SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL PROTEINS PROFILE OF RED PALM WEEVIL- AND MECHANICALLY-WOUNDED OIL PALM SEEDLINGS

Hazlina Ahamad Zakeri
School of Fundamental Science
Universiti Malaysia Terengganu (UMT)
21030 Kuala Terengganu, Terengganu, Malaysia
Email: hazlina@umt.edu.my

Fahmeeda Mohamad Jazamuddin
School of Fundamental Science
Universiti Malaysia Terengganu (UMT)
21030 Kuala Terengganu, Terengganu, Malaysia
Email: uk28862@student.umt.edu.my

Wahizatul Afzan Azmi
School of Marine and Environmental Sciences
Universiti Malaysia Terengganu (UMT)
21030 Kuala Terengganu, Terengganu, Malaysia
Email:wahizatul@umt.edu.my

ABSTRACT

In this study, we reported the proteins expressed by oil palm seedlings using a one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (1D SDS-PAGE) analysis. This study was carried out as a preliminary study on the effects of an invasive palm pest, the Red Palm Weevil (Rhynchophorus ferrugineus) on oil palm. In order to determine whether the trees can differentiate between the weevil (RPW)- and mechanical-inflicted (MW) wounds, proteins of both types of wounds were compared. Treatments were carried out within four time frames: one, two, three and four weeks. For each week, new 12 months old oil palm seedlings were used and cabbage tissue samples were analyzed for their protein content and profile. Protein content was determined using Bradford assay while SDS-PAGE analysis was used to construct the protein profile of the samples. Protein content of MW seedlings gradually increased from week one until week four while for RPW, the proteins significantly increased in week one, decreased in week two and three but then increased again in week four. Thirteen protein bands were newly formed on the gels of RPW while 12 bands were newly formed for MW. Additionally, 19 bands were missing from the profiles of RPW while 3 bands were missing for MW. From the results obtained, it can be concluded that oil palm seedlings triggered different defense and regulatory mechanisms depending on the types of inflicted wound. New and missing proteins observed can be used as potential biomarkers to detect the early infestation of RPW on oil palm. Further analysis will be carried out using 2D gel electrophoresis for better understanding of the mechanisms triggered by the seedlings.

Key words: Protein Biomarker, Red Palm Weevil, Oil Palm, Insect Herbivory, Mechanical Wounding.

Introduction

Half of the nearly one million insect species known to date feed on plants (Wu and Baldwin, 2010). When it comes to natural and planted crops, this can be a major ecological and economic impacts as millions of ringgits are lost each year in agriculture due to these pests. Agriculture in Malaysia is one of the important contributor to economic growth (i.e. contributes about 11.9% of the 2012 country's Gross Domestic Production) since it can increase the income of farmers, fishermen, agro-based industries and ensure the nations food supply remain sufficient. However, having a tropical climate, Malaysia is suitable for phytophagous insects to realize their large increase capacity. Some of the major insect crop pests in Malaysia that have been identified include the moth, *Conopomorpha cramerella* which infect the cocoa trees (Alias et al., 2004) and the chironomid, *Chironomus kiiensis* which invade the rice field (Al-Shami et al., 2010). Plant–insect interactions have resulted in the evolution of sophisticated mechanisms in plants that respond to insect attack. The study of plant defences against herbivory is important, not only from an evolutionary view point, but also in the direct impact that these defences have on agriculture (Kerchev et al., 2012). According to Wu and Baldwin (2010), when plants perceive herbivore-derived physical and chemical cues, plants dramatically reshape their transcriptomes, proteomes, and metabolomes. Proteins, for example, are the functional unit of the cell and, as such, they are responsible for setting in motion the wide array of cellular events that occur in response to abiotic and biotic factors. Studying the proteins directly gives us a more accurate representation of the molecular phenotype and tells us more about the functional and regulatory aspects of the cellular stress response.

Malaysian palm oil industry is a significant contributor to Malaysia's overall economy, providing both employment and income from exports. About 4.49 million ha of land in Malaysia is under oil palm (*Elaeis guineensis*) cultivation; producing 17.73 million tonnes of palm oil and 2.13 tonnes of palm kernel oil. Additionally, Malaysia is one of the largest producers and exporters of palm oil in the world, accounting for 11% of the world's oils and fats production and 27% of export trade of oils and fats (MPOC, 2013). Oil palm can be affected by a wide range of pests and this will affect the productivity of this important crop. Examples of most common pests to oil palm are boring insects such as rhinoceros beetles (*Oryctes rhinoceros* and *Scapanes australis*), red palm mite (*Raoiella indica*) (UNCTAD, 2012) as well as a leaf beetle known as *Brontispora longissima* (Takano et al., 2013).

In the early 2007, an unidentified cryptic species of weevil known as the Red Palm Weevil (RPW) was first sighted in some small land holdings in Rhu Tapai, Setiu, Terengganu (Wahizatul et al., 2013). Little is known about its origin and how they came to Terengganu but its devastating damages were clearly seen along the coastal area. This weevil or its scientific name *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), is an important invasive tissue borer that has a broad host range restricted to palm trees infesting about 17 palm species worldwide including oil palm (EPPO, 2015). RPW is reported to usually attack young palms, mostly below the age of 20 years (Kaakeh, 2005). It is very difficult to detect the presence of RPW at the early stages of infestation. This hidden enemy that strikes to kill from within is the most dreadful because once the palm is attacked, it succumbs within 6 to 8 months and death of the palm is certain.

This study, thus, was conducted in order to understand more about the oil palm-RPW interaction in terms of proteins expressed in oil palm. The proteins expressed can be used as biomarkers to detect the early infestation of RPW and for oil palm protection. To date, no previous studies on the effects of RPW herbivory on oil palm proteins were reported. The results reported here can be used as baseline study to find the biomarkers for early detection of RPW attack on oil palm and for oil palm protection. Since insects usually feed on the tissues of plants, comparison between wounding caused by RPW and simple mechanical was also conducted to determine whether the oil palm can differentiate between these two.

Research design

Healthy and non-infested 12-month old oil palm seedlings were obtained from Felda Tenang, Besut, Terengganu, Malaysia. The seedlings were covered with mesh until the treatment applied. The seedlings were watered four times a week prior to treatments. Samples of red palm weevil (RPW) were collected around the Universiti Malaysia Terengganu area by using pheromone traps in 10-liter polypropylene buckets according to that explained in detail by Wahizatul et al. (2013).

For the mechanical wounding treatment, three holes of 2 cm wide x 2 cm long x 2 cm high for each seedlings were made using appropriate tools. Similar holes were made for the RPW wounding treatment, except that two RPW (one male and one female) were placed in each hole. The seedlings were then left on the field, covered with wire net to avoid RPW from escaping. Controls used were healthy and non-infested seedlings. Cabbage tissues from treated and untreated seedlings were sampled every one week, two weeks, three weeks and four weeks. For each time frame, new seedlings were used. During treatments, the seedlings were watered four times a week. All treatments were done in triplicates.

Protein crude extracts were prepared according to Lippert et al. (2007) with modifications. Cabbage tissues (~6.0 g) were ground in liquid nitrogen into fine powder. The powder was dissolved in Tris-HCl extraction buffer consisting of 200 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 0.1% MgCl, 15% glycerol, 28 mM phenylmethylsulphonyl fluoride (PMSF) and 10 mM mercaptoethanol. The samples were then kept in ice for 20 mins before centrifugation at 10000 rpm for 20 mins in 4℃ to pellet all insoluble material, which was then subsequently discarded. The supernatant was then transferred to a fresh tube and was centrifuged again at 10000 rpm for 10 min in 4℃. Supernatant was collected and the crude extract obtained was stored at -80℃ before further analyses.

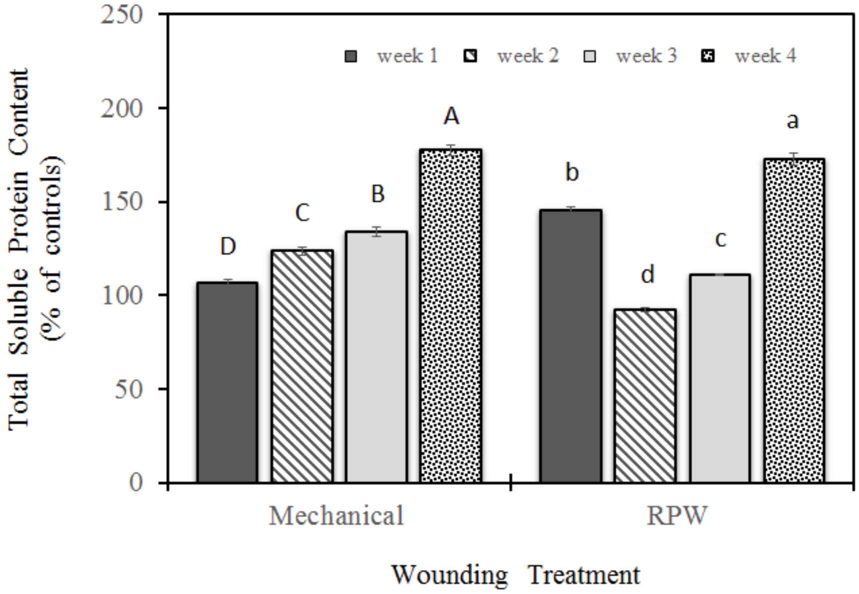
The protein content in the extract was determined using Bradford assay (Bradford, 1976). The crude extract was mixed with Bradford reagent and left for about 10-15 mins before absorbance measurement at 595 nm. The absorbance was compared with that of a calibration curve constructed from a range of 1.0 mg/L bovine serum albumin (BSA) concentration. Total soluble protein (TSP) content of the cabbage tissue was expressed as mg TSP in 1 g fresh weight (FW) of tissues. Data were presented as mean±SE based on percentage of controls. The statistically significant differences of TSP content between different timeframes within similar wounding treatment were analysed using a one-way ANOVA followed by Tukey's HSD post hoc test at probability level of 0.05. Software used was Daniel's XL Toolbox ver. 6.53, an Add-in for Microsoft Excel.

Protein profile was constructed according to that of a discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) system (Laemmli, 1970) using a 12% acrylamide gel for resolving and 4% acrylamide gel for stacking. Samples were heated with loading buffer (ratio of 1:1) at 95°C for 4 mins before being loaded into the gel. SDS-PAGE was performed at constant voltage of 80 volt. Gels were stained with Coomassie brilliant blue dye G-250 and documented. Relative mobility of the protein bands formed was also calculated by comparing with that of a standard molecular weight protein markers of 12-225 kDa (Amersham ECL Rainbow Molecular Weight Markers, RPN800E).

Results and discussion

It was observed that the seedlings reacted differently in terms of their total soluble protein (TSP) content as well as proteins expressed against wounding caused by mechanical or that of RPW herbivory (Figures 1 and 2). In week one, TSP of the seedlings did not show any significant changes compared to controls (Figure 1). But then, TSP of the seedlings gradually increased from 24% of controls in week two to 78% in week four. Comparatively, an induction of TSP can be observed even in week one of the RPW wounded seedlings with a 46% increment from controls. However, TSP of the seedlings suddenly decreased to an 8% lower from controls in week two. TSP of the seedlings started to increase again in week three until it reached the highest in week four with a 76% of controls.

Figure 1: Total soluble protein content of mechanically and Red Palm Weevil (RPW) wounded oil palm seedlings after treatment. Means with different letters are significantly different within similar wounding treatment at $p<0.05$.



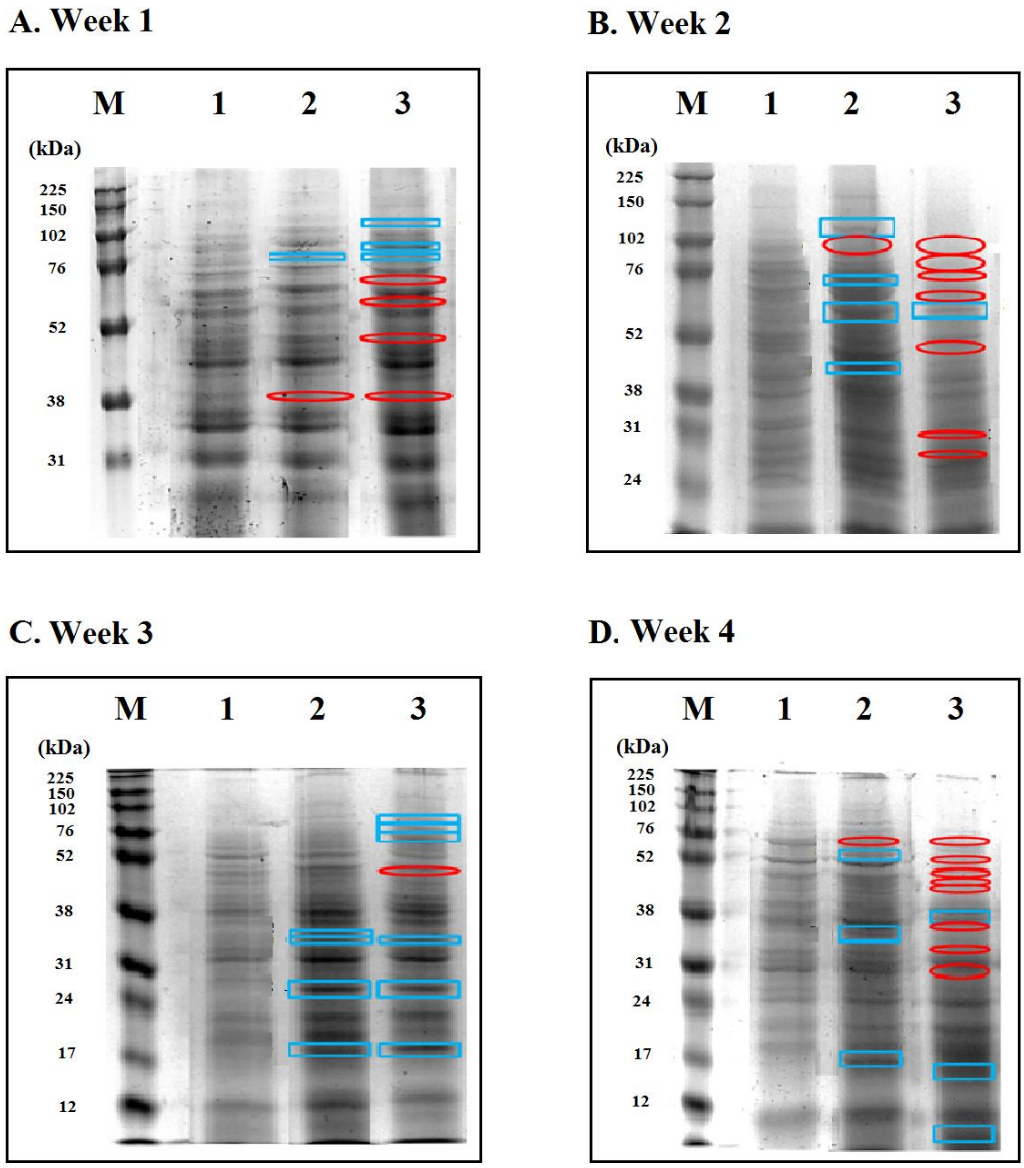
SDS-PAGE analysis of the extracted proteins also produced different profiles between that of mechanically and RPW wounded seedlings (Figure 2). Differences in the profiles were also evident according to weeks of exposure for both wounding treatments. Collectively, 12 (i.e. with molecular weight of 15-120 kDa) new proteins were detected on the gels of mechanically wounded seedlings while 13 (i.e. 10-143 kDa) new proteins were detected for RPW wounded seedlings. Of these 25 new proteins, 5 (i.e. 15-88 kDa) were shared between the two wounding treatments. Some of the proteins that were visible on the gels of controls were also found to be missing in wounded seedlings. In general, 3 proteins of about 43, 91 and 95 kDa appeared to be missing for mechanically wounded seedlings while 19 proteins of between 34 and 95 kDa were missing for RPW wounded seedlings. The 3 proteins missing from mechanically wounded seedlings were also missing from the RPW wounded seedlings.

Injury by either mechanical wounding or insect herbivory may trigger inducible responses within the damaged plants resulting in the appearance of new defensive proteins and enzymatic activities (Sánchez-Serrano, 2001; Howe and Jander, 2008; War et al., 2012). Thus, the increased in TSP within the mechanically and RPW wounded oil palm seedlings observed in this study can be explained by these responses (Figure 1). In fact, proteins are known to be very sensitive to abiotic and biotic stress imposed on plants (Atkinson and Urwin, 2012; Suzuki et al., 2014).

The change in protein content was also reflected in its profile (Figure 2) which can be due to the transcriptional activation of their corresponding genes. According to Sánchez-Serrano (2001), plants react to mechanical damage by activating a set of wound-responsive genes, whose function is devoted to plant healing and prevention of subsequent pest attacks. Hence, the responses induced by mechanical wounding may be similar to that of insect attack since both involves damage to the cell wall of the plants. These wound-induced responses are both rapid, such as the oxidative burst and the expression of defence-related genes, and late, such as the callose deposition, the accumulation of proteinase inhibitors and of hydrolytic enzymes (i.e., chitinases and glucanases) (reviewed by Savatin et al., 2014). Maybe this is the reason why we observed shared proteins being expressed or lost between the mechanically wounded and RPW wounded seedlings in Figure 2.

However, responses can be highly dynamic, very specific and activated more rapidly by damage from insect herbivory. For example, caterpillar feeding on maize, induces a type of protease inhibitor called the ribosome-inactivating protein 2 or RIP2 which when ingested by the insect, can inhibits the insect's digestion (Chuang et al., 2014). This type of protein was induced only when triggered by insect herbivory upon chewing of the tissues. In addition, sweet potato employed different mechanisms in the expression of sporamin, a storage protein which has multiple biological functions in response to stress caused by mechanical wounding and *Spodoptera littoralis* (a leafworm) attack (Rajendran et al., 2014). Other proteins that are being induced by insect herbivory include production of plant lectins (Macedo et al., 2015), and, production of enzymes involving in host plant resistance such as peroxidases, lipoxygenases and polyphenol oxidases (War et al., 2012).

Figure 2: SDS-PAGE analysis of mechanically and RPW wounded oil palm seedlings after treatment. Lane M, molecular weight protein marker; lane 1, control; lane 2, mechanical wounding; lane 3, RPW wounding. Blue squares, new proteins; red circles; missing proteins.



In conclusion, the changes in the TSP content as well as proteins being expressed in wounded seedlings compared to controls indicated that the seedlings have evolved sophisticated mechanisms to promptly respond to wounding. These mechanisms are triggered differently depending on the type of wounds, either mechanical or RPW attack. Since SDS-PAGE analysis separates the proteins based on the molecular weight and proteins with similar weight will migrate at similar rate on the gels, we could only assumes that some of the proteins discussed above can also be found within the obtained protein bands being separated. A proteomics analysis using a 2D gel electrophoresis is currently being undertaken in our lab for further characterization of the proteins of interest (i.e. new and missing proteins shown in Figure 2). These proteins can be used as biomarkers for early detection of RPW attack on oil palm and for oil palm protection.

Acknowledgements

The authors acknowledge the Malaysian Ministry of Higher Education for funding opportunity under the Fundamental Research Grant Scheme (Phase 2/2013) and the School of Fundamental Science, UMT for providing the facilities. The authors also acknowledge the members of RPW Research Group, UMT for their help and support.

References

Alias, A., Sadao, W. & Tay, E. B. (2004). Efficacy of mating disruption using synthetic sex pheromone for the management of cocoa pod borer, *Conopomorpha cramerella* (Snellen) (Lepidoptera: Gracillariidae). *Malaysian Cocoa Journal*, 1, 46-52.

Al-Shami, S. A., Che Salmah, M. R., Hassan, A. A. & Siti Azizah, M. N. (2010). Temporal distribution of larval Chironomidae (Diptera) in experimental rice fields in Penang, Malaysia. *Journal of Asia-Pacific Entomology*, 13, 17-22.

Atkinson, N. J. & Urwin, P. E. (2012). The interaction of plant biotic and abiotic stresses: from genes to the field. *Journal of Experimental Botany*, 63, 3523–3544.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248-254.

Chuang, W-P., Herde, M., Ray, S., Castano-Duque, L., Howe, G. A. & Luthe, D. S. (2014). Caterpillar attack triggers accumulation of the toxic maize protein RIP2. *New Phytologist*, 201, 928–939.

European and Mediterranean Plant Protection Organization (EPPO) (2015) EPPO A2 list of pests recommended as quarantine pests. http://www.eppo.int/QUARANTINE/listA2.htm. [3 August 2015]

Howe, G. A. & Jander, G. (2008). Plant immunity to insect herbivores. *Annual Review in Plant Biology*, 59, 41–66.

Kaakeh, W. (2005). Longevity, fecundity, and fertility of the red palm weevil, *Rynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) on natural and artificial diets. *Emirates Journal of Agricultural Science*, 17, 23-33.

Kerchev, P. I., Fenton, B., Foyer, C. H. & Hancock, R.D. (2012). Plant responses to insect herbivory: interactions between photosynthesis, reactive oxygen speciesand hormonal signaling pathways. *Plant, Cell and Environment*, 35, 441-453.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

Lippert, D., Chowrira, S., Ralph, S. G., Zhuang, J., Aeschliman, D., Ritland, C., Ritland, K. & Bohlmann, J. (2007). Conifer defence against insects: Proteome analysis of Sitka spruce (*Picea sitchensis*) bark induced by mechanical wounding or feeding by white pine weevils (*Pissodes strobi*). *Proteomics*, 7, 248-270.

Macedo, M. L. R., Oliveira, C. F. R. & Oliveira, C. T. (2015). Insecticidal activity of plant lectins and potential application in crop protection. *Molecules*, 20 2014-2033.

Rajendran**,** S. K., Lin**,** I-W**.,** Chen, M-J., Chen, C-Y. & Yeh, K-W. (2014). Differential activation of sporamin expression in response to abiotic mechanical wounding and biotic herbivore attack in the sweet potato. *BMC Plant Biology*, 14, 112-132**.**

Sánchez-Serrano, J. J. (2001). Plant responses to wounding. *Encyclopaedia of Life Sciences*, New York: Wiley and Sons.

Savatin, D.V., Gramegna, G., Modesti, V. & Cervone, F. (2014). Wounding in the plant tissue: the defence of a dangerous passage. Frontiers in Plant Science, 5, 470-480.

Suzuki, N., Rivero, R. M., Shulaev, V., Blumwald, E. & Mittler, R. (2014). Abiotic and biotic stress combinations. *New Phytologist*, 203, 32–43.

Takano, S., Mochizuki, A., Takasu, K., Konishi, K., Alouw, J. C., Pandin, D. S. & Nakamura, S. (2013). Rapid discrimination of two cryptic species within *Brontispora longissima* (Gestro) (Coleoptera: Chrysomelidae) by PCR-RFLP. *Journal of Pest Science*, 86, 151-155.

United Nations Conference on Trade and Development (UNCTAD) (2012) AAACP Products – Coconut. http://www.unctad.info/en/Infocomm/AACP-Products/COMMODITY-PROFILE---Coconut2/. [25 July 2015]

Wahizatul, A. A., Zazali, C., Abdul Rahman, A. R. & Nurul Izzah, A. G. (2013). A new invasive coconut pest in Malaysia: the Red Palm Weevil (Curculionidae: *Rhynchophorus ferrugineus*). *The Planter*, 89, 97-110.

War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S. & Sharma, H. C. (2012). Mechanisms of plant defence against insect herbivores. *Plant Signaling and Behavior*, 7, 1306-1320.

Wu, J. & Baldwin, I. T. (2010). New insights into plant responses to the attack from insect herbivores. *Annual Review of Genetics*, 44, 1-24.