Enzymatic Status of Germinating Wheat Grains under Heavy Metals Stress
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Abstract
In the recent years, the looming food scarcity problem has transformed plant sciences as an emerging discipline committed to devise new strategies for enhanced crop productivity. The major factors causing food scarcity are biotic and abiotic stresses which substantially limit crop productivity worldwide. For enhancing the crops productivity under these unfavorable conditions we firstly understand the effect of these conditions on the crops. In this work the effect of different heavy metals (Cd, Cu, Pb and Zn) on enzymatic status through the germination of wheat grains is investigated. All studied heavy metals caused inhibition in the wheat grains germination at concentration 10,50 mM, while the low concentration 1mM did not cause any significant change in case of Cu and Zn but cause significant inhibition in case of Cd and Pb. It was found that different concentrations of different heavy metals increase the activity of the antioxidant enzymes in all studied stages compared to the control, on the other hand the studied hydrolysis enzymes activity (amylase, protease, acid and alkaline phosphatase) decreased in their all studied stages compared to the control.

Key words- Amylase; Antioxidant enzymes; Protease; Phosphatases; Wheat

I. INTRODUCTION
Toxic heavy metals have no function to organism and can be highly toxic when their concentrations are exceeded threshold value. Other heavy metals at low doses are essential micronutrients for plants, but in higher doses they may cause metabolic disorders and growth inhibition for most of the plants species [1, 2].

Heavy metals are transition metals having an incompletely filled d-orbital present at cations at physiological conditions. The aerobic cells have physiological redox between ~420 mV and +800 mV. Therefore, heavy metals can be divided into redox active and inactive metals. Metals with lower redox potential than those of biological molecules cannot participate in biological redox reactions. One of the mechanisms causing toxicity involves autoxidation of redox active metals such as Fe^{2+} or Cu^{2+} in O^{2-} formation and subsequently to H_{2}O_{2} and OH^{-} via Fenton-type reactions. Cellular injury by this type of mechanism is well demonstrated for iron, copper, and for other metals. The up-regulation of ROS induced by Cd stress may contribute to inhibitory effects on the activities of antioxidative enzymes including superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2) [3, 4].

Another mechanism of heavy metal toxicity is their ability to bind strongly with oxygen, nitrogen, and sulfur atoms [5]. This binding affinity is related to free enthalpy of the formation of the product of metal and ligand. Thus, due to these activities, heavy metals can inactivate the enzymes by binding to cysteine residues [6].

Seed germination is one of the earliest events in the plant life cycle, and the transition from seed to seedling is one of the most drastic developmental processes in plants. Plants change from heterotrophic to autotrophic and from quiescent to active, and although they are initially protected
within a seed coat, they are then exposed to ambient conditions. Any adverse effect on seed germination process would be detrimental for the establishment and healthy growth of seedlings vis-a-vis crop productivity[7].

Hydrolysis enzymes play an important role in degrading the storage materials during germination process. Enzymes associated with starch degradation, sucrose synthesis and utilization of glucose play a key role in seedling growth which further determine the final yield of the crop. α-Amylase (EC 3.2.1.1) catalyses hydrolytic cleavage of internal α-1,4-glucan bonds of starch releasing fragments that can be further broken down by β-amylase (EC 3.2.1.2) into maltose [8, 9].

Regulated proteolysis is directly or indirectly involved in most cellular processes [10]. Among the functions assigned to proteolysis are: (a) the supply of amino acids needed to make new proteins and (b) the removal of abnormal proteins. These molecular mechanisms are related to numerous developmental events including seed germination during which storage protein mobilization is a fundamental component. Thus, the role of proteases in germination is essential. [11].

Phosphate plays an extremely important role in a variety of reactions in germinating seed including energy metabolism and synthesis of nucleic acids and membranes, where it appears primarily in a linked organic form and very little seems to be present as free inorganic orthophosphate[12].

Acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) are enzymes that catalyze the removal of inorganic phosphate (orthophosphate) from organic phosphate esters. Acid and alkaline phosphatases in plants play a major role in the supply and metabolism of inorganic phosphate for the maintenance of cellular metabolism [13, 14].

Wheat is an important staple food of the world population having high nutritional quality. Many different studies evaluate the effects of different heavy metal concentrations on living plants. Most of these studies deal with seedlings or adult plants. In a few studies, the seeds have been exposed to the metals. The present study test the effects of some heavy metals (Essential metals Cu and Zn, Non-essential metals Pb and Cd) with different concentrations on enzymatic status in germinating wheat grains

II. MATERIALS AND METHOD

A- Plant Materials and Heavy Metal Treatments

The heavy metals used in this study (Essential metals Cu and Zn, Non-essential metals Pb and Cd) were in the form of sulphates. five concentrations of each metal were used in this study. The selected concentrations were 1, 10, 50 and 100. For each treatment, the pH was adjusted to 6.5.

Wheat grains were purchased from a local seed market. seeds were sterilized in 10% Nahypochlorite solution for 20 min to prevent fungal growth, washed with distilled water for several changes.

B- Determination of Germination Parameters

Grains germination test on filter paper was carried out in glass Petri dishes (90 × 15 mm) with three layers of filter paper on the bottom. Each dish contained 10 mL of metal solution or 10 mL of distilled water (control), and 20 seeds, covered by lid. Effluents were applied every alternate day. Petri dishes were maintained at 25 ± 1_C with a 16 h period of light in a growth chamber after 3 days of darkness. This in vitro germination was designed with three replicates and all parameters were recorded at 24, 72 and 120 hr.
C- Total cellular enzyme extraction
5 g Germinated grains was homogenized in 1.5 ml extraction buffer (a potassium phosphate buffer 0.1 M, pH 7.5 mL: 0.5 g fresh weight) in ice cold condition. The homogenates were centrifuged at approximately 5000g for 10 min at 4 °C and the supernatant was used as enzyme sample [15].

1- Antioxidant enzymes assay:
1.1- Assay of super oxide dismutase (SOD): SOD activity was measured by the nitro blue tetrazolium (NBT) reduction method [16], the SOD activity is represent as % percent.

1.2- Assay of polyphenol oxidase activity (PPO) peroxidase activity (POX): According to [17] the PPO activity is represent as change in Optical density (OD) min\(^{-1}\) mg\(^{-1}\) protein.

2- Hydrolysis enzymes assay:
2.1- Amylase enzyme assay:
The amount of starch hydrolyze by the action of amylases was measured according to [18] The amylase activity expressed as amount of starch hydrolyzed min\(^{-1}\) mg protein\(^{-1}\).

2.2- Protease enzyme:
The Protease activity was calculated by using method of [19]. The protease activity was expressed as mg hydrolyzed protein min\(^{-1}\) mg\(^{-1}\) protein.

2.3- Acid and Alkaline Phosphatase
About 5 g from freshly germinated grains were used for enzyme extractions and assays. The activities of all the two enzymes were assayed according to the method of [20]. One nkat of enzyme activity is defined as one nmol p-nitrophenol liberated min\(^{-1}\) and specific activity as nkat mg\(^{-1}\) protein.

D- Statistical analysis:
Data in all experiments were statistically analyzed using paired T test for testing compare means in relation to control with the help of Spps 18. Statistically significant differences at the p=0.01 level are indicated.

III. RESULTS AND DISCUSSION
A- Germination ratio:
Heavy metals such as Cu, Zn, Ni, Hg, Cd, Cr and Pb present in soil and water naturally or as contaminants from human activities can cause bioaccumulation affecting the entire ecosystem and pose harmful health consequences in all life forms [21].

All heavy metals in the study (Cu, Zn, Pb and Cd) caused significant inhibition in germination ratio at the 10 mM and 50 mM concentrations the lowest inhibition ratio was in case of Zn 16 and 36% at 10 and 50mM respectively and Cd caused the highest inhibition ratio 58% at 10 and 82% at 50mM Cd concentration . The low concentration 1 mM caused non-significant inhibition in case of the Cu and Zn (2 and 1% respectively), while cause significant inhibition (17 and 26%) in case of the Pb and Cd respectively (Table 1 and Figure 1).

| Table 1: Effect of different heavy metals concentration on germination ratio |
|---|---|---|---|---|---|---|
| Control | Cu (1) | Cu (10) | Cu (50) | Zn (1) | Zn (10) | Zn (50) | Pb (1) | Pb (10) | Pb (50) | Cd (1) | Cd (10) | Cd (50) |
| Germination ratio (%) | 100 | 99 | 94 | 84 | 83 | 65 | 22 | 74 | 42 | 18 |
| Inhibition ratio (%) | 0 | 2 | 20 | 55 | 1 | 16 | 36 | 17 | 35 | 78 | 26 | 58 | 82 |
The same results were obtained [2] reported the retardation of germination of horse gram seeds under cadmium stress, [22] also emphasized that the mustard seed germination was severely damaged by cadmium. Other work of [23] showed the retardation effect of Pb on pea seeds germination. Also another work showed that CuCl2 delay germination and biomass mobilization from storage tissues of bean seeds[11]. The higher concentrations of heavy metals (Cu, Zn, Mg and Na) inhibit seed germination and early growth of barley, rice and wheat seedlings significantly compared to control [24].

The reduced germination can be explained by the decrease in physiological and metabolic activities [25]. The reduced germination rate may be related to the restricted oxygen uptake and physiological disorders in the supply of food reserves as explained by[26].

heavy metals such as Co, Cu, Fe, Mn, Mo, Ni, V, and Zn are required in minute quantities by organisms, excessive amounts of these elements can become harmful to organisms. Heavy metals such as Pb, Cd, Hg, and as (a metalloid but generally referred to as a heavy metal) do not have any beneficial effect on organisms and are thus regarded as the “main threats” since they are very harmful to both plants and animals [27] this explain that low concentration of Cu and Zn did not signifigantly change the germination ratio of wheat grains.

B- Antioxidant enzymes:

One of the mechanisms causing toxicity by heavy metals is the production of excess reactive oxygen species this excess ROS are degraded by the activation of antioxidant enzymes, such as SOD, APX, POD and CAT [28].All the studied antioxidant enzymes activity(SOD, PPO and POX) increased underall heavy metals treatment and this activity increased with the increased concentrations, the enzymes activity decreased with time but remained higher than the control. The increase in enzyme activity was in the following range Cu > Zn >Cd >Pb as the highest activity of antioxidant enzymes was recorded at 50mM of Cu. On the other hand the lowest concentration (1mM) of Pb treatment caused the lowest activity of the antioxidant enzymes (Figure 2).
The same results were recorded by [29] as antioxidant enzymes SOD and POX increased in wheat seedling under Cd and Pb stress. Also the increase in the antioxidant enzymes activity in radish seedling under heavy metals stress was reported [30]. Another work reported the increase in the antioxidant enzymes activity in germinating Vignamungo seeds under Cu and Zn stress[31].
Enhanced antioxidant activities observed upon heavy metals exposure are further suggestive of their protective role against heavy metal-induced stress since these enzymes are involved in constitutive as well as inducible plant defense [32, 33].

**C- Hydrolysis enzymes:**

In contrast to the antioxidant enzymes the heavy metals treatments caused significant reduction in the hydrolysis enzymes activity (amylose, protease, acid and alkaline phosphatase) (as shown in Figure 3). Inhibitions of these important hydrolyzing enzymes results in an impaired hydrolysis of storage products, which, in turn, leads to starvation of the germinating embryo [25]

![Figure 2: Effect of different heavy metals concentration on hydrolysis enzymes, amylase, protease, acid and alkaline phosphatase in germinating wheat grains at 24, 72 and 120hr](image)

Amylase enzyme activity in the control germinated grains increased with time during the study as it reached the highest value, while in case of the heavy metals treatment this activity was inhibited highly than the control and decreased with increasing the age of the germinating grains the lowest activity of amylase was in case of the highest concentration of cadmium (50 mM) with inhibition ratio about 89.6%, while under Zn (1 mM) after 24hr germination the lowest inhibition ratio was
recorded 22.3%. Reduced starch mobilization from reserve tissues was also observed in germinating *Phaseolus vulgaris* L. seedlings under cadmium (34) and copper toxicities (35) which was attributed to reduced amylase activities [23]. Earlier, [36] reported the Pb decreased activity of proteases and α-amylases in rice endosperm.

Protease activity in the control germinating grains showed different pattern to the amylase as under no heavy metals the protease activity increased till 72 hr germinating grains but at the 120 hr germinating grains the activity decreased, this can be explained as protease is strongly active in the beginning of germination, to initiate the breakdown of storage proteins, before the massive de novo biosynthesis of the new protein needed in the various biochemical and physiological process which decrease the protease activity again [11, 37]. On the other hand the protease activity under heavy metal treatments increased with germination time but remain lower than the control as the highest inhibition ratio was shown after 24 hr germinating grains in Cd (50 mM) 86.48% and the lowest inhibition ratio caused by the the lowest concentration of Cu (1 mM) at 120 hr germinating grains about 23.07%). The work of [37, 38] has pointed out that Cu stress inhibits protease activity in bean seeds. It was reported the inhibition of the amylase and protease during germination under heavy metal stress [25, 39]. AlsoPb exposure inhibits radicle emergence from *B. campestris* by interfering with the biochemical processes linked to protein and starch metabolism through decreasing amylase and protease activity [40].

Phosphatases catalyze the hydrolysis of phosphate monoesters producing inorganic phosphate (Pi) required for energy metabolism and metabolic regulation [41], and plant differentiation and development [42]. Therefore, decreased activity of phosphatases and thus reduced Pi level relate positively to reduced growth.

In case of acid and alkaline phosphatase in the present study showed the same as amylase under the normal germinating grains as the activity increased with the germination time increase, but in case of the heavy metal treatment the acid and alkaline phosphatase increased also with germination time but remain lower than control.

Alteration in the activity of acid and alkaline phosphatase in plants has been observed under variety of stressful conditions including under toxicities due to various metals [43,44]. It is noteworthy that acid and alkaline phosphatase is also inhibited by higher concentrations of the metals Zn^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Mo^{6+}[45]. Later, heavy metals like Cd, Cu, and Hg have been demonstrated to be strong inhibitors of phosphatases in vitro [46]. One possible reason for metal-induced inhibition of alkaline phosphatase activity appears to be the capability of metal ions to replace Zn^{2+} from the active site of alkaline phosphatase resulting in changes in enzyme conformation and consequently inhibition of activity[47]. Nevertheless, the higher activities of acid and alkaline phosphatases seen under non-stressed conditions indicate that higher phosphorolytic activity is necessary to fulfill the needs of germinating wheat grains [14].

### IV. CONCLUSION

Pollution of the environment by toxic metals in recent years has accelerated dramatically due to rapid industrial progress. Heavy metals when taken up in amounts in excess of the normal concentration produce lethal effects on plants, on microbes, and directly or indirectly on the human health. This study showed the deleterious impact of metals (Cu, Zn, Pb and Cd) on germination of the wheat grains as the metals decreased the hydrolysis enzymes activity and so the embryo starved and could not have the energy to germinate and as a defense mechanism against heavy metals the antioxidant enzymes increased in the germinating grains under heavy metals stress. Rapid germination is an important for successful establishment of healthy plants. It is a crucial stage that from seed germination to fully developed seedling and limit species distribution under stress conditions. Therefore, the study not only has theoretical significance in physiology, but also has a practical significance for crop production.
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BIBLIOGRAPHY


