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TO INVESTIGATE THE ACTIVITY OF ANTIOXIDATIVE ENZYMES IN GERMINATING CHICKPEAS UNDER VARYING CONCENTRATION OF MANGANESE

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ABSTRACT

Metal contamination issues are becoming increasingly common in India particularly in areas with high anthropogenic pressure as metal toxicity is increasing by mining industries, smelters, coal-burning power plants and agriculture. Being non-degradable in nature it enters into the food chain and subsequently reaches to human and animals. In humans, it can cause neurodegenerative diseases, increased allergic reactions, high blood pressure, depression, irritability, poor concentration, sleep disabilities etc. In plants, metal toxicity is manifested through an array of physiological and metabolic alterations, growth reduction, lower biomass production and metal accumulation. Plant tissues with most of metals accumulated in it cause increased generation of reactive oxygen species. In organisms, antioxidants help to deal with oxidative stress caused by free radical damage; as the main characteristic feature of an antioxidant is its ability to trap free radicals. The present work was done to investigate the activity of antioxidative enzymes in germinating chickpeas under varying concentration of manganese. In lower concentration of manganese, a considerable amount of growth is shown as it is required by the plant in trace amount. Antioxidative systems in plants in relation to high Manganese amounts has also been reported as a defense mechanism, as the concentration increases the growth of plant is inhibited due to free radical produced during stress condition of manganese. Trace elements are necessary for the normal metabolic function of the plants, but at higher concentrations, these metals are toxic and may severely interfere with physiological and biochemical functions.

KEYWORDS: Antioxidative enzyme, metal stress, chickpeas, germination.

INTRODUCTION

Chemical waste resulting from industrial activities and agricultural effluents such as fertilizer, herbicides and pesticide has contributed to the increased accumulation of heavy metals in soil (Fu et al. 2014; Hu et al. 2013; Nicholson et al. 2003; Neilson and Raja karuna 2015). During past few decades, unlike organic pollutants which can be easily degradable toxic heavy metals, such as cadmium, copper, lead, chromium and mercury constitute important groups of environmental pollutants worldwide, are immutable by biochemical reactions (Malar et al., 2014), hence, it is difficult to remediate these metals form the soil and water. Soil pollution has recently been attracting considerable public attention since the magnitude of the problem in our soils calls for immediate action (Garbisu & Alkorta 2003). The hot spots of soil contamination are located in the regions of large industrial activities, where surrounding agricultural lands are affected by the deposition of heavy metals and also agricultural practice, e.g., application of sewage sludges, phosphate fertilizers, liming, irrigation water and pesticides has lead to increased heavy metal concentration in soils (Puschenreiter et al., 2005). Cadmium (Cd), Hg, Pb, Cu and As are some of the most toxic heavy metals or metalloids found in soil (Dago et al. 2014;Clemens 2006; Hadi et al. 2013). Cadmium, for example, is nonessential for plant metabolic activities and a known phytotoxicant (Grata oetal. 2005).

The toxicity of metals can affect functioning of all type of living organism from microorganism to higher organisms. The threshold of manganese injury and the tolerance to an excess of this metal highly dependent on the plant species and cultivars or genotypes within a species (Foy et al., 1988, Horst, 1988). In humans, it can cause neurodegenerative diseases and other symptoms like, increased allergic reactions, high blood pressure, depression, mood swings, irritability, poor concentration, aggressive behavior; sleep disabilities, fatigue, speech disorders, high blood pressure, neuropathy, autoimmune diseases, and chronic fatigue are just some of the many conditions resulting from exposure to toxins. About 5% or more of the inhaled oxygen is converted to reactive oxygen species (ROS) such as O_2^-, H_2O_2 and OH^- by univalent reduction of O_2. With the presence of metal ion pollution the synergistic effect of metals and ROS is dangerous to body.

Antioxidant is any substance that, when present at low concentrations, significantly delays or prevents oxidations of cell contents like proteins, lipids, carbohydrates and DNA.

Antioxidants neutralize the effect of free radicals through different ways and may prevent the body from various diseases (Gupta and Sharma 2006). Anti oxidants can act by scavenging reactive oxygen species like Superoxide Dismutase (SOD) removes O_2^-, by inhibiting their formation i.e. by blocking activation of phagocytes, by binding transition metals ions and preventing formation of OH^- or decomposition of lipid hydroperoxides, by repairing damage. Some of the antioxidant enzymes that are found to provide protection against the ROS are superoxide dismutase, catalase, peroxidase, and ascorbate peroxidase (Rani et al., 2004).

Chickpeas is a very popular pulse crop in Asian countries, due to which it was selected for the present study. The current research was an attempt to understand the effect of Mn on growth and on antioxidant enzymes of chickpeas.

MATERIALS AND METHODS

Plant material and seed collection

A cultivar of Chickpeas (C*icer arietium*) was used as the source material in the present investigation. The seeds of the cultivar were obtained from Ambala, Haryana (India).

Germination of seeds

Chickpeas seeds were taken and washed with distilled water. After that the seeds were surface sterilized with 0.1% $HgCl_2$ for 5 min and rinsed with sterile distilled water for 5-6 times. Surface sterilized seeds were germinated aseptically in petri plates having sterilized wet filter paper. The experiments were performed in triplets.

Extraction of enzymes

Enzymes were extracted by homogenizing germinated seeds (1 g) in 10 ml of the extraction buffer (50mM potassium phosphate buffer, pH 7.0) using a chilled mortar and pestle. The resulting homogenate was centrifuged at 10,000 x *g* for 15 min at 4ºC and the supernatant was used for the determination of activities.

Estimation of soluble proteins

Soluble proteins were estimated following the method of Lowry *et al*. (1951). The amount of soluble proteins was calculated in mg g^{-1} FW with the help of standard plot of BSA (0-150 µg).

Estimation of ascorbate peroxidase (APX) activity

Ascorbate peroxidase (EC 1.11.1.11) activity was determined according to the modified method of Zhu *et al.* (2004). The 3.0 ml of assay mixture containing 1.0 ml potassium phosphate buffer (50 mM, pH 7.0), 1.0 ml H_2O_2 (39 mM), 0.8 ml ascorbic acid (0.5 mM) and 0.2 ml of the enzyme extract. The reaction was started by the addition of H_2O_2. A blank was run without addition of the enzyme extract. The activity of APX was measured by monitoring the rate of ascorbate oxidation at 290 nm for 3 min at 30s intervals in a double beam UV-VIS spectrophotometer. One unit of APX activity was defined as the amount required to decompose 1µmol ascorbic acid oxidized min^{-1} calculated from the extinction coefficient of 2.6 mM^{-1} cm^{-1}.

Estimation of peroxidase (POX) activity

Peroxidase (EC 1.11.1.7) activity was assayed adopting the method of Shannon et al. (1966). The assay mixture comprised o-dianisidine (2.4µmol), H_2O_2 (20 µmol), crude extract (0.05-0.5 mg protein) and 0.05 M citrate buffer (pH 4.8) to make the final volume of reaction mixture 3 ml and omission of H_2O_2 from the incubation mixture served as a blank. The enzyme activity was measured by following the absorbance at 430 nm at intervals of 15 sec.

Estimation of H_2O_2

The amount of hydrogen peroxide was estimated according to the method given Sinha (1972). Supernatant was diluted to 2 ml with 10 mM potassium phosphate buffer (pH.7.0). 2ml 5% potassium dichromate and glacial acetic acid (1:3 v/v) was added to the reaction mixture. The absorbance was read at 570 nm against the reagent blank without sample extract .The quantity of H_2O_2 was determined from the standard curve prepared by taking different concentrations of H_2O_2 ranging from 20 to 100 moles.

Estimation of malondialdehyde (MDA)

Malondialdehyde (MDA) was estimated by following the method of Moshaty *et al.* (1993). To 1 ml of supernatant was added an equal volume of MDA reagent (20% TCA in 5% thiobarbituric acid) and kept in a water bath at 95°C for 40 min and immediately chilled on ice for 15 min. The mixture was centrifuged at 10,000 x g for 30 min and the absorbance of the supernatant was measured at 520 nm and 600 nm. The non-specific absorbance at 600 nm was subtracted from that of 520 nm. The content of MDA was calculated using the extinction coefficient 155 mM^{-1} cm^{-1}.

Estimation of Superoxide Dismutase (SOD)

Superoxide Dismutase (SOD) was estimated using pyragallol (5% v/v). To 0.5 ml of 0.1 M Tris-HCl (pH 8.2), equal volume of distilled water, pyragallol and enzyme extract was added to give a final reaction volume of 2 ml. The activity of SOD was measured by monitoring the autooxidation of pyragallol at 420 nm for 3 min at 30s intervals in a double beam UV-VIS spectrophotometer.

Estimation of catalase (CAT) activity

Catalase (EC 1.11.1.6) activity was determined according to the modified method of Aebi (1984). The assay mixture for determining CAT activity containing 1.4 ml potassium phosphate buffer (50mM, pH 7.0), 1.5 ml of H_2O_2 (12.5 mM) and 0.1 ml of enzyme extract was used to give a final reaction volume of 3 ml. The reaction was started by the addition of H_2O_2. A blank was run without addition of the enzyme extract. The decrease in H_2O_2 was followed by recording the decrease in absorbance at 240 nm for 3 min at 30s intervals in a double beam UV-VIS spectrophotometer. One unit (U) of CAT activity was defined as the amount of enzyme catalyzing the decomposition of 1 µmol H_2O_2 per min at 240 nm.

Statistical Analysis

All the experiments were performed in triplicates and their mean values are given.

RESULT AND DISCUSSION

Plant possess a well organized Reactive Oxygen Species (ROS) scavenging systems comprising enzymatic such as Catalase, Ascorbate peroxidase and Superoxide Dismutase, and non-enzymatic antioxidants. Relative to control seedlings, growth of chickpeas seedlings was reduced due to Mn at tested concentrations. Increased level of antioxidative enzymes protects the cell against the oxidative damage by removal of free radicals or reactive oxygen species. Catalase activity was calculated in control and stressed seeds at alternate days of germination. It was observed that the activity of catalase increased with increase in time as compared to unstressed seedlings. Increased level of catalase was observed, which showed that it is major enzyme in scavenging cellular H_2O_2 (Vranova et al., 2002)**.** This enzyme is regarded as bioindicators of heavy metal toxicity and play important roles in scavenging ROS like H_2O_2 to reduce oxidative damage.

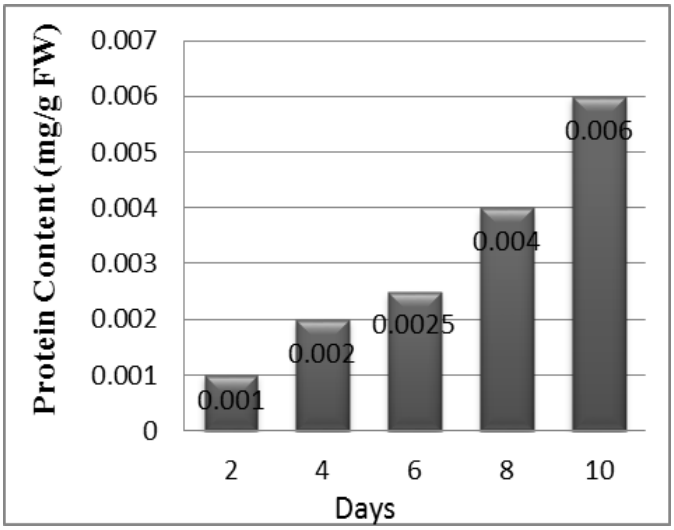
Ascorbate peroxidase activity was calculated in control and stressed seeds at alternate days of germination. It was observed that the activity increased with increase in time as compared to unstressed seedlings.

Activity of peroxidase increased with increase in time as compared to unstressed seedlings at alternate days of germination. The POX has been implicated in the synthesis of lignin and other phenolic polymers. So the enhancement in POX activity might defend the cells against harmful concentrations of hydroperoxides thereby protecting cellular components such as proteins and lipids against oxidation.

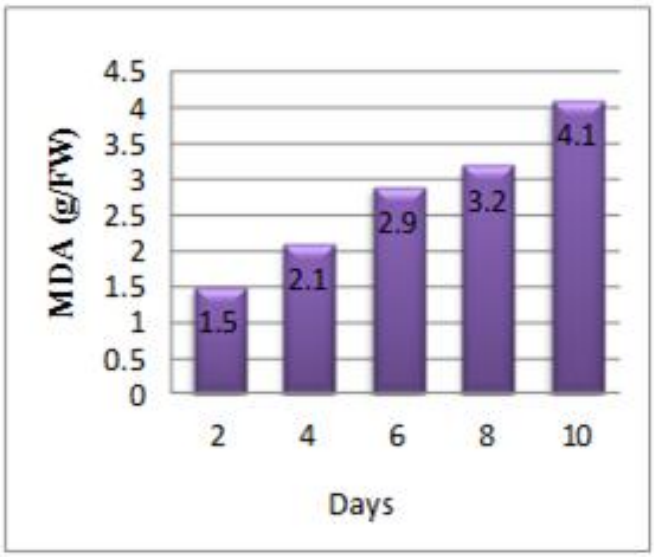
Lipid peroxidation occurs in plant tissues leading to production of free radicals, which are normally detoxified by antioxidative enzymes. Under stress conditions, more production of free radicals takes place. MDA is product of lipid peroxidation which can be estimated by measuring the level of MDA. In this study, it was observed that MDA content was high in germinating chickpeas when treated with Mn as compared to control. So, it was suggested that involvement of free radicals in membrane lipid peroxidation in the seeds subjected to Mn-stress could be a reason for increase in MDA content. Generally, free radical generation and membrane damage would be low in tolerant plants and thereby the formation of lower levels of MDA content.

SOD is a key enzyme in cell which plays a vital role against oxidative damage and severe environmental conditions. It was observed that at alternate days of germination the activity increased with increase in time as compared to unstressed seedlings and also it was observed that at alternate days of germination the Hydrogen peroxide content decreased with increase in time as compared to unstressed seedlings.

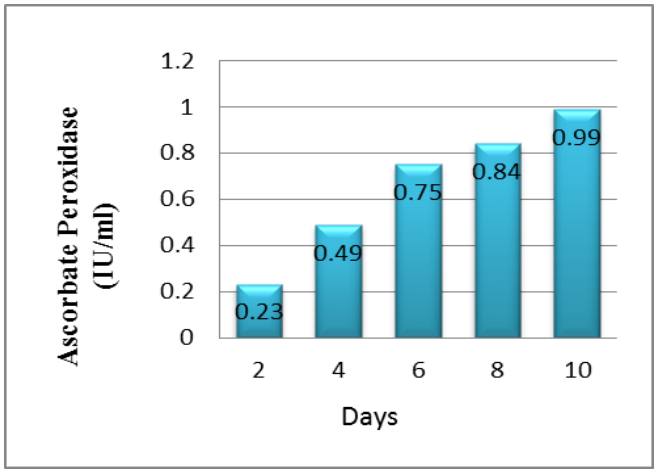
Protein content in Mn-treated seedlings was increased, indicating that heavy metal stress may induce production of stress proteins (Sanita di Toppi and Gabbrielli, 1999) including some heat shock proteins (Namjooyan et al., 2012). It may be suggested that protein content increased due to de novo synthesis of stress proteins provoked by metal exposure (Verma and Dubey, 2003).


Protein Content (mg/g FW)
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
0.001
0.002
0.0025
0.004
0.006
2
4
6
8
10
Days

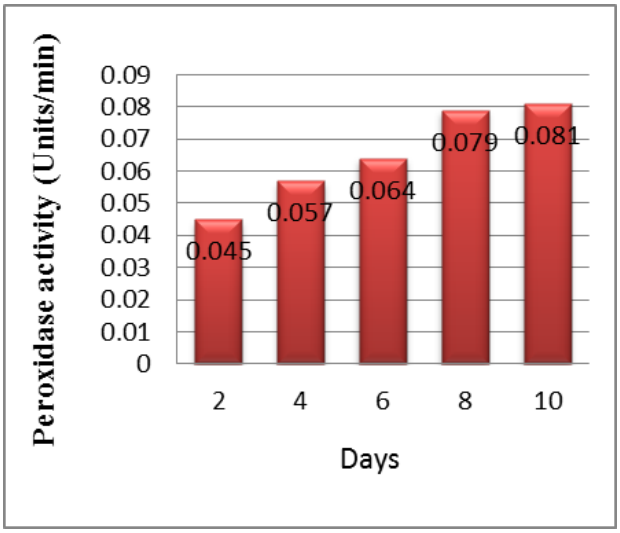
(a)


MDA (g/FW)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1.5
2.1
2.9
3.2
4.1
2
4
6
8
10
Days

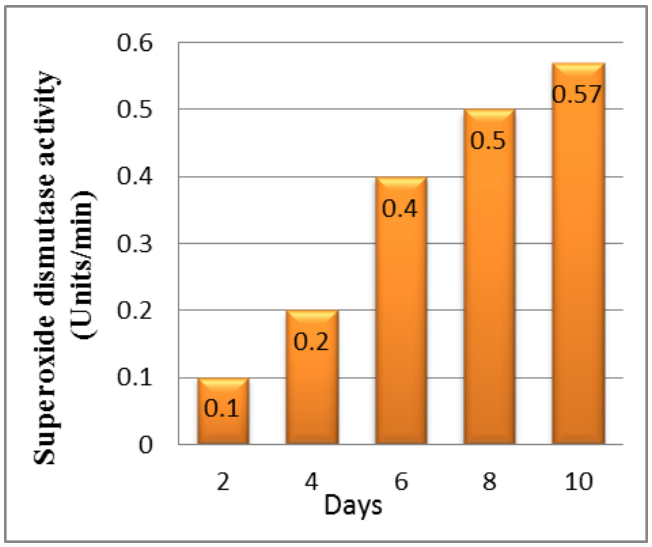
(b)


Ascorbate Peroxidase (IU/ml)
1.2
1
0.8
0.6
0.4
0.2
0
0.23
0.49
0.75
0.84
0.99
2
4
6
8
10
Days

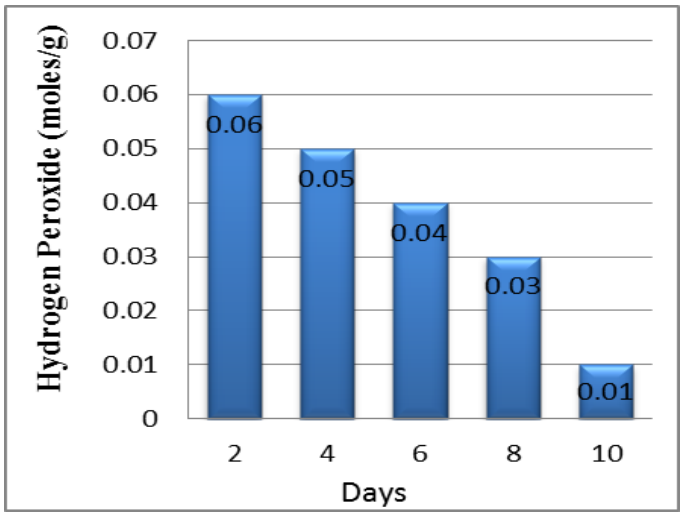
(c)


Peroxidase activity (Units/min)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0.045
0.057
0.064
0.079
0.081
2
4
6
8
10
Days

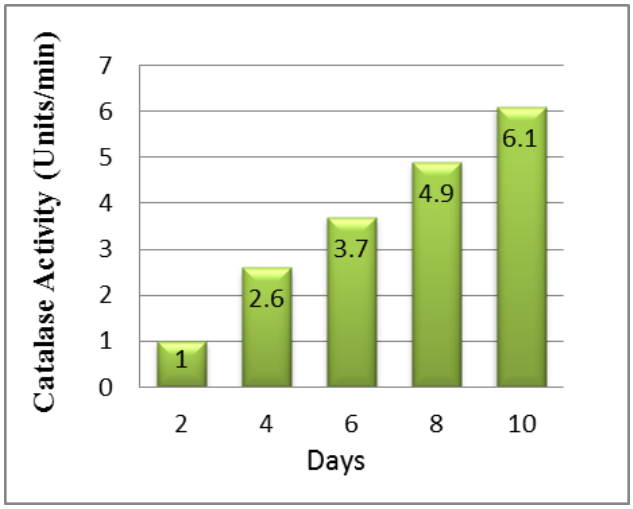
(d)


Superoxide dismutase activity (Units/min)
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
0.2
0.4
0.5
0.57
2
4
6
8
10
Days

(e)


Hydrogen Peroxide (moles/g)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0.06
0.05
0.04
0.03
0.01
2
4
6
8
10
Days

(f)



(g)

Figure: Effect on antioxidant enzymes from germinated chickpeas germinated under stress conditions (a) Protein content (b) MDA (c) Ascorbate peroxidase (d) Peroxidase (e) SOD (f) Hydrogen peroxide (g) Catalase.

CONCLUSION

In conclusion, antioxidant plays an important role in Manganese-stress tolerance of plants, which is considered as an essential micronutrient for the metabolic process in plants. In the present study, we hypothesized that enhanced levels of antioxidants and MDA content on Manganese exposure activate the multi tolerance mechanism of antioxidative enzymes under stress. The results of the present study also demonstrate the effect of Manganese exposure, the cultivar capability to activate multi defense mechanism against oxidative damage caused by Manganese ions may be a key factor in the detoxification mechanism of plant tolerance to unfavourable conditions. Nevertheless, both its deficiency and excess use in plants and animals alter processes. The mechanisms of this will contribute in improving quality and yield of cultivated plants in soil contaminated with heavy metals.

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