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THE EFFECT OF MANGANESE ON ANTIOXIDANT ENZYMES ACTIVITY ON THE WHEAT SEEDLING

Anuradha Mittal[1*], Sumit Chhibber[2] and Zeenat Madan[3]

[1] Department of Biotechnology, S.D. College, Ambala Cantt
[2] Department of Botany, S.D. College, Ambala Cantt
[3] Department of Zoology, S.D. College, Ambala Cantt

*Corresponding Author Email: mittalanuradha7@gmail.com

ABSTRACT

A microelement 'Manganese' which is important for plant metabolism, when gets accumulated it can act as a stress factor. Such action of manganese is not fully documented and is currently being intensively studied. Most of elements when accumulate in plant tissues 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 influence of manganese, at different concentrations, on the wheat seedlings was studied. Seedlings were subjected to concentration as control, and 5mM, 10mM and 20mM Manganese for 10 days. It was observed that the germination of seeds depends on the concentration of manganese, under low concentrations; a considerable amount of growth is shown as it is required by the plant in trace amount. But 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.

KEY WORDS

Antioxidant enzyme, wheat, manganese, germination

INTRODUCTION

Manganese (Mn) is an essential trace element required for the growth and survival of most, if not all, living organisms. In cells, Mn is a component of metalloenzymes engaged in the antioxidant defense system and in addition, is required for carbohydrate, lipid and protein biosynthesis [1]. Manganese deficiency is a widespread problem, typically more pronounced in cool and wet conditions [2]. Numerous crops, including wheat, barley and oats, are sensitive to Mn deficiency, as revealed by high decline in yield. The reduced Mn content in the cells substantially impairs photosynthesis, resulting in a marked decrease in soluble sugar concentration in various parts of plants. The excess of Mn is commonly extremely toxic to the majority of plant cells, inducing oxidative stress through the production of reactive oxygen species (ROS) [3].

Antioxidants neutralize the effect of free radicals through different ways and may prevent the body from various diseases [4]. Anti-oxidants can act by scavenging ROS 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 [5]

Under conditions of Mn excess, apoplastic deposition of its oxidized forms occurs and it is suggested that peroxidases are involved in this reaction [6]. Besides

these enzymes, other antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), may be engaged in the scavenging system, minimizing cellular damage caused by ROS [7][8]. Higher activities of antioxidant enzymes in response to Mn excess were found in woody plants [9], herbs such as white clover (*Trifolium repens* L.) and in rye grass (*Lolium perenne* L.) [10] [11]. However, the changes in the activity of these enzymes were dependent on the plant tolerance to oxidative stress. In sensitive plants, a decrease of antioxidative enzymes activity (especially SOD), as a result of protein damages by ROS, was registered [12] [13]. High Mn concentrations stimulate cytotoxic effects in cell structure causing extensive modifications in the content of cytoplasmic components and plasma membranes [14]. An increase of membrane lipid peroxidation is accepted as an indicator of oxidative stress intensity [15] [16]. It was also found that Mn excess can inhibit the uptake of other essential elements, such as calcium (Ca), magnesium (Mg), iron (Fe) and phosphorus (P) due to the similarity of ionic size or binding strength in ligands [1] [17].

Wheat (*Triticum aestivum* L.) is one of the most important crop which plays a special role in people's nutrition. But unfortunately stresses, such as metal and salinity, decrease wheat growth and productivity and cause nutrient disorders and ion toxicity.

The aim of this study was to check the sensitivity of wheat seedlings with respect to changes in the activity of antioxidant enzymes which were adopted as indicators of Mn stress action. As mentioned before, manganese has the most important role in decreasing wheat growth. In this research, wheat grains were germinated under manganese stress with a control for a period of 10 days. With the elapse of this period, the activity level of catalase, peroxidase, lipid peroxidation, superoxide dismutase etc. was measured.

MATERIALS AND METHODS

Plant material and seed collection

A cultivar of Wheat (*Triticum aestivum* L.) was used as the source material in the present investigation. The seeds of the cultivar were obtained from Ambala, Haryana (India).

Germination of seeds and Extraction of enzymes

Wheat grains were taken and washed with distilled water. After that grains were surface sterilized with 0.1% $HgCl_2$ for 5 min and rinsed with sterile distilled water for 5-6 times. Surface sterilized grains were germinated aseptically in petri plates having sterilized wet filter paper. Various Mn concentrations were applied ranging from 5-20mM. A control was run simultaneously. Enzymes were extracted by homogenizing germinated wheat (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. Extraction was done at alternate days for 10 days. The experiments were performed in triplicate.

Estimation of catalase (CAT) activity

Catalase (EC 1.11.1.6) activity was determined according to the modified method of Aebi [18]. 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 µ mol H_2O_2 per min at 240 nm.

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.* [19]. 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*. [20]. 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. One unit of enzyme activity represents a change of 1 unit/min of absorbance at 430 nm.

Estimation of H_2O_2

The amount of hydrogen peroxide was estimated according to the method given by Sinha [21]. 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.* [22]. 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. One unit of enzyme activity represents a change of 1 unit/min of absorbance at 420 nm.

Estimation of soluble proteins

Soluble proteins were estimated following the method of Lowry *et al*. [23]. The amount of soluble proteins was calculated in mg g^{-1} FW with the help of standard plot of BSA (0-150 µg).

Statistical Analysis

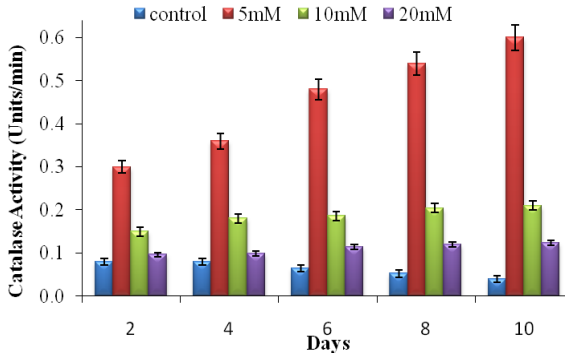
All the experiments were performed in triplicates and their mean values are given.

RESULT AND DISCUSSION

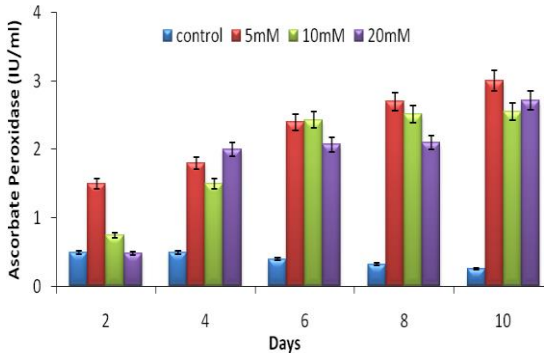
Plant possess a well-organized Reactive Oxygen Species (ROS) scavenging systems comprising enzymatic such as Catalase, Ascorbate peroxidase and Superoxide Dismutase, and non-enzymatic antioxidants. A coordinated function of these systems plays an important role in scavenging ROS and maintaining redox status of the cell. Relative to control seedlings, growth of wheat showed an increased effect but with a variation due to Mn at tested concentrations.

The cells are protected against the oxidative damage by increased level of antioxidative enzymes 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 at a particular concentration of manganese as compared to unstressed seedlings (Figure 1a). Increased level of catalase showed that it is major enzyme in scavenging cellular H_2O_2 [24]. However, a decrease in activity was observed with increase in concentration of manganese at that time. This enzyme is regarded as bioindicators of heavy metal toxicity and plays important role 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 (Figure 1b). It was observed that the activity increased with increase in time as compared to unstressed seedlings. Higher activity of Catalase and Ascorbate peroxidase decrease H_2O_2 level in cell and increase the stability of membranes, as a high level of H_2O_2 directly inhibits CO_2 fixation [25]. Peroxidase, Catalase and Ascorbate peroxidase are three important H_2O_2 scavenging antioxidant enzymes functioning in different sub-cellular compartments [26]. Activity of peroxidase generally increased with increase in time as compared to unstressed seedlings at alternate days of germination (Figure 1c) and also increased with increase in concentration of manganese but drastically decreased at high concentrations. The enhancement in POX activity might defend the cells against harmful concentrations of hydroperoxides as POX has been implicated in the synthesis of lignin and other phenolic

polymers, thereby protecting cellular components such as proteins and lipids against oxidation.



(a)



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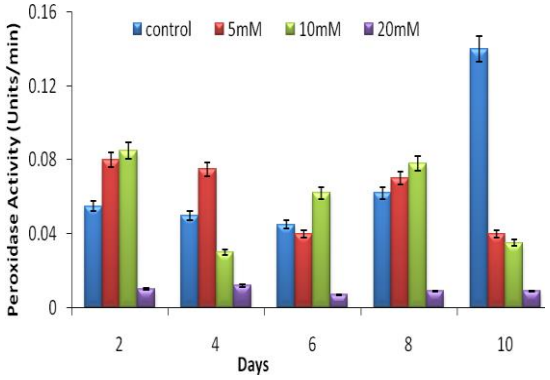

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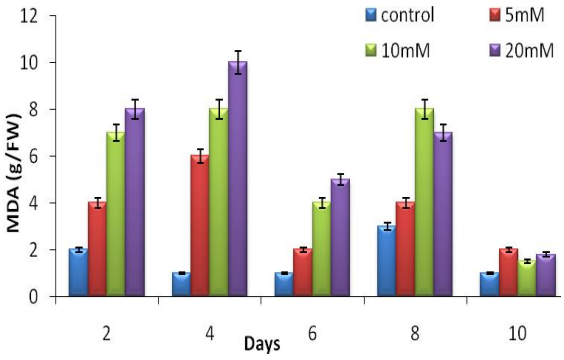

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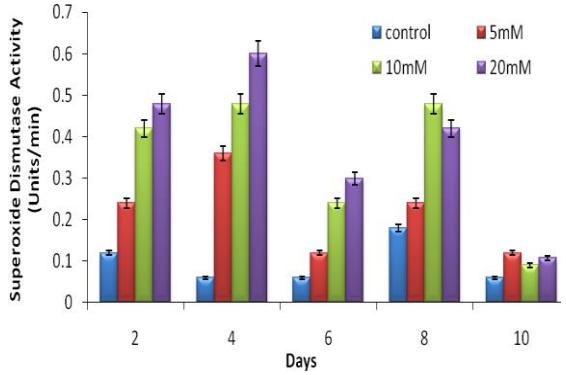

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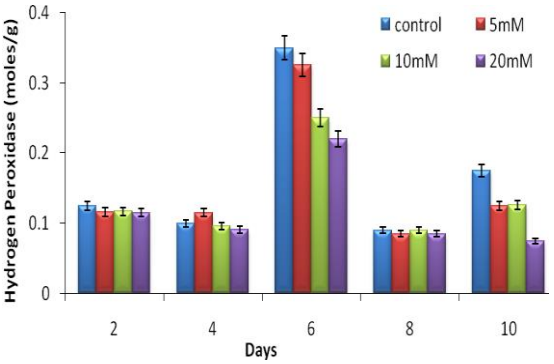

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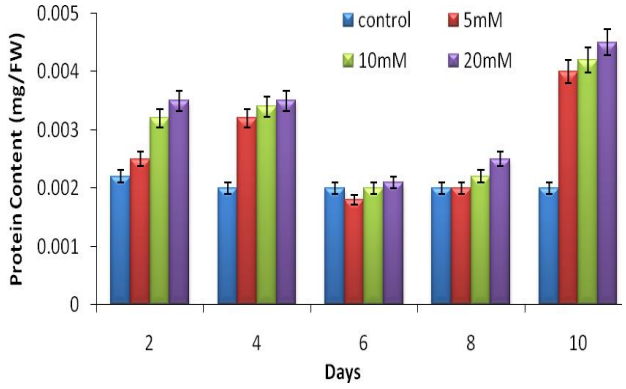

(g)

Figure 1: Effect on antioxidant enzymes from wheat germinated under stress conditions (a) Catalase, (b) Ascorbate peroxidase, (c) Peroxidase, (d) MDA, (e) SOD, (f) Hydrogen peroxide, and (g) protein content

Lipid peroxidation occurs in plant tissues leading to production of free radicals, which are normally detoxified by antioxidative enzymes. MDA is product of lipid peroxidation. So, lipid peroxidation can be estimated by measuring the level of MDA. Under stress conditions, more free radicals are produced, and it was observed, in the present study, that MDA content was high in germinating wheat when treated with Mn as compared to control (Figure 1d). Generally, if free radical generation and membrane damage would be low, there is formation of lower levels of MDA content. So, it can be concluded 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.

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 (Figure 1e). Also, SOD increased with increase in concentration but decrease in activity was observed on 10th day. When SOD activity increased, free radicals scavenging was done properly and thus, damage to membranes and oxidative stress decreased, leading to the increase of tolerance to oxidative stress. Vital biomolecules are disturbed, if the radical is not scavenged by SOD [27]. Esfandiari *et al.* [28] and Zhao *et al.* [29] had expressed that the increase in SOD activity and decrease in oxidative damage were closely related. It was observed that at alternate days of germination the Hydrogen peroxide content decreased with increase in time as compared to unstressed seedlings (Figure 1f).

Protein content in Mn-treated seedlings was increased (Figure 1g), indicating that heavy metal stress may induce production of stress proteins [30] including some heat shock proteins [31]. It may be suggested that protein content increased due to de novo synthesis of stress proteins provoked by metal exposure [32]. Several researchers found increased protein synthesis under metal stress.

CONCLUSION

In conclusion, antioxidant machinery plays an important role in Mn-stress tolerance of plants. For successful scavenging of ROS by a scavenging system, some antioxidant enzymes must cooperate with each other. In the present study, we hypothesized that enhanced levels of antioxidants and MDA content on Mn exposure activate the multi tolerance mechanism of antioxidative enzymes under stress.

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***Corresponding Author:**
Anuradha Mittal*
Email: **mittalanuradha7@gmail.com**