting operations, use of lead arsenate, use of tetramethyl lead as antiknocking additive to petrol (Foy *et al.,* 1978). Lead is common heavy metal and can be found in batteries, ceramics, chemicals and fertilizers. It is also used in a number of products including gasoline, hair dyes, leaded glass, newsprint, paints, pesticides, and pottery and rubber toys. Inhibition to germination and retardation of plant growth has been reported due to lead toxicity (Jaffer *et al.,* 1999; Wierzbicka and Obidzinska, 1998).

The purpose of this study is to examine the effects of Cd and Pb on the seed germination, growth, oxidative stress, and antioxidant response of chick pea (*Cicer arietinum* L.). The possible mechanisms of chick pea seedlings response to heavy metal stress involving lipid peroxidation and antioxidant changes are also discussed. Information from this study will be helpful to identify toxic critical values of Cd and Pb based on chick pea's response and the changes in biochemical parameters; it will also provide a reference for eco-toxicity assessment of these heavy metals.

MATERIAL AND METHODS

Reagents and Chemicals

Cadmium carbonate ($CdCO_3$) obtained from CDH chemicals, Mumbai and Lead Nitrate pure [Pb $(NO_3)_2$] was obtained from Sisco Research Laboratories (SRL) Pvt. Ltd, Mumbai, India. All other chemicals and reagents used were of analytical grade and highest purity.

Collection and Surface Sterilization of Seeds

Seeds of *Cicer arietinum* L. were obtained from Tamil Nadu Government Agri- Horticulture Department, Villupuram, India. They were surface sterilized with 1% $HgCl_2$ for 30 minutes and rinsed with tap water followed by double distilled water. The experiments were carried out using uniform size seeds.

Seed Germination and seedling growth Experiment

The effect of cadmium and lead on chick pea seed germination was carried out as follows: The seeds were plated in Petri dishes with four filter-paper discs containing 0, 0.25, 0.50, 1.0, 2.0 and 4 mM l^{-1} each of Cd and Pb (All of these metals were added as single metal solution, i.e. one experimental set contained a single metal in particular concentration), soluble from Cadmium carbonate and Lead nitrate respectively and germinated for 2 days at 30 ± 2 ºC in the darkness in an incubator. The Petri plates also contained streptomycin at a final concentration of 25 µg ml^{-1} to suppress microbial growth. For root and shoot growth experiments, seedlings were cultivated in Petri dishes with four layers of filter paper containing 20 ml of afore mentioned heavy metal concentrations at 30 ± 2 ºC under a light irradiance of 300 mmol m^{-2} s^{-1} (12±2 h light-dark cycles) for 12 days. All the experiments were carried out in triplicate and each replicate was carried out on 25 seeds for germination and 20 seedlings for growth measurements. At the end of experimental period, the Radical and Hypocotyls length were measured using thread and scale.

Determination of plant heavy metal content by AAS

Fresh plant material was washed with dilute (1M) hydrochloric acid, rinsed with distilled water and dried in direct sunlight. The dried plant samples were decomposed to ash by heating at 250°C for 12 hours in a muffle furnace. The ashed plant samples were digested in nitric acid-sulphuric acid till a clear solution was obtained and the volume was maintained with double distilled water. Lead and cadmium were estimated at 283.3nm and 228.8nm respectively by atomic absorption spectrophotometer *(Instrument: Perkin Elmer; Model: 2380; Light source: hollow cathode lamp; Absorption cell: graphite furnace)*. The cadmium and lead content of plant materials were expressed as µg g^{-1} FW.

Lipid peroxidation analysis

The effect of varying concentrations of Cd and Pb on lipid peroxidation was measured by the method of Zhou (2001) with some modifications. 0.5g of seedlings (germinated for 2 days) were homogenized in 5ml of 0.25% thiobarbituric acid, then heated at 98 ºC for 30 min, quickly cooled on ice and then centrifuged at 10,000 rpm for 10 min at 4 ºC, the absorbance of the supernatant was measured at 532nm.

Measurement of activities of Antioxidant enzymes

Enzymatic antioxidants (SOD, CAT, and GPx) were measured. Enzymes were extracted according to Cho and Seo (2005) with a small modification. Samples of frozen leaves (about 0.5 g fresh weight) were ground with liquid nitrogen and homogenized in 2.0 ml ice cold 100 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 1.0% poly vinyl pyrrolidone (PVPP). The homogenates were centrifuged at 12000g for 20 min at 4 ºC and the supernatants were used for the assay of antioxidants and the protein content. The protein content was determined using the method of Bradford (1976). Bovine serum albumin (BSA) was used to construct the calibration curve. SOD activity was assayed by its ability to inhibit the reduction of the nitroblue tetrazolium (Dhindsa *et al.,* 1980). The absorbance was recorded at 560 nm, and one unit of enzyme activity was defined as the amount of enzyme causing 50% inhibition of NBT reduction under the assay conditions. CAT activity was assayed according to the method of Cakmak *et al.,* (1993). Adding H_2O_2 started the reaction, and the decrease in absorbance at 240 nm was recorded for 1 min. Enzyme activity was calculated by using the extinction coefficient of 39.4 mM^{-1} cm^{-1}. Peroxidase activity was assayed by the method of Kar and Mishra (1976). The reaction mixture contained 100 mM Tris-buffer (pH 7.0), 10 mM pyrogallol and 5 mM H_2O_2. The reaction was started by adding 25 µl enzyme solution and stopped after 5 min incubated at 25 ºC by adding 1.0 ml 2.5 N H_2SO_4. The amount of purpyrogallin formed was measured spectrophotometrically at 425 nm. The enzyme activity was expressed as change in absorbance units g^{-1} fresh weight min^{-1}.

RESULTS

Effects of Cadmium and Lead on germination and growth parameters

The effect of Cadmium and Lead on Chick pea seed germination under laboratory conditions after 12 days was observed and given in Table 1. The results indicated that seed germination rate had a slightly upward trend up to 0.5 mM l^{-1}, where as, at 1 - 4 mM l^{-1} of metal stress showed inhibitory effects, when compared to their relevant controls. The radical lengths of seedlings exposed to various concentrations of Cd and Pb were presented in Fig. 1 From the results it was observed that upto 0.5 mM $l^{-1}$of Cd and Pb promoted the radical growth by 7.22% and 8.64%, respectively, when compared to the radical length of the control experiments. The

Table 1. Effect of Cadmium and Lead on percentage of Chick pea (*Cicer arietinum* L.) Seed germination

Heavy metal Concentration (mM l^{-1})	% of Seed Germination	
	Cd	Pb
0	89.66 ± 1.88	91.62 ± 1.96
0.25	92.66 ± 2.69	94.06 ± 2.36
0.5	93.54 ± 3.23	94.62 ± 2.92
1.0	83.82 ± 3.65	84.65 ± 3.69
2.0	76.39 ± 3.44	77.69 ± 3.62
4.0	69.36 ± 2.89	71.63 ± 2.58

All the data are mean of three values ±SD.

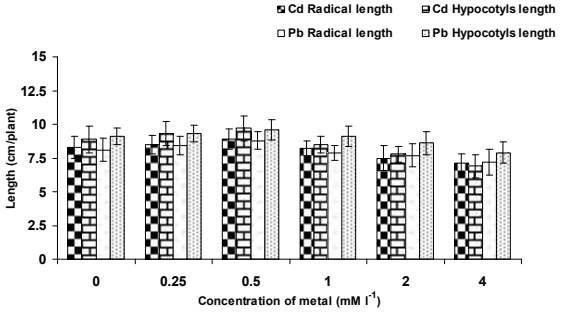


Fig. 1. The effect of Cd and Pb addition on radical and hypocoyls length of *Cicer arietinum* seedlings (12 days). (Data points and error bars represent mean ± S.D. of three replicates (n = 3).

Effect of Cd and Pb on hypocotyls length of chick pea during seed germination was observed and the results shown in Fig. 1. The length of hypocotyls was increased with increasing metal concentrations upto 0.5 mM l^{-1} and the percentage of hypocotyls length increased with 8.98 and 5.49 for Cd and Pb at 0.5 mM l^{-1}, over the control experiments. Further increase in metal stress showed the concentration dependent inhibition of radical and hypocotyls lengths between 1 and 4 mM l^{-1} doses.

Heavy metal accumulation in Plant

The metals accumulation in the plants was estimated at the end of 12 days, the Cd and Pb concentration of plants increased with the increase of metal concentration in solution (Table 2). The increase in the Cd and Pb concentration of plant were by far higher between 1 and 4mM l^{-1} of metal concentration than between the lower levels. Thus, in chick pea, the highest Cd and Pb concentrations were estimated at 4 mM l^{-1} and were 91.6 and 198.3 µg g^{-1} FW respectively.

Table 2. Cadmium and Lead content of Chick pea seedlings treated for 12 days with different concentrations of metal

Heavy metal Concentration (mM l^{-1})	Plant content of Cd and Pb (µg g^{-1})	
	Cd	Pb
0	0.53 ± 0.08	1.3 ± 0.7
0.25	8.6 ± 1.6	12.4 ± 1.3
0.5	17.3 ± 1.8	35.6 ± 1.8
1	26.3 ± 2.7	56.7 ± 2.4
2	39.4 ± 2.9	126.8 ± 3.9
4	91.6 ± 3.6	198.3 ± 4.2

All the data are mean of three values ±SD.

Estimation of Lipid peroxidation

The effects of Cd and Pb on lipid peroxidation of the germinated seeds were estimated through malondialdehyde

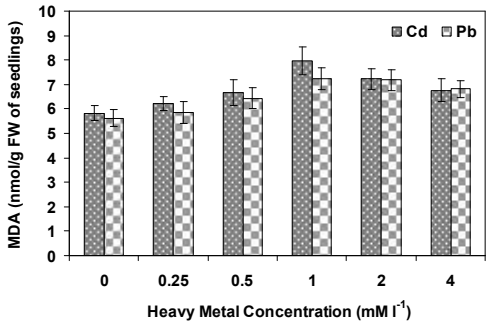
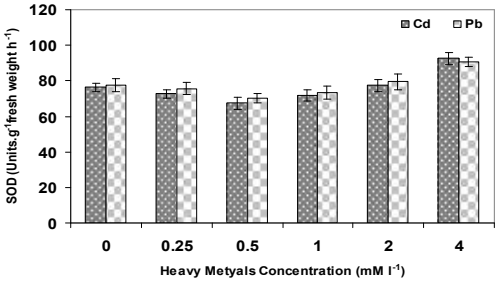


Fig. 2 The effects of Cd and Pb on lipid peroxidation of the germinated seeds (2 days) were estimated through malondialdehyde (MDH) concentration present in plantlets.
Data points and error bars represent mean ± S.D. of three replicates (n = 3).

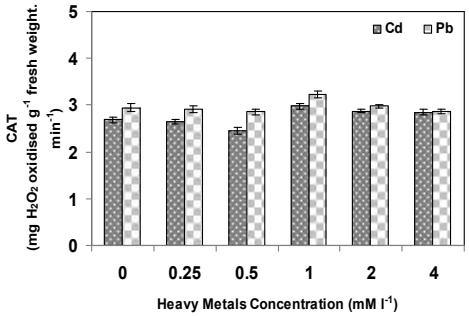
concentration present in plantlets. The malondialdehyde (MDO) content of the plants was elevated due to metal toxicity and the magnitude of elevation ranged from 1.07 to 1.36 folds for Cd and 1.04 to 1.28 for Pb over controls at 0.25 to 4 mM l^{-1} of heavy metal stress. The accumulation was significant at the 1 mM l^{-1} of heavy metal treatment level, where it was approximately 28-36% more than that of the control (Fig. 2).

Determination of Antioxidant enzymes (SOD, CAT and GPx)

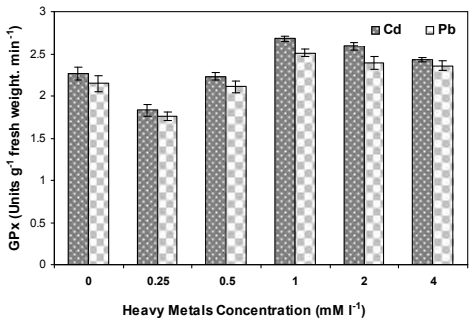
The antioxidant enzymes during metal stressed conditions were estimated (SOD, CAT and GPx). At the lower levels of metal exposure, the antioxidant enzyme activities were not significantly altered when compared to the control (Fig. 3a-3c), whereas high levels of metals stimulated enzyme activity. Superoxide dismutase activity was increased due to metal stress and the maximum enzyme activity was observed at 4 mM l^{-1} of metal treatment compared to controls and increased by 21% for Cd and 16.8% for Pb. The maximum activity of CAT and GPx was observed at 1 mM l^{-1} of metal exposure, where enzyme activity levels increased by 9.5-10.7% for CAT and 16-18% for GPx.



(3a)



(3b)



(3c)

Fig 3a-3c. Changes in the activity of SOD, CAT and GPx under different levels of Cd and Pb for 12 days. Data points and error bars represent mean ± S.D. of three replicates (*n* = 3).

DISCUSSION

Seed Germination and seedling growth

From the Fig. 1 the heavy metals at lower concentrations (0.25-0.5 mM l^{-1}) slightly enhanced the percentage of *Cicer arietinum* seed germination, radical and hypocotyls length, whereas the stimulatory effects declined at higher levels (1-4 mM l^{-1}). This stimulatory effect on growth observed at morphologic levels could be ascribed to the enhancement of cell division and proliferation at microcosmic levels, which were observed for both animals and plants in cell culture studies (Beyersmann and Hechtenberg 1997). Similar trend in hydroponics experiments were observed by Stolt *et al.,* 2003. According to Sobkowiak and Deckert (2003), the stimulation of cell proliferation by low doses of Cd could be connected to the capacity of Cd to functionally substitute Zn^{2+}, which allows the binding of multiple transcription factors to the regulatory regions of the genes and is also a component of key enzymes involved in replication and translation. Declining pattern in growth of plants at high metal stress could be due to the obstruction of metabolic processes which allied with regular growth of plants. Similar experiments conducted by Ahsan *et al.,* (2007) reported that the elevated levels of toxic metals affects the seed germination, this is due to the high level of metals significantly decreases the photosynthesis process in the plants.

Metals content of Plant

An increase in Cd and Pb content in the plants studied was noticed with increase in the strength of metal stress. Generally, the toxicity of metals within the plant occurs when metals move from soil to plant roots and get further transported and stored in various sites in the plant (Verma and Dubey 2003). When these toxic metals are taken up by the plants, toxicity symptoms occur within the tissues. During the uptake process, the root cell walls initially bind metal ions from the soil and thereafter via high affinity binding sites and plasma membrane localized transport system; metal ions are taken up across plasma membrane (Mishra and Dubey, 2006). After up taking the toxic heavy metals get stored in all the parts of the plant systems and which, triggers lot of physiological and biochemical changes in the plants. In the present study, the chick pea seed germination was carried out with toxic heavy metals, Cd and Pb. The metal content was estimated in 12 days grown plants and the results were shown in Table 2. The metal uptake is depends on the oxidative states of the metal ions. The high levels of Pb accumulated in the plants was observed when compared with Cd, this is due to the Pb metal ions actively transported in to the roots. After treatment at 0.25, 0.5, 1.0, 2.0 and 4.0 mM l^{-1} doses of metals, the amounts of these metals in tissues differed even more, since the total amount of lead was 2.2 fold higher than that of cadmium (91.6 ± 3.6 and 198.3 ± 4.2 µg g^{-1}). These points to a difference in toxicity between cadmium and lead.

The results obtained on miscanthus previously, the toxicity threshold could be set more exactly between 0.5 and 0.75 mg l^{-1} for Cd (Arduini *et al.,* 2004). Although our results indicate that chickpea is sensitive to cadmium concentrations higher than 0.5 mM l^{-1} in the exernal solution, a similar degree of sensitivity was found by Landberg and Greger (1994) in willow clones and by Salt *et al.,* (1995) in *Brassica juncea,* plants that are both considered suitable for the phytoextraction of cadmium. An increase in lead content in the plant studied was noticed with increase in the intensity of metal stress. The percent increase in lead concentration was more when compared to cadmium. Plants absorb lead in its soluble form from soil through roots and there may be a limited translocation to the shoots. Based on comparative studies of metal content in plant parts Baker and Walker (1990) suggested that uptake, translocation and accumulation mechanisms differed for various heavy metals and for the species. Mostly, the plants with highest tolerance take-up the smallest proportion of the total soil-metal and had the lowest shoot metal contents (Sudhakar *et al.,* 1992 and Liu *et al.,* 2004).

Lipid peroxidation

Application of different concentrations of Cd and Pb resulted in membrane damage and lipid peroxidation in plant under investigation, which was obvious from significant increase in the content of malondialdehyde (MDA) in a concentration dependent manner (Fig. 2). The induction of oxidative stress by Cd has been considered to be partially responsible for Cd phytotoxicity (Mithofer *et al.,* 2004 and Smeets *et al.,* 2005). The significant increase in MDA concentration observed at higher Cd concentrations also suggests that Cd induced the increasing oxidative damage. However, the growth parameters in these groups were still higher than those in the control, suggesting a temporary stimulatory effect in the early growth period for these groups.

Further, plants usually experience oxidative stress when they are exposed to Pb and other heavy metals (Fargasova 1994 and MacFarlane 2003). The reactive oxygen species produced as a result of oxidative stress causes a variety of harmful effects in plant cells including lipid peroxidation. In the present study an increase in the MDA content reflects that ROS caused lipid peroxidation in planlets, which was more severe above 0.5 mM l^{-1} of Pb. This is due to increased level of H_2O_2 is produced either due to action of SOD on superoxide radicals or by direct formation in biochemical pathways viz., photorespiration. lipid peroxides are formed by direct action of redox-active metals or indirectly by lipoxygenase-mediated lipid peroxidation by non-redox active metals (Mishra *et al.,* 2006). To cope and repair the damage caused by ROS, plants

have evolved complex antioxidant (both enzymatic and non-enzymatic) systems. However, the severe stress of reactive oxygen species always causes oxidative damage to antioxidant enzymes and, hence, the antioxidant enzymes activities are diminished (Gallego *et al.,* 1996).

Antioxidant Enzymes during Metal stress

Reactive oxygen species (ROS) are by-products of aerobic metabolism, which are inevitably generated by a number of metabolic pathways. These are partially reduced forms of molecular oxygen (O_2) and typically result from the transfer of one, two or three electrons to O_2 to form superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), respectively (Mittler 2002). Under normal growth conditions plants, invariably generate ROS as by-products from the metabolic process and this is the predominant source of ROS in plants (Shah *et al.,* 2001). However, when the plants are subjected to metal stress conditions ROS production was increased, these overproduced ROS was scavenged by the antioxidants, oxidative damage and lipid peroxidation mechanisms by the plants.

In the present study, the effect of Cd and Pb on antioxidants were observed and given in Fig.3a-3c. Based on biological significance the Cd and Pb are considered as non-redox group of metals, these metals can directly cause oxidative injury in plant tissues, because they initiate hydroxyl radical production. Schutzendubel *et al.* (2002) studied the effect of plants to non-redox-active metals and concluded that the oxidative stress elevated to the plants, due to lipid peroxidation, protein oxidation and an oxidative burst. In these lipid peroxidation is the major indicator of the oxidative stress, due to excessive production of ROS by the plants against toxic heavy metals. Sharma and Dubey (2005), reported that the overproduced ROS and involves oxidative degradation of polyunsaturated fatty acyl residues of membrane lipids during metal stressed conditions.

In this study, a significant increase of SOD activity in leaves was observed only at the Cd treatment level of 4mM l^{-1} (Fig. 3a). This may be attributed to the increased production of superoxide, resulting in the activation of existing enzyme pools or increased expression of genes encoding SOD (Mishra *et al.,* 2006). Increased SOD activity caused by heavy metals has been previously observed in several plant species, and is routinely considered to be an adjustment response to stress (Verma and Dubey 2003). A slight drop or no change in SOD activity under low levels of Cd stress indicates no excess accumulation of superoxide anion in seedlings, since SOD activity is mediated by superoxide level (Somashekaraiah *et al.,* 1992). Furthermore, in the present investigation, the H_2O_2-scavenging enzymes (CAT and GPx) showed similar responses to heavy metal stress (Fig. 3b-3c), probably due to their co- regulation (Mishra *et al.,* 2006). No significant variance compared to the control was observed at low levels of Cd treatment, possibly indicating no excess accumulation of H_2O_2 in tissues. In contrast, the significant induction of enzyme activity in the group treated with 1.0 mM l^{-1} Cd and Pb might indicate excessive accumulation of H_2O_2. However, the reduction observed with above 1.0 mM l^{-1} treatment might be attributed to enzyme protein damage due to excessive H_2O_2 or Cd and Pb.

Conclusion

The present investigation indicates that the Chick pea seed germination exposed to Cd and Pb concentrations from 0.25 to 4 mM l^{-1} did not cause any visible toxic symptoms. At lower metals concentrations (0.25-0.5 mM l^{-1}) slightly enhanced the percentage of *Cicer arietinum* seed germination, radical and hypocotyls length, but the stimulatory effects declined at higher levels. The level of lipid peroxidation measured as MDA content and antioxidant enzyme activities were significantly induced in Chick pea seedlings, when the heavy metals concentration exceeds 1mM l^{-1}. The study reveals that the exposure of plants to unfavourable environmental conditions such as heavy metals stress can increase the production of ROS e.g., superoxide radical ($O_2^{\circ-}$), hydrogen peroxide (H_2O_2) and hydoxyl radical (OH°), which can exert their toxic effects to the cells. These species may affect cell membrane properties and cause oxidative damage to nucleic acids, lipids and proteins that may make them nonfunctional. Thus, it is evident from this study that incisive use and presence of heavy metals have toxic effects on plants, animals and other living organisms and affects the same after certain limits. Further investigation on cellular or molecular level is needed for better understanding of heavy metal toxicity on plants and allied areas to maintain the ecological harmony of the globe.

Acknowledgement

The authors like to express their thanks to the Management, Dean and Principal of Mohamed Sathak College of Arts & Science, Chennai – 600 119, Tamil Nadu, India for giving necessary permission and facilities to carry out this research work.

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