Copper toxic stress on *Aloe barbadensis* and its antioxidant response

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Abstract

Toxic levels of copper (Cu) can cause deleterious effects such as protein denaturation and oxidative stress. Plants could respond to heavy metal tolerance through various mechanisms including alteration of their enzymatic antioxidant activity which acts as a toxicity tolerance mechanism. The aim of this study was to investigate the relationship between *Aloe barbadensis* enzymatic antioxidants and its response to different concentrations of Cu (control, 1ppm, 3ppm and 5ppm). *A. barbadensis* were treated with different concentrations of Cu on a 4 days cycle for a period of 21 days. Activity of the antioxidant enzymes namely superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) were determined after 21 days. SOD exhibited changes in activity in response to increased Cu concentration and showed significantly higher ($P<0.05$) at 3ppm and 5ppm Cu compared to 1ppm. CAT exhibited changes in activity as Cu concentration increased and showed no significant difference among the three levels of Cu. APX exhibited changes in activity as Cu concentration increased and showed significantly higher ($P<0.05$) at 1ppm when compared to 3ppm and 5ppm. GPX exhibited changes in activity as Cu concentration increased and showed no significance within three levels of Cu. In conclusion, the enzymatic antioxidant exhibited relationship in activity. SOD antioxidant was found to be the most effective in scavenging the reactive oxygen species as it increases during copper stress and shows consistency in activity.

Introduction

Medicinal plants have been used to relieve and treat human diseases. Their use has been widespread and favored over the synthetic drugs because of their mild features and low side effects. However, contamination has been found from cultivation, processing, and the deliberate introduction of heavy metals as part of the therapeutic ingredients.

Aloe barbadensis is more commonly known as *Aloe vera* and widely used for alternative medicine during the ancient times and the recent times; treating diseases through its therapeutic properties (Juneby, 2009). *A. barbadensis* possess many properties such as anti-tumor, anti-septic, anti-inflammatory, anti-diabetic properties as well as therapeutic properties for AIDS (Kumar *et al.*, 2010).

Copper is an essential metal in plants. Plants under metal stress such as copper stress react could adapt by increasing antioxidants as a shield. During metal stress, biochemical pathways occur where reactive oxygen species (ROS) is produced. ROS is dangerous to the plant because they oxidize cellular components such as proteins, lipids and nucleic acids leading to detrimental alterations in the

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plants physiology (Kumar *et al*., 2010). Upon ROS production, plants exhibit defensive mechanism through enzymatic antioxidants, namely peroxidase (POX), Catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD), and polyphenol oxidase (PPO). These enzymatic antioxidants scavenge the ROS, their increase and decease in activity during metal stress indicates the adaptive reaction of a plant in tolerating metal toxicity (Haribabu & Sudha, 2011).

Content of heavy metals such as copper in medicinal plants, are of great interest and concern to scientists. According to WHO regulations, maximum limits for heavy metals including copper that accumulated in medicinal plants amounts is up to 10 ppm (WHO, 1996). Investigating the adaptive characteristics of *A. barbadensis* against stresses from copper can enhance the understanding of the medicinal plant properties. Moreover, the increase or changes in the levels of enzymatic antioxidants can act as biomarkers for the heavy metal toxicity of *A. barbadensis.* Hence, the aim of this study is to investigate the relationship between *Aloe barbadensis* enzymatic antioxidants and its response to different concentrations of Cu (control, 1ppm, 3ppm and 5ppm).

Methodology

Copper treatment

The *A. barbadensis* plants were procured from the Nilai Plant Nursery, Malaysia. The plants were acclimatized for one week and watered twice per week before the commencement of the copper treatment. The plants were treated with different concentration of copper namely the control (no copper treatment), 1ppm, 3ppm and 5ppm of copper. Plants were treated for every four days with different concentration of copper and watered once a week for a period of 21days.

Plant extracts preparation

On day 21, leaves from each control and treated plants weighing 0.5g were harvested using a sharp knife. The leaves were cut with a point of a knife at the constricted base near the stem to avoid undue leakage and allow the leaf to self-heal and remain good condition (Juneby, 2009). Three replicates were extracted for each treatment.

The leaf samples were first washed with distilled water and then homogenized with 0.16 M phosphate buffer pH 7.5 (prepared from 0.2M solutions of KH_2PO_4 and K_2HPO_4) using pestle and mortar at 4˚C in an ice box for protein extraction. 4.5ml of the protein extracts from each of the 12 samples were transferred to Eppendorf tubes and centrifuged at 10,000rpm for 15minutes using Thermo Scientific microcentrifuge. The supernatant was transferred to another Eppendorf tube and stored at 4ºC. The supernatant was used for the antioxidant assays and protein determination (Hassan & Mansoor, 2014). Unit to express the activity as specific activity used was nmol/mg/g.

Total protein content

The total protein assay was carried following the method established by (Bradford, 1976). Standard Bovine Serum Albumin (BSA) was prepared at different concentrations (0, 40, 80, 120, 160 and 200 µg/ml). The absorbance was measured using a UV-spectrophotometer at 595 nm. The total protein content was determined as mg of BSA equivalent by using the equation obtained from the standard curve of BSA.

Superoxide Dismutase (SOD)

The activity of SOD was measured based on Gunes *et al*. (2009). The measurement is on the basis of SOD inhibition of NBT under fluorescent light. The experiments were carried out in two sets of test tubes for 10 minutes; one set of test tubes was under illumination while the other set of test tubes was covered with aluminium foil and used as a control. After 10 minutes, absorbance readings were taken at 560nm. Buffer solution pH 7.8 was used as a blank. Extinction coefficient of $0.0436 mM^{-1}cm^{-1}$ was used to calculate the specific activity.

Catalase (CAT)

The activity of CAT was measured according to Aebi (1984) method. The absorbance reading was taken every 30 seconds at 240nm at a minute interval to observe the trend of activity. Buffer solution pH 7.0 was used as a blank. Extinction coefficient of 0.036 mM^{-1} cm^{-1} was used to calculate the specific activity.

Ascorbate Peroxidase (APX)

The activity of APX was measured according to Nakano and Asada (1981). Absorbance readings were taken every 30 seconds at 290nm at 2 minute intervals to observe the trend of activity. Buffer solution pH 7.0 was used as a blank. Extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ was used to calculate the specific activity.

Guaiacol Peroxidase (GPX)

The activity of GPX was measured according to Roy *et al.* (1996). The assay was based on the oxidation of guaiacol by GPX. Absorbance readings were taken every 30 second at 470nm at 2 minute intervals to observe the trend of activity. Buffer solution pH 7.0 was used as a blank. Extinction coefficient $26.6 mM^{-1}cm^{-1}$ was used to calculate the specific activity.

Statistical analysis

Mean comparison of antioxidant activity at three different Cu concentrations was made by Student-Newman-keuls (SNK) and analysis of variance (ANOVA) at the $P<0.05$ level of significance (Steel & Torrie, 2006). SNK and ANOVA was done using SPSS software version 20.

Result and Discussion

Total Protein Determination

Based on Figure 1, standard graph plotted shows the absorbance of BSA concentrations at 0, 40, 80, 120, 160, and 200 µg/µl at 595nm. The linear equation with R^2= 0.9908 was generated to calculate the concentrations of the unknown enzyme samples taken from the *A. barbadensis* plants.

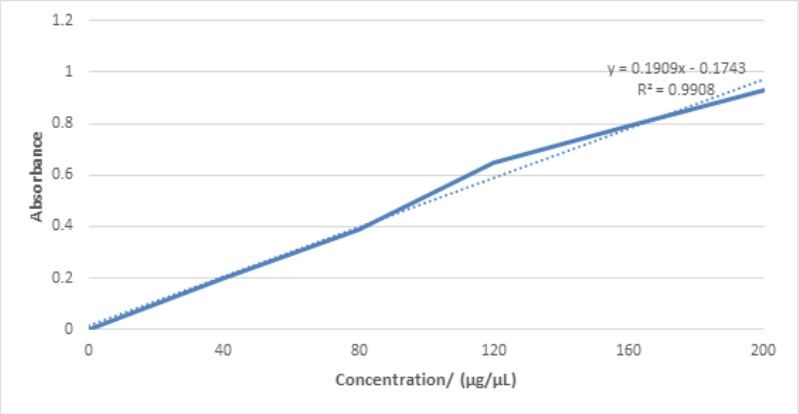

Figure 1: Absorbance of Bovine Serum Albumin (BSA) at concentrations of 0, 40, 80, 120, 160, and 200 µg/µl at 595nm.

Superoxide Dismutase

According to Figure 2, SOD antioxidant activity was significantly higher ($p<0.05$) at 3ppm (0.3574±0.0177) and 5ppm (0.3445±0.0119) than 1ppm (0.2275±0.0011). Cu ions like other metal ions act as catalyst in the formation of reactive oxygen species (ROS). These ROS interact with biological molecules by modulating them in cells of the plant specifically in mitochondria, chloroplast, peroxisomes, and at the extracellular side of the plasma membrane (Mittler & Suzuki, 2006).

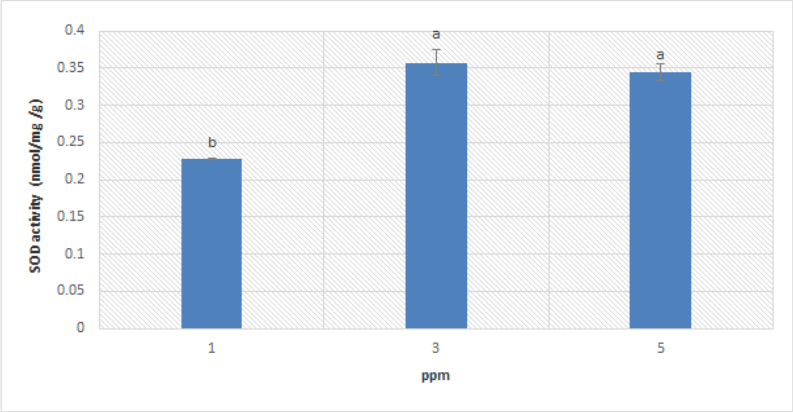

Figure 2: SOD activity (mean ± standard deviation, nmol/mg/g) in *A. barbadensis* plants at Cu concentrations of 1ppm, 3ppm, and 5ppm after 21 days.

Note: a,b: different alphabets in each column show the different significant means (SNK test, $P<0.05$).

The increase in SOD activity observed is probably due to the effect of higher Cu treatment (3ppm and 5ppm) on SOD synthesis in *A. barbadensis* plants. It has also been reported that Cu (II) ions triggers SOD gene in yeast species such as *saccharomyces cereviciae* by acting directly on the SOD gene (Li *et al*., 2004). The gene induction could have occurred in all Cu treated plants, which is concurrent with the hypothesis that Cu (II) ions are cofactors to the synthesis and stability of the SOD enzyme (Li *et al*., 2004). Upon synthesis of SOD, the SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide.

At 1ppm, the activity of SOD is significantly lower than at 3ppm and 5ppm as shown in Figure 1. This is probably due to lower amount of Cu uptake by the plant which weakly stimulates the SOD activity. The amount of ROS formed was lower and therefore the SOD activity was lower compared to 3ppm and 5ppm in Figure 1. The increased SOD activity contributed to the removal of O_2^- . Another reason for the low activity is because the weak oxidative stress was greatly alleviated due to the strong capability of the *A. barbadensis* plant to recover (Benavides *et al*., 1996).

At 3ppm, the activity of SOD was relatively high and significantly higher than at 1ppm. This is probably due to the increased concentration of the Cu uptake by *A. barbadensis* which strongly stimulated the SOD activity. The induction of the oxidative stress was much higher than at 1ppm. More Cu ions resulted in more induction of SOD which scavenged the ROS O_2^- and H_2O_2 (Erturk, 1999).

At 5ppm, the activity of SOD was relatively high and significantly higher than at 1ppm, but no significant difference when compared to 3ppm. This suggests that there is no drastic change in the SOD activity between 3ppm and 5ppm. This is because the SOD activity has become consistent.

Catalase

CAT activity is highest at 3ppm (15.7522±5.5692) followed by 1ppm (15.2770±3.6008) and lowest at 5ppm (13.2692±10.4252) (Figure 3). Catalase activities from the various treatments in the plants do not show significantly differences with each other. CAT is known to be a major H_2O_2, scavenging antioxidant enzyme. CAT works more effectively at scavenging the H_2O_2 converting it into oxygen and water (Li *et al*., 2004).

According to Figure 3, CAT activity in 5ppm was lower compared to the activity at 1ppm and 3ppm. The low activity was caused mostly by the replacement of the Fe^{2+} component of the CAT antioxidant enzyme by the Cu (II) ions (Li *et al*., 2004). The Fe (II) ions are found in the prophyrin heme group in the catalase enzyme which allows the enzyme to react with hydrogen peroxide (H_2O_2). The plant may have stopped growing well due to the high Cu concentration. In addition, the high copper concentration at 5ppm resulted in high production of H_2O_2 which lowered the CAT activity by the binding of the copper ions readily to the thiol groups thereby inactivating the enzymes (Li *et al*., 2004).

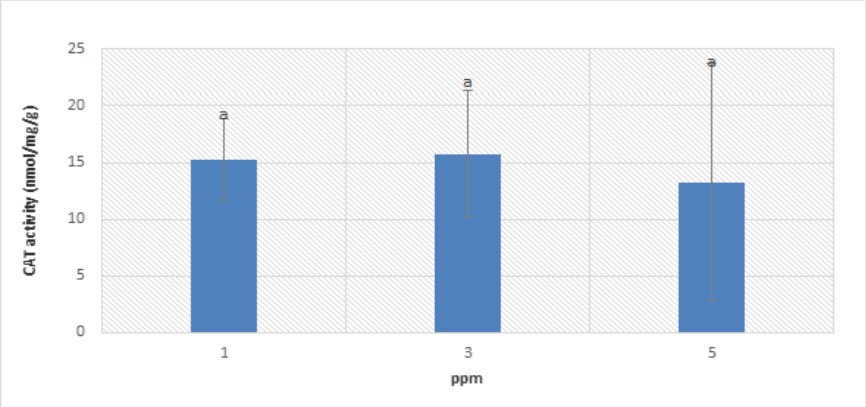

Figure 3: CAT activity (mean ± standard deviation, nmol/mg/g) in *A. barbadensis* plants at Cu concentrations of 1ppm, 3ppm, and 5ppm after 21 days.

Note: a,b: different alphabets in each column show the different significant means (SNK test, P<0.05).

Ascorbate Peroxidase

According to Figure 4, APX is significantly higher at 1ppm (0.0310±0.0067) than 3ppm (0.0068±0.0029) and 5ppm (0.0042±0.0018). The antioxidative capacity stimulated by Cu was involved in APX conversion of H_2O_2 to water and O_2 (Li *et al*., 2004). APX is a component of the ascorbate-glutathione pathway, which plays a role in scavenging H_2O_2 (Asada, 1992). H_2O_2 is a systemic signal for the induction of APX (Kaminaka *et al*., 1999).

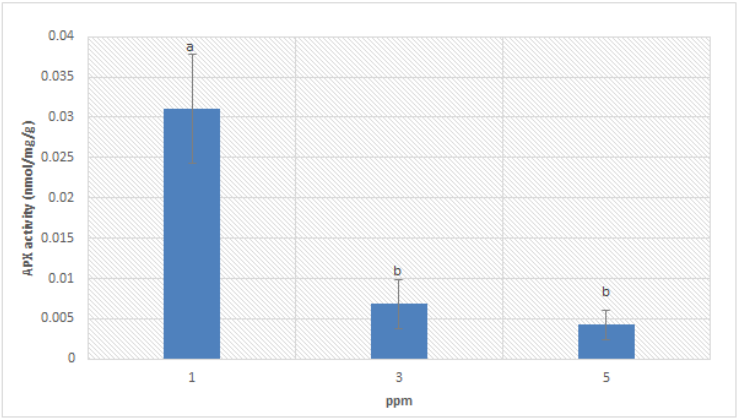

Figure 4:APX activity (mean ± standard deviation, nmol/mg/g) in *A. barbadensis* plants at Cu concentrations of 1ppm, 3ppm, and 5ppm after 21 days.

Note: a,b: different alphabets in each column show the different significant means (SNK test, P<0.05).

The maximum activity of APX occurred at 1ppm. The high activity of APX was seen at low levels of heavy metals. For instances, maximum increase in APX is observed in *Cicer arietinum L*. (Chickpea plants) at low levels of aluminum (9.5ppm and 20ppm) followed by a decrease at Al concentrations greater than 20ppm (Dubey *et al*., 2012).

Levels of SOD which increased under copper stress caused an accumulation in peroxide H_2O_2 in the plant. The H_2O_2 accumulated results in the induction of peroxidases like APX. APX was shown to increase initially to balance the SOD levels (Fasidi & Odjegba, 2007). SOD actually eventually decreased which explained the low activity of SOD at 1ppm compared to 3ppm and 5ppm. APX was shown to decrease at high levels of heavy metals like Al (Schützendübel & Polle, 2001). There was constantly low APX activity at 3ppm Cu concentration and even lower activity at 5ppm as shown in Figure 4.

Glutathione Peroxidase

GPX activity changed in response to copper stress from 1ppm to 3ppm and decreased from 3ppm to 5ppm as shown in Figure 5. GPX activity was highest at 3ppm (0.0155±0.0087), followed by 1ppm (0.0082±0.0027) and least at 5ppm (0.0044±0.0041). GPX activity from all treatment plants did not significantly differ among each other.

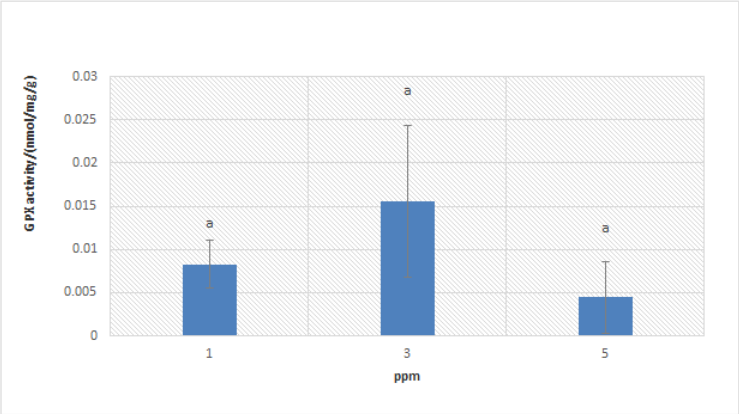

Figure 5: GPX activity (mean ± standard deviation, nmol/mg/g) in *A. barbadensis* plants at Cu concentration of 1ppm, 3ppm, and 5ppm after 21 days.

Glutathione Peroxidase (GPX), similar to APX, was triggered in all Cu treated plants. The maximum enzyme activity was seen at 3ppm. From 1ppm to 3ppm, the activity increases indicating that GPX activity increased as Cu concentrations in *A. barbadensis* increased. This increase correlated with the Cu concentration and the other enzymatic antioxidants. However, at 5ppm the activity was lower than 3ppm suggesting that the GPX enzyme had reached saturation level after it was exposed to 5ppm Cu concentration which eventually led to its inhibition due to high load of oxidative stress (Dubey *et al*., 2012). The insignificant increase in activities of the enzyme at all three levels of Cu might greatly be contributed to the degradation of H_2O_2 by APX and converting it to H_2O.

The trend of activity from 1ppm to 3ppm is consistent with the trend of GPX activity in *Bacopa monnieri* where it has been reported that GPX increases in correlation to metal concentration and exposure time, but at highest concentration its activity reduces (Singh *et al*., 2006). The increase in activity at the initial levels of metals is due to the high availability of H_2O_2 produced by the metal stress (Cristina *et al*., 2011).

Conclusion

The enzymatic antioxidants of *Aloe barbadensis* exhibit a relationship in activity. Based on the results, the trend of APX and SOD activity supports the hypothesis that APX increases initially to balance the SOD levels. APX is highest initially at 1ppm whereas SOD is lowest initially at 1ppm. CAT has demonstrated no significant change in activity from 1ppm to 5ppm. GPX and APX exhibited low activity at 5ppm indicating Cu toxicity intolerance in *A. barbadensis* plant at 5ppm. Both APX and GPX have served as ideal biomarkers of Cu toxicity at 5ppm concentration in *A. barbadensis* plants. However, further studies on the genetic structure on this species are needed in the future in order to confirm it being an ideal biomonitor. In summary, SOD is most effective in scavenging the reactive oxygen species produced as it increases during copper stress and shows consistency in activity.

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