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Total amylase activity of *in vitro* germinated *Cyperus esculentus* (L.) tubers treated with salt and heavy metals

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ABSTRACT: The growth of seeds and tubers is a major concern due to increased salt stress and widespread accumulation of heavy metals in agricultural land. Amylase is an important enzyme involved in germination processes and seedling development. This study was carried out to determine the effects of salt stress and heavy metals on the activity of amylase in *in vitro* germinated *Cyperus esculentus*. Sodium chloride (NaCl), at concentrations of 0, 50, 100, 150, 200, 250 and 300 mM, were used to evaluate the effect of salt stress. Four heavy metals, cadmium, zinc, lead and arsenic, at concentrations of 0, 50, 100, 150, 200, 250 and 300 ppm were used to determine the effect of heavy metals, on the total amylase activity of *in vitro* germinated *Cyperus esculentus* (L.) (tiger nut). Total amylase activity was determined eight days after treatment (8DAT). The results obtained showed that NaCl marginally increased total amylase activity. The mean values obtained for total amylase under 0, 50 and 250 mM NaCl treatments were 118.967±1.073, 118.540±0.523, and 120.127±0.318 µM glucose released/min/g FW respectively. For 0, 250 and 300 ppm arsenic ion (As) solutions mean values for total amylase activity were 38.77±0.54, 39.21±0.95 and 30.07±0.41 µM glucose released/min/g FW. Cadmium (Cd) solutions increased total amylase activity as the concentration increase. Lead (Pb) solutions did not show any profound differences in the enzyme activity. Total amylase activity in 0, 200 and 300 ppm Pb solutions were 38.77±0.54, 38.89±0.65 and 38.47±0.61 µM glucose released/min/g FW. It was observed that total amylase activity under As and Zn solutions were similarly patterned.

Keywords: Amylase activity, *Cyperus esculentus*, heavy metal, stress, *in vitro*

Introduction

Environmental deterioration has generated an increase of stress in all forms of life (Bhardwaj *et al*., 2009). Of these, stress on agricultural crops is of prime importance since agriculture is the live wire of global society (Bhardwaj *et al*., 2009). Abiotic stresses include water stress, salinity stress, high temperature stress and heavy metal stress (Vwioko *et al*., 2008).

In the present technological drive by many nations, the environment is heavily polluted by various toxic metals which create danger for all living beings. Contamination of agricultural lands by heavy metals has become a critical environmental concern due to their potential adverse effects on growth and ecology. Heavy metals cannot be degraded and this makes their presence in agricultural environment a serious concern (Pristch *et al*., 2006; Yadav, 2010; Morsy *et al*., 2012; Sardar *et al*., 2013;). Agricultural soils in many parts of the world suffer mild to moderate contamination by heavy metal from metals such as Cd, Cu, Zn, Ni, Co, Cr, Pb and As (Pristch *et al*., 2006). These toxic elements, at high levels are considered as soil pollutants due to their widespread occurrence and their acute and chronic toxic effects on plants grown in such soils (Yadav, 2010).

Heavy metals comprise of essential (for example, Fe, Mn, Mo, Ni, Zn, Cu) and non-essential (Cd, Pb, Cr, Hg) micro-nutrients (Morsy *et al*., 2012). Elevated amounts of both non-essential and essential heavy metals can lead to

toxicity symptoms and growth inhibition in most plants (Mudgal *et al*., 2010; Aidoobie and Beltagi, 2013). They have been reported to inhibit physiological processes such as respiration, photosynthesis, cell elongation and affect plant water relationship as well as mineral nutrition (Morsy *et al*., 2012). These toxic chemicals have also been reported to affect biochemical reactions (Aidoobie and Beltagi, 2013). The activities of isoenzymes, esterases, peroxidase, anti-oxidants, amylase, and proteases have also been reported to be affected by these toxic metals (Fayiga *et al*., 2004; Bhardwaj *et al*., 2009; Pant *et al*., 2011).

The progressive salinization of agricultural environment is a severe worldwide problem affecting agricultural production (Sidari *et al*., 2008). The effects of salinity on crop plant have been a focus of many researches. The responses of crop plants are complex but involve several physiological and biochemical changes (Haouari *et al*., 2013). Among the biochemical changes are the inactivation or inhibition of enzyme activities. Amylase has been implicated as one of the enzymes affected by salinity and this may have a direct link to the usual observed reduction in germination of seeds and poor seedling establishment of agricultural crops (Sidari *et al*., 2008). Sidari *et al*. (2008) reported reduction in amylase activities with increase in the concentration of sodium chloride applied to four Lentil (*Lens culinaris* M.) cultivars. Similar results were obtained by Haouari *et al*. (2013) who worked with two genotypes of wheat (*Triticum monococcum* and *Triticum aestivum*) although response differed between the genotypes.

Starch is the main storage form of carbohydrate in plants. One of the most important enzymes of carbohydrate degradation in plants is amylase which rapidly degrades starch into soluble substrates such as maltose and glucose for other enzymes to metabolise (Agoreyo and Fregene, 2014). The activities of amylases have been reported in seeds, fruits and other vegetative tissues (Gana *et al*., 1998; Agoyero and Fregene, 2014). Amylase activity varies with plants and plant parts (Doehlert and Duke, 1983). Amylases (alpha amylase) play a key role in the metabolism of the plant by hydrolyzing starch in the germinating seed and in other tissues (Huang *et al*., 1992). The best known amylolytic enzymes are the α-amylases, β-amylases and the glucoamylases (Horvathova *et al*., 2000). Alpha (α) - amylase has been implicated in starch degradation in the endosperm during germination (Stanley *et al*., 2005).

Tiger nut (*Cyperus esculentus)* is an emergent grass-like plant belonging to the sedge family, Cyperaceae. This family has members that are found to be cosmopolitan perennial crop of the same genus as the papyrus plant that is common in seasonally flooded wetlands. It is widely distributed in the temperate zones within South Europe as its probable origin, and has become naturalised in Ghana, Nigeria and Sierra Leone. Tiger nut is one of the earliest domesticated crops and in fact, was found in vases and was used to embalm bodies of the Egyptian Pharaohs. In Nigeria, farmers transport tubers of tiger nut in fresh, semi-dried and dried form to local markets where it is sold for consumption. Tigernut tubers are eaten raw. Tiger nut tubers serve as rich source of sugars, amino acids and dietary fibre(Bamishaiye and Bamishaiye, 2011).

The objective of this study was to investigate the activities of amylase in the germinating tubers growing in abiotic stress conditions.

Materials and Methods

Plant Material

Fresh tubers of *Cyperus esculentus* (L.) were obtained from a local market, along Sakponba road, in Benin City. The fresh tubers were taken to the laboratory in the Department of Plant Biology and Biotechnology, University of Benin, where the experiment was conducted. The tubers were washed with clean water to remove soil particles. Viable tubers were selected using floatation method. The viable tubers were employed in the *in vitro* germination experiment that was carried out. Germination of tubers to measure the enzyme activity was done in different concentrations of NaCl, zinc, lead, arsenic, and cadmium solutions.

Experimental Setup

Five experiments were set up and four of them involved treating the tubers with solutions of zinc ($ZnSO_4$. $7H_2O$), lead ($Pb(NO_3)_2$), arsenic (AsO_3) and cadmium ($CdCl_2$. H_2O) and the fifth one involved treatment with sodium chloride (NaCl). Concentrations of metal treatments applied in the study were 0 ppm (control), 50 ppm, 100 ppm, 150 ppm, 200 ppm, 250 ppm and 300 ppm. Each concentration of the treatments was replicated three times. For NaCl, the concentrations of prepared solutions were 0 mM (control), 50 mM, 100 mM, 150 mM, 200 mM, 250 mM and 300 mM. Distilled water was used as the control (0 ppm / 0 mM).

Five tubers of *Cyperus esculentus* were carefully placed on the filter paper mounted in Petri dishes and moistened with the respective treatments. The Petri dishes were kept moistened by augumenting with respective solutions. The control and treated tubers were allowed to germinate producing plumule and radicle, for eight days

after treatment. Plant tissues were carefully harvested from the different treatments (including control) and were used for the extraction of amylase.

Preparation of the plant materials for the determination of amylase activity

Plant tissue from all treated tubers were rinsed and isolated from accompanying substratum. A weight of 1.5 g of plant tissues was obtained from the respective petri dishes. The excision of tissues was done with a blade. The weighed tissues were put in test tubes and kept in the refrigerator (0ºC).

Extraction of the crude amylase enzyme

The plant samples (1.5g each) of the different treatments were removed from the tubes and each was homogenized using a ceramic mortar and pestle. Enzyme stabilization was achieved by the addition of 10 ml of citrate phosphate buffer (pH 6.0 containing 20 mM $CaCl_2$) to the homogenized plant samples. The homogenate was then filtered by the use of a clean white cheese cloth. The filtrate was transferred into small tubes, sealed and properly labeled. The filtrate was then preserved in the refrigerator for centrifugation to commence. The filtrate was centrifuged at a speed of 4000 g for 30 minutes. The supernatant was re-filtered using a clean white cheese cloth and the pellets were discarded. The filtered supernatant was then transferred into clean fresh micro-tubes. The supernatant served as the crude extract for the enzyme assay. The supernatant was then utilized for the total amylase assay.

Enzyme assay for total amylase

The method of Agoreyo and Fregene (2014) for total amylase activity was employed with slight modifications. The assay mixture was prepared containing 5 mg/ml of starch, 250 ml of 0.1 M sodium fluoride and was adjusted to a pH of 4.8 with 0.2 M sodium acetate buffer. A volume of 1 ml of the assay mixture was added to a micro-tube. The reaction was started by adding 3 ml of the crude enzyme extract to the micro-tube. The reaction, already started, was allowed to stand for 15 minutes under room temperature after which 2 ml of 2 mM sodium hydroxide was added to the test tube to stop the reaction. A blank was made containing every component of the reaction mixture excluding the crude enzyme extract. The reducing sugar liberated was measured by the method of Agoreyo and Fregene (2014) adapted from an earlier work by Nelson (1944) and Somogyi (1952).

For the liberation of the reducing sugar, 1 ml of Nelson-Somogyi's R4 reagent was added to 0.5 ml of the reaction mixture and boiled in a water bath for 20 minutes. After this it was cooled and 1 ml of R3 solution was added. It was shaken vigorously to remove carbon (IV) oxide and allowed to stand for 10 minutes. A volume of 5 ml of distilled water were added and the absorbance read at 600 nm. A blank was prepared containing the assay mixture and Nelson-Somogyi's reagent but excluding the crude extract. The release of the reducing sugar in each reaction mixture was quantified using a standard glucose calibration curve. Amylase activity was expressed as µmole glucose equivalent released min^{-1} g^{-1} fresh weight.

Statistical analysis

The analysis of variance (ANOVA) was evaluated by the SPSS statistical package for computer systems. Duncan Multiple Range Test (DMRT) was utilized for the determination of statistical differences among the means.

Results

The total amylase activities in germinating tubers of *Cyperus esculentus* treated with different concentrations of arsenic, zinc. cadmium, lead and sodium chloride solutions are shown in Figures 1-5. Figure 1 shows the amylase activities of germinating tubers of *C. esculentus* in arsenic solutions. The arsenic solutions did not show visible effect on amylase activities. The mean values recorded for amylase activity in As-treated tubers were between 37-40 µmole glucose released/minute/g FW for 0-250 ppm As solutions. A decrease in amylase activity was recorded for 300 ppm As-treated tubers. The differences in enzyme activities between the control and As-treated tubers were insignificant ($p>0.05$). The effect of zinc solutions on amylase activity is shown in Figure 2. The effect of the zinc solution was very pronounced at 300 ppm Zn-treated tubers, as it caused a decrease in amylase activity. Total amylase activity observed in arsenic and zinc solutions treated tubers were similar. The mean amylase activity in tubers treated with cadmium increased as the concentration of cadmium solution increase. This is shown in Figure 3. The may be described as a positive effect, where cadmium solution increased the activities of amylase in *C. esculentus* tubers. For lead solutions, mean amylase activities recorded were 37.3 - 38.8 µmole glucose released/minute/g FW, as shown in Figure 4. The differences in activities among the treatments were not statistically

significant. The highest mean value of amylase activity was recorded in tubers treated with 200 ppm Pb solutions. The amylase activity in tubers treated with sodium chloride solutions increased as the concentrations increase. This is a positive effect observed where the activity of amylase was enhanced by NaCl solutions.

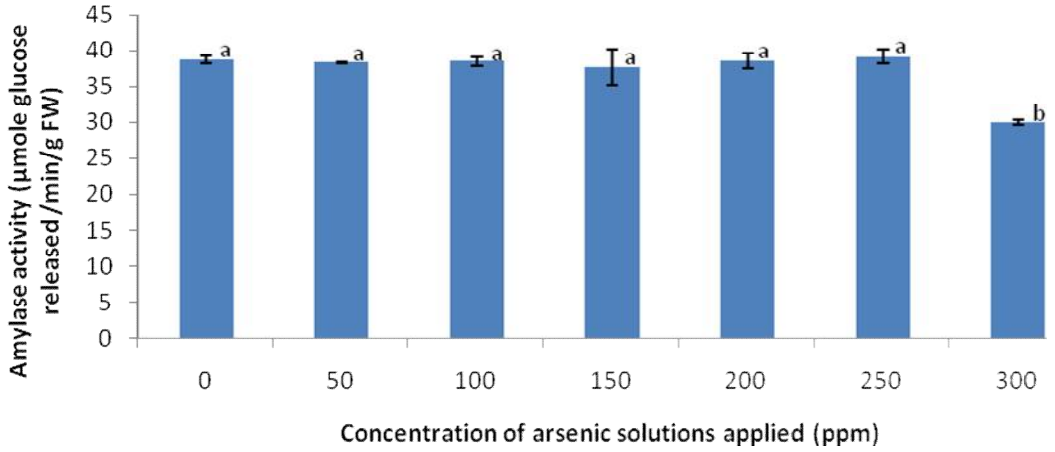

Figure 1: Amylase activity of seed-tubers of *Cyperus esculentus* subjected to different concentrations of arsenic solution (error bars represent standard deviation, similar alphabets on bars indicate no significant difference at α=0.05)

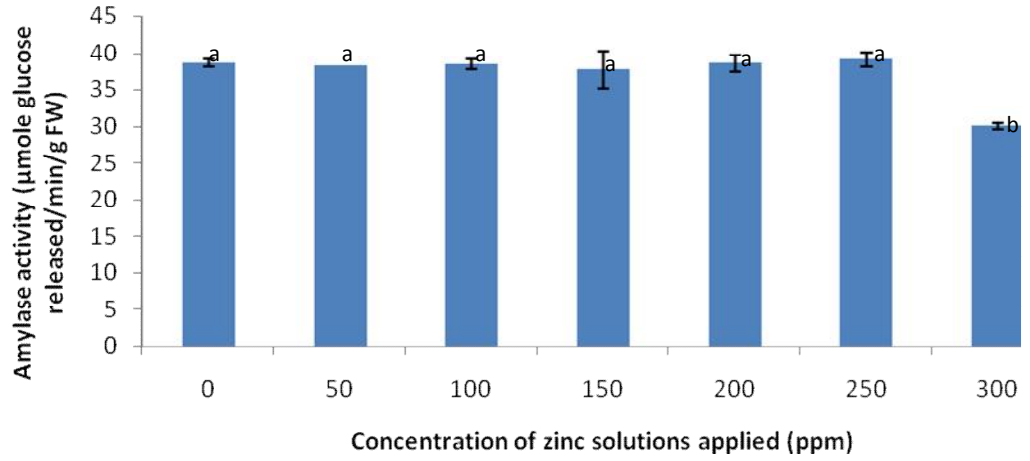

Figure 2: Amylase activity in tubers of *Cyperus esculentus* subjected to different concentrations of zinc solution (error bars represent standard deviation, similar alphabets on bars indicate no significant difference at α=0.05)

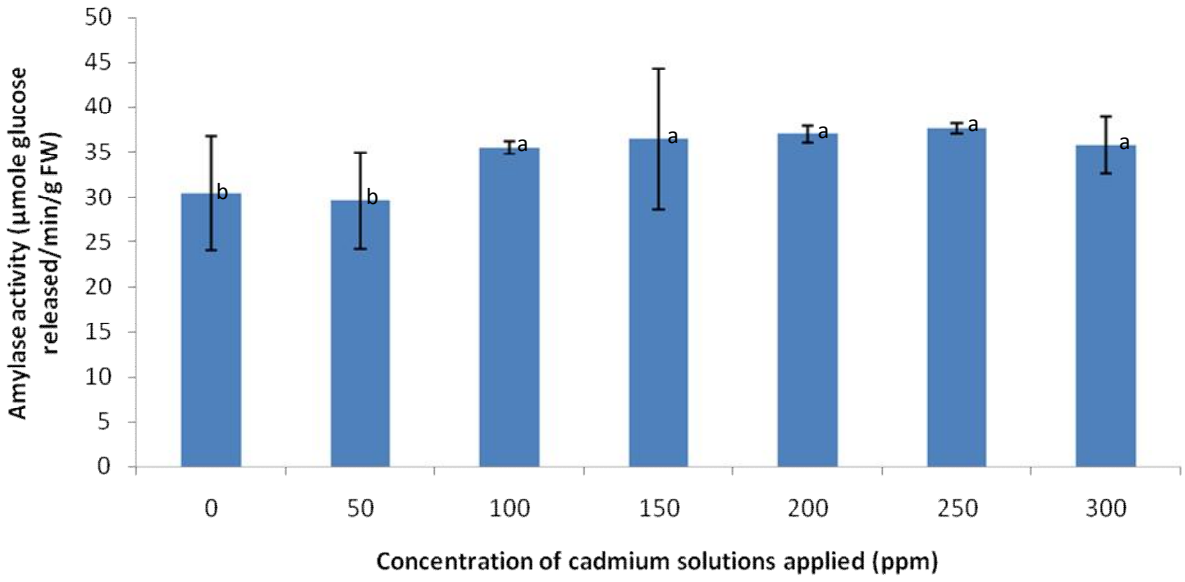

Figure 3: Amylase activity of seed-tubers of *Cyperus esculentus* subjected to different concentrations of cadmium solution (error bars represent standard deviation, similar alphabets on bars indicate no significant difference at α=0.05)

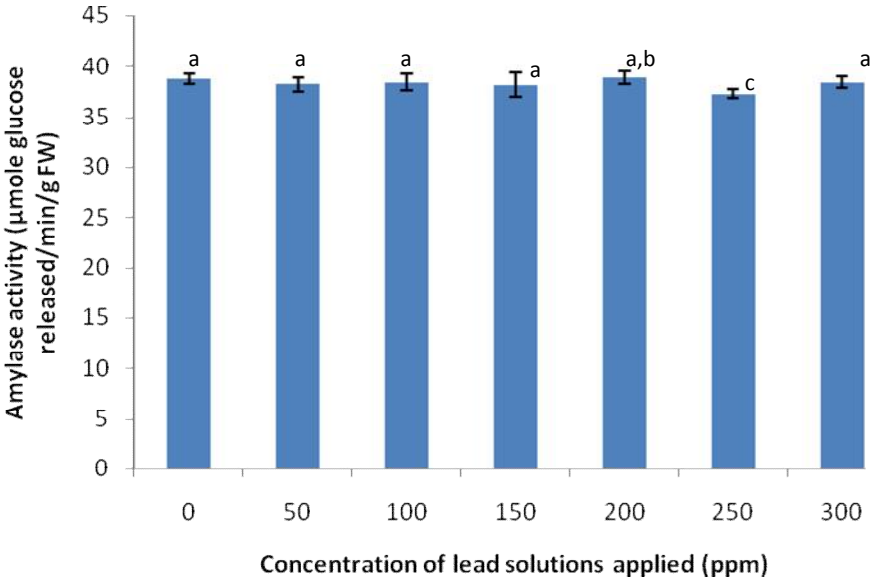

Figure 4: Amylase activity in tubers of *Cyperus esculentus* subjected to different concentrations of lead solution (error bars represent standard deviation, similar alphabets on bars indicate no significant difference at α=0.05)

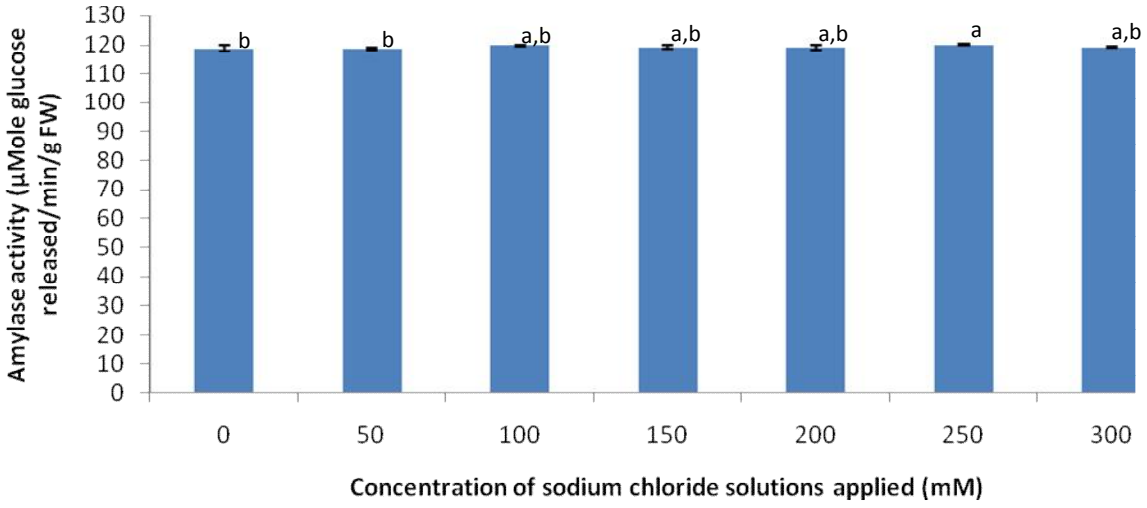

Figure 5: Amylase activity in tubers of *Cyperus esculentus* subjected to different concentrations of sodium chloride solution (error bars represent standard deviation, similar alphabets on bars indicate no significant difference at α=0.05)

Discussion

Stress in plants interferes with vital growth processes such as mineral nutrition uptake, photosynthesis, biosynthesis of chlorophyll and nucleic acid, seed germination and enzyme activities related to metabolism (Aras *et al*., 2012).

Morsy *et al*., (2012), Bhardwaj *et al*. (2009) and Yadav (2010) are some of the authors who reported the effects of metal stress on activities of enzymes. Salt stress following application of NaCl solutions inhibited amylase activity in two mung bean (*Vigna radiate*) reported by Shakeel and Mansoor (2012). This inhibition increased with increasing concentration of the salt solution. Shereen *et al*., (2011) stated that alpha amylase activity was reduced in salt treated rice seeds during germination and that the decreased activity was concentration dependent. The reduction in alpha amylase activity was connected in some way to weak seedling vigour and development observed (Shereen *et al*., 2011). In this study the total amylase activity was stimulated marginally at concentrations that between 100 – 250 mM NaCl treatments. The amylase activities recorded were significantly different. Sidari *et al*. (2008) reported that amylase activities in plants under saline conditions vary with cultivar and species and that low salt concentrations stimulated amylase activities in Lentil (*Lens culinaris* M).

Total amylase activities in metal treated-tubers showed some variation. In this study As and Zn treated tubers exhibited similar pattern of enzyme activity where inhibition of total amylase was observed at 300 ppm treatments. Zeid *et al*. (2013) Co and Cr stimulated α- and β- amylase activities while high concentrations inhibited activities. Solanki and Dhankhar (2011) reported that amylase activities were inhibited by Cu and Mn treatments in maize seeds. Further, Mittal *et al*. (2015) stated that Fe, Cu and Zn inhibited the activities of amylase and this inhibition was concentration dependent in *Vigna radiata*.

Misra *et al*. (2010) reported amylase activities following the application of nickel and lead solutions to young leaves of *Saccharum officinarum*. Nickel and lead solutions stimulated amylase activity and this effect increased as the concentration of nickel solution increased. Inhibition was observed for beta-amylase activity as the concentration of Pb increased. In this experiment, concentrations of 0 ppm to 250 ppm As and Zn neither stimulated nor inhibited total amylase activities (Figures 1 and 2). Although Jahan *et al*. (2012) reported that Zn inhibited amylase activity in *Raphenus sativus* L. Arsenic has been reported to affect growth of plants negatively. In this study, 300 ppm As concentration inhibited amylase activity.

Cadmium is known to be phytotoxic but in this study increased amylase activities were recorded for Cd solutions of 100-300 ppm concentrations. Amylase activities under these treatments were significantly different from control.

Concentrations of 50 – 200 ppm Pb solutions did not show significant differences in amylase activity from the control. Amylase activities were unaffected by the Pb solutions. Pb is known to be phytotoxic to plants. This study has shown that though Cd and Pb are phytotoxic their effects on amylase activity vary. Low concentrations of metals such as cobalt, copper, iron and nickel contribute to the functions of many enzymes and proteins (Mehes-Smith *et al*., 2013). High concentration of heavy metals in plant tissues set up oxidative reactions that cause the protein degradation (Mittal *et al*., 2015). This suggests a reason for the decrease in activity of amylase at 300 ppm concentrations of metals in this study. Mittal *et al*. (2015) stated that delays in seed germination is linked to the performance of hydrolyzing enzymes and amylase activity is important for germination. Amylase is involved in the supply of sugar to developing embryo axes in seeds and release of glucose and fructose from stored starch and sucrose to primary roots and shoots. The study has shown that delays in the germination of *C. esculentus* tubers observed following treatments with metal solutions of As, Pb, Cd and Zn may not be connected with total amylase activity.

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