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Antioxidative Activities in Coconut Cultivar against the Infestation of Red Palm Weevil (*Rhynchophorus ferrugineus* Olivier)

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ABSTRACT

The Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* Olivier is a pest which targets coconut palms in Malaysia. The RPW-coconut interaction leads to a toxic reactive oxygen species (ROS), predominantly hydrogen peroxide (H_2O_2) and superoxide radicals ($O_2^{\cdot -}$), thus activating its protective antioxidant system. This study looks at the catalase; CAT, ascorbate peroxidase; APX and guaiacol peroxidase; g-POD specific activities as well as ascorbic acid, α-tocopherol, and carotenoids content in the most commonly planted coconut cultivar, MATAG. Fourteen months old MATAG plants were infested with RPW for 28 days. The antioxidant assays were carried out at 0, 7, 14, 21 and 28 days of infestation at upper and lower parts of the stem. The CAT activities were significantly higher ($p<0.05$) in the upper part of infested coconuts (11.72 ± 0.78 units/mg protein) compared with its control (1.68 ± 0.55 units/mg protein), especially at 7 days of treatment. G-POD specific activities were also significantly higher ($p<0.05$) in the upper part of infested coconuts (484.12 ± 31.30 units/mg protein) compared with its control (160.21 ± 47.58 units/mg protein). In contrast, APX specific activities were induced to 46.94 ± 2.26 units/mg protein, especially at 14 days of infestation at the lower part whereas the APX activities were slowly increased at the upper part of stem. The RPW infestation managed to significantly increase ($p<0.05$) the carotenoids content in infested MATAG whereas there were no significant changes in ascorbic acid and α-tocopherol in infested

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and control plants. This study revealed that different antioxidants have different role in combating the oxidative stress induced by RPW in MATAG cultivar.

Keywords: Coconut cultivar MATAG, Enzymatic antioxidants, Non-enzymatic antioxidants, Oxidative stress, Red Palm Weevil

INTRODUCTION

The coconut palm is grown abundantly in many tropical countries. It is a source of food, drink, fibre, vitamins, minerals and electrolytes associated with impressive health benefits. There are 12 different varieties of coconut in Malaysia, however, only 3 hybrids are recommended by Department of Agriculture (DOA), including Kelapa Wangi or Pandan (Aromatic Dwarf), MAWA, and MATAG (DOA, 2011). This study focused only on MATAG cultivars, a hybrid between seedlings from Malaysia and Philippines, Dwarf/Malayan Red Dwarf X Tagnanan Tall, as it is capable of producing more coconuts (about 10 to 22 coconuts per time) compared with other cultivars (Mohd. Taufik & Md. Akhir, 2014). This coconut hybrid begins to fruit on the third year of cultivation and can be harvested at 48 months (DOA, 2016). The MATAG shows high production of nuts as it can yield approximately 25,000-30,000 nuts per hectare per year which is more than the normal coconut or other hybrid coconut tree (PRESSREADER, 2016).

The coconut plantations in Malaysia are now under attack by the invasive red palm weevil, *R. ferrugineus* (DOA, 2011). Belonging to the order of Coleoptera, the adult RPW can grow more than 25 mm in length, and has red and black spots (Figure 1). This weevil can fly up to 1 km in distance uninterrupted. Completing one of the compulsory general characteristic of pest, this *R. ferrugineus* is said to be a fast breeder and able to breed in a wide range of climates (Rajamanickan, Kennedy & Christopher, 1995).

Figure 1. Adult red palm weevil

The RPW-coconut interaction has contributed to a great loss in the coconut industry. In 2011, an intensive three month-survey throughout Terengganu in over 800 ha of coconut plantations indicated that RPW attacked as many as 550,000 coconut trees, indicating a drastic increase and rapid spread of RPW population (Wahizatul, Zazali, Abdul Rahman & Nurul Izzah, 2013). El-Mergawy & Al-Ajlan (2011) reported that, *R. ferrugineus* spread slowly and attacked many palm species, especially in the Middle East and several countries of the Mediterranean Basin. In Malaysia, the first RPW infestation was detected in

2007 by the Department of Agriculture in all seven Terengganu districts. Currently, the infestation rate has drastically increased and RPW is found spread throughout the country. Wahizatul et al. (2013) reported that the RPW attack coconut palms in three ways: through the shoot and straight to the cabbage (edible palm pith) of the coconut, through the trunk and through the root system. Symptoms of RPW attacks are hard to detect as RPW is a concealed tissue borer. At severe infestation stage, the coconuts show signs of wilting, drooping of the leaves (like an umbrella or skirt-shaped leaves) (Figure 2).

Figure 2. Wilting and drooping of the infested leaves

In 2016, RPW attacked coconut plantations in 5 states of Peninsular Malaysia including Terengganu, Kelantan, Kedah, Penang and Perlis (DOA, 2016). Infestation of *R. ferrugineus* is believed to trigger the oxidative stress response in coconut plants, resulting in the overproduction of highly reactive oxygen species (ROS) (Gill & Tuteja, 2010). Infested coconut plants activate both enzymatic and non-enzymatic antioxidants as defence mechanisms against the infestation process. This study elucidates the defence mechanism in coconut-RPW interaction by measuring the enzymatic (catalase; CAT, ascorbate peroxidase; APX and guaiacol peroxidase; g-POD specific activities) and non-enzymatic antioxidants (ascorbic acid, α-tocopherol, and carotenoids contents) in MATAG stems. This study contributes to the understanding of the defence mechanism in coconut cultivars leading to the development of possible antioxidative markers in coconut-RPW infestation.

MATERIALS AND METHODS

Plant Materials

Thirty, 14-month-old plants of MATAG coconut cultivars were obtained from Kompleks Pertanian Negeri, Ajil, Hulu Terengganu, Malaysia. They were planted in Kampung Bukit Berangan, Tepuh, Kuala Nerus, Terengganu (Figure 3). The average temperature at the study site was between 30 to 32°C, humidity of 75-76%, sandy soil type and pH 5.8. For each infested and control treatment, three replicates of coconut plants were used. Each plant was infested with 10 red palm weevils per plant (Figure 4). Each control and treated plants was covered with a few layers of green mesh net (54cm X 54cm X 126cm) (Figure 5). The infestation was carried out for 28 days. The antioxidative defence mechanisms of the stem were evaluated by

measuring the enzymatic and non-enzymatic antioxidants in both control and infested plants at different distance. The upper part was measured 30cm above the soil line and lower part was 15cm above the soil line. The assays were carried out at 0, 7, 14 and 28 days of infestation. The experiments were repeated two times using Randomized Complete Block Design (RCBD).

Figure 4. Treated plant infested with 10 alive RPWs

Figure 3. Fourteen months old MATAG cultivars

Figure 5. Plants covered with green mesh net

Antioxidant Assays

Enzymatic antioxidants. The CAT specific activity was extracted based on Clairbone's method (1985). Stem tissues (0.15g) was ground with 1.0ml of 50mM phosphate buffer (pH 7.4) and clean sand at 0-4°C in pre-chilled mortar and pestle. The mixture was then centrifuged at 10000rpm (Eppendorf Centrifuge 5804R, Germany) at 4°C for 10 minutes. A total of 3.0ml reaction buffer containing 19mM H_2O_2 in 50mM phosphate buffer, pH 7.0 and 100µl of supernatant (enzyme extract) was added. The rate of changes in absorbance was measured at 240nm for 3 minutes using spectrophotometer (Shimadzu UV-1601A, Japan). The CAT specific activity was expressed in µmoles of H_2O_2 consumed per minutes per mg protein.

The APX specific activity was analysed following the method of Sairam, Shukla, & Sayena (1998) and Nakano & Asada (1981). Approximately 0.15g stem tissue was extracted with 1.0ml of 100mM phosphate buffer (pH 7.0) containing 1.0mM ascorbic acid in pre-chilled mortar and pestle at 0-4°C. Then, it was centrifuged at 10000rpm (Eppendorf Centrifuge 5804R, Germany) for 10 minutes at 4°C. Approximately 0.5ml 3mM ascorbic acid, 1.5ml 100mM phosphate buffer (pH 7.0), 0.1ml 3mM EDTA, 0.4ml enzyme extract and 0.3ml

distilled water were added. Finally, 0.2ml 1.5mM H_2O_2 was added into the above mixture to induce the reaction. The changes in absorbance were monitored at 290nm at 3 minutes and were expressed as moles ascorbate oxidised per hour per mg protein.

g-POD specific activity was estimated based on the method of Agrawal & Patwardhan (1993). Stem tissue (0.15g) was ground with 1.0ml of 100mM phosphate buffer (pH 7.0) in pre-chilled mortar and pestle at 0-4°C. The homogenate was then centrifuged at 10000rpm (Eppendorf Centrifuge 5804R, Germany) at 4˚C for 10 minutes. The reaction mixture consists of 3.0ml of solution containing 1.0ml 50mM phosphate buffer (pH 7.5), 1.0ml 20mM guaiacol, 1.0ml 30mM H_2O_2 and 100µl enzyme extract. The changes in absorbance were monitored at 470nm for 3 minutes and POD specific activity was expressed as µmoles of H_2O_2 consumed per minute per mg protein.

The total protein concentration was measured according to the method proposed by Bradford (1976). Coomassie Brilliant Blue G-250 (100mg) was dissolved in 50ml 95% ethanol. Then, 100ml concentrated phosphoric acid was added and the mixture diluted to 1.0L with distilled water. The solution was later filtered through a filter paper and stored at room temperature in light-proof bottles. A total of 100µl of enzyme extract was added to 3ml of Bradford's reagent and the absorbance measured at 595nm after 10 minutes. The protein standard curve was prepared with various concentrations (0 to 1.0mg/ml) of Bovine Serum Albumin (BSA).

Non-Enzymatic antioxidants. The procedure based on Jagota & Dani (1982) was followed to determine the amount of ascorbic acid. A total of 0.15g stem tissue was ground with pre-chilled mortar and pestle in 1.0ml of 10% trichloroacetic acid (TCA) and clean sand under low light intensity at 0-4°C. The ground sample was then centrifuged (Eppendorf Centrifuge 5804R, Germany) at 10,000 rpm for 10 minutes at 4°C. The supernatant obtained (300µl) was added into test tube containing 200µl 10% Folin reagent and 1700µl distilled water. After 10 minutes, the absorbance of the mixture was measured at 760nm. The amount of ascorbic acid in the sample was calculated based on the standard curve prepared using ascorbic acid at the range of 0 to 60µg/ml.

α-Tocopherol was extracted based on the method proposed Hodges, Andrews, Johnson and Hamilton (1996) and Kanno and Yamauchi (1977). A total of 0.15g stem tissue was ground with 1.5 ml acetone and clean sand in a mortar and pestle at 0-4°C. Then, 0.5 ml hexane was added. The mixture was vortexed for about 30 seconds followed by centrifuging at 10,000 rpm (Eppendorf Centrifuge 5804R, Germany) for 10 minutes. After the centrifugation, the top layer was discarded and the hexane extraction was repeated twice. The hexane-extract (0.5ml) was added into 0.4ml 0.1% (w/v) PDT (3-(2-pyridyl)-5,6-diphenyl-1,2,4 triazine),

0.4ml 0.1% (w/v) ferric chloride and 1.7ml absolute ethanol. The mixture was gently swirled and left for four minutes for colour development. Following this, 0.2 ml of 0.2 M orthophosphoric acid was added and the mixture was allowed to stand for 30 minutes at room temperature. The absorbance was measured at 554nm using spectrophotometer (Shimadzu UV-1601A, Japan). Amount of α-tocopherol was calculated based on the standard curve prepared using α-tocopherol (Sigma, type V) at various concentrations (0-1.4 µg/ml).

The carotenoid content was analysed based on the method proposed by Lichtenthaler (1987). Stem tissue (0.15g) was ground up with 3 ml of 80% (v/v) acetone and clean sand in a mortar and pestle. The mixture was centrifuged at 10,000 rpm (Eppendorf Centrifuge 5804R, Germany) for 10 minutes. Supernatant obtained was measured spectrophotometrically (Shimadzu UV-1601A, Japan) at three different wavelengths, i.e. 663.2, 646.8 and 470nm.

Statistical analysis. Data obtained were analysed using analysis of variance (TWO WAY ANOVA) of Statistical Package for Social Science software (SPSS) version 20. Multiple comparisons were performed using Duncan Multiple Range Test (DMRT) at $\alpha=0.05$ as significant level.

RESULTS AND DISCUSSION

MATAG hybrid was selected as it produces very high yield nuts per year with multifarious uses including coconut water, coconut milk production and also for grated coconut. Besides, one MATAG coconut tree can produce high quality copra eight times per year in the long term where it has thicker plump compared with other hybrid coconut as well as normal coconut (Sivapragasam, 2008).

Recently, RPW is reported as a pest of more than 40 palm species worldwide which not only destroy the coconut tree but also greatly affects its quality and quantity (Cangelosi, Clematis, Curir & Monroy, 2016). The activity of RPW must be monitored to prevent infestation (Vacas, Primo, & Navarro-Llopis, 2013). Infestation may alter the equilibrium between free radical production and defence mechanisms in favour of free radical production. The balance between the formation and detoxification of ROS is critical to plant cell survival. However, the degree of vulnerability and defence mechanisms of palm species against the RPW are still poorly known. Thus, the study of RPW-coconut interaction is very important to understand the responses of coconut against the RPW infestation.

Figure 6 shows the effect of coconut-RPW infestation on CAT specific activity in (A) upper part and (B) lower part of infested and non-infested MATAG stem at 28 days of infestation. The CAT specific activities of infested upper stem were significantly increased ($p<0.05$) from day 0 to 7 days of infestation compared with control (Figure 6A). Results of this study confirm the findings of Arutselvi, Balasaravanan, Ponmurugan & Muthu (2012). The authors reported that infested

turmeric leaves by *Udaspes folus* increased CAT activities in the leaves compared with control plants which could have been the result of higher production of ROS, particularly H_2O_2, as a response to the infestation. This is the earliest defence of the plant (Wojtaszek, 1997). Thus, the H_2O_2 may be removed by CAT and therefore, the formation of hydroxyl radical damage will be avoided. No significant difference ($p>0.05$) was observed in the infested and control plants after 21 days of treatment. The maximum activity was observed at 7 days of infestation (11.72 ± 0.78 units/mg protein). Higher activities in infested plants indicated that CAT enzyme involved in defence response and decreased the toxicity of ROS produced during the infestation process (Khorshidi & Sherafatmandjour, 2013). However, CAT specific activities in control dropped significantly ($p<0.05$) after day 7 in addition to reduced infestation of the lower stem (Figure 6B). This might be due to the inactivation and degradation of CAT (Feireabend, Schaan, & Hertwig, 1992). Lower CAT specific activities in lower stem might also be related to low concentration of H_2O_2 produced. Zamocky, Janecek and Koller (2002) stated that CAT has been proven to be inefficient in converting low concentrations of H_2O_2 compared with APX. Figure 6 shows CAT specific activities were significantly higher in upper stem compared with the lower stem. It may be suggested that the infestation site might influence the antioxidative responses in this plant. Wahizatul et al. (2013) suggested that the infestation in coconut trees could occur through the shoot, trunk and root system. Since young MATAG plants were used in this study, the infestation started at the root of the plants. The distance between the infestation sites to the lower part of the stem was only about 12cm, so excess production of H_2O_2 may be produced, thus enhancing the CAT activities at an early phase of infestation. The increase of H_2O_2 could act as signalling molecules to trigger the CAT activities in the upper part of the infested stem.

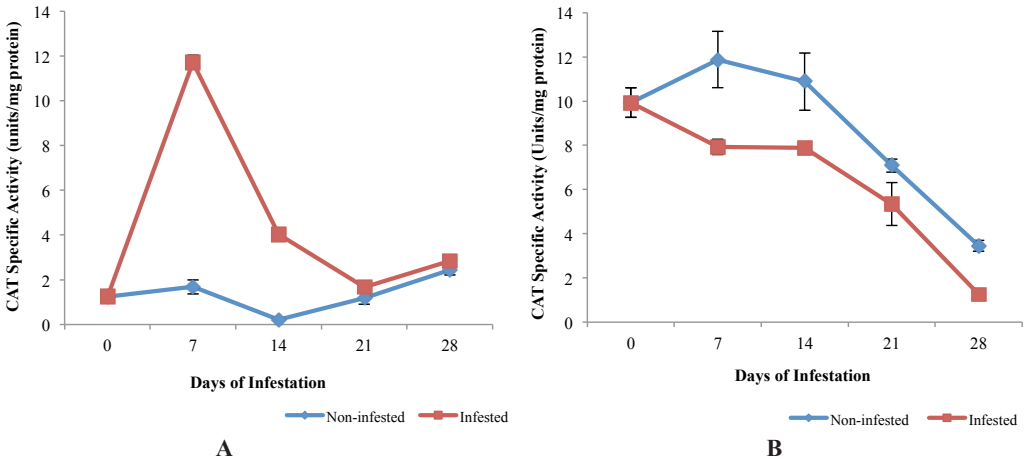

Figure 6. The effect of coconut-RPW infestation on CAT specific activity in (A) upper part and (B) lower part of MATAG stem at 0, 7, 14, 21 and 28 days of infestation. Vertical bars represent standard errors (n=5)

The APX is a key enzyme that plays an important role in ascorbate-glutathione cycle, main H_2O_2-detoxification system in plant chloroplasts (Asada, 1992). In particular, APX has higher affinity for H_2O_2 and utilises ascorbate as specific electron donor to reduce H_2O_2 to water in chloroplasts, cytosol, mitochondria and peroxisomes, as well as in the apoplastic space (Sofo, Scopa, Nuzzaci & Vitti, 2015). The APX-specific activities in infested upper stem slowly increased throughout the experiment, whereas the non-infested stem showed significantly ($p<0.05$) higher APX specific activities at 21 days of experiment (27.45 ± 0.28 units/mg protein) (Figure 7A). Higher APX specific activities were observed in the infested upper stem of coconuts compared to with control, especially at initial and later stages of experiments (Figure 7A). The infested lower stem showed the same pattern at 14 days of infestation (Figure 7B). The considerable increase in APX activity observed can protect plants, which, under stress conditions, present sustained electron flows and are the main producers and targets of ROS action (Foyer & Shigeoka, 2011). Enhanced APX specific activities in the lower part at 14 days of infestation might be also related to decrease the toxicity of ROS, particularly H_2O_2 as APX play a secondary role in H_2O_2 scavenging as observed by Gondim, Filho, Costa, Alencar & Prisco (2012) in salt stress maize and Khorshidi & Sherafatmandjour (2013) in fennel. In addition, continuous increases in the antioxidant enzyme activities may protect the cell structure by eliminating the ROS produced (Ahn, Oke, Schofield & Paliyath, 2005).

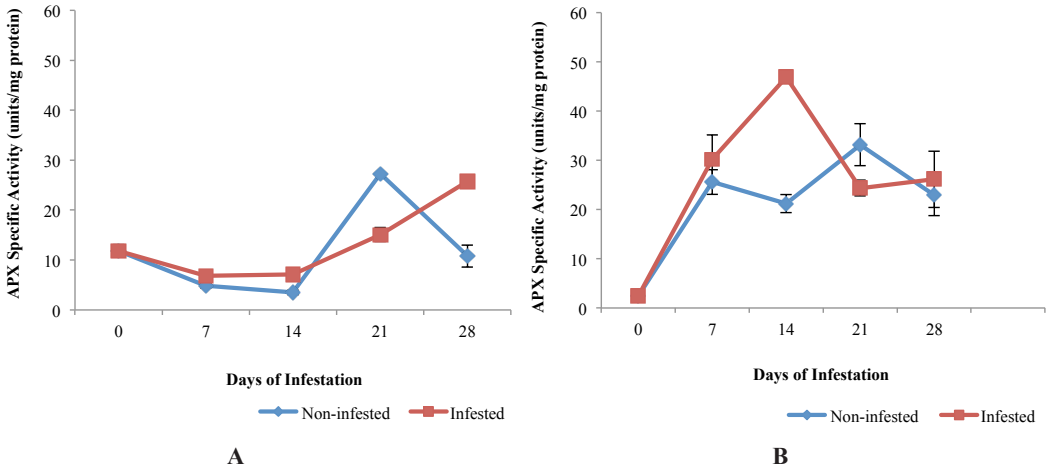

Figure 7. The effect of coconut-RPW infestation on APX specific activity in (A) upper part and (B) lower part of MATAG stem at 0, 7, 14, 21 and 28 days of infestation. Vertical bars represent standard errors (n=5)

The PODs are major enzymes associated with defence related pathways in plants and pathogens (Van Loon, Rep & Pieterse, 2006). Almagro et al. (2009) reported the importance of PODs in auxin metabolism, cross-linking of cell wall components, lignin, suberin and phytoalexin synthesis, as well as in metabolism of ROS. The PODs decompose H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants. In this study, infested upper stem significantly enhanced ($p<0.05$) the g-POD specific activity to a maximum activity at day 7 (484.12 ± 31.30 units/mg protein) compared with non-infested stem (160.20 ± 47.58 units/mg protein) (Figure 8A). Similarly, infected lower part of stem induced higher g-POD specific activities after 7 to 21 days of infestation compared with its control (Figure 8B). Thus, the increase in g-POD specific activity in this study may help to reduce the oxidative stress generated by the imbalanced production of ROS in coconut due to RPW infestation. Previous study on the induced POD activities also noted infested cabbages of PANDAN, MATAG and MAWA coconut cultivars as a response to RPW attacked (Norhayati, Afzan, Jannah, & Nurul, 2016). Similar elevated POD specific activities were also observed in infestation of *Coccus hesperidum* on its host plant *Nephrolepis biserrata* (Golan, Rubinowska, & Gorska-drabik, 2013) and of *Spodoptera litura, Aphis craccivora* and *Bemisia tabaci* on cowpea, cotton and tomato (Singh, Dixit, Singh, & Verma, 2013).

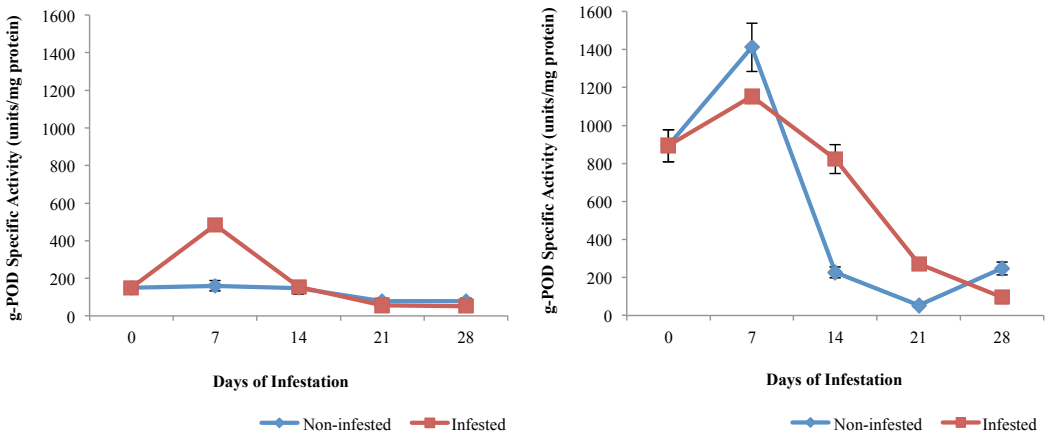

Figure 8. The effect of coconut-RPW infestation on g-POD specific activity in (A) upper part and (B) lower part of MATAG stem at 0, 7, 14, 21 and 28 days of infestation. Vertical bars represent standard errors (n=5)

Ascorbic acid is the most important vitamin in fruits and vegetables. This substance helps to protect organism from cell membrane damage and other structures by neutralising free radicals that occur as a result of oxidative stress (Rekha et al., 2012). Amount of ascorbic acid in infested and non-infested upper stem remained unchanged throughout

the experiments except at day 21, where the amount of ascorbic acid in infested plants were lower (388.60±4.87 µg/g fwt) compared with the non-infested plants (Figure 9A). In this study, the antioxidant activity of ascorbic acid might be associated with resistance to oxidative stress in coconut plants. Infested lower stem has significantly ($p<0.05$) higher amount of ascorbic acid at day 14 of infestation compared with non-infested stem (Figure 9B). Niki (1987) suggested that the ascorbic acid increment is to help the scavenging of free radicals in plants. This also indicated that ascorbate acid as the hydrogen donor had eliminated the generation of H_2O_2 in MATAG plants. Sarkar, Srivastava & Dubey (2009) reported that ascorbate acts as a specific antioxidant by donating an electron and convert the free radicals to more stable products and thus, terminating free radical initiated chain reactions.

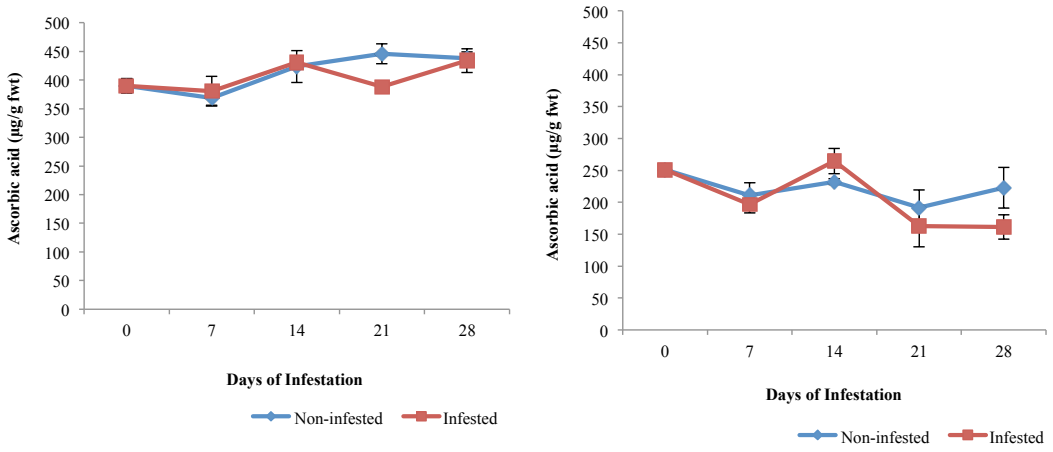

Figure 9. The effect of coconut-RPW infestation on ascorbic acid content in (A) upper part and (B) lower part of MATAG stem at 0, 7, 14, 21 and 28 days of infestation. Vertical bars represent standard errors (n=5)

Tocopherol is a well-known nature's major lipid soluble chain-breaking antioxidant that helps to protect biological membranes and lipoproteins from oxidative stress (Serbinova, Kagan, Han, & Packer, 1991). The main biological function of α-tocopherol is its direct inducing of cellular responses to oxidative stress through modulation of signal transduction pathways (Azzi, Boscoboinik, & Hensey, 1992). Based on Figure 10, infested upper and lower MATAG stem shows no significant differences ($p>0.05$) in the amount of α-tocopherol along the experiments. However, the α-tocopherol drastically reduced to 14.52 ± 3.17 µg/g fwt and 12.78 ± 0.35 µg/g fwt in control of upper and lower stem respectively. After seven days, the α-tocopherol level was comparable with infested plants. This might be due to the adaptation of the plants towards the environmental conditions on the site at early stages of experiment. Munne-

Bosch (2005) reported that amount of α-tocopherol also changed during plant growth and development as well as in response to oxidative stress. These changes were due to the altered expression of pathway related genes, degradation and recycling. This also indicated that α-tocopherol level and its composition vary during cell development and in response to biotic stress (Collakova & Dellapenna, 2003).

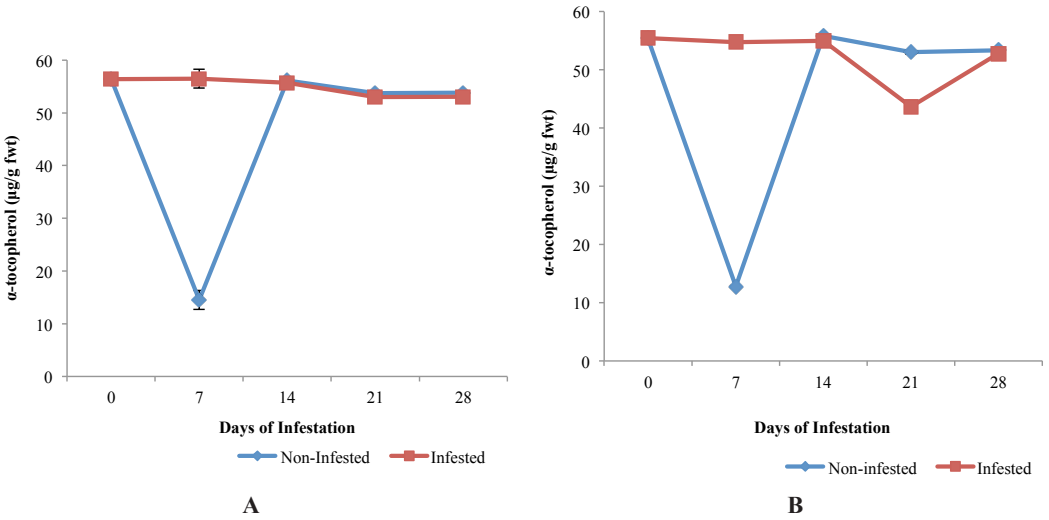

Figure 10. The effect of coconut-RPW infestation on amount of α-tocopherol in (A) upper part and (B) lower part of MATAG stem at 0, 7, 14, 21 and 28 days of infestation. Vertical bars represent standard errors (n=5)

Carotenoids play an important role in both photobiological and non-photobiological systems. The fact that carotenoids can be readily oxidised, and thus inhibit other oxidation reactions has been known for many years (Krinsky, 1979). Burton & Ingold (1984) proved that carotenoids can function directly as antioxidants by reacting with active oxygen species. Generally, the carotenoids content fluctuated in both control and infested upper stem of MATAG cultivar. However, significantly higher ($p<0.05$) carotenoids content were produced in infested upper stem compared with the non-infested plants (Figure 11A). Lower carotenoids content was observed at the infested lower stem during the later stages of infestation (Figure 11B). Carotenoids protect photosynthetic organisms against potentially harmful photooxidative processes (Bartley & Scolnik, 1995). Thus, this helps the cell to encounter the over production of ROS resulting in damage to the photosynthetic apparatus that can causes photoinhibition (Breusegem, Vranova, Dat, & Inze, 2001).

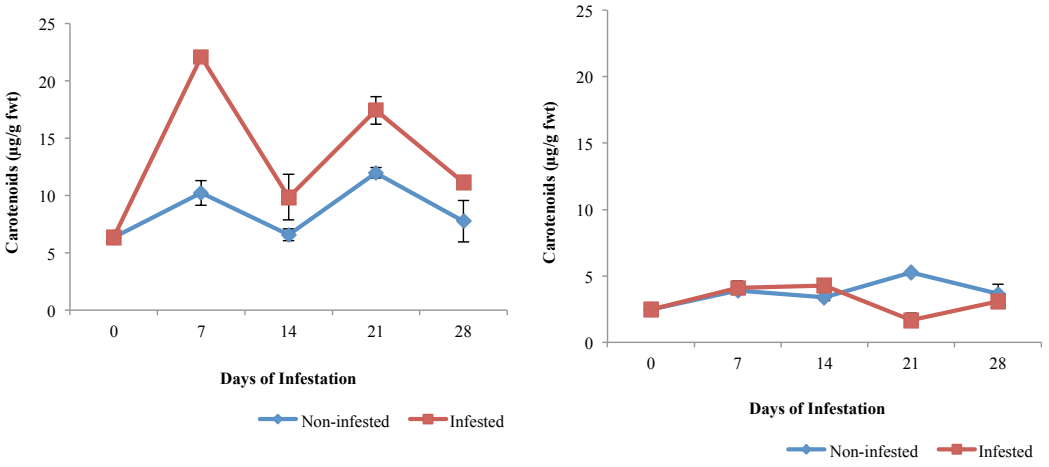

Figure 11. The effect of coconut-RPW infestation on carotenoids content in (A) upper part and (B) lower part of MATAG stem at 0, 7, 14, 21 and 28 days of infestation. Vertical bars represent standard errors (n=5)

CONCLUSION

Results indicated that most of the antioxidants studied i.e. CAT, APX and g-POD specific activities as well as ascorbic acid, α-tocopherol and carotenoids content showed significant increase as a response to the oxidative stress induced by the RPW infestation. Most of the antioxidants were higher at the lower part of infested stem at early stages of experiment but then signalled the infested upper stem after 14 days of infestation. The activation of antioxidants may enhance the resistance of MATAG cultivar toward RPW infestation. Further studies need to be undertaken to better understand the tolerance level of MATAG plant against RPW infestation.

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