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ANTIOXIDANTS ACTIVITY IN PINEAPPLE CV. N36 CULTURE UNDER ALUMINIUM STRESS

AZIZ, A.* and NURHALIM, M.S.

Department of Biological Sciences, Faculty Science and Technology
University Malaysia Terengganu Kuala Terengganu, Terengganu, 21030 Malaysia.
**Email: aaziz@umt.edu.my; Phone: 609-6684165; Fax: 6096684167*

ABSTRACT

Aluminium is an important stress factor for plants in acidic environments. In the present study, the effect of aluminium-stress on the oxidative enzymes and antioxidant activities in pineapple cv. N36 plantlets was investigated. The plantlets were cultured in MS medium containing 100 μM Al (pH 4.0) for 30 days. Results showed that fresh and dry weight, ascorbic acid content and catalase (CAT) enzyme activity were not significantly ($p>0.05$) affected by Al-stress. Al did significantly ($p<0.05$) reduce the α-tocopherol content (2.76 mg/fwt.) and guaiacol peroxidase (GPX) enzyme activity (3.39 units/mg protein), and increased the enzyme ascorbate peroxidase (APX) activity (7.16 units/mg protein). These results indicated that long-term Al-stress did change antioxidant substances content and oxidative enzymes activities in the leaves of pineapple cv. N36 plantlets.

ABSTRAK

Aluminium merupakan faktor tekanan utama bagi tumbuhan di kawasan berasid. Dalam kajian ini, kesan tekanan-aluminum ke atas enzim oksidatif dan aktiviti aktioksidan dalam Ananas cv. N36 telah dikaji. Anak benih telah dikultur dalam medium MS mengandungi 100 μM Al (pH 4.0) selama 30 hari. Keputusan menunjukkan berat basah dan kering, kandungan asid askorbik dan aktiviti enzim katalase (CAT) tidak dipengaruhi ($p>0.05$) oleh tekanan Al. Al memberi kesan yang bererti ($p<0.05$) terhadap penurunan kandungan tokoferol (2.76 mg/berat basah) dan aktiviti enzim guaikol peroksidase (GPX) (3.39 unit/mg protein), dan peningkatan aktiviti enzim askorbat peroksidase (APX) (7.16 unit/mg protein). Keputusan ini menunjukkan tekanan jangka panjang Al telah mengubah kandungan sebatian antioksidan dan aktiviti enzim oksidatif dalam daun-daun anak pokok Ananas cv. N36.

Key words: catalase, peroxidase, ascorbate peroxidase, carotenoids, ascorbic acid, α-tocopherol

INTRODUCTION

Aluminium (Al) is the most abundant metal in the earth's crust, comprising about 7% of its mass. It is one of the major factors limiting growth and productivity of important crops in many acidic soils throughout the world. Considerable research has been carried out to reveal the mechanisms of Al toxicity and plant tolerance (Radic *et al*., 2010; Darko *et al*., 2004; Yamamoto *et al*., 2002). Al seems to affect structure and functions of plasma membrane by strong binding to phospholipids, consequently altering the total and relative loads of phospholipids and other membrane lipids (Ishikawa and Wagatsuma, 1998). Evidence showed that Al toxicity can cause excessive generation of reactive oxygen species (ROS) and increase in peroxidation and/or breakdown of membrane lipids (Yamamoto *et al.*, 2002). A significant effect was demonstrated on modification in membrane permeability (Ishikawa and Wagatsuma, 1998), ion transport, the activity of membrane bound enzymes and intracellular metabolism by triggering secondary-messenger pathways (Matsumoto, 2000) which is correlated with the inhibition of cell growth and the inhibition of root elongation (Yamamoto *et al.*, 2002). Plants possess a cellular protection mechanism to respond and maintain the ROS below the toxicant level by shifting the activity of enzymatic antioxidants and/or metabolites (Sharma and Dubey, 2007). Enzymatic antioxidants include a variety of scavengers or detoxifying enzymes such as superoxide dismutase, catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidise (POD),

* To whom correspondence should be addressed.

monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase. Meanwhile, the non-enzymatic antioxidants include ascorbate, α-tocopherol, carotenoids, flavonoids and glutathione (Gill and Tuteja, 2010). The antioxidant defence system provides adequate protection against ROS and is responsible for clearing up excessive free radicals caused by environmental stress (Sairam *et al*., 1998). However, regulation of ROS levels depends on generation rate, reaction with other metabolites, degradation and scavenging/ neutralizing by the enzymatic and/or non-enzymatic antioxidants (Mittler, 2002).

Although Al is present and not harmful to plants in many soil types, it is considered as a major growth-limiting factor for plants on acidic soils (Matsumoto, 2000). Decrease in soil pH shows a great increase in active aluminium in soil, resulting in toxicity to plants. In acidic soils the Al can dissolve into the soil solution and become bio-available to plants by inducing Ca and Mg deficiency. Pineapple is the most important representative of the Bromeliaceae family cultivated worldwide; tropical and subtropical considered as tolerant to soil acidity, or low pH. The question is how this plant survives and manages the abiotic stresses particularly Al. Although the implication of the oxidative stress in aluminum toxicity is well documented in the roots of other plant species (Boscolo *et al*., 2003; Matsumoto, 2000; Ishikawa and Wagatsuma, 1998), to date very little information is available on the involvement of oxidative stress in the photosynthetic organ, i.e., leaf. Furthermore, study focusing on a comparison between oxidative stress in plants representing the three main modes of carbon dioxide assimilation, i.e. C_3, C_4 and Crassulacean acid metabolism (CAM) is lacking. Most of the available results described studies in C_3 plants (Sharma and Dubey, 2007). Therefore the objectives of this work were to evaluate the influence of Al stress on enzymes and non-enzymatic antioxidants production and/or degradation in leaf of pineapple CV. N36 culture, a CAM plant.

MATERIALS AND METHODS

Plant Material and Treatment Condition

The plant material used was *in vitro* plantlets of pineapple (*Ananas comosus*) cv. N36, a hybrid of "Gandul" (Singapore Spanish) and Sarawak ("Smooth Cayenne"). The plantlets were proliferated in MS basalt medium (Murashige and Skoog, 1962). The two months old plantlets were aseptically passed into treatment medium consisting of MS basic salt and 100 µM Al at pH 4.0 adjusted with 1M HCl and citric acid. Al-free medium at pH 4.0 was used as the control. Three replications were used for each condition. Cultures were incubated at 28ºC for 16h day-light provided by white fluorescent light for 30 days.

Biomass and Non-enzymatic Antioxidants

At harvest the fresh leaves were measured and subsequently homogenised for biochemical assay or dried at room temperature until constant weight. Only fresh leaf was used for biochemical assay. α-Tocopherol was assayed according to the method introduced by Kanno and Yamauchi (1977). Under dim light and ice condition, 0.1 g fresh sample was homogenized in 1.5 ml acetone then extracted with 0.5 ml hexane for about 30 seconds and centrifuged at 1000rpm for 10 min. The top layer was then removed and the hexane extraction was repeated twice. The assay mixture consisted of 0.5 ml hexane extracts mixed with 0.4 ml of 0.1% (w/v) PDT (3-(2-pyridyl)-5, 6-diphenyl-1, 2, 4 triazine (prepared in ethanol) and 0.4 ml of 0.1% (w/v) ferric chloride (prepared in ethanol). The volume was made up to 3.0 ml using absolute ethanol and the mixture was gently swirled and left for 4 min for colour development. Subsequently, 0.2 ml of 0.2 M orthophosphoric acid (prepared in ethanol) was added to the mixture and left for 30 min at room temperature and absorbance measured at 554 nm using spectrophotometer (Shimadzu, Japan). The blank was absolute ethanol. The amount of α-tocopherol in the samples was calculated based on the standard curve prepared using α-tocopherol (Sigma, type V) at 0 to 1.4 µg/ml concentration.

The ascorbic acid content was assayed using the method described by Jagota and Dani (1982). One hundred mg of fresh sample was homogenized in 1.0 ml of 10% trichloroacetic acid (TCA) under dim light and ice cold conditions and centrifuged at 5320 rpm for 10 min at 4ºC. The assayed mixture consisted of 300 µl extract, 1700 µl distilled water and 200 µl of 10% Folin reagent. The mixture was gently swirled and left on the bench under dim light for 10 min and the absorbance at 760 nm was measured using spectrophotometer (Shimadzu, Japan). The amount of ascorbic acid in the samples was calculated based on the standard curved, 0-60 µg/ml of ascorbic acid.

The carotenoids content was assayed according to Lichtenthaler (1987). About 0.10 g of fresh tissue was homogenized in 3 ml of 80% (v/v) acetone and centrifuged at 10,000 rpm for 10 minutes. The absorbance of the supernatant obtained was measured at 663.2, 646.8 and 470 nm using spectrophotometer (Shimadzu, Japan). 80% acetone was used as a blank. Carotenoids content was calculated using the following equation:

$C_a=12.25A_{663.2} - 2.79A_{646.8}$
$C_b=21.50A_{646.8} - 5.10A_{663.2}$
$C_{x+c} =1000A_{470} - 1.82C_a - 85.02C_b198$

Where, C_a = chlorophyll a (mg/l);
C_b = chlorophyll b (mg/l);
C_{x+c} = carotenoids (mg/l).

Protein and Enzymatic antioxidants assay

One hundred mg fresh leaf tissues were homogenized in 1.0 ml of 100 mM phosphate buffer (pH 7.0) containing 1 mM of ascorbic acid at 0-4ºC. Homogenates were centrifuged at 15 000 *x g* for 20 min at 4ºC and the supernatant used for protein determination. Protein concentration in the supernatant was measured according to the method of Bradford (1976), with bovine serum albumin as a standard.

All enzyme activities were measured spectrophotometrically (UV-1200; Shimadzu, Japan) at 25ºC. The ascorbate peroxidase (APX: EC 1.11.1.11) activity was assayed according to Sairam *et al*. (1998). The reaction mixture consisted of 1.5 ml of 100 mM phosphate buffer (pH 7.0), 0.5 ml of 3 mM ascorbic acid, 0.1 ml of 3 mM EDTA, 0.4 ml enzyme extract, 0.3 ml deionized water and 0.2 ml of 1.5 mM hydrogen peroxide was added to initiate the reaction. The oxidation of ascorbic acid in the reaction mixture was measured using the rate of decrease in absorbance at 290 nm for 3 min; enzyme activity was calculated using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$.

The guaiacol peroxidases (GPX: EC 1.11.1.7) activity was assayed according to the method of Agrawal and Patwardhan (1993). The reaction mixture contained 1.0 ml of 50 mM phosphate buffer (pH 7.5), 1.0 ml of 20 mM guaiacol, 1 ml of 30 mM H_2O_2 and 200 µl of enzyme extract. The polymerization of guaiacol in the reaction mixture was measured using the rate of decrease in absorbance at 470 nm for 3 min; enzyme activity was calculated using an extinction coefficient of 26.6 $mM^{-1}cm^{-1}$.

The catalase (CAT: EC 1.11.1.6) specific activity was assayed according to Claiborne (1985). The reaction mixture consists of 3 ml reaction buffer (19 mM of hydrogen peroxide in 50 mM of phosphate buffer, pH 7.0) and 100 µl of enzymes extract. The disappearance of H_2O_2 in the reaction mixture was measured using the rate of decrease in absorbance at 240 nm for 3 min. The enzyme activity was calculated using an extinction coefficient of 0.036 $mM^{-1}cm^{-1}$.

All enzyme activities were expressed as units per milligram of protein. APX and CAT activity, one unit was defined as the amount of enzyme to decompose 1 µmol min^{-1} of each substrate at 25ºC. One GPX unit was defined as the amount of enzyme to produce 1 µmol min-1 of tetraguaiacol at 25ºC. Data obtained were analysed by One-Way Analysis of Variance (One-Way ANOVA) using Statistical Package for Social Sciences (SPSS). Multiple comparisons were performed using Tukey test at $p<0.05$ as a significant level.

RESULTS AND DISCUSSION

Biomass and Non-enzymatic Antioxidants

In present study, the dry weight was slightly reduced in the Al treated plant, but comparatively the biomass was not significantly affected by the Al stress. Normally, Al exhibit severe effect on root growth compared to other plant parts (Darkó *et al.,* 2004). Furthermore, plant with availability of organic carbon, might represent an attempt to avoid the over-reduction of the photosynthetic apparatus and the transfer of electrons to O_2 rather than for CO_2 fixation. This condition might also be contributing to a lesser effect of Al on both dry and fresh weight of pineapple plantlets grown in tissue cultures. The recovery process might have been transpired in the pineapple tissue during the 30 days of Al stress.

Al had a significant effect ($p<0.05$) on the α-tocopherol concentration in the leaf of pineapple cv. N36 (Fig. 2), which 2.6-times lower than in the control. Endogenous α-tocopherol levels were

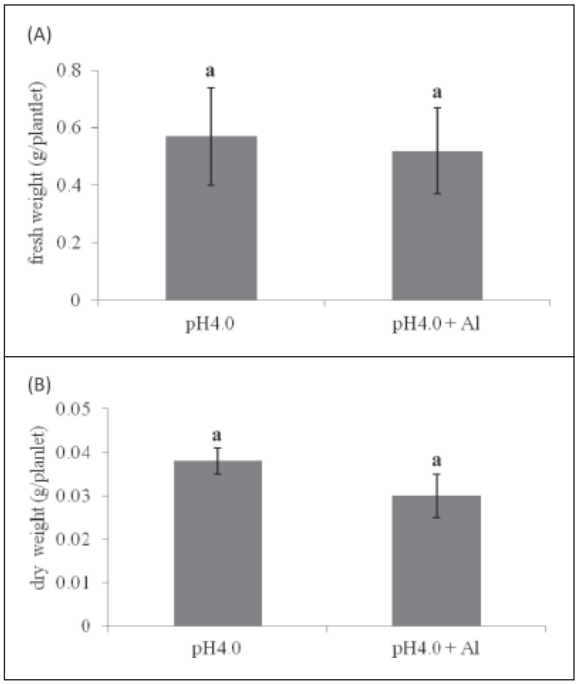

Fig. 1. The fresh (A) and dry weight (B) of pineapple plantlets after 30 days grown in culture medium containing 100 mM Al. Histogram with same small letters is not significantly different using Turkey's test ($p>0.05$), data mean for n= 3.

severely affected by the extent of its degradation and recycling under stress (Munne-Bosch, 2005). An increment in tocopherol content in the shoots was associated with the enhancement of lipid peroxidation, which indicates that tocopherol may be involved in the protection of the shoot tissues against oxidative stress (Gill and Tuteja, 2010). As stress is more severe and the amounts of ROS in chloroplasts increased, α-tocopherol concentrations tend to decrease. However, α-tocopherol is not essential for plant survival under optimal conditions, its deficiency leads to only a slightly increased susceptibility to photooxidative stress (Munne-Bosch, 2005). Under long-term Al stress in pineapple i.e. after 30 days, tocopherol and carotenoids might be utilised and act as the first line of defence which converted O_2^- into H_2O_2.

The endogenous ascorbic acid in Al-stress is slightly higher than control (Fig. 2), but no significant difference ($p > 0.05$). Ascorbic acid is considered as the most popular and powerful ROS detoxifying substance to scavenging the H_2O_2 and O_2^-. It is found at high concentration in chloroplast, mitochondria, cytosol, peroxisome and apoplast (Mittler, 2002). Thus, higher level of endogenous ascorbic acid in pineapple leaves (> 200 μg/g fwt.) is necessary to counteract oxidative stress in addition to regulating other plant metabolic processes. Ascorbic acid might be involved throughout the Al stress period or possibly act as a buffer which scavenged both O_2^- in the first line defence and H_2O_2 thereafter. Furthermore, ascorbic acid is required as electron donor for many key metabolic functions including the ascorbate peroxidase reaction and regeneration of tocopherol from tocopheroxy radical (Gill and Tuteja, 2010).

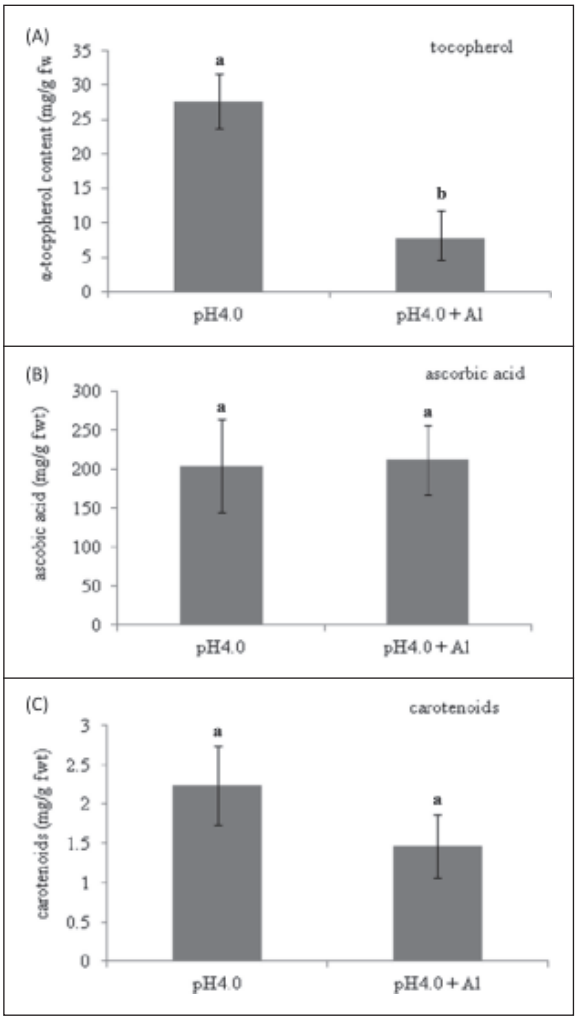

Fig. 2. The content of (A) α-tocopherol, (B) ascorbic acid and (C) carotenoids in pineapple leaf after 30 days grown in culture medium containing 100 mM Al. Histogram with same small letters is not significantly different using Turkey's test ($p>0.05$), data mean for n=3.

Carotenoids content in Al-stress pineapple leaf exhibited slightly lower than control (Fig. 2), but not significantly ($p>0.05$) different. This finding suggested that the reduction of carotenoids content in pineapple is not solely altered by Al, but might be also contributed by other factors such as mineral nutrient in the medium. Previous study showed that the presence of metal such as Zn and Al altered the carotenoids content in plant (Radic *et al.*, 2010; Asmat and Hasan, 2008). Nonetheless, carotenoids serve an important photoprotective role similar as tocopherol, β-carotene and zeaxanthin. Its three major functions in plants are absorb light at wavelength between 400 and 550 nm and it transfers to the chlorophyll, protecting the photosynthetic apparatus from free radicals naturally formed during photosynthesis, and the PSI assembly and stability of light harvesting complex protein (Gill and Tuteja, 2010).

Enzymatic antioxidants

Al triggered a significant increment of APX activity (Fig. 3) in pineapple leaf, which 22.2-times higher than in control. APXs are keys enzymes of the most active antioxidant that scavenging the formation of H_2O_2 localized in many organelles of plant cells includes chlorophyll, mitochondria, peroxisome, glyoxysome, cytosol and apoplast (Gill and Tuteja, 2010; Mittler, 2002). They detoxify the H_2O_2 using ascorbate as an electron donor in an oxidation reduction reaction regenerated the monodehydroascorbate radical (MDA). The MDA spontaneously dissociate into ascorbate and dehydroascorbate (Sharma and Dubey, 2007). This mechanism might be contributed to a constant ascorbic acid or slightly higher in Al treated than control (Fig. 2). Al also increased APX activity in root of tea (Ghanati *et al*., 2005), rice (Sharma and Dubey, 2007), maize (Boscolo *et al*., 2003) and wheat (Darko *et al*., 2004).

On the other hand, CAT activity in pineapple was not altered by Al, no significant difference

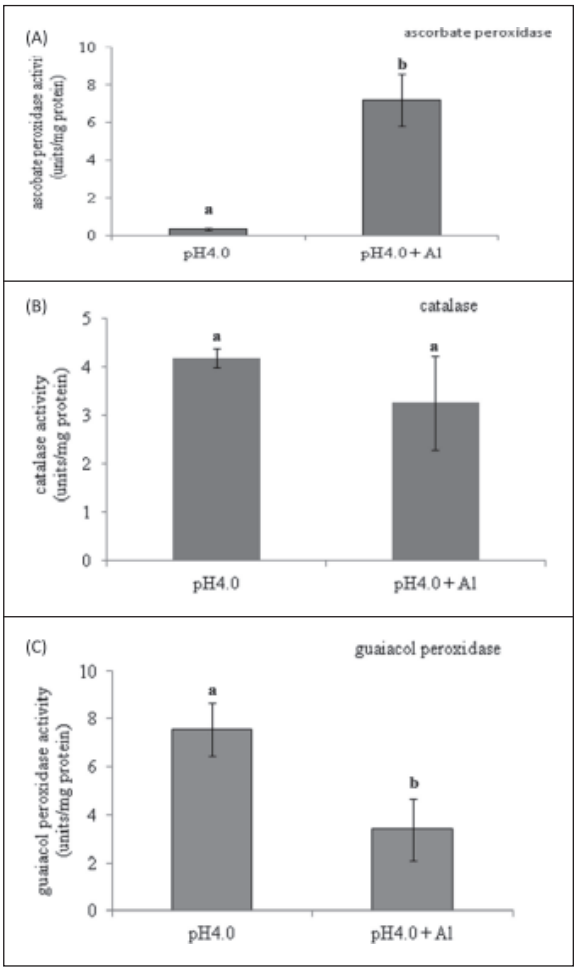

Fig. 3. The activity of (A) ascorbate peroxidase, (B) catalase and (C) guaiacol peroxidase in pineapple leaf after 30 days grown in culture medium containing 100 mM Al. Histogram with same small letters is not significantly different using Turkey's test ($p>0.05$), data mean n=3.

($p>0.05$) was observed in Al treatment and the control (Fig. 3). A similar result was reported on CAT activity under Al toxicity in maize (Boscolo *et al.*, 2003). Catalase has a high reaction rate but a low affinity for H_2O_2, thereby only removing the bulk of H_2O_2 (Radic *et al.*, 2010). Although CAT affinities towards H_2O_2 similar to APX, CAT might be responsible for the removal of excess oxygen intermediates during stress, whereas APX might be responsible for the fine modulation of reactive oxygen intermediates signalling scavenging enzymes (Mittler, 2002). The CAT might act as a buffer zone to control the overall level of ROSs that reaches different cellular compartment during stress and normal metabolism. The variable response of CAT activity in plants under Al stress has been observed. Its activity increases in tea (Ghanati *et al*., 2005) whereas, declines in rice (Sharma and Dubey, 2007).

Meanwhile, guaiacol peroxidases (GPX) specific activity significantly ($p<0.05$) decreased in Al stress, 2.2-fold lower than control. Guaiacol peroxidases are ubiquitous enzymes involved in the scavenging of soluble H_2O_2 in plants vacuole and apoplast like an APX by oxidation of co-substrates such as phenolic compounds and/or antioxidants. This oxidation is prevented and even reversed in the presence of ascorbic acid (Gill and Tuteja, 2010). In such, long-term Al stress exhibited a significant reduction of GPX activity in pineapple leave, where higher ascorbic acid is present. In this situation, hydrogen peroxide is reduced with the phenolic compound acting as the primary reducing agent and ascorbate acting as the secondary and ultimate reductant (Mittler, 2002; Matsumoto, 2000). The peroxidases can serve as an effective ascorbate dependent H_2O_2-scavenging enzymes if ascorbate is available in sufficient concentrations. This result is in agreement with suggestion by Yamamoto and co-workers (2002) that APXs and guaiacol-type peroxidases not as mutually exclusive in their functional capacities.

CONCLUSION

Many abiotic stresses are accompanied by an enhanced rate of ROS production, avoiding or alleviating the effects of stresses such as Al on plant metabolism will reduce the risk of ROS production. The present study indicated that some antioxidant enzymes, especially APX is activated during Al stress, but GPX and CAT are not. Under long-term Al stress, APX is increased to reduce the ROS risk and recover the depletion of á-tocopherol, carotenoids and GPX which are utilised at the early phase of stress. As such, pineapple possessed the CAM metabolism that was not severely affected during long-term Al-stress.

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