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Biodiversity & Conservation **BC-01**

PREDICTING THE HABITAT SUITABILITY OF A POTENTIAL INVASIVE TROPICAL FERN, *Cyclosorus afer* IN LAFIA, NIGERIA

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ABSTRACT

The vast colonization of some wetlands by *Cyclosorus afer* in Lafia, Nigeria has been a serious concern to ecologists and indigenous dwellers. In this study, we used the Maximum Entropy (Maxent) modeling technique to predict the habitat suitability of this fern in Lafia, Nigeria. We obtained the presence data of this fern in three already invaded wetlands of size 500 x 500 m^2 each using multiple 200 m transect. Bioclimatic and elevation variables which were obtained from different databases were imputed into the model as predictor variables of *C. afer*. Thereafter, Maxent model was run with 70% of the presence data as training and 30% as test data. Our model result revealed that the area under curve for receiver operating characteristics of training is 0.847 while and test data is 0.970. The model's sensitivity was observed to be 100%. The model was assessed based on a jackknife test of individual contributions of each predictor variable to the model. Therefore, the environmental predictors of occurrence of *C. afer* in this study area include precipitation seasonality, Precipitation of driest quarter, precipitation of coldest quarter and elevation.

Keywords: *Cyclosorus afer*; fern; invasion; Lafia; maxent; modelling

1. INTRODUCTION

The pattern of successful invasions of invasive plants has always been by occupying new geographical locations, geometrical growth increase, rapid spread, eventually posing threats to the native biodiversity and economy of the areas invaded (Galil et al. 2015). This is why monitoring and predicting the habitats suitable for their spread is very important to ecologists (Coro et al. 2018). Apart from the prediction of habitat suitability, concerted efforts towards assessments of their potential negative environmental effects and control measures are highly recommended (Hulme 2006).

Over the years, the use of ecological niche models (ENMs) or species distribution models (SDMs) in predicting invasive species distributions has generated a lot of interests among ecologists, particularly using different techniques in establishing habitats suitability differences between the native and invaded regions of invasive plants (Guisan et al. 2014). One of the most commonly used presence-only ENMs is the maximum entropy (MAXENT) machine learning model (West et al. 2016). Predictions of the ecological factors influencing the establishments of potential invasive plants which is a function of the climatic similarities between native and invaded communities, is very important in preventing their future spread and establishments (Thuiller et al. 2005).

Therefore, in this study, we aimed at predicting the habitat suitability of a wetland fern *Cyclosorus afer* (Christ) Ching, also known as *Pneumatopteris afra* (Christ) Holttum in Lafia, Nigeria, using Maximum Entropy (Maxent) as the species distribution model. This fern has

displayed its invasive potential in many wetlands in Lafia, Nigeria by forming large covering and disrupting the flow of water (Akomolafe et al. 2017).

2. MATERIALS AND METHODS

2.1 Study Area and Sampling Technique

The study was carried out at Lafia, Nigeria. Lafia lies between latitude 8°25’40°N to 8°34’15°N and longitude 8°24’25°E to 8°39’19°E of North Central Nigeria. We chose three invaded wetlands in Lafia, Nigeria as sampling sites. These wetlands have been heavily colonized by *C. afer* thereby forming homogeneous colonies. 500 m x 500 m plot was demarcated in each site for the study. A minimum distance of 1000 m was maintained between each site.

2.2 Spatial data mapping and Distribution Modelling

2.2.1 Presence data

The local presence data of *C. afer* in the three sites was taken on the field by marking the spatial reference points using a GPS device (Garmin Etrex 10). This was done when the biomass of *C. afer* has reached the peak of growth in all the sites. We maintained a minimum distance of 20m between each reference point of *C. afer* along 200 m transect at each site. A total of 95 presence spatial reference points were documented in all the study sites.

2.2.2 Environmental Variables used for the Model

Bioclimatic and elevation variables were input into the model as environmental variables. These variables have been reported to influence the productivity of plant species. Out of 19 bioclimatic variables, we selected 5 through multicolinearity test of linear regression model using IBM SPSS 24 (Table 1). These variables were also chosen based on their importance in the species ecology. All these variables were extracted individually by superposing them on Lafia boundary map using ArcMap 10.2.1 tools.

2.2.3 Species distribution modelling

We used maximum entropy (Maxent) model to predict the habitat suitability and distribution of *C. afer* in Lafia, Nigeria. Maxent model has been tested and reported to be a reliable model that utilizes few occurrence data (Phillips et al. 2006). Maxent is able to predict species niche by determining the probability distributions closest to uniform (maximum entropy) from pool of species occurrence data and environmental variables. Maxent 3.4.1 software was used to actualize this. The species presence data were divided into training and test proportions. We used 30% of it as test data while the remaining 70% was used as training data. The environmental variables were loaded into the model as ASCII file type.

2.2.4 Assessment of Individual Variables contributions

The contribution of individual environmental variable to the model was assessed using the Jacknife test in the Maxent software. The Jacknife test sequentially isolated each variable out of the model and produced a model using other variables. It also produced a model using only the isolated variable alone.

2.3.5 Evaluation of the Model

Area under curve (AUC) of the Receiver Operating Characteristics (ROC) curve plotted by the Maxent model was used to evaluate the model.

3. RESULTS AND DISCUSSION

The prediction of habitat suitability of *C. afer* in this study was based primarily on the influence of bioclimatic factors which usually play significant roles in species establishments (Woodward et al. 2004; Roura-Pascual et al. 2004). Maxent prediction as compared with random prediction is revealed by the receiver operating characteristics (ROC) curve, whereby the area under curve (AUC) for training and test data are 0.847 and 0.970 respectively. The AUC for random prediction is 0.5 (Figure 1). Our Maxent model predictions performed better than random prediction due to higher area under curve (AUC) of both training and test data as compared with random. Invariably, this is an indication of a higher accuracy and sensitivity of the maxent model (Anderson et al. 2003) and this also means that *C. afer* exhibited high tolerance to environmental factors (Thuiller et al. 2005).

The Maxent model showed that all the local presence points of *C. afer* are found within the predicted areas of high probability (Figure 2). Considering this model, areas with higher predictions are represented by warmer colors, training data are represented by white dots and test data are denoted by violet dots. This Maxent model has 100% sensitivity for occurrence of *C. afer,* since the predicted and observed points are the same. The predicted spatial extent of *C. afer* in Lafia, Nigeria is between $8^{o}21'0''$ – $8^{o}55'0''$ N and $8^{o}5'0''$ - $8^{o}55'0''$ E. The total area predicted was found to be 1784.07 Km^2. The predicted geographical extent of spread of *C. afer* which is almost half of the total land cover in Lafia is really worrisome and should be a thing of concern to environmentalists as it has implications for future loss of biodiversity in the area.

The most important predictor variables of *C. afer* distribution in Lafia whose AUC values are greater than 0.5 include precipitation seasonality (bio15), Precipitation of driest quarter (bio17), precipitation of coldest quarter (bio19) and elevation (Figure 3). The positive relationship established by the occurrence of *C. afer* and precipitation of the driest quarter in this model, means that areas with high precipitations during the driest three months of the year will have high probability of occurrence of *C. afer.* On the contrary, from our model, *C. afer* exhibited a negative relationship with precipitation of coldest quarter, precipitation seasonality and elevation.

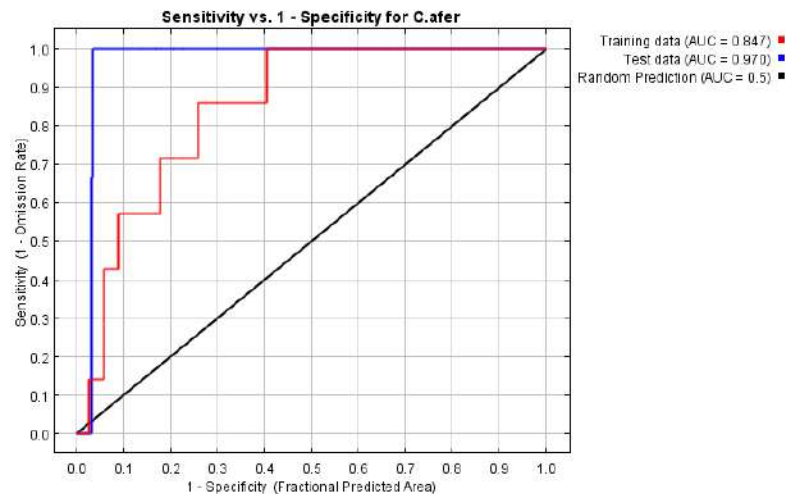

Figure 1. The Receiver operating characteristics (ROC) curve of the training and test data

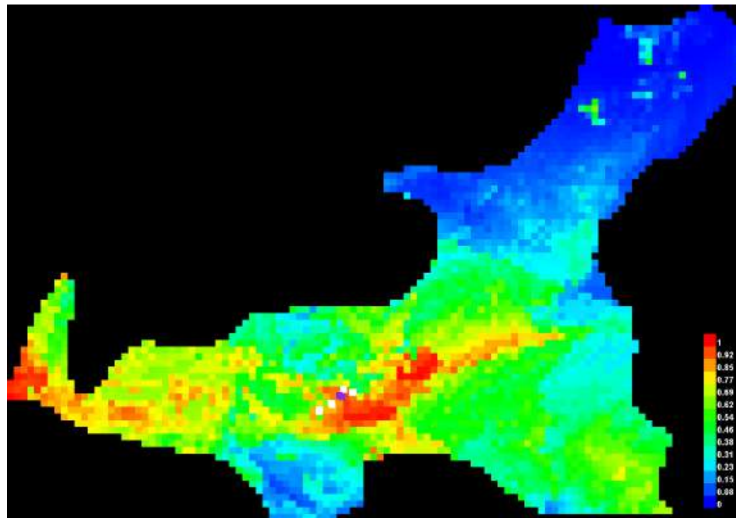

Figure 2. The species distribution Maxent model of *C. afer* in Lafia, Nigeria.

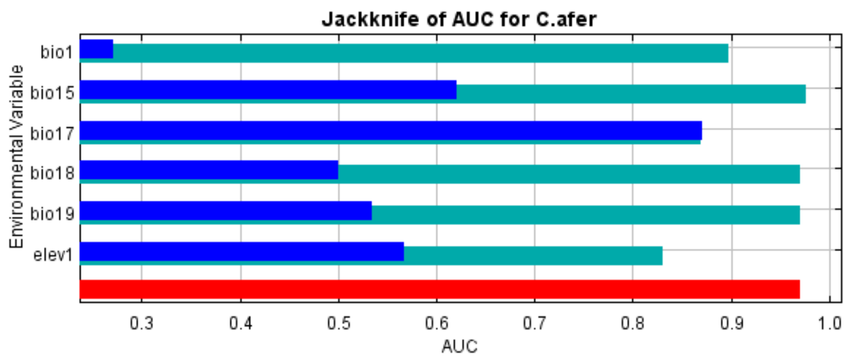

Figure 3. Jackknife plot for AUC of *C. afer*

Table 1. Environmental variables used in the model

S/N	Code	Description
1	Elevation	Elevation
2	Bio1	Annual mean temperature
3	Bio15	Precipitation seasonality (Coefficient of Variation)
4	Bio17	Precipitation of driest quarter
5	Bio18	Precipitation of warmest quarter
6	Bio19	Precipitation of coldest quarter

4. CONCLUSION

In comparison with random predictions, our Maxent model is more accurate. Occurrence and distribution of *C. afer* in Lafia, Nigeria is influenced by limiting environmental factors. Areas having similar environmental ranges are more prone to risk of *C. afer* invasion in the future.

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Biodiversity & Conservation **BC-02**

The Diversity and Abundance of Jellyfish (Scyphozoa and Cubozoa) in the Northern Penang Waters, Malaysia

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ABSTRACT

Jellyfish blooms have been a threat to mankind over the decades. Jellyfish pose risks to human health and socioeconomical activities which include severe stings, death, collapse of fisheries industries and aquatic systems. Jellyfish diversity and abundance in the northern Penang waters were studied using the towing method. Water quality parameters were recorded and its correlation with jellyfish abundance was determined using Principle Component Analysis (PCA). Eight jellyfish species were identified in this study, namely *Chrysaora chinensis*, *Phyllorhiza punctata*, *Aurelia* sp., *Rhopilema hispidum*, *Acromitus flagellatus*, *Cyanea capillata*, *Chiropsoides buitendijki* and *Morbakka* sp. The most abundant and dominant species was *C. chinensis*. The highest diversity of jellyfish was recorded in the waters off Tanjung Tokong. Abundance of jellyfish showed positive correlation with water temperature, salinity and concentration of nutrients, whereas it revealed a negative correlation with dissolved (DO) level. Other factors such as current effects, concentration of chlorophyll-*a*, and abundance of mesozooplankton should be taken into considerations for future researches on abundance of jellyfish.

Keywords: jellyfish, species, diversity, abundance, Northern Penang

1. MATERIALS AND METHODS

Six study sites off the northern Penang waters were selected; Teluk Bahang, Batu Ferringhi, Tanjung Bungah, Tanjung Tokong, Georgetown, and Pantai Bersih. The sampling trip was conducted once a month during spring tide. The tow net was trailed behind the boat with a steady speed of approximately 2.0 knots & towing distance of each study site was recorded using flowmeter and the total volume coverage was calculated. Jellyfish specimens were collected after the tow and all the data were recorded in the data collection sheet as per study sites. The numbers of individual and species of jellyfish caught are recorded in a data sheet. Three diversity indices namely Shannon's Index (H'), Pielou's Evenness Index (J'), and Simpson's Index (D) were used to quantify the diversity of jellyfish in terms of species richness, species evenness, and dominance. Abundance of scyphozoan and cubozoan jellyfish for each study site was calculated separately by dividing the total number of jellyfish, N (individuals) by total volume towed, V (m^3). Total volume towed, V (m^3) is computed by multiplying the area of the metal frame, A (m^2) by the distance towed, D (m). This method was used by previous studies on jellyfish in California (Suchman & Brodeur, 2005).

Water quality parameters such as water temperature, salinity, dissolved oxygen (DO), and pH of seawater at the study sites were measured *in-situ*. Nutrient analysis of the collected water samples on ortho-phosphate, nitrate-nitrogen, and ammonia-nitrogen was carried out in

laboratory. The procedures of analysing the nutrients were conducted following the manual provided. Principal Component Analysis (PCA) was then used to determine the effects of water quality on the abundance of jellyfish.

2. RESULTS AND DISCUSSION

A total of eight jellyfish species were found in the northern Penang waters from July to December 2017, which comprises eight different genera and two classes. Six species of scyphozoan jellyfish were identified in this study namely *Chrysaora chinensis*, *Phyllorhiza punctata*, *Aurelia* sp., *Rhopilema hispidum*, *Acromitus flagellatus*, and *Cyanea capillata.* A specimen of *Cyanea capillata* or lion's mane jellyfish (with bell diameter of 60cm) was first recorded in Malaysian waters. Two species of cubozoan jellyfish identified were *Chiropsoides buitendijki* and *Morbakka* sp.. The most abundant jellyfish species was *C. chinensis*, which was found in every study site. The other scyphozoan jellyfish only occurred in one or two of the study sites. For cubozoan jellyfish, *C. buitendijki* was found in three study sites, i.e., Batu Ferringhi, Tanjung Bungah, and Tanjung Tokong whereas *Morbakka* sp. was only found in Tanjung Tokong. Five jellyfish species including both cubozoan jellyfish were found in Tanjung Tokong, three jellyfish species were found in Pantai Bersih whilst the other study sites had only two jellyfish species found.

Diversity indices of the jellyfish community at each study site including Shannon's Index (H'), Pielou's Evenness Index (J'), and Simpson's Index (D) were calculated. Tanjung Tokong has the highest J' (0.81) and H' (0.50), and the lowest D (0.61). In converse, Teluk Bahang has the lowest J' (0.08) and H' (0.12), and the highest D (0.97). Overall, J' and H' for all study sites are lower than 0.50 except Tanjung Tokong. D of all study sites are high values exceeding 0.80 except Tanjung Tokong. The abundances of jellyfish (scyphozoan and cubozoan) from July to December 2017 were plotted in Figure 1 according to six study sites. Both scyphozoan and cubozoan jellyfish hit the peak in November at Batu Ferringhi. The abundance of scyphozoan jellyfish peaked in November with 15.50 $x10^{-3}$ ind./m^3 at Batu Ferringhi followed by 12.92 $x10^{-3}$ ind./m^3 at Teluk Bahang. In contrary, scyphozoan abundance hit the lowest in September which they were only present at one study site, i.e., Pantai Bersih. Overall, scyphozoan jellyfish were more abundant at Teluk Bahang, Batu Ferringhi, and Tanjung Bungah.

Cubozoan jellyfish had a much lower abundance compared to scyphozoan jellyfish. They only occurred in July and November. In July, *Morbakka* sp. appeared as a single specimen (0.25 $x10^{-3}$ ind./m^3) at Tanjung Tokong. In November, there was a total of six *C. buitendijki* caught at Batu Ferringhi, Tanjung Bungah, and Tanjung Tokong with abundance of 2.58 $x10^{-3}$ ind./m^3, 1.44 $x10^{-3}$ ind./m^3, and 0.62 $x10^{-3}$ ind./m^3 respectively. Bungah, and Tanjung Tokong with abundance of 2.58 $x10^{-3}$ ind./m^3, 1.44 $x10^{-3}$ ind./m^3, and 0.62 $x10^{-3}$ ind./m^3 respectively.

PCA was carried out to find out the correlation between water quality and the abundance of jellyfish in the northern Penang waters. A two-dimensional PCA plot for the correlation between log concentration of seven parameters (i.e., DO, salinity, water temperature, pH, ammonia, phosphate, and nitrate) and the abundances of *C. chinensis* is shown in Figure 4.5. The most abundant jellyfish species, *C. chinensis* was chosen as it is the only jellyfish species that shows a significant trend using this plot. Meanwhile, the factor in the plot is month rather than study site as trends were not significant between study sites. Two principal components which cover 44.7% of variation in the data are used in the plot for easier visualization, however, three principal components are needed to explain 61.0% of the variation in the data.

The abundances of jellyfish (i.e., *C. chinensis*) amongst the months are manipulated by multiple parameters rather than any single parameter acting as a main factor. The jellyfish abundance is higher when pH, water temperature, and concentration of ammonia are higher. Besides, the abundance of jellyfish is also higher when the concentrations of nitrate and phosphate are higher. The high abundance of jellyfish is further aided by low level of DO. The

abundance of jellyfish in September was the lowest albeit high concentrations of nitrate and phosphate due to low salinity.

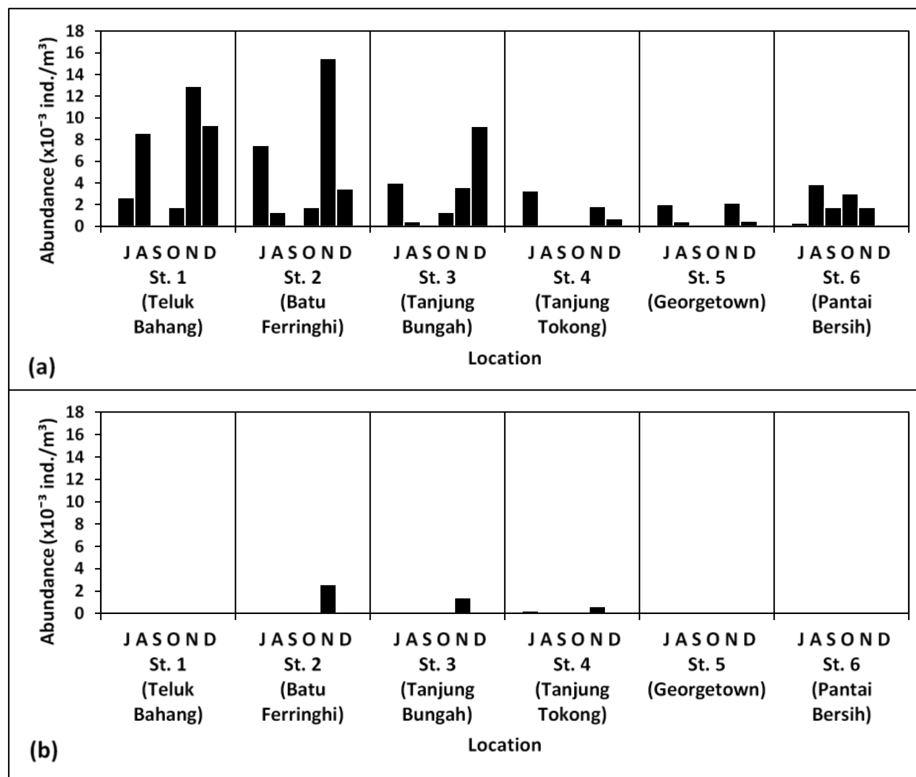

Figure 1. Abundance of jellyfish in six study sites from July to December 2017.
(a) Scyphozoan & (b) Cubozoan.

3. CONCLUSION

Abundance of jellyfish was found to be a combined result of multiple factors of water quality parameters. Jellyfish abundance was found to be higher when there were higher water temperature, higher salinity, lower level of DO, and higher concentration of nutrients including phosphate, nitrate, and ammonia. However, there are a few factors that were not investigated in this study. Recommendations to future researches on jellyfish abundance in the northern Penang waters include the studies on effect of currents, abundance of chlorophyll-a, and abundance of mesozooplanktons.

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Biodiversity & Conservation **BC-03**

It is in the Details: Simple Structural Complexity Modification Could Restore Ecological Function on Seawall

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ABSTRACT

Over the last decades, climate change and increased human use has put huge pressure on coastal area. To shield coastal settlements and facilities from hazardous events, the most common management strategy used is the building of hard structures such as seawalls and breakwaters. However, hard engineered structures often have low ecological values and interrupts dynamic coastal processes. We tested the effectiveness of a novel tool in green engineering to modify the structural complexity of ordinary seawalls, to promote the growth of native biodiversity and thus rehabilitate ecological function of hard engineered structures. Seventy eco-concretes with three different degrees of complexity: a flat, 2.5 cm and 5.0 cm complex enhancements were installed at mid-water level on seawall of Penang Port and Straits Quay Marina in Penang, Malaysia. Monitoring was carried out trimonthly for one year. Results suggested relative richness and abundance were highest at 5.0 cm > 2.5 cm > flat and lastly, seawall. Although there was no significant difference in net productivity between 5.0 cm, 2.5 cm, and flat eco-concrete, all of them were relatively higher than that of seawall. The results provide an insight on how addition of complexity on the seawall could restore a certain degree of biodiversity.

Keywords: green engineering, structural complexity, coastal biodiversity

1. INTRODUCTION

The coast today is heavy populated. An estimated 3 billion or 40% of total population lives within 100 km of the coast (Neumann *et al.* 2015). Population growth, sea level rise, and climate change cause the flourish of coastal protection structures transforming natural coastal landscapes into a series of hard engineered structures. In Malaysia, particularly Penang Island, where rapid and intensive coastal development is taking place, 20% of its 107 km of shoreline is fortified with artificial hard engineered structure (Chee *et al.* 2017). The extent of artificial shoreline is expected to increase again after completion of several reclamation projects which started in 2017. Many coastal ecosystems such as mangroves, intertidal mudflat, and rocky shore was converted into man-made environments. Man-made environment consists of mostly hard engineered structures provide little to no habitat for coastal fauna and flora to attach on, resulting in “grey zones” where little life can be found. Meanwhile, there is growing interest globally in ecological engineering which enable improvement on these hard, engineered structures to

maximize bio-supporting capacity (Chapman & Underwood 2011). Many projects executed in temperate countries involved simple and cost-effective interventions, such as drill-cored artificial rock pools and marine flowerpots to create artificial intertidal pools on seawalls (Evans *et al.* 2015; Morris *et al.* 2017). In Asia, potential of ecological engineering project is still in an early stage and hard engineering approach have been used extensively. Conventional seawall construction lack novel concepts of green engineering which, proven from past studies, could enhance ecological performances on seawalls (Firth *et al.* 2013). The addition of green engineering features could be done even on existing hard engineered structure by introducing supplementary surface complexity. The application of simple characteristics such as pits and grooves are manageable and less costly with the benefit of retaining the original purpose of the structure (Loke *et al.* 2014). In this study, we apply concrete tiles with specially designed ridges and grooves to modify surface complexity of existing seawall and compare biodiversity and productivity performance with that of the seawall.

2. MATERIALS AND METHODS

Experiment was replicated on 30 m seawalls at Penang Port (5.416 N 100.344 E) and Straits Quay Marina (5.459 N 100.315 E) with five replicates per treatment: flat plate, 2.5 cm high enhancements, 5 cm high enhancement, and flat seawall plate as control. The settlement plates were installed at mid-tide level with an aluminium framework hung over the vertical seawall (Figure 1). The cover of algae and sessile invertebrates were photo documented using GoPro Hero 5 at month 0, 1, 3, 6, and 12. At the end of month 12, all plates were removed and transported in zip-lock bags to laboratory immediately. The plates were placed in a seawater tub to measure day- and night-time dissolved oxygen using a portable galvanic dissolved oxygen meter (Hanna HI9147) over a 1-hour duration to estimate productivity.

Figure 1. Aluminium framework used to support settlement plates on seawall

3. RESULTS AND DISCUSSION

The community showed a positive response to complex habitat enhancement as cumulative species richness over 12-months period increased on seawall (17 species), flat (19 species), 2.5 cm (26 species), and 5 cm plates (25 species). The species composition include that from Class Bivalvia (15 species), Class Sessilia (5 species), Class Cheilostomatida (1 species), Class Chondrillida (1 species), Class Bryopsidales (2 species), Class Corallinales (1 species), Class Polychaeta (1 species) and one unidentified microalgal mat. Of these, colonization from Class Bivalvia and Sessilia was most stable, creating small oyster reefs along the grooves or

forming clusters on the flat surfaces of plates (Figure 2). The presence of green algae Bryopsidales, which was lacking on seawall plate, contributed to lowest productivity of all (Figure 3). However, there is no significant differences between productivity of all treatments (Kruskal-Wallis X^2 = 6.085, P = 0.108, df = 3). Nonetheless, higher community richness and productivity output from complex plates indicated higher degree of reestablishment from marine community, allowing organisms to colonize a new man-made environment in a just a few weeks.

Figure 2. Month 1 and 12 comparison of (Upper row, clockwise) seawall, flat, 2.5 cm, 5 cm plates.

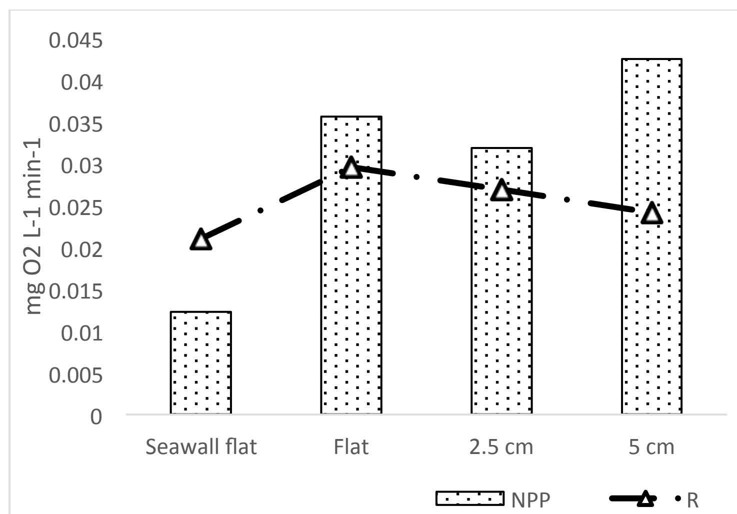

Figure 3. Average net productivity (NPP) and respiration rate (R).

4. CONCLUSION

The addition of several grooves and pits maybe simple, yet it is able to make a difference among the marine community. The species richness and productivity performance were increased by 50% when comparing complex plates with ordinary seawall plate. However, the difference was not significant and this could be caused by sampling effort. The community composition between the treatments can be further examined in the future to comprehend the role of simple structural complexity modification in changing the succession pattern of marine assemblage.

ACKNOWLEDGEMENT

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Biodiversity & Conservation **BC-04**

Descriptive Event of Flowering and Fruiting of Non-Climbing Rattan *Calamus castaneus* in Segari Melintang Forest Reserve, Perak

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ABSTRACT

Calamus castaneus Griff. (subfamily Calamoideae) or "rotan cucor" is a dioecious and non-climbing rattan. *C. castaneus* is one of the commonest palms in Malaysia that prefers to grow in lower hillslopes and near stream sides. Although it flowers and fruits all year round, yet little is known about its flowering and fruiting duration especially in Malaysia. This study was aimed to observe the duration of anthesis in male and female inflorescences and fruiting duration of *C. castaneus* over one year. Segari Melintang Forest Reserve, Perak was chosen as the study site. Five square plots of 10 m x 10 m were established at the site. A total of 32 individuals were found in the five study plots. All individuals of *C. castaneus* within the study plot were tagged and the variables such as microclimate reading, any flowering or fruiting, presence of pollinators and possible seed dispersers were recorded. From observations, due to higher degree of flower branching, male plants tend to produce larger and longer duration of flowering compared with female plants. Female plants on the other hand, was less rewarding for pollinators. Insects and pollinators such as *Heterotrigona itama* and *Vespa tropica* were among the frequent visitors of *C. castaneus* inflorescence. Meanwhile, the fruits of *C. castaneus* were favoured by macaques. Duration of fruiting can be as short as one month to as long as one year. The fruit size and colour range vary according to the vegetation type and microclimate of the study site. This study helped contribute information regarding the population dynamics of *Calamus castaneus* in Peninsular Malaysia.

Keywords: Rattan, palm, Calamoideae, Perak

1. INTRODUCTION

As most of rattans are climbers, *Calamus castaneus* Griff. on the other hand, is a non-climbing rattan (Dransfield 1979; Kidyoo and McKey 2012; Ruppert et al. 2016). It shows no tendency to climb due to the lack of climbing organs, which are flagellum and cirrus (Dransfield, 1979). The selection of *C. castaneus* as the preferred rattan species to be studied were due to certain reasons. First, this species is the commonest rattan in Malaysia (Dransfield, 1979) hence, it is easy to find it and it also fruits all year round. Besides that, *C. castaneus* is a convenient model, although its non-climbing character could lead to some ecological differences from the rest of rattans. Moreover, this rattan is relatively well preserved since it is less exploited by humans (Kidyoo & McKey, 2012). Their cane are too short to be used, hence the leaves were used for "atap" (roof material) and the immature fruit are used as cough medicine by indigenous people (Dransfield, 1979; Sunderland & Dransfield, 2002). Despite of low commercial value as cane, this species produced inflorescences continually and would provide food for animals, birds and insects as reward for pollination or seed dispersal (Watanabe & Suzuki, 2008). As stated by Ruppert et al. (2016), their scaly chestnut brown coloured fruit with sweet and acidic taste were favoured by primates especially macaques. This study was aimed to observe the duration of flowering and fruiting of *C. castaneus* over a year.

2. MATERIALS AND METHODS

2.1 Study site

The sampling was carried out at Segari Melintang Forest Reserve (04° 19' 34.7" N, 100° 34' 57.9" E) situated at Manjong district, Perak in northern region of Peninsular Malaysia. The forest reserve (total area 2741.79 ha) is a coastal dipterocarp forest. Mean relative humidity in the study area is 64% with mean temperature approximately 29°C and light intensity about 0.7kLux. Microclimate parameters such as relative humidity, light intensity and air temperature were recorded every month. This study was done for one year starting from March 2017 until March 2018. Sampling were done twice a month in every first week and third week of the month.

2.2 Population structure and sex determination

Since *C. castaneus* is a common palm, the plants were easily found alongside small streams. A total of 5 plots with 10 m x 10 m each had been established. The plants within the study plot were marked with numbered plastic tags. Only the plant that had borne inflorescences or infructescences were chosen. The distance between plot were made sure to not be further than 50 m apart from each other to ensure that sampling for whole population can be finished in a day. The study population comprised of 32 plants (16 males, 16 females). Sex of each plant were determined by observations of flowering and fruiting from previous seasons, noting that only female plants would bear infructescences.

2.3 Number of flowers produced by male and female plants

Number of flowers produced by each individual were estimated by comparing the number of inflorescences produced by each flowering individual and the mean number of flower per male and female inflorescences.

2.4 Fruiting frequency and stages

The number of fruit borne by each infructescences were estimated by number of fruit per rachillae. The fruit width and length were measured each month using ruler. Several fruits were sampled for cross sectioning to observe the growth stages inside the fruit.

2.5 Floral visitors and seed dispersers

Insect pollinators that regularly visited flowers of both sexes were observed and recorded. We also noted the behaviour of insect visitors such as pollen collection, nectar collection and contact with anthers of staminate flowers or stigma of pistillate flowers. Possible seed dispersers within the plot were examined by the tracks that they had left such as footprints or leftover fruits and seeds. Photographs were taken as evidence.

3. RESULTS AND DISCUSSION

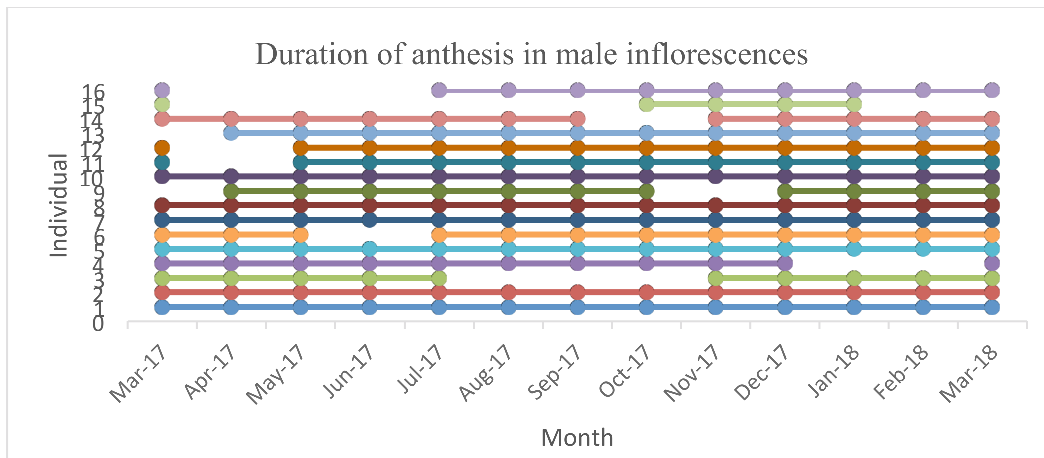

Figure 1. Duration of anthesis in male *Calamus castaneus* inflorescences.

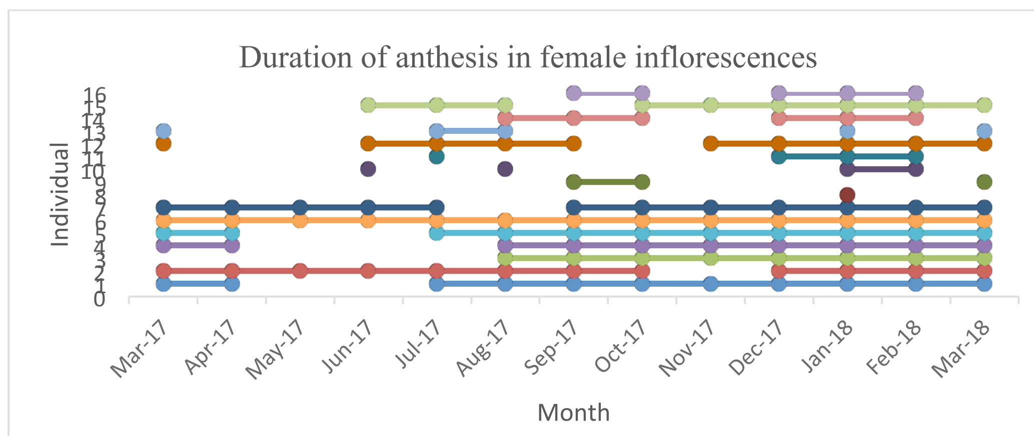

Figure 2. Duration of anthesis in female *Calamus castaneus* inflorescences.

Calamus castaneus displayed a pleonanthy mode of flowering. The axillary inflorescences are produced continually thus flowering and fruiting do not result in the death of the stem (Sunderland & Dransfield, 2002). Based on Figures 1 and 2, it was cleared that plants of both sexes flowered throughout the year. Independent sample t-test for equality of means shows that there was a significant difference between male and female primary branches per inflorescences with $p<0.003$. Due to higher degree of flower branching, male plants tend to produce larger and longer duration of anthesis compared with female plants. Female plants on the other hand, was less rewarding for pollinators (Dransfield, 1979; Kidyoo & McKey, 2012). 32.5% of male inflorescences were recorded to bloom all year round (individuals number 1, 2, 5, 7, 8 and 10) compared with only 6.25% from female inflorescences (individual number 6). Least male individual flowered in April. However, all of them were recorded flowering in December (Figure 1). On the other hand, most of female individual were recorded to flower in January and least flowered in May (Figure 2). From observations, insects and pollinators such as a few species of stingless bee for example *Heterotrigona itama*, greater banded hornet (*Vespa tropica),* blank ant (*Camponotus* sp.), wasps, honey bees and fruit flies were among the frequent visitors of *C. castaneus* inflorescence.

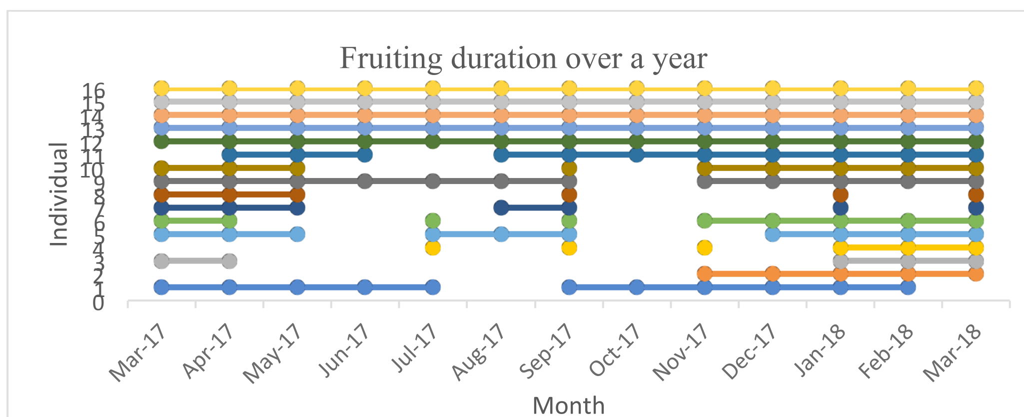

Figure 3. Fruiting duration of *Calamus castaneus* over a period of one year

C. castaneus produced fruits all year round, non-seasonally and one individual would exhibit different fruiting stage; unripe, ripe, budding and flowering all at the same time. In addition, fruits of *C. castaneus* were favoured by macaques (Ruppert et al. 2014). Duration of fruiting may differ due to different fruiting stages displayed by an individual. Based on Figure 3, the longest fruiting duration can reach for a year (individual number 12 to 16), meanwhile the shortest duration was a month (individual number 4, 6, 7, 8 and 10). Most of the individual fruited from January until March, meanwhile October was the least fruiting month. *C. castaneus* fruit are chestnut coloured. However, we found that the fruit size and colour range vary in different plot. We assumed that it was due to the vegetation type and microclimate of the study site. We also observed the macaques eating the ripe fruit based on the leftover fruit skins inside the plot. Macaques helped in the dispersals of *C. castaneus* seeds. From traces of footprints on the ground, we assumed that wild boar also favoured the fruits. Wild boar played an important role in loosening the soil, giving the seed a chance to grow as seedling.

4. CONCLUSION

In this study, there was a significant difference between male and female primary branches per inflorescences. The duration of anthesis for one year in male plants were longer than female plants. Besides that, the duration of fruiting can last for a year long, while the shortest duration can be for a month. Although *C. castaneus* was low in commercial value, most of them produced inflorescences and infructescences continually that would provide food for pollinators and seed dispersers. This study contributed to the information regarding the population dynamics of *C. castaneus* especially in Peninsular Malaysia.

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Biodiversity & Conservation **BC-05**

The Relationship between the Gaster Size, Transverse White Band and Ovary Development in the Workers of Yellow Crazy Ant, *Anoplolepis gracilipes*

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ABSTRACT

In the event of food scarcity and queen absence, the workers of yellow crazy ants *(Anoplolepis gracilipes* [Fr Smith]) are capable of developing viable ovaries and laying eggs to feed the colony. These individuals are commonly found within proximity to the queens and brood. They usually have larger gaster size and have conspicuous transverse white bands between tergite plates. In this study, we examine the relationship between gaster size, transverse white band and ovary development in corpulent (workers with larger gaster and visible bands) and regular workers of the yellow crazy ants. No correlation ($P > 0.05$) between gaster size, ovariole number, and oocyte number was found using Spearman's Rank-Order Correlation analysis. A similar finding was observed between the mean width of the transverse band and ovariole number and oocyte number. Comparison of ovary presence between corpulent and regular worker ants was significantly different ($P < 0.05$) in independent sample Mann-Whitney test. Kruskal-Wallis analysis of colony differences was found to be significantly different ($P < 0.05$) in both ovariole number and oocyte number, suggesting that there could be other contributing factors toward ovary development in the workers of the yellow crazy ants.

Keywords: ovary development; worker ants; corpulence; gaster size.

1. INTRODUCTION

The yellow crazy ant *(Anoplolepis gracilipes* [Fr Smith]) is listed as one of the worst 100 invaders among the International Union for Conservation of Nature (IUCN) (Lowe et al., 2000). Invasive species are the primary drivers of biodiversity loss, and it has been shown in the case of yellow crazy ant invasion to cause the population decline of the native endemic red crab in Christmas Island (Green, 1997). It creates further ecological damage as it forms a mutualistic relationship with scale insects that infest the trees (O'Dowd et al., 1999). It is also a predator of birds, reptiles and small mammals (Davis et al., 2008; Mezger & Pfeiffer, 2011). Yellow crazy ants also reduce ants diversity at the invaded site (Bos et al., 2008).

Lee et al. (2017) stated that reproduction and ovary development in yellow crazy ant workers might be a contributing factor to its survival and reproduction, hence this enhance its success rate of invasion at an introduced site. The workers possessed functional ovaries and were able to lay two types of eggs, the trophic and viable egg (Lee et al., 2017). Trophic eggs served as food for the colony members, especially when food is scarce and conditions are unfavourable (Lee et al., 2017). It was found that the ants can undergo asexual reproduction by laying the viable egg

that subsequently developed into a male that possessed viable reproductive organ and viable sperm (Lee et al., 2017). Corpulent worker ants with distended gaster and white transverse band were shown to have a significantly higher number of ovarioles than the regular worker (Lee et al., 2017). This study aims to determine whether the gaster width and mean width of transverse band are indicators of ovary development in worker ants, as the early dissection results showed that developed ovaries were also found in some regular workers and some corpulent workers have no developed ovaries (Figure 1). We are also interested in the difference of ovary development between corpulent and regular worker and the difference in ovary development between sampled colonies to determine whether variation among colonies exist.

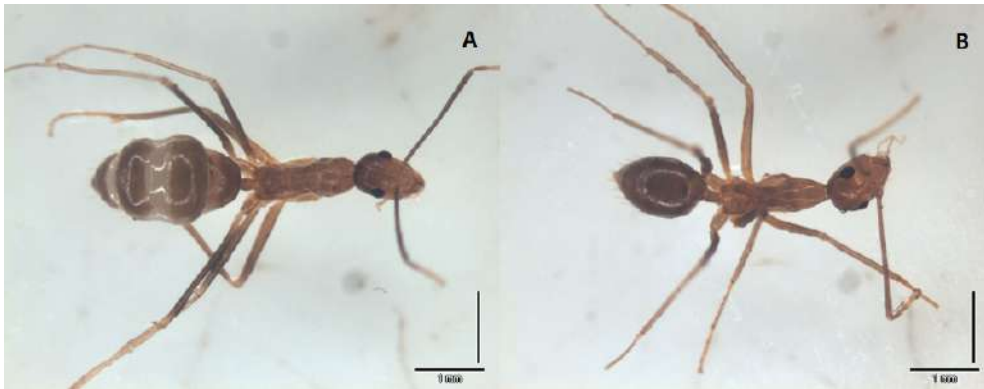

Figure 1. A) Corpulent yellow crazy ant worker conspicuous transverse white band on its larger gaster. B) Regular yellow crazy ant worker with smaller gaster.

2. MATERIALS AND METHODS

Ant colonies were collected from different locations in Penang Island, Malaysia (Bukit Jambul, Balik Pulau and Youth Park). All colonies were queenright. Both regular and distended workers were randomly selected. Distended workers were identified by the presence of white transverse band across the gaster. The ants were killed by placing it in the freezer at <0 °C for 30 minutes.

2.1 Measurement of gaster width and mean width of the transverse band

The collected ants were observed under stereomicroscope Olympus SZ61. Gaster width was defined as the largest transverse width at the dorsal view of gaster. The width of transverse bands was measured when whitish transverse band was present on the ant gaster.

2.2 Dissection of the ovary

The samples were kept in the refrigerator and dissected within two weeks. Standard phosphate buffered saline (PBS) was used as dissection solution. The number of ovarioles and yolky oocyte were observed and recorded after the ants were dissected. Undeveloped ovary that looked like a fine thread will not be counted.

3. RESULTS AND DISCUSSION

3.1 Correlation between gaster width and oocyte number and ovarioles number.

Spearman's Rank-Order Correlation (two-tailed) analysis shows there was no correlation between the gaster width and oocyte number (r = 0.086, n = 133, p = 0.323). There was also no correlation between the gaster width and ovariole number (r = 0.082, n = 133, p = 0.348). In *Pheidole morrisi* Forel, corpulent worker ants with distended gaster serve as nutritional storage for the colony (Yang, 2006). The lack of correlation stated above might be due to the gaster width is also affected by the amount of nutritional reserve stored in the gaster. Age is another possible factor that affects the ovary development (Billen, 1982) that might be not related to gaster width.

3.2 Correlation between the mean width of the transverse band and oocyte number and ovarioles number.

Mean width of the transverse band measures the extent of corpulence more accurately as it measures the distance between the abdominal plates as the result of expansion from increasing content in the gaster without being affected by variation of gaster size within the colony. However there was no correlation between the average gaster band and oocyte number (r = 0.152, n = 133, p = 0.080). There was also no correlation between the average gaster band and ovariole number (r = 0.161, n = 133, p = 0.065), as shown in the analysis of Spearman's Rank-Order Correlation (two-tailed). Having similar results with the previous result of gaster width, this observation might hint that other factors have confounded the results, such as the age and amount of nutritional reserve (Billen, 1982; Yang, 2006).

3.3 Presence of ovary in regular and corpulent workers

Independent samples Mann-Whitney test (two-tailed) at 95% confidence interval were carried out to compare the presence of ovary between regular and corpulent workers. The number of the distended worker with the presence of developed ovary was significantly higher (U= 1839.5, p= 0.040) that that of the regular worker. The findings agree with those of Lee et al. (2017) that corpulent worker usually has more developed ovarioles.

3.4 Colony effect on oocyte number and ovariole number of worker ants

Independent-samples Kruskal-Wallis test showed that there was a significant difference in oocyte number and ovariole number between different colonies (n=133), (p<0.001, adjusted using Bonferroni correction) (Table 1). The post hoc pair-wise test used was Dunn's pairwise test.

Table 1. Mean oocyte number per individual of yellow crazy ant worker between colonies collected from various locations.

Colony	Mean no. oocytes ± std. dev
Bkt. Jambul	2.27 ± 2.94^{a}
Balik Pulau	1.65 ± 3.26^{ab}
Youth Park	0.40 ± 1.81^{b}

Table 2. Mean ovariole number per individual of yellow crazy ant worker between colonies collected from various locations.

Colony	Mean no. ovarioles/individual ± std. dev
Bkt. Jambul	0.90 ± 0.96^{b}
Balik Pulau	0.43 ± 0.79^{a}
Youth Park	0.12 ± 0.54^{a}

For oocyte number per individual, the comparison between colony from Bkt. Jambul was significantly different from Youth Park, but not with that of the Balik Pulau colony (Table 2). The oocyte numbers per individual for Balik Pulau and Youth Park colonies were significantly different ($P < 0.05$). One of the factors between these intracolonial variations may be possibly linked to genetic variability. In honey bees colony, genetic variability affected worker egg laying production and larvae care (Robinson et al., 1990). Thus, ovary development may reflect as traits of egg-laying traits being expressed. Furthermore, trophic eggs were being fed to colony members of yellow crazy ant (Lee et al., 2017), hence the nutritional status of the colony could be another possible factor that contributed the ovary development to feed their colony members, especially when food became scarce.

4.CONCLUSION

Gaster width and mean width of the transverse band could not indicate the presence and extent of ovary development. Corpulent worker ants had more developed ovaries than regular worker ants, and interestingly there was colony difference in ovary development that may suggest the involvement of other factors. The contribution of the physiological development of invasive ant and its advantages that help the species to secure their new territory in an introduced placed remained as a captivating subject. Since egg production of worker ant in the field is yet to be quantified, and it is essential to learn how the ant manages its egg production rate in response to new environment and also the egg type produced, this will be a plausible research area waiting to be explored.

ACKNOWLEDGEMENT

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Biodiversity & Conservation **BC-06**

Mimosa pigra: Variation in Morphology, Phenology and Nutrient Acquisition under Different Densities of Monospecific Stand

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ABSTRACT

Plant-plant interactions are not only competitive but can be facilitative or neutral. Competition among same species (intraspecific) might occur when the plants within the population interfere with the growth and success of each other. In plant interactions, it is varied as different plant categories and species have different resource requirement (i.e., nutrient, space and water), growth intensity and growth adaptation. However, the intraspecific competition will be intense as same plant species may require similar resources and performed similar phenology. In this study, *Mimosa pigra* that was grown under four different monospecific densities was studied on the morphological adaptability, nutrient acquisition and phenological performances. Results has shown that the individual grown from low density stand had the best morphological performances and acquired substantial amount of nutrient in comparison with the individual grown from high-dense population intensity. However, as the population density increased, the imposed condition had induced the plant fitness (rapid fruiting) but did not improve the fruit productivity. This study suggested a strong influence of plant community on the growth and fitness of *M. pigra*. The plasticity of *M. pigra* is density dependent which suggested that fitness was accelerated as the carrying capacity of the growth environment and species interaction were above average.

Keywords: *Mimosa pigra*; intraspecific; morphology; phenology; nutrient uptake.

1. INTRODUCTION

Number of studies on measuring traits of invasiveness did a comparative experiment on the species interaction between congeneric and conspecific invasive species, with the native plant species (Hulme 2008; Gioria & Osborne 2014) incorporated with additional of environmental factors that was subjected to the experimental plants (Skalova *et al*. 2013). Those designed studies had apparently divulged and highlighted the important role of phenotypic plasticity in plant invasions which pointed out several traits implied species invasiveness. Previous studies had found that invasive species shown greater plasticity across environmental gradients particularly in response to competition relative to non-invasive species (Skalova *et al*. 2013). However, there has been a limited study on the interaction and the performance of invasive weed species within a mass conspecific species. In this study, one single species (*Mimosa pigra)* had been studied for its phenotypic plasticity as McNutt *et al*. (2012) had suggested to manipulate the intraspecific density to obtain the overall evolutionary effects of competition to the plant. A few questions had arisen; either *M. pigra* will perform a similar pattern of plasticity when it's grown among others non-invasive or native species, and either this species performed differently at multilevel of conspecific densities. Therefore, the aims of this study were to monitor the *M. pigra* morphological, physiological and phenological performances. Thus, a single factor of each plant performance can be solely monitored.

2. MATERIALS AND METHODS

The experiment was carried out from November 2015 to July 2016 at a vacant former orchard land at Batu Pahat, Johor (1° 50' 55" N, 103° 3' 4"E). The plot was designed with a four of 10 m x 5 m split plots system. Each split plot was haphazardly planted with different weight of *M. pigra* seeds sown as; 25 g, 50g, 75 g, and 100 g where each split plot was divided by 1 m pathway along the plots. During the plants growth, the phenological stages ;1) days taken to 50% flowering, 2) the onset of flowering, 3) days to 50% physiological maturity, 4) onset of maturity 5) number of mature pod production and 6) weight of 1000 pure seeds of the plants in each plot were denoted. Plants were harvested at approximately 8 months after sowing as more than 50 % of plants of each sub plot had completed their life cycle. 10 randomly plants of each sub plot with slightly similar height and stem diameter were harvested to measure morphological performances (i.e., height, stem diameter, biomass partitioning and relative growth rate (RGR)). While three plants out of 10 were analysed on nutrient uptake and concentration. Comparison on the functional traits data was made between each density by using One-way ANOVA which further analysed using Tukey post-hoc test.

3. RESULTS AND DISCUSSION

Morphological: Apparent finding showed that, plants grown from low-density population (plot 25 g) had shortest height with the thickest and sturdy stem and the pattern were vice versa as the plants grown from low to dense intraspecific competition (Figure 1a). Similarity pattern of plants morphological plasticity was reported by Nur-Zhafarina and Asyraf (2017). The plasticity of both traits (height and stem diameter) help the plant to mitigate the overtopping of the adjacent plants as a response of stressed avoidance particularly in assessing light, in nutrient uptake and food production for adaptation in constrain and intense competitive area (Pires *et al*. 2012).

Biomass: In Figure 1b, the total biomass production decreased proportionately with the increasing seed density. There was a big difference on biomass production from plants in 25 g plot (approximately 43.31% difference) compared with the other three plots. There were no big differences of total biomass in plants from 50, 75 and 100g (299.7, 291.3 and 286.4 g, respectively). Under high density conditions, the plants intensely grow upright (Figure 1a) and the plants were actually losing the matter inside instead of gaining mass (McNutt *et al.* 2012), which is resulting in biomass decreasing along with the increasing of density gradients. Therefore, the *M. pigra* biomass production was mitigated by the increasing of population density.

RGR: In intraspecific interactions or competition, the density of the neighbouring populations will influence the growth rate the individual (Orcutt & Nilsen, 2000). The significant reduction of relative growth rates often occurred when the competition became intense (Burton & Bazzaz 1995). Under low intraspecific density, plants showed fastest growing rate and gradually decreased with the increased of dense population intensity (Figure 1c). Fast growing plants is often associated by initially allocating more biomass to the roots (Orcutt & Nilsen 2000), which was observed in the root biomass of plants from 25g plot (Figure 1b). Good roots systems may facilitate the species growth by resources pre-emption as less interference between the root systems of competing species as compared with the high density population (Orcutt & Nilsen 2000).

Nutrient uptake: In Table 1, *M. pigra* grown from low density population had acquired the highest amount of N and K, and the amount was decreased as the plant densities increases. Meanwhile, the pattern of N, P and K acquirement was partly similar as the plants grown from moderate (50 g seed densities) to high density populations. Intense competition had caused high inter-root competition which likely minimized the nutrient uptake by the roots in the deficiency areas (Marschner, 1995). In low density, the plants might take advantage from the benign environments by maximizing the uptake of nutrients and performed well by vigorously grown. Instead, as density increases, the individual had to overcome the nutrient limitation by

economically uptake the nutrient with others neighbouring plant by maintaining high fitness under unfavourable condition. The adaptive plasticity might be one of the reason that facilitate the establishment of invasive species by allowing the plant to take advantage of the benign environment (such as in low density population) or maintain high fitness under high density population to fit with the environments (Hulme 2008).

Phenology: The onset of each phenological stages was significantly different among all plants in four density degrees (Table 2). The common onset of the plants to produce first mature pod is at the earliest of 4 to 5 months after germination (Binggeli 2005). In this study, *M. pigra* grown from the two highest density degrees had the fastest to reach maturity (approximately 5 months) than others two low density plants (approximately 9 months). Plants that grown under limited resources might exhibit a competitive resources avoidance owing to escape from the stressor condition by accelerating the pace of phenological stages. Thus, unfavourable growth conditions had ability to promote the evolution of the plants to adapt with the situation which induce the growth performances (Lee & Yoe 2015). In this study, it was either govern by the physiological of the plant (Jochner *et al*. 2013) and/or the soil nutrient status (Orcutt & Nilsen 2000; Funk 2013) which influence the timing of the onset growth stages.

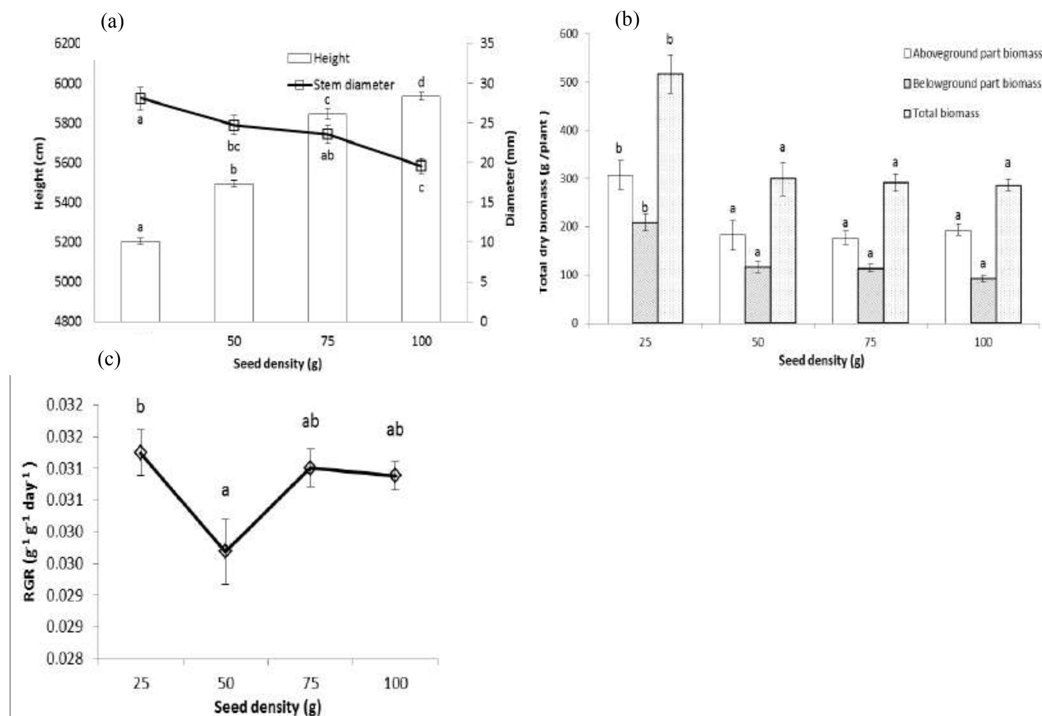

Figure 1. Morphological performances; a) height and stem diameter, b) above- and belowground parts, and total biomass and c) relative growth rate (RGR) of *M. pigra* grown at four different seed densities at the end of cultivation (mean ± 1 s.e). Different alphabet represented at the graph of each seed density shown significant differences ($p < 0.05$) at Tukey's multiple comparison test (n=3).

Table 1. Means of nitrogen (N), phosphorus (P) and potassium (K) uptake by *M. pigra* grown from four different plots of different seed densities. Values in the same column followed by different letters are significantly different at $p < 0.05$ at Tukey's multiple comparison tests (n=3).

Plot	N uptake (kg/50m2)	P uptake (kg/50m2)	K uptake (kg/50m2)
25 g	30.33 a	1.03 a	3.63 a
50 g	12.86 b	1.12 a	2.03 ab
75 g	10.72 b	0.92 a	1.83 b
100 g	12.79 b	0.97 a	2.03 ab

Table 2. Effects of different seed densities on the total days taken to the onset of each phenological stages. Values are the means, n = 3 per plot. Means in a column without a similar letter differ ($p < 0.05$), as analysed by one-way ANOVA and Tukey's post hoc test.

	Days			
Plot	Onset of 50% flowering	Onset of flowering	Onset of 50% physiological maturity	Onset of maturity
25 g	64 b	185 b	198 b	221 b
50 g	99 a	200 a	221 a	274 a
75 g	63 b	99 c	115 c	141 c
100 g	63 b	91 d	113 d	139 c

4. CONCLUSION

This study suggested a strong influences of plant community on the growth and fitness of *M. pigra*. The plasticity of *M. pigra* is density dependent which suggested that fitness was accelerated as the carrying capacity of the growth environment and species interaction. Further study is recommended by incorporating various species interaction with *M. pigra*.

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Biodiversity & Conservation **BC-08**

Comparison of Sex Identification Using Morphological and Molecular Methods for Sex Identification of the Southeast Asian Barn Owl, *Tyto alba javanica*

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ABSTRACT

Accurate sexing is vital for ecology, behavior and breeding strategy studies of birds. However, most bird species are sexually monomorphic, making sexing using morphological traits to be inaccurate, and barn owls are among the many of such types of birds. Sexing of barn owls is vital information for captive propagation, breeding and translocation programs of this species. To improve the success rate of sexing the barn owl, we evaluated six morphological traits to sex the owls; namely, the shape and colour of the facial disc, the colour of the throat area, the tail plumage, the colour of tarsus, the back plumage, and the frequency of spotting on the chest and underside of wings, and compared these results with those from a molecular sexing method using primers 2718R/2550F. Our comparison showed that sex identification using morphological traits only have an accuracy of 72.73%.

Keywords: Barn owls; sexing; morphological traits; molecular sexing.

1. INTRODUCTION

In birds, it is difficult to determine the sex of sexually monomorphic adults and most juvenile chicks. Early sex identification used plumage colour, body weight and shape of the beak differences to determine sex. Behavioural observations, laparotomy or laparoscopy examinations, sex chromosomes and morphometric measurements were also used to identify the sex of birds before modern molecular sexing techniques were developed to sex various avian species. The barn owl has a worldwide distribution (Taylor 1994) and shows variation in their reddish colouration and black feather spots. Plumage is often the most obvious way to tell the difference between male and female barn owls, with male birds often having bright and whiter plumage, while female birds generally have drab and dull coloured plumage (Marti 1990, Taylor 1994, Roulin 1999, Roulin *et al*. 2008, Van den Brink *et al*. 2011). We investigated the accuracy of sexing barn owls using morphological traits by comparing morphological features with molecular sexing techniques. Our focus was the Southeast Asian barn owl subspecies, *Tyto alba javanica*. Sex identification of barn owls is important for more successful breeding, introduction and conservation programs.

2. MATERIALS AND METHODS

2.2 Barn owl samples

Barn owls were harvested from oil palm plantations in Jengka 24, Bandar Jengka, Pahang, Malaysia (3° 46' N, 102° 26' E) then transferred to the aviary located on the main campus of Universiti Sains Malaysia (USM), Penang, Malaysia (5° 21' N, 100° 18' E). The owls were allowed to acclimatize to their new environment for one month before sampling was done.

2.2. Sexing using morphological traits

Pictures of selected traits of the owls were taken for sex identification purposes. Photos were taken of; i) the facial disc, ii) the chest and throat area, iii) the ventral (underside) view with wings extended, iv) the dorsal (topside) view with wings extended, v) the nape, vi) the dorsal view of their tail; and, vii) tarsus of owls. These traits were used to sex individual barn owls following the identification used in other studies (Colvin 1984, The Barn Owl Trust 1989, Marti 1990, Taylor 1994, Roulin *et al*. 2008, Hamid *et al*. 2010).

2.3. Molecular sexing

Blood was collected from the owls for molecular sexing. The procedure for bloodletting followed that of Salim et al. (2014). DNA was extracted using a Qiagen DNeasy® Blood and Tissue Kit and the standard protocol provided was followed to extract genomic DNA. DNA was amplified via polymerase chain reaction (PCR). The primers used were 2718R/2550F (Fridolfsson & Ellegren 1999). Two bands in PCR results indicate that the sample is female while a single band indicated the sample is male (Fridolfsson & Ellegren 1999).

3. RESULTS AND DISCUSSION

Our comparison between sex identified using both morphological traits and molecular sexing showed that morphological traits had a 72.73% accuracy for sexing barn owls. All barn owls which were inaccurately sexed using morphological traits were sexed as females but molecular sexing showed these owls were males. These owls had mostly female identifying morphological traits, i.e. a smudged face, brown colouring on their chest and throat area, and frequent spotting on their underparts and chest. We also analysed each morphological trait for accuracy by comparing the trait to sex of the bird confirmed through molecular sexing. From our results, the most accurate morphological trait for sexing of barn owls was frequency of spotting on the chest and underside of wings (81.82% accuracy), and the next accurate traits were the colour of their facial disc and throat area (63.64%). The less effective trait to sex barn owls was the colour of their back plumage.

Molecular sexing of barn owls using primers 2718R/2550F produced bands of 600 bp in size and a 1000 bp size when a double band was present. A single band indicates a male sample while a double band indicates a female. Fridolfsson and Ellegren (1999) tested 2718/2550F primers on two Strigiformes owls: *Aegolius funereus* and *Strix nebulosi* and the tested owl species produced band fragments of 600-650 bp in size and 1200 kb in size. In our study, there was no obvious differences in colour of the tail, colour of the tarsus and the back plumage of barn owls. All these traits are subjective to observer and additionally, it is hard to compare the colour of the tail and tarsus from afar.

For morphological traits, we conclude that the best indicator for sexing owls was the frequency of spotting on the chest and underparts of the owls. Additionally, these two traits are easier to discern and make identification faster compared with all the other traits whose differences are subtle in the Southeast Asian barn owl. Other studies observe that females have more pronounced flecking in underparts than males (The Barn Owl Trust 1989, Taylor 1994, Roulin *et al*. 2008, Hamid *et al*. 2010), an observation seen in our samples as well. In our samples, females typically had spotting extending along their wings and till their leg feathers. Female owls in our study had white throat area with brown patches and some even had

completely brown feathered throat areas. Meanwhile, the male owls had white throat areas with some having few light brown patches. This difference in plumage colouration of the throat area was similarly reported by The Barn Owl Trust (1989).

4. CONCLUSION

From our results, the best morphological characteristic for sexing barn owls is the frequency of spotting on their chest and underparts of their wings and colour of the throat area. These traits can be seen from afar and allows sexing to be easily done in the field. When accurate results are needed, sex of owls should be determined using molecular sexing, as sexing using morphological traits are only 72.73% accurate.

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Biodiversity & Conservation **BC-09**

Effect of Silica Concentrations on the Growth Rate and Photosynthesis of Tropical Diatom *Actinocyclus octonarius*

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ABSTRACT

A study was conducted to determine the growth and photosynthesis in tropical diatom *Actinocyclus octonarius* under three different silica concentrations of 0 µM (Si-deplete), 11 µM (Si-medium) and 106 µM (Si-replete). Dominant diatom species, *A. octonarius* was isolated from Teluk Bahang, Penang and the growth rate of diatom was estimated by cell count, chlorophyll *a* and biomass (F_o) of Pocket-PAM. Meanwhile, maximum quantum yield (F_v/F_m), maximum relative electron transport rate ($rETR_{max}$), photosynthetic efficiency (α) and photoacclimation index (E_k) derived from rapid light curves (RLCs) were measured using Pocket-PAM. The exponential phase was between Day 2 and Day 4 with a specific growth rate of 1.52 d^{-1}. The specific growth rate during exponential phase in Si-deplete condition was significantly higher ($P < 0.05$) by 58% compared to Si-replete condition. The phototrophic growth of *A. octonarius* displayed similar trends in F_o and chlorophyll *a* biomass but they were not significant ($P < 0.05$) among silica concentrations. However, with the absent of silica (0 µM), in particular at Day 16, the culture had 18% higher F_o and 36% higher chlorophyll *a* than 106 µM silica concentration. Cell density, rETR and F_v/F_m values were either 0 or undetectable as low biomass of chlorophyll *a* was obtained. Silica, phosphate, ammonia and pH in the cultures did not show significant changes ($P > 0.05$) among silica concentrations. However, nitrite was significantly different ($P < 0.05$) at silica concentration between 0 and 11 µM and between 0 and 106 µM. Meanwhile, nitrate was significantly higher ($P < 0.05$) at silica concentration of 106 µM compared to 0 µM throughout the days. Nitrite showed significant increase from Day 1 to Day 20 whereas nitrate decreased. The cell density, F_v/F_m and rETR values were not observed. In general, nutrient concentrations (silica, phosphate, nitrate and ammonia) decreased from Day 1 to Day 20. This meant nutrients had been used up by the cells for cellular processes or in building frustules. In short, *A. octonarius* could grow under silica limitation but their photosynthetic rate would be low.

Keywords: culture; microalgae; nutrient; silica.

1. INTRODUCTION

The effect of silica limitations is often studied in diatoms (Valenzuela et al., 2012; Chu et al., 2013; Schnurr et al., 2013). According to Bienfang et al. (1982), silica-depletion causes higher sinking rates compared to silica-replete cultures in diatoms *Skeletonema costatum*, *Chaetoceros gracile*, *Ditylum brightwellii* and *Coscinodiscus wailesii*. In eutrophic waters, excess nitrogen and phosphorus usually increase diatom growth and sedimentation. This may enhance silica build-up in the sediments and thus limiting the silica concentration in the water (Papush & Danielsson, 2006). This condition may affect diatom species composition (Olli et al. 2008) as silica limitation will promote the growth of flagellates rather than diatoms (Smayda, 1990; Wasmund & Uhlig,

2003; Conley et al., 2008). Eventually, changes in microalgal composition may lead to major shifts in the entire food web and may cause harmful algal blooms (Smayda, 1990; Conley et al., 1993, Conley et al., 2008).

2. MATERIALS AND METHODS

Dominant diatom species, *Actinocyclus octonarius* was isolated from Teluk Bahang using micromanipulation technique. The isolation procedure was conducted under non-axenic environment in a clean workspace. Cells were grown in f/2 medium (Guillard & Ryther, 1962) with CSIRO modification in either sterile 50 mL yellow-capped bottles or 100 mL glass test tubes (Favorit) at 26 ± 1°C under 12:12 light:dark cycle. Cultures were illuminated with cool white fluorescent lights at 35 - 40 μmol photons m^{-2} s^{-1} on the surfaces of the culture. They were maintained under these conditions until they were ready to be used in preliminary and experimental cultures. The growth rate of diatom was estimated by cell count, chlorophyll *a* biomass and F_o of Pocket-PAM. Meanwhile, photosynthesis was measured using Pocket-PAM by obtaining the rapid light curves (RLCs) and maximum quantum yield (F_v/F_m).

2.1 Experimental setup

To investigate the photosynthesis and growth rate of *A. octonarius*, experimental cultures were then conducted by adjusting three silica concentrations. Experiments were carried out in triplicate of 250 mL cultures in a 500 mL polycarbonate container (Tarsons, India). All experiments started with an inoculation of approximately 1000 cells mL^{-1} *A. octonarius* grown in f/2 without silica medium.

Cultures were incubated at a constant temperature of 24 ± 2°C under 35 - 40 μmol photons m^{-2} s^{-1} measured on the surface of the cultures with a calibrated light meter (LI-250A, Licor Biosciences, USA). All experiments were carried out under 12:12 h light:dark cycle under cool white light fluorescence.

For silica treatments, 5 L of f/2 media was adjusted to different silica concentrations of 0 μM (Si-deplete), 11 μM (Si-medium) and 106 μM (Si-replete) prepared in sterile Schott bottles. The range of silica concentrations was improvised after conducting five silica concentrations for polar experiment and no significant differences were found among five silica concentrations. For every fourth day, samples were taken for measurements of cell count, pH, water quality analyses and photosynthesis at about 10.00 am each morning. Temperature, pH and salinity were monitored using a pH meter (Starter 300, OHAUS, USA) and handheld refractometer (Atago Brix N1, 0.32% Brix, USA) every sampling day.

Chlorophyll *a* analysis for tropical diatom *A. octonarius* was based on spectrophotometric monochromatic method modified from Marker (1994) using a UV-vis spectrophotometer (UVmini-1240, Shimadzu, Japan).

Each day, the F_v/F_m value of *A. octonarius* was monitored using a Pocket- PAM. For each sampling day or every fourth day, $rETR_{max}$, α and E_k were measured. Gain setting was set at 1. All samples were dark adapted for at least 15 minutes at 26 ± 1°C before taking measurements.

3. RESULTS AND DISCUSSION

To investigate the effect of three silica concentrations on the growth and photosynthesis of *A. octonarius*, experimental culture was conducted. The phototrophic growth of *A. octonarius* displayed similar trends in F_o and chlorophyll *a* biomass but they were not significant ($P < 0.05$) among silica concentrations (Figure 1). However, with the absent of silica (0 μM), in particular at Day 16, the culture had 18% higher F_o (Figure 1a) and 36% higher chlorophyll *a* (Figure 1b) than 106 μM silica concentration. Cell density, rETR and F_v/F_m values were either 0 or undetectable as low biomass (F_o or chlorophyll *a*) was obtained (data not shown).

In experimental culture, the growth based on minimal fluorescence (F_o) and chlorophyll *a* was not significant in silica concentrations. This might be due to silica concentrations supplied

was growth saturating. Chu et al. (1996) stated that the growth saturating for diatom *Nitzschia inconspicua* was at silica concentrations of 8.8 to 172 µM. In this study, the initial silica concentration was 45 µM and they were pre-acclimated in f/2 medium without silica for 10 days so that silica would be taken up by the cells. Perhaps, *A. octonarius* has already adapted to culture without silica (0 µM) that the cells showed no significant changes in moderate (11 µM) and high (106 µM) silica concentrations. Another possibility was that *A. octonarius* did not require high silica concentration for growth. However, silica concentrations might not have direct effect on the growth of diatoms but they did seem to have a direct effect on silicification in order to build their frustules (Martin-Jézéquel et al., 2000; Ragueneau et al. 2000). Hence, in Si-deplete conditions, they can maintain division rates near maximum (Paasche 1973, Ragueneau et al. 2000) but the silica uptake is limited by concentration of silicic acid resulting in less silica being deposited during cell cycle (Paasche 1973). This could be observed morphologically in diatoms as Si-depleted cells had thinner frustules (Brzezinski et al. 1990). Similarly, in African upwelling coastal water which had low ambient silica concentration (i.e. 1 - 10 µM), growth of diatoms had thinner frustules or weakly silicified which was more susceptible to dissolution (Ragueneau et al., 2000). In contrast, field studies at coastal waters of Pantai Jerejak and Teluk Bahang, Penang coastal waters recorded relatively higher silica concentrations at 22 µM and 42 µM, respectively compared to African coastal water. In addition, silica concentrations in the sediment was much higher at 348 µM and the frustule of diatoms observed were thicker.

The cell density, F_v/F_m and rETR values in experimental culture were not observed. Pocket-PAM was used to measure the photosynthetic parameters in the experiment but since it had a high detection limit (500 µg Chl *a* L^{-1}; Figueroa et al., 2013), it could not detect the low biomass of *A. octonarius*. Having culture in higher biomass using Pocket-PAM would alleviate this problem. Furthermore, batch culture system was used, hence, the quality of the harvested cells was less predictable since the cells might vary from one replicate to another during inoculation. Also, the time of harvesting varied when large samples were involved. In general, nutrient concentrations (silica, phosphate, nitrate and ammonia) decreased from Day 1 to Day 20. This meant they had been used up by the cells for cellular processes or in building frustules. Nitrate, was affected by silica concentrations which might indicate co-limitation in the diatom. In short, *A. octonarius* could grow under silica limitation but their photosynthetic rate would be low.

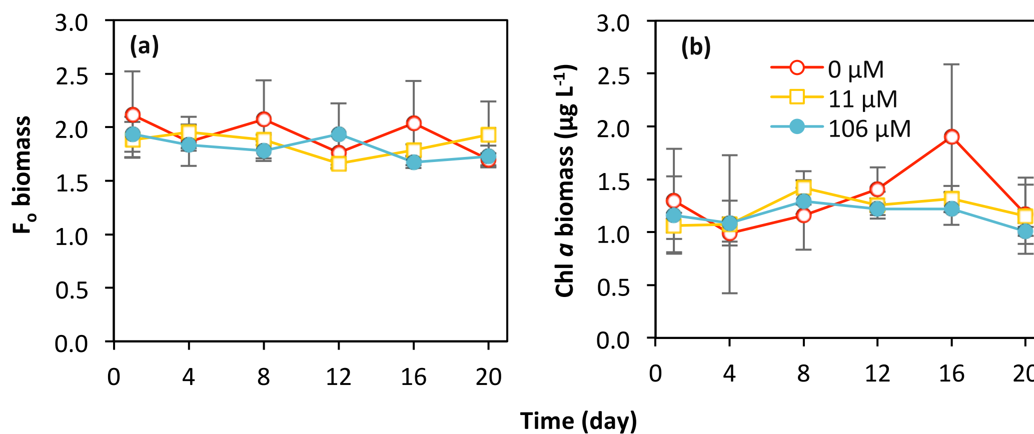

Figure 1. Semi-log experimental curve of (a) F_o and (b) chlorophyll *a* biomass *Actinocyclus octonarius* at three silica concentrations of 0 µM (○), 11 µM (□) and 106 µM (●) media. Sampling for cell count was conducted every four days. Values are means ± SD (n = 3).

4. CONCLUSION

Study on the effect of silica was carried out on dominant species of tropical diatom, *Actinocyclus octonarius* isolated from Teluk Bahang. At low silica concentrations, the culture could survive but they experienced low photosynthetic activity.

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Biodiversity & Conservation **BC-11**

Diversity and Morphological Description of Octocorals from the Family Alcyoniidae at Pahang and Johor Islands in the Lower South China Sea

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ABSTRACT

Little is known on the studies of diversity and taxonomy of octocorals in Malaysia. Previous studies of ocotocoral surrounding Malaysian waters include; Verseveldt, 1960 (Indo-Pacific); Mahadi, 2004 (Malaysia); Goh, Tan & Tan, 2009 (Singapore); Chanmethakul *et al.*, 2010 (Thailand). During the Universiti Malaysia Terengganu (UMT) Scientific Expedition in August 2015, five islands in Pahang and Johor waters were surveyed to determine the diversity of octocorals in the area. The octocorals were collected at the shallow (3-6 meters) and deep (9-20 meters) reef area. This study documented 4 genera in family Alcyoniidae: *Dampia*, *Lobophytum*, *Sarcophyton,* and *Sinularia*. The structure of octocorals polyp (autozooid and siphonozooid) is important in differentiating between genera in this family. This character will divide the family into two groups: monomorphic polyp group (*Dampia* & *Sinularia*) and dimorphic polyp group (*Lobophytum* & *Sarcophyton*). Within these groups, the character of colony shape and the type of sclerites will further distinguished the members of the group.

Keywords: diversity, soft coral, *Sinularia*, zooxanthellate.

1. INTRODUCTION

The family Alcyoniidae was first established by Verrill, (1869). Alcyoniidae is categorized into a sterile stem and a polyp-bearing disc or capitulum whose outer surface may appear, branched, lobed, mushroom like or otherwise provided with short or long processes. Usually there is polyp dimorphism, but in certain cases the siphonozooids, as a result of retrograde development, may be absent. The commonest sclerite are spindles, double spindles, sticks, and barrels or cylindroids with warts that may be in the form of girdles. The Alcyoniidae octocorals inhabit warm, shallow tropical waters from the coast of Southeast Asia, Red Sea, East Africa, and to the Central Pacific (Benayahu, 2004). Their presence in the Indo-Pacific and the islands of Malaysia has received limited investigation (Mahadi et al., 2004). The octocoral of Malaysia was a subject of a beginning stage, including taxonomic studies of the Pulau Pemanggil (Mahadi et al., 2004) and of the northern region of Straits of Malacca (Mohammad et al., 2016). In the year 2015, a survey was conducted throughout the lower part of South China Sea in the Pahang and Johor islands to assess the diversity of alcyoniid octocorals. Field identification of alcyoniids to the species level is not possible given the need for modern scanning electron microscopic (SEM) analysis of the sclerites or skeletal elements. Samples were collected and examined in the laboratory following in the in-situ observations. These samples are currently housed in the collections of the South China Sea Repository and Reference Centre (RRC), Universiti Malaysia Terengganu (UMT). The preliminary identification results are presented here.

2. MATERIALS AND METHODS

2.1 Study Sites

Samples were collected during UMT Scientific Expedition Series 1 in August 2015. Study locations were in the southern part of South China Sea, East Coast of Peninsular Malaysia at Pahang, and Johor Islands, as shown in Figure 1. Coral reef here are fringing reef and there is distinct habitat in reef habitat preference in between inshore and offshore islands. Altogether there were six study area, two of the study locations were located at the inshore islands namely Tokong [=Little bay] Sanggul and Tokong Bahara East while at the offshore islands there were four locations which was Tokong Bahara West, Pulau [=Island] Jahat, Letak Layar in Pulau Pemanggil and Pulau Yu. The coral reef extent can be up to 15 meter in depth at the inshore islands and 25 meter in depth at the offshore islands.

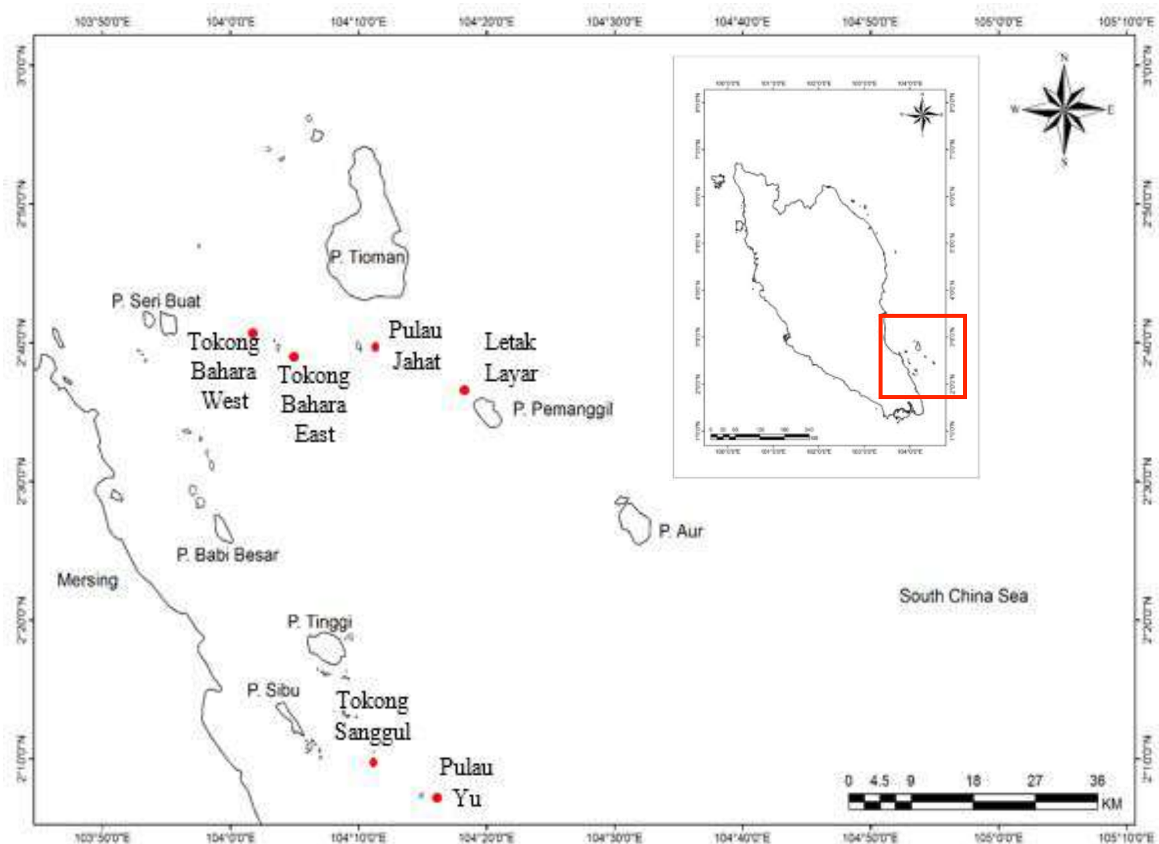

Figure 1. Location of the Study Sites in the Southern Part of South China Sea, East Coast of Peninsular Malaysia at Pahang, and Johor Islands.

Samples were collected during neap tide by scuba diving with two depth zonation the shallow (1-7 meter) and deeper (8-25 meter) reef area. All the specimens were relaxed until the specimens fails to react to stimuli immersing specimens in 5% Magnesium Chloride ($MgCl_2$) seawater. According to Janes (2013) this method is required to maintain the polyps in their natural states of expansion. After the sample was totally anaesthetize, photo was taken immediately. Next the specimens were fixed and preserved in 70% ethanol. The samples were kept in a glass jar accompanied by a waterproof label paper with information of the specimens (species name, date,

location, depth, and name of collector). Specimens were deposited in RRC, UMT with a maintain room temperature.

2.2 Sclerite Extraction

Sclerites was extracted using the method described by Bayer (1956), Benayahu *et al*., (2004), Janes and Wah, (2007) and Janes (2008). Tissues from the polyp and stalk of the colony, were extracted and dissolved in 6% of sodium hypochlorite to obtain the sclerites. Sclerites were then washed several times with deionized water and lastly absolute alcohol. They were then air dried and mounted on scanning electron microscope (SEM) stub. The sclerites were coated with gold and observed under a Leo Supra 50 VP Field Emission SEM at 15kV acceleration voltage.

3. RESULTS AND DISCUSSION

A total of four species from four genera were recorded from the Pahang and Johor islands. All specimens collected were identified up to genus level. All specimens were examined morphologically by observing colony growth form, type of polyp presence and sclerite characters (Table 1). The distinct part in separating between genera in this family is their type of polyp presence in the colony. As for *Dampia* and *Sinularia*, both were having autozooid polyp only in their colony, while for *Lobophytum* and *Sarcophyton* both were presence of autozooid and siphonozooid. Figure 2 shows the colony of *Dampia*, *Sinularia*, *Lobophytum*, *Sarcophyton* and their sclerites presence on surface and interior of the colony. In the colonies of *Sinularia*, they are thick, massive, and upper surface lobate with digitate processes. Sclerite identification of *Sinularia* followed by Fabricius and Alderslade, (2001), with club sclerites covered the predominant large and tuberculate spindles sclerites in the interior lobes. *Dampia* colonies were thickly encrusting with a basal portion devoid of polyps. Sclerites of the deeper coenenchyma contain large warty spindles and the surface layer of the *Dampia* colony contain small club sclerites.

Table 1. Summary of Identified Genera Collected from the Pahang and Johor Islands

Genera	Colony Shape	Polyps	Type of Sclerites
Dampia Alderslade, 1983	Colony thickly encrusting with a basal portion devoid polyp, distinctly differentiated from a lobed polyparium	Monomorphic, retractile	Colony surface contains small clubs, interior colony packed with spindles
Sinularia May,1998	Thick, massive, upper surface lobate & digitate processes	Monomorphic, retractile	Predominant sclerites are large, tuberculate spindles covered by superficial layer of small clubs
Lobophytum Marenzeller, 1886	Low sterile stalk not sharply delimited from capitulum, which is lobed	Dimorphic, retractile	Interior lobes contain spindles, interior base contain ovals with the warts nearly always arranged in several girdles
Sarcophyton Lesson, 1834	Colonies with distinct sterile stalk and rounded, often marginally folded capitulum	Dimorphic, retractile	Surface of the polyparium and stalk are characteristically well-formed clubs, occasionally very long and thin, the colony interior contains sticks and spindles

Colonies of *Lobophytum* were flattened with low sterile stalk and simple unbranched lobes. Sclerites of the interior lobes contains spindles and sclerites of the interior base were thicker and more robust than those in the interior lobes and were ovals with the warts nearly always arranged in several girdles. *Sarcophyton* colonies have a mushroom-shaped polyparium consisting of a smooth and marginally folded disc, which projects beyond a clearly differentiated

base or stalk. Surface sclerites were long-handled clubs with poorly differentiated heads and fairly sparse, simple ornamentation while the colony interior contains sticks and spindles sclerites. A detailed account of the species identification of Alcyoniids from Pahang and Johor islands is forthcoming (Muhammad Lutfi et al., in preparation).

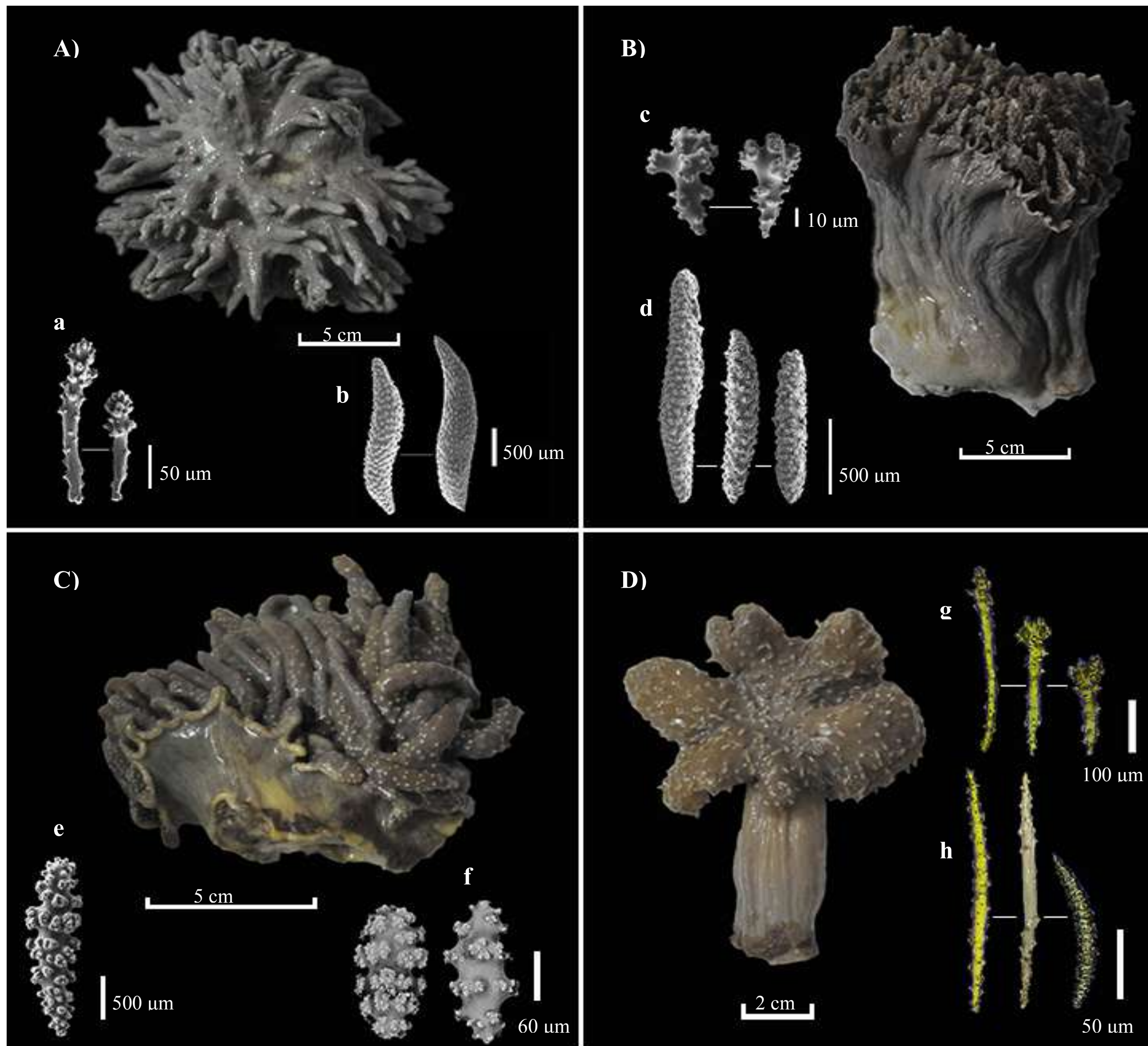

Figure 2. The Octocorals of Family Alcyoniidae from Pahang and Johor islands. A, *Sinularia* May, 1898, UMTCnid 01430; a, Clubs from the surface of the Polyp-bearing part; b, Spindles from the Interior Lobes of *Sinularia*; B, *Dampia* Alderslade, 1983, UMTCnid 01463; c, Clubs from the Surface of the Colony; d, Spindles from the Interior of the Colony; C, *Lobophytum* Marenzeller, 1886, UMTCnid 01431; e, Spindle from the Interior of the Lobes; f, Ovals from the Interior of the Base; D, *Sarcophyton* Lesson, 1834, UMTCnid 01422; g, Clubs from the Surface of Polyparium; h, Stick and Spindle from the Colony Interior.

Previously, 11 species from four genera of the family of Alcyoniidae were note by Mahadi (2004) to occur in the Pulau Pemanggil from Johor island on the lower part of South China Sea. This study has revealed a similar number of genera found from previous studies in Malaysia. Identification of morphological characters diagnostic for each of the genera allow us to distinguish among the genera in Alcyoniidae. For instance, among species of *Sinularia*, Verseveldts, (1980) came out with five taxonomic identification keys to a distinct group of species according to the colony form and morphology of club-shaped sclerite in the surface layer of the colony. However, many of the morphological characters used for higher level classification

in these taxa have now been shown to be conflicting with molecular phylogenetic evidence, hence they are likely homoplacious (McFadden et al., 2009). Although the morphological characters identified here (Table 1) distinguishes only to genera level, consideration of these characters in future taxonomic studies will also improve our ability to determine species boundaries.

4. CONCLUSION

This survey yielded four species of octocorals from four genera in the family Alcyoniidae. These results highlight the importance of such surveys in the region which undoubtedly will continue to elucidate patterns of biodiversity and understanding the ecology of octocorals and their adaptability in the changing environment. Future studies will integrate both alpha taxonomy and molecular approaches to further study the taxonomy, diversity and distribution of octocorals in Malaysia.

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Biodiversity & Conservation **BC-12**

Establishment, Home Range and Core Area of Introduced Barn Owls in Urban-Garden Areas of Minden Campus, Universiti Sains Malaysia

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ABSTRACT

The purpose of this study was to determine the establishment, home range and core area of introduced barn owl, *Tyto alba javanica* in urban-garden area of Minden Campus, USM Penang. The owls were introduced in three different release methods; the first was using wild owls (caught from their habitat and simply released in the campus); acclimatized fledglings (harvested from their nest and acclimatized in aviary for few weeks and released in artificial nest box in the campus); and acclimatized adults (harvested in fledgling stage and acclimatized in aviary for more than a year and released in the campus). The radio telemetry tracking recorded all (*n* =4) of introduced wild owls were not stayed and flew off from the campus immediately after their release program. No home range and core areas were calculated since the owls were not established in the campus even in a short period. A total of 9 acclimatized fledglings were introduced in the campus. Three of the owls were managed to radio-tracked up to 3 weeks in the campus. The average home range sizes and core area of these owls based on the 95% Mean harmonic calculations were at 8.32 ha and 6.39 ha. However, the owls were not sustained and dispersed away from the campus after few weeks of their release day. Next, a pair of acclimatized adults (*n*=2) was released in the campus. The pair were managed to radio-tracked for more than three months and were seen hunting, perching and grooming around the campus throughout the tracking activities. The average of home range and core area of the owls were recorded at 22.43 ha and 9.78 ha respectively. The persistent presence of owls with the established home range and core area indicated the third method was successful to introduce barn owl in the campus.

Keywords: barn owl, home range, translocation, radio telemetry

1. INTRODUCTION

The use of wild barn owls to control rodent pests has been implemented in many regions in the world, and studies in Malaysia have reported that this bird of raptor, *Tyto alba javanica* is highly efficient in controlling rats in agricultural lands (Duckett *et al.*, 1990; Hafidzi *et al.*, 1999). Studies have shown that rats can comprise up to 98% of their diet. Besides, barn owls have several unique traits such as an attraction to artificial nest boxes, high rate of reproduction, a voracious appetite towards pests rodent and highly resilient towards human habitation. The potential of barn owls as a rat biological control in urban areas has been studied by Meek *et al.* (2003). They carried out a 21-year study on barn owl release and discovered that barn owls are adapted to live in close proximity to human habitation. While researchers in Johannesburg, South Africa conducted a study on released barn owls in semi-urban area and discover that the survival rate of the owls was high, up to 50% and could establish their breeding population in released area (Meyer, 2008). However, the potential of barn owls as biological control agent against rat

population in urban area in Malaysia is largely unexplored. Thus, this study is the first initiative to introduce barn owls in urban-garden areas in university campus of USM. The USM campus is rich with tropical plants and forests habitat and this unique environment in urban areas is ideal for introduction purpose of barn owls.

2. MATERIALS AND METHODS

2.1 Study site

A total of 15 barn owls was released and introduced in USM Minden Campus. The barn owls were harvested from three different locations, namely Pusat Penyelidikan Pertanian Tun Razak, Bandar Jengka Pahang, Jabatan Pertanian Bumbung Lima, Kepala Batas, Pulau Pinang and Pejabat Pertanian Daerah Kerian, Parit Buntar, Perak, Malaysia and all the birds were translocated to the campus. The owls were introduced in three different release methods; the first was using wild owls (caught from their habitat and simply released in the campus); acclimatized fledglings (harvested from their nest and acclimatized in aviary for few weeks and released in artificial nest box in the campus); and acclimatized adults (harvested in fledgling stage and acclimatized in aviary for more than a year and released in the campus). This duration allowed the barn owls to pair and establish their home territory in aviary prior to release in the campus.

2.2 Radio telemetry

All barn owls were tagged with numbered metal bands attached around their feet. The study relied on radio telemetry, whereby the spatial locations of individual owls were mapped. These radio locations were used to analyse individual home range sizes and core area. The radio telemetry set consisted of radio receiver TRX-48S (Wildlife Materials Inc.), with a frequency coverage of 150-152 MHz, a 9g transmitter which attached at the back of the owl using back-pack technique powered by 1.5V battery and a 3 element Yagi antennae. By tuning the radio receiver to the desired frequency and following the path of the strongest signal, the position of the owl can be ascertained. Each owl was followed for at least 10 cumulative days, starting from dusk (2000 hours) to dawn (0630 hours). Home range size and core area were calculated using the Minimum Convex Polygon and the Mean Harmonic method. Calculation were done by using the Biotas® Software.

3. RESULTS AND DISCUSSION

The results of the study are shown in Table 1. The radio telemetry tracking recorded all of four introduced wild barn owls did not stay and flew off from the campus immediately after release program. No home range and core areas were calculated since the locations of these four owls were barely detected and not established in the campus even in a short period. These findings is concomitant with Bunn *et al.* (2010) where the adults deserted their release site after being translocated into new area for introduction program. The second release method which was by using acclimatised fledglings (5 females and 4 males) showed that three of the owls were managed to be radio tracked up to 2 weeks in the campus. The other six owls were traceable up to 3-6 days after their release. The average home range and core area from this category were 8.32 ha and 6.39 ha. However, all of the introduced acclimatized fledglings were not sustained and dispersed away from the campus. Their small area of home range and core area and fewer number of days tracked indicated that these barn owls were not able to establish their home territorial range in the campus, thus they dispersed and were untraceable. Next, the third method was the acclimatised and paired adult barn owls. This pair was managed to be radio tracked for more than three months and were seen hunting, perching, roosting and grooming around the campus throughout the tracking activities. The average home range and core area of the owls were recorded at 22.43 ha and 9.78 ha respectively. The persistent presence of owls with established

home range and core area indicated that the barn owls were successfully introduced and remarks the success of biological control programs (Puan, 2013).

Table 1. Home range, core area and survival of released barn owls.

Method of release (sample size)	Home range (ha) (mean)	Core area (ha) (mean)	Days tracked (mean)	Distance from release (m)	Status *
Wild adults (n=4)	0	0	1	No record	X
Acclimatised fledglings (n=9)	8.32	6.39	4.37	80.08	X
Acclimatised adults (n=2)	22.43	9.78	77	3329.11	Y

*status of barn owls after a month of released: X: untraceable, Y: survived and established in the campus.

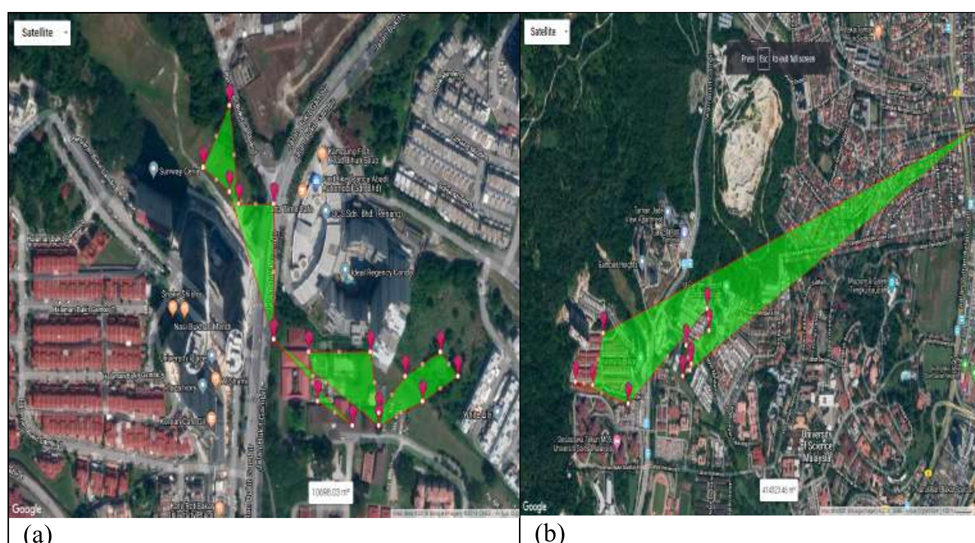

Figure 1. Foraging ground and ranging behaviour of successfully introduced barn owls in the campus. (a) Female (b) Male

4. CONCLUSION

From this study, the most suitable release method for establishing barn owls in urban-garden areas is acclimatised adults. From our study, the average home range and core area of acclimatised adults are 22.48 hectares and 9.78 hectares. The success of this release method is most probably because prolonged acclimatisation attributed to the owls familiarization in an urban setting and establishing homing instinct at release site induce the owls to stay within university campus.

ACKNOWLEDGEMENT

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Biodiversity & Conservation **BC-14**

Preliminary Checklist of *Hoya* (Asclepiadaceae) in the Northern Region of Peninsular Malaysia

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ABSTRACT

Hoya, an epiphytic herbaceous climbers is a genus that commonly found in Peninsular Malaysia. This waxy plant usually have milky white sap, star-shaped corona and colorful inflorescences. The diversity of *Hoya* species in Peninsular Malaysia is incomplete due to the scarcity of recent collections. After 40 years since *Hoya* complete recorded by Rintz (1978) in Peninsular Malaysia, the preliminary checklist of *Hoya* is getting up for more than eight species were newly recorded with 27 species to date. Thus, the aim of this study is to update the number of species including a new record by focusing mainly on the northern part of Peninsular Malaysia which is from Kedah and Perak. Based on the result obtained, 11 species found from Sungai Pedu, 12 species from Lata Celak, 17 species from Sungai Rui and 11 species from Belukar Semang. This study will promote researchers to explore and expand more on this genus diversity and the updating data will be valuable for future studies including the conservation purposes.

Keywords: *Hoya*; Apocynaceae; taxonomy; Peninsular Malaysia

1. INTRODUCTION

Species of *Hoya* R. Br. the wax flower genus, are among the most commonly cultivated indoor plants in Europe, the United States and Australia. Named in 1810 by Robert Brown in honor of his friend, the gardener Thomas Hoy. Currently, the genus *Hoya* is the largest genus of Apocynaceae (Endress *et al*. 2014) with more than 500 published species names (IPNI 2018). However, according problems with species delimitation abound, and the species number estimate for the genus may therefore now lie in between 350 and 450 accepted species found throughout tropical Asia, tropical Pacific islands and NE Australia (Liddle & Forster 2008; Rodda 2015) with new ones being added every year. Most species of *Hoya* are lactiferous epiphytic climbers with slender twining stems and succulent, opposite leaves. Their remarkable flowers are sympetalous, pentamerous and complex in morphology (Wanntorp *et al*. 2011). The flowers are grouped in umbelliform- extra-axillary inflorescences (Omlor 1998) that can be concave and positively geotropic, convex and positively geotropic or convex and negatively geotropic (Rintz 1978). *Hoya* also can bear flowers multiple times a year. Malaysia is one of the world's mega diverse countries according to the National Biodiversity Index, which is based on estimates of country richness and endemism because of its natural resources. The diversity of Malaysian forest can be seen in this case as we can see *Hoya* are well distributed in a good-lighted and moist tropical environments. Trees along the rivers and limestone are often occupied with members of this genus. The genus is particularly diverse in the island of Borneo where Lamb *et al*. (2014) estimated that 60–70 species may occur in the state of Sabah alone. The diversity of the genus in Sarawak and Kalimantan is less known and extensive field explorations will be required before

compiling a comprehensive revision. For Peninsular Malaysia, *Hoya* was revised three times, first by King and Gamble (1908), who recorded 23 species, then by Ridley (1923) (25 species) and, as mentioned, by Rintz (1978) (25 species). *Hoya endauensis* Kiew (Kiew 1989) and *Hoya mappigera* Rodda et Simonsson (Rodda and Simonsson Juhonewe 2012), bringing the number of species to 27. The known distributions of the 27 species of Peninsular Malaysian *Hoyas* are currently quite vague and likely to remain so for some time. Based on Rintz (1978), there are currently six species of *Hoya* recorded only from Peninsular Malaysia and an additional three species known only from Peninsular Malaysia and the Thai-Burmese isthmus. A single species is known elsewhere only from South Sumatera, another only from Sarawak and single species extends in Thailand. The remainder of Malaysia's *Hoyas* occur widely in the islands to the south and east but generally no further than Java and Borneo. Presently, no comprehensive taxonomic revision is available for the genus (Meve 2002). Due to the lack of taxonomic studies on the whole genus, the number of species in *Hoya* is difficult to estimate. Nomenclature issues are still unresolved for various taxa in Peninsular Malaysia certainly rich in *Hoya* species remain up to the present study insufficiently studied.

2. MATERIALS AND METHODS

2.1 Study sites

The *Hoya* study was carried out in four different sites around lowland and limestone forest in two states (Kedah and Perak) of the northern region of Peninsular Malaysia. The list of study sites as in Table 1.

Table 1. The Details of Sampling Locations used in *Hoya* Study

State	Study site	Gps coordinate	Forest Type
Kedah	Sungai Pedu	5.4700° N, 100.3500° E	Limestone
Kedah	Lata Celak	5.5637° N, 100.8670° E	Lowland Dipterocarp
Perak	Sungai Rui	5.4590° N , 101.1737° E	Lowland Dipterocarp
Perak	Belukar Semang	5.5739° N, 100.9920° E	Limestone

2.2. Sampling Collection

Standard collecting materials and methods were used (Bridson & Forman 1992). Species identification was done in the field and the assistance of local and taxonomist was sought for unfamiliar species. All voucher and herbarium collections are lodged in Herbarium Unit of the Universiti Sains Malaysia (USM). Living collections were grown in the School of Biological Sciences, USM nurseries. Collection of living material was necessary as many of the plants did not flower during the survey period. Establishing these plants in the nurseries allowed close monitoring for flowering activities, which enabled species identification. Field photographs were taken during these field trips, although subsequent photographing were also carry of field-collected plants flowering in cultivation.

3. RESULTS AND DISCUSSION

A total of 24 species of *Hoya* were enumerated from the four locations indicated 11 species found from Sungai Pedu, 12 species from Lata Celak, 17 species from Sungai Rui and 11 species from Belukar Semang (Table 2). These four study sites were categorised into two different types of ecology and habitat. Sungai Pedu, Kedah and Belukar Semang, Perak were limestone forest while Lata Celak, Kedah and Sungai Rui, Perak were lowland forest. The similarity between the existences of *Hoya* species at the four sites was, most of the species were found near

to riverbanks and shaded area. This may show that most of the *Hoya* species grow on the lower altitude areas to prevent the direct exposure to the sunlight in order to avoid the water loss despite some of them have really waxy and succulent leaves as strategies to prevent the water starvation. There are six species commonly found on these four sites including *H. revoluta*, *H. forbesii*, *H. lacunosa*, *H. parviflora*, *H. elliptica* and *H. finlaysonii*.

Although each of the sites differs in the numbers and types of species found but each site has some of the species that were abundantly found, for example, *H. forbesii* were numerous at Lata Celak and Belukar Semang. Another two sites resulted in an abundance of *H. lacunosa*. The finding of *H. ignorata* proved to be a new record in Peninsular Malaysia and also was considered a rare species to be found. The existence of *H. beccarii* strengthens the study done by Rodda and Simonsson Juhonewe (2013) that it is a different species than *H. revoluta based* on our taxonomic and ecological comparison. The previous finding of *H. beccarii* in Peninsular Malaysia was about 42 years ago which misidentified by Rintz in 1976 as a similar species to *H. revoluta*.

Table 2.0. The Species List from Four Sampling Sites

	Sungai Pedu, Kedah	Lata Celak, Kedah	Sungai Rui, Perak	Belukar Semang, Perak
H. revoluta	√	√	√	√
H. forbesii	√	√**	√	√**
H. lacunosa	√**	√	√**	√
H. parviflora	√	√	√	√
H. obtusifolia	√®		√	
H. elliptica	√®	√	√	√
H. finlaysonii	√	√	√**	√
H. diversifolia	√			
H. coronaria	√		√	√
H. erythrina	√		√	√
H. flagellata	√®			
H. caudata				√
H. coriacea		√	√	
H. mitrata			√	√
H. ignorata		√®	√	
H. javanica		√		
H. multiflora			√	
H. curtisii		√®		
H. erythrostemma			√®	
H. beccarii			√®	√®
H. lasiantha		√	√	
H. latifolia			√	
H. verticillata var hendersonii		√		
H. imperialis			√	

** Abundant

® Rare/ Harder to find

4. CONCLUSION

Information from this study is very important to determine the taxonomic revisions in terms of an updating the checklist of *Hoya* genus in Peninsular Malaysia. This research shall be continued by expanding the study sites and should be supported by molecular study. The molecular study are very significant in proving the recording of new species and may also shows variation in same species which resulted because of the environmental and ecological factors.

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Biodiversity & Conservation **BC-15**

Preliminary Results on the Taxonomy of the "*Barbodes binotatus* Species Complex" (Cyprinidae): First Record of *B. rhombeus* in Peninsular Malaysia

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ABSTRACT

The cyprinid freshwater fishes of the "*Barbodes binotatus* (Valenciennes, in Cuvier & Valenciennes 1842) species complex" comprise currently three valid species, *B. binotatus* and *B. banksi* (Herre 1940) that are distributed in Sundaland whereas *B. rhombeus* (Kottelat 2000) is endemic to Indochina. These species are difficult to identify from each other in absence of unambiguous diagnostic morphological character. This causes much confusion regarding their reciprocal validities and obscures their respective geographical distribution. The goal of this study is to determine whether a genetic approach can help to resolve the taxonomy of the "*Barbodes binotatus* species complex." We focus our study on Peninsular Malaysia where only *B. binotatus* and *B. banksi* have been recorded in lowland streams. We preliminary sampled24specimens of this complex from different localities in Peninsular Malaysia. For comparison, we have included specimens of each three species collected at, or nearby, their respective type locality. To estimate the genetic distinctiveness of each species, we sequenced the mitochondrial gene cytochrome *b*. Results reveal a cryptic pattern of genetic diversity within this group, with the presence of at least five lineages. For the first time, we show evidence for the presence of *B. rhombeus* in Peninsular Malaysia.

Keywords: freshwater fish; species complex; genetics; *Barbodes rhombeus*

1. INTRODUCTION

The genus *Barbodes* Bleeker 1859 (Teleostei; Cyprinidae) comprises of more than 40 species, small to medium size (5 to 10 cm) and distributed in Southeast Asia (Kottelat, 2013). Species of *Barbodes* were classified in the large, but ill-defined, genus *Puntius* Hamilton 1822 before Kottelat (2013) split this genus into different genera and resurrected *Barbodes* for species sharing similar body marking characteristics at juvenile and adult stages. In Peninsular Malaysia, four species of *Barbodes* [i.e. *Barbodes* cf. *binotatus*, *B.* cf. *banksi*, *B. dunckeri* (Ahl 1929) and *B. lateristriga* (Valenciennes, in Cuvier & Valenciennes 1842)] were commonly documented in lowland freshwater habitats (Ambak *et al.*, 2012). These fishes are locally known as "Ikan Tebal Sisik" for *B.* cf. *binotatus* and *B.* cf. *banksi*, and "Ikan Baguh" for *B. dunckeri* and *B. lateristriga*.

Some species of *Barbodes* are difficult to identify because of their overall morphological appearance and the presence of local populations with slightly different phenotypes. This is particularly true for one group of species which we refer to as the "*Barbodes binotatus* species complex" (Bariche, 1998) in which we include three valid species (Figure 1): *B. binotatus*, *B. banksi* and *B. rhombeus*. These three species share a similar pattern of colouration with the presence of a black dorsal mark. Whereas *B. binotatus* is recorded from almost everywhere in Sundaland, Kottelat (2000) suggested that the real *B. binotatus* is restricted to "Java (type locality), Bali, Lombok and highlands of Sumatra." *Barbodes rhombeus* (described from the Trat Province, Thailand) is endemic to Indochina, north to Isthmus of Kra. *Barbodes banksi* is known

from Sarawak (Type locality), Peninsular Malaysia and Sumatra. Regional genetic investigations in Sumatra and Java showed large cryptic diversity in this group (Roesma *et al*. 2018; Hutama *et al.* 2017). In this preliminary study, we assess the genetic differentiation of the "*Barbodes binotatus* species complex" in Peninsular Malaysia using the mitochondrial cytochrome *b* gene.

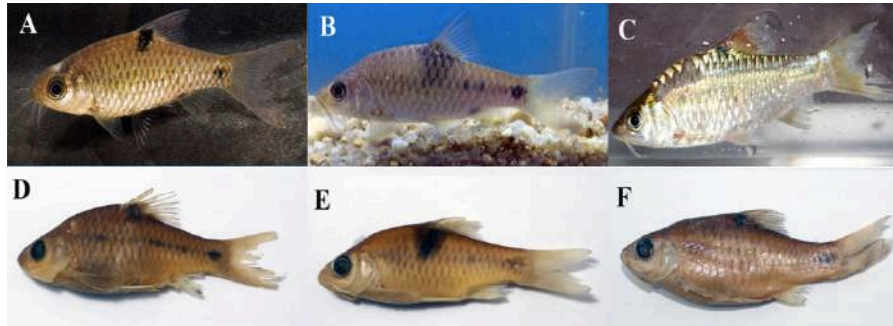

Figure 1. Photos of live (top) and preserved (bottom) specimens of the three species of the "*Barbodes binotatus* species complex" in Peninsular Malaysia: A) *B.* cf *binotatus* (not preserved; Standard Length [SL] 42 mm; from Selangor); B) *B.* cf *banksi* (USMFC 4314; SL 76 mm; from Selangor); C) *B. rhombeus* (USMFC 4300; SL 74 mm; from Kelantan); D) *B. cf binotatus* (UMKL 12132; SL 55 mm; from Pulau Pinang); E) *B. cf banksi* (UMKL 12096; SL 89 mm; from Johor) ;F) *B. rhombeus* (USMFC 4300; SL 74 mm; from Kelantan).

2. MATERIALS AND METHODS

2.1 Specimen sampling

Fresh specimens were collected from several river drainages in Peninsular Malaysia (Figure 2, Table 1) using a battery powered backpack electro-shocker and hand fishing net. Fin clips were taken from the fish and preserved in 95% alcohol and stored at room temperature. All specimens were fixed in 10% formalin for at least seven days, then soaked in water for another seven days before their transfers into 70% denatured ethyl alcohol. A total of 16 individuals of *B.* cf. *binotatus* and eight individuals of *B.* cf. *banksi* were examined in this study. Both species were initially identified by the size and shape of their black dorsal mark (Figure 1): in *B.* cf. *binotatus*, the mark is small, covering only one or few lateral scales and it does not reach the lateral line. In *B. banksi*, the mark is larger and form a triangular saddle blotch covering at least three lateral scales and often reaching the lateral line.

2.2 DNA Extraction, Amplification and Sequencing

Total genomic DNA were extracted from fin using a modified CTAB (Cetyl Trimethyl Ammonium Bromide) method. The cytochrome *b* (cyt*b*) gene was selected to estimate the phylogenetic structure because its genetic properties appear informative at this taxonomic level. The complete sequence of cyt*b* gene was amplified with the forward primer L14735Glu (5'- AAC CAC CGT TGT TAT TCA ACT A -3') and reverse primer H15973Pro (5'- TTG GGA GTT AGK GGT RRG AGT T -3'). PCR reaction were carried out in a total volume of 25 µl containing 1.0 µl genomic DNA, 3.5 µl 10X PCR Buffer, 2.5 µl 25 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.5 µl of each 5 µM primer and 0.2 µl of 5 U/µl i-Taq™ DNA Polymerase (NHK Bioscience Solutions Sdn. Bhd.). Only the forward direction of each PCR product was sequenced at "1st BASE Sequencing Service Sdn. Bhd." Sequences will be deposited in Genbank.

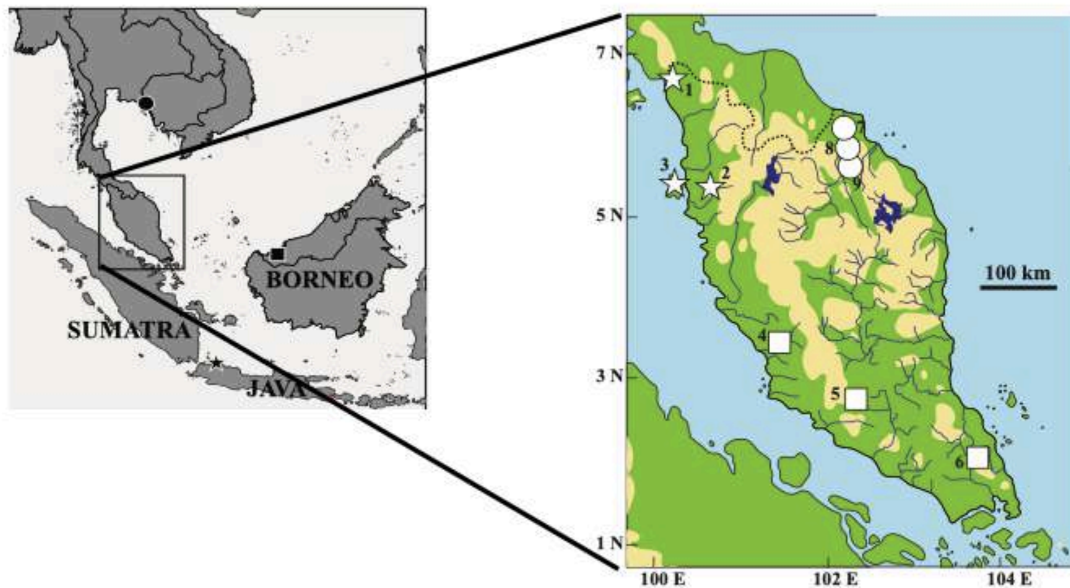

Figure 2. Map of Peninsular Malaysia on which is indicated the sampling localities

Table 1: List of specimens examined study along with their geographical origin. "*" localities not shown in Figure 2; "A" Initially identified as *B.* cf. *binotatus*

Species	Localities	No. of specimen	Specimen code
B. cf. *binotatus*	Small Stream to Wang Burma, Taman Negeri Perlis, Wang Kelian, Perlis (locality "1" in Figure 2)	3	PWBb1-3
	Tributary of Sungai Sedim, Sungai Riau, Kedah (2)	2	KSSb1-2
	Taman Rimba, Teluk Bahang, Pulau Pinang (3)	3	GTBb1-3
B. binotatus	**Timur, Indonesia***	**3**	**INDb1-3**
B. rhombeus[A]	Sungai Jedok, tributary of Sungai Golok, Kelantan (7)	3	DSJr1-3
	Hulu Jeram Perahu, tributary of Sungai Golok, Kelantan (8)	3	DJPr1-3
	Sungai Keding, tributary of Sungai Golok, Kelantan (9)	1	DSKr1
B. rhombeus	**Chantaburi, Thailand***	**1**	**THAr1**
B. cf. *banksi*	Sungai Gombak, Selangor (4)	3	WSGn1-3
	Sungai Langkap, Kampung Langkap, Kuala Pilah, Negeri Sembilan (5)	2	NSLn1-2
	Air Terjun Kota Tinggi, Johor (6)	3	JKTn1-3
B. banksi	**Sibu, Sarawak***	**2**	**SSBn1-2**
B. lateristriga	Air Terjun Kota Tinggi, Johor (6)	1	JKTg1

2.3 Sequence editing and Phylogenetic analysis

Chromatograms were edited using MEGA v7.0 (Kumar *et al*. 2016). Alignment was done using ClustalW v1.6 in Mega. A specimen of *B. lateristriga* was used as outgroup. Phylogenetic relationships were reconstructed using the Maximum Likelihood method under the model of sequence evolution "Hasegawa-Kishino-Yano with a gamma distribution of rates across sites" (HKY+G). One thousand bootstrap replications were conducted to assess node robustness.

3. RESULTS AND DISCUSSION

Phylogenetic tree (Figure 3) revealed five well supported (by Bootstrap values) lineages: 1) all specimens of *B.* cf. *binotatus* from northwest Peninsular Malaysia (PM), 2) all specimens of *B.* cf. *banksi* from west and south PM, 3) all specimens we initially identified as *B.* cf. *binotatus* from northeast PM along with the specimen of *B. rhombeus* from Thailand (THAr1). The last two lineages comprise specimens of *B. banksi* from Sarawak and specimens of *B. binotatus* from Timur, respectively. The relationships among these five lineages are not well supported; we only note that *B.* cf *binotatus* and *B. binotatus* are not closely related to each other; and *B.* cf *banksi* and *B. banksi* are also not closely related to each other.

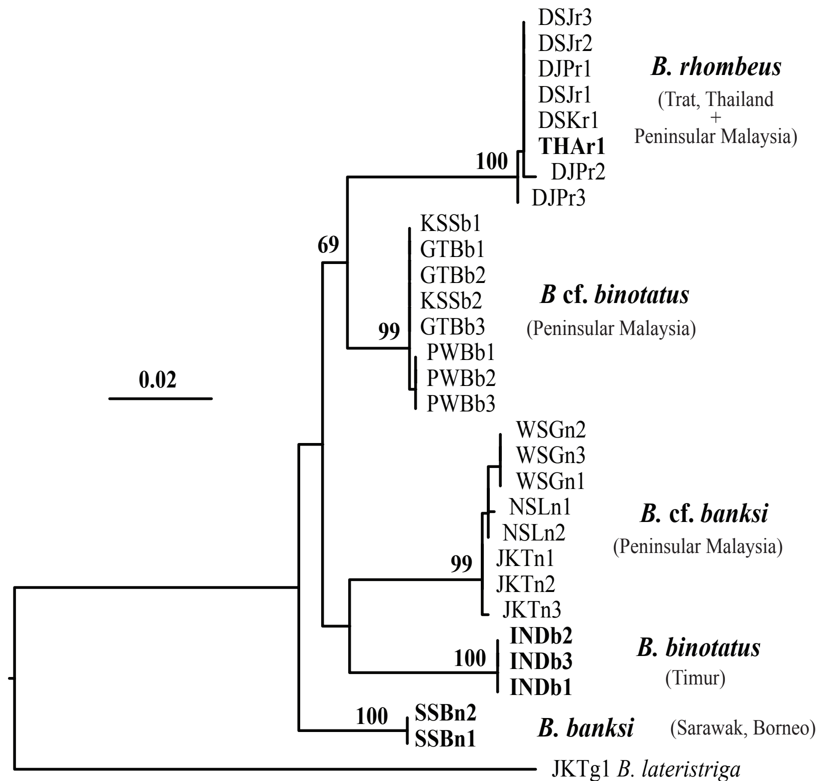

Figure 3: ML phylogenetic tree of the "*Barbodes binotatus* species complex" in Peninsular Malaysia. Numbers at node are Bootstrap percentage values (if >50%). This tree is rooted with the outgroup *B. lateristriga*

We found strong phylogenetic evidence that commonly identified specimens of *B.* cf *binotatus* in PM belong to two species. The specimens of *B.* cf. *binotatus* of northeast PM are genetically identical (or nearly identical) with one specimen of *B. rhombeus* from its type locality. Kottelat (2000) diagnosed (in part) *B. rhombeus* by the presence of 1) a small black spot immediately below dorsal fin origin, 2) a small black spot at caudal-fin base, 3) a faint longitudinally elongate blotch immediately behind upper extremity of gill opening, followed along body midline by a dark spot below dorsal and one or two above anal fin, 4) black crescent at the base of scales. A closer examination of our specimens of *B.* cf. *binotatus* of northwest PM reveals they share a similar marking pattern with *B. rhombeus*. This is, therefore, the first record of *B. rhombeus* in PM and this represents a significant range extension for this species, south of Isthmus of Kra.

Genetically, *B.* cf. *binotatus* and *B. rhombeus* are distinct lineages that are allopatrically distributed in PM. These two species, however, exhibited a lot of intra- and inter-population variability in their marking patterns which makes difficult to distinguish them. Furthermore, our phylogenetic results confirm the suggestion of Kottelat (2000) that the populations of *B.* cf

binotatus in PM are not conspecific to *B. binotatus* described from Java and possibly occurring in Timur, Lombok and Sumatra, as well. Similarly, our results indicate that *B.* cf *banksi* in PM is not conspecific of *B. banksi* described from Sarawak. Our study adds one line of evidence that there are several undescribed species under the "*Barbodes binotatus* species complex" (Bariche, 1998; Kottelat, 2000; Roesma *et al*. 2018; Hutama *et al.* 2017).

4. CONCLUSION

In this study, we demonstrate: 1) the presence of *B. rhombeus* in Peninsular Malaysia (PM) and 2) *B.* cf *banksi* and *B.* cf *binotatus* in PM are not conspecific to *B. banksi* in Sarawak and *B. binotatus* in Timur. A denser taxonomic sampling both within and outside PM along with more molecular and morphological characters are needed to reveal the identities of all species of *Barbodes* occurring in PM.

ACKNOWLEDGEMENT

We thank the School of Biological Sciences (USM) for the transportation to the sampling sites. We extend our appreciation to the Zoological Museum of the Institute of Biological Sciences (University of Malaya) and the School of Marine and Environmental Science (Universiti Malaysia Terengganu) for their permission to examine fish collection. We also thank to Department of Aquatic Science (UNIMAS) for their permission to conduct research on biological resources in Sarawak (Permit No. NCCD.907.4.4(JLD.12)-217. This study was funded by FRGS 203.PBIOLOGi.6711522 and MyBrain PhD.

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Biotechnology & Food Technology **BFT-01**

Meristem Culture of *Ficus carica* L. cv. Panachee

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ABSTRACT

Ficus carica L. is a deciduous fruiting plant and it is drought tolerant which has nutritional and economic values. It is commonly known as fig. *Ficus carica* L. cv. Panachee is chimera which gives mature yellow fruit with green stripes. Fig is commonly propagated through micropropagation technique due to its low survival rate through grafting and air-layering methods. Moreover, fig plant is easily infected by fig mosaic virus (FMV). Meristem culture which uses actively dividing meristem tissue is proved to be efficient to produce diseases-free plantlets. In this study, the effects of 6-Benzylaminopurine (BAP) with different concentrations will be determined based on the height of the shoot using meristem culture. After 8 weeks, it was confirmed that BAP at 30 μM was the best concentration which produced the height at 0.817 ± 0.257 cm.

Keywords: *Ficus carica*; meristem culture; BAP.

1. INTRODUCTION

Ficus carica L. is a deciduous subtropical flowering woody plant from the family Moraceae (Somashekhar *et al.* 2013). *Ficus carica* L. cv. Panachee is found as a chimera plant and it is commonly known as tiger fig. The fig is cultivated worldwide because it has nutritional value to humans as it is fat as well as cholesterol-free with excellent sources of vitamins, minerals, and carbohydrate. It is also used for medicinal purposes and Mawa *et al. (*2013) highlighted that the consumption of fig has medicinal effects on respiratory, gastrointestinal, inflammatory as well as cardiovascular disorders. It can also be used to treat constipation (Ali *et al.* 2012; Barolo *et al.* 2014). Sirisha *et al.* (2010) reviewed that the leaves of *F. carica* have pharmacological actions such as anti-ulcer, anti-diabetic and anti-fungal activities. It is susceptible to many diseases such as rust and fruit rot. *F. carica* cultivated in Malaysia was found to be difficult to be propagated through grafting and air-layering due to low survival rate and lack of specific pollinator in nature. Therefore, it is important to apply plant tissue culture technique on fig in agricultural prospects. By using plant tissue culture technology, *F. carica* L. cv. Panachee can be propagated as a disease-free plant at high survival rate in shorter period thus the markets on fig can be established for local supply. Micropropagation is an improved protocol for clonal propagation and a short-term conservation for *F. carica*.

2. MATERIALS AND METHODS

2.1 Plant materials

The plant sources were one-year-old *F. carica* L. cv. Panachee which were supplied by FigDirect Sdn Bhd., Kedah, Malaysia. The plants were planted near the building D32 Biotechnology Industrial Research Laboratory, Universiti Sains Malaysia. The plants were taken care under horticultural practices. Any diseased buds and leaves were cut off then discarded. Healthy apical buds were selected based on judgement sampling method and surface sterilized.

2.2. Culture conditions

The cultures were cultured in jars containing gelled Murashige and Skoog (MS) medium (Murashige & Skoog, 1962). Basal MS media were supplemented with various cytokinins (BAP, Kinetin, 2-iP) at different concentrations (0 as control, 10, 20, 30, and 40 µM). The pH of the media was adjusted to the range 5.7 - 5.8 (EUTECH Instrument pH 700 (pH/mV/ ºC/ F meter)) using 1 N Sodium hydroxide, NaOH or 1 N Hydrochloric acid, HCl. Gelling agent, 3 g/L Gelrite® was added prior to wet heat sterilization using autoclave (TOMY High Pressure Steam Sterilizer ES-315) at 121ºC for 15 minutes. The explants were incubated in plant tissue culture room under a 16 hours photoperiod with 16 hours of light and 8 hours of dark conditions (White LED Philips TLD, 36 W, 60 µmol m^{-2} s^{-1}) at 55 % humidity and 25 ºC ± 2 ºC.

2.3. Multiplication and maintenance of *in vitro* stock culture

Aseptic apical buds were transferred and incubated onto gelled MS media containing 30 µM BAP for shoots multiplication. Meanwhile, *in vitro* shoots differentiated from meristem tissues were transferred and incubated onto gelled MS media fortified with 30 µM BAP after eight weeks of incubation on liquid MS media. In shoots multiplication, newly formed shoots were excised and subcultured onto gelled MS media containing 30 µM BAP. These steps were repeating to maintain and multiply the *in vitro* plants of *F. carica* L. cv. Panachee which act as *in vitro* stock cultures for further experiments.

2.3.1. Effects of cytokinins

After two months of subculture from the stock, newly formed shoots were excised with 1 cm height were then transferred and incubated onto MS media supplemented with cytokinin (BAP, Kin, and 2-iP) in various concentrations. Control treatment is MS media without any plant growth regulators or other additional supplements. Cytokinins (BAP, Kin, and 2-iP) were added into media at 10, 20, 30, and 40 µM prior to wet heat sterilization using autoclave. Each jar containing five *in vitro* shoots incubated onto MS media supplemented with cytokinin at various concentrations for four weeks. After four weeks of incubation, their data and observations were recorded. Percentage of response for each treatment was calculated by using the following formula:

$$\text{Percentage of response, \%} = \frac{\text{Explant(s)that formed new shoot(s)}}{\text{Total number of explants tested}} \times 100\ \%$$

Increased in plant height of the aseptic apical buds was obtained by using the following formula:
Increased in plant height, cm = Final plant height – initial plant height (1.0 cm)

The number of shoots produced, the average shoot height produced, the presence of the callus formation and the types of callus were recorded. The average shoot height produced were calculated by using the formula as followed:

$$\text{Average shoot height produced, cm} = \frac{\text{Sum of the height of newly formed shoot(s)}}{\text{Total number of newly formed shoot(s)}}$$

The experiment was repeated ten times for each treatment.

3. RESULTS AND DISCUSSION

After 4 weeks of incubation, there was a significant difference among the growth parameters which were increased in explant height, number of shoots produced, and averaged shoot length produced (p-value of Wilks' Lambda = 0.352). For the mean increased in height, control treatment in the absence of PGR shown significantly lowest increment which was 1.03 ±

0.24 mm and meanwhile 40 µM Kinetin gave the largest increment at 9.90 ± 0.74 mm. Kinetin was observed to stimulate cell elongation thus giving larger increment of shoot height as compared to that of BAP and 2-iP. For the mean number of shoots produced, control treatment devoid of any PGR gave the least number which were 1.60 ± 0.31 while 30 µM BAP produced the largest number of shoots which was 7.23 ± 0.59. For the mean average shoot height produced, control treatment devoid of any PGR and 40 µM BAP shown significantly least height which was 1.08 ± 0.19 and 1.78 ± 0.11 mm respectively while 30 µM BAP shown significantly highest which were 3.89 ± 0.30 mm but no significantly different from 10 and, 20 µM BAP, 10 µM Kinetin, 10, 20 and, 30 µM 2-iP. The 2-iP at various concentrations was shown to induce shoots in the form of sucker rather than axillary shoots. The BAP at 30 µM is the best cytokinin as it is cost effective and induce highest number of shoots and axillary shoots and averaged shoot height produced as compared to BAP itself at other concentrations and other cytokinins tested at various concentrations.

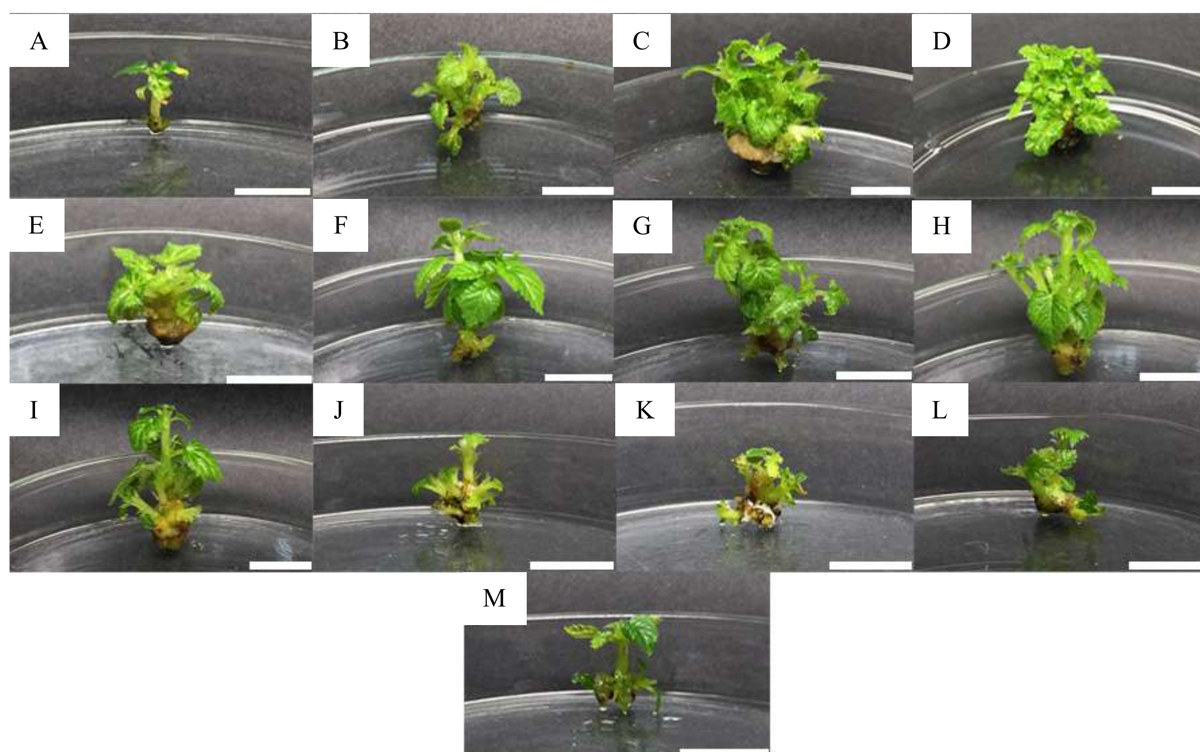

Figure 1. Shoot Multiplication. (A) Control, (B) 10 µM BAP, (C) 20 µM BAP, (D) 30 µM BAP, (E) 40 µM BAP, (F) 10 µM Kinetin, (G) 20 µM Kinetin, (H) 30 µM Kinetin, (I) 40 µM Kinetin, (J) 10 µM 2-iP, (K) 20 µM 2-iP, (L) 30 µM 2-iP, and (M) 40 µM 2-iP.

Table 1. Effects of Different Concentrations of Cytokinins On Mean Increased In Height, Number of Shoots Produced and Averaged Shoot Height Produced.

Hormone	Concentration (µM)	Mean increased in height, mm	Mean number of shoots produced	Mean averaged shoot height produced, mm
BAP	0 (Control)	1.03 ± 0.24^{a}	1.60 ± 0.31^{a}	1.08 ± 0.19^{a}
	10	6.17 ± 0.54^{de}	4.53 ± 0.41^{cdef}	3.26 ± 0.27^{bc}
	20	6.80 ± 0.70^{ef}	5.40 ± 0.66^{efg}	3.09 ± 0.38^{bc}
	30	7.93 ± 0.90^{fg}	7.23 ± 0.59^{h}	3.89 ± 0.30^{c}
	40	2.60 ± 0.26^{ab}	6.33 ± 0.53^{gh}	1.78 ± 0.11^{a}
Kinetin	0 (Control)	1.03 ± 0.24^{a}	1.60 ± 0.31^{a}	1.08 ± 0.19^{a}
	10	8.33 ± 0.61^{fgh}	3.00 ± 0.26^{abc}	3.43 ± 0.33^{bc}
	20	9.47 ± 0.65^{gh}	5.50 ± 0.67^{efg}	2.90 ± 0.22^{b}
	30	9.57 ± 0.77^{gh}	5.07 ± 0.77^{defg}	2.59 ± 0.32^{b}

	40	9.90 ± 0.74^{h}	5.77 ± 0.70^{fgh}	2.70 ± 0.23^{b}
2-iP	0 (Control)	1.03 ± 0.24^{a}	1.60 ± 0.31^{a}	1.08 ± 0.19^{a}
	10	2.60 ± 0.36^{ab}	3.97 ± 0.46^{bcde}	3.30 ± 0.28^{bc}
	20	4.57 ± 0.64^{cd}	3.87 ± 0.42^{bcde}	3.33 ± 0.20^{bc}
	30	2.47 ± 0.29^{ab}	3.60 ± 0.35^{bcd}	3.23 ± 0.19^{bc}
	40	3.40 ± 0.43^{bc}	2.80 ± 0.39^{ab}	2.90 ± 0.32^{b}

4. CONCLUSION

BAP at 30 µM concentration is the best cytokinin tested on *in vitro* shoots based on highest mean number of shoots and averaged shoot height produced.

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Biotechnology & Food Technology **BFT-02**

Isolation and Properties of Collagen From the Indonesian Local "Kacang" Goat Skin

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ABSTRACT

The objectives of the research was to isolate and characterize of pepsin soluble collagen (PSC) from Indonesia local goat which could be utilized for food or pharmaceutical. Indonesian local "Kacang" goat skin was obtained from local slaughter house and used as a new source of PSC. Isolation of collagen process was using 0.1% pepsin for 24 h at 38°C and extract was filtered and precipitated with NaCl at final concentration of 2.6 M. The pellet was collected by centrifuging at 7000 g for 30 min at 4°C and then re-dissolved in 0.5 M acetic acid. It finally were dialyzed against 0.1 M acetic acid for 24 h and distilled water sequentially. The properties of it were characterized by differential scanning calorimetry (DSC), fourier transform infrared spectroscopy (FT-IR), SDS-polyacrylamide gel electrophoresis (PAGE), and acid amino composition. DSC curve is showed with two thermal peaks (T_{max1} = 104.17°C and T_{max2} = 185.2°C). FT-IR spectra showed regions of amides A, B, I, II, and III were 3297.14, 2943.36, 1630.48, 1547.27, and 1238.81 cm^{-1}. SDS-PAGE patterns of protein profile showed that the molecular weight of the extracted collagen was 15 to 75 kDa and describing the molecular weight continuing increase after dialysis and lyophilized process. In conclusion the collagen isolated from the Indonesia local Goat skin have high temperature stable and more susceptible in the solution.

Keywords: Indonesia Local Goat Skin, Collagen, Isolation, Physical Characteristics

1. INTRODUCTION

Collagen is one of the important protein utilize in various functional food, medical, pharmaceutical and biodegradable packaging. Lee et al (2001) stated that collagen a well-known fibrillar protein obtained from connective tissues of animals, has received considerable attention because of its abundance and many important biological functions such as tissue formation and cell adhesion with excellent biodegradability and biocompatibility.

One of the frequent used of the type 1 collagen was manyfounded in skins from animal tissues. Study of collagen preparation showed the most used of skin from fish, bovine, and pig skin. Using collagen from bovine and pig skin has many carries potential risks such as some disease bovine spongiform encephalopathy (BSE) and religious problems (Jongjareonrak et al., 2005; Benjakul et al., 2007). Alternative of a raw material to produce collagen should be founded from different sources. Goat is one of the popular animal for the moslem country and was slaughtered millions head especially in the celebration day. Consequently the goat skin are available in millions annually and should be processed for valuable product. Based on the literature review, study of collagen extraction from goat skin was very limited. Therefore the first study needs to explore the collagen isolation and preparation using goat skin which has a high production in Indonesia.

Many previous research applied the establish methods of isolation collagen from skin are using chemical methods and enzymatic methods. Extraction of collagen using chemical methods with acetic acid has low produce yield, and some researcher was using enzymatic methods. One of the enzyme used in extraction of collagen is pepsin. Pepsin is enzyme can hydrolysis the non collagenous proteins and reduce of the antigenicity (Benjakul, et al., 2012; Regenstein and Zhou, 2007). Recently, Roy et al. (2017) applied the various times of heat and pepsin enzyme concentrations to extract gelatin from bovine heart collagen and describe their characteristics compare to other sources of gelatin, but there was no information study about collagen extraction and characterization from goat skin. Therefore, the objective of this study was to investigate the effects of initial incubation time and pepsin treatment during subsequent enzymatic digestion on collagen quality from local goat skin (GS) connective tissue.

2. MATERIALS AND METHODS

2.1 Extraction by pepsin soluble collagen

One hundred grams of skin was precisely weighed. The skins was extracted with 0.5 M acetic acid containing 0.1% pepsin for 24 at 38°C. The extract was filtered with Whatman No.1 paper. The collagen was precipitated by adding NaCl at final concentration of 2.6 M. The pellet was collected by centrifuging at 7000 g for 30 min at 4°C and then re-dissolved in 0.5 M acetic acid. The resulting solutions were dialyzed against 0.1 M acetic acid for 24 h with a change of solution once per 3 h, and finally, a change used distilled water sequentially. The collagen was obtained by freeze-drying.

2.2 Differential scanning calorimetry (DSC)

DSC studies were done using the DSC-60 Plus (Shimadzu) which was calibrated for temperature and enthalpy using indium as the standard.

2.3 Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra were obtained from 2 mg collagen in approximately 100 mg potassium bromide (K-Br) and analysed using a MBB3000 FT-IR spectrophotometer from 4000 to 1000 cm-1.

2.4. SDS-polyacrylamide gel electrophoresis (PAGE)

Electrophoresis patterns were measured according method of Laemmli (1970).

2.5 Scanning electron microscopy

The samples were coated with palladium for about 8 min with the sputtering system (JEOL. JEC-3000PC). The image was taken by using a scanning electron microscopy from JEOL (JSM-6510LA) under vacuum at 20 kV with a magnification of 100x and 1000x was applied.

3. RESULTS AND DISCUSSION

3.1 Differential scanning calorimetry

DSC curve of PSC from Indonesian local "Kacang" goat skin is shown in Figure 1 with two thermal peaks (T_{max1} = 104.17°C and T_{max2} = 185.2°C). Safandowska & Pietrucha (2013) report that the first value peak is related with temperature of thermal denaturation of collagen and second peak is destruction of materials of collagen which connected with continued conformation changes of superhelix structure. According to report study collagen from skin of Brama australis by Sionkowska et al. (2015), the first one peak (T_{max1} = 78.2°C) is related degradation of collagen triple helix structure and releasing of the water bound in collagen molecule. The second peak

(T_{max2} = 189.2°C) indicated the crosslinked collagen parts occuring of melting temperature. Kozlowska et al (2015) report that PSC from Nothern pike (Esox lucius) has peak of collagen T_{max1} = 80.1°C and T_{max2} = 203.1°C. The T_{max} values of collagen from Indonesian local "Kacang" goat skin was higher than that of fish collagen. Different thermal transition depend on raw material sources and method of collagen, amino acid, habitat and temperature species.

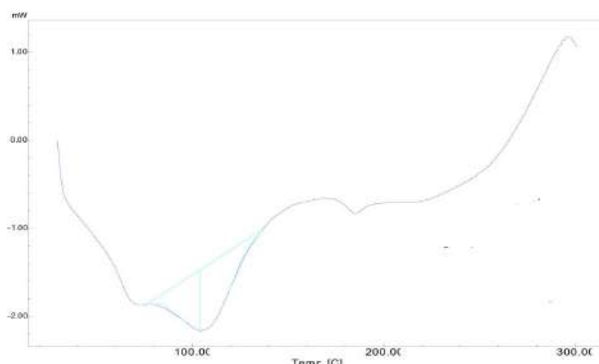


Figure 1. Differential scanning calorimetry PSC from Indonesian local "Kacang" goat skin

3.2. Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra PSC from Indonesian local "Kacang" goat skin is shown in Figure 2 with maximum of absorption peak was found at 3297.14 cm^{-1} (amide A of wave number range 3279 cm^{-1}) is related to N-H strecthing vibrations (Liu et al., 2007). Amide B and amide I peak was found at 2943.36 cm^{-1} and 1630.48 cm^{-1}. Amide B peak (~ 30796 cm-1) is related to C-H strecthing and amide I (1600-1660 cm^{-1}) is the most important factor investigating the secondary structure of protein with strecthing vibrations of C=O in polypeptide backbone of protein (Sionkowska et al. 2015). Amide II and III peak in this study was found 1547.27 cm^{-1} and 1238.81 cm^{-1}. Previous study reported that amide II has wave number range ~1548 cm-1 with N-H deformation and amide III (1235-1237 cm-1) resulting from C-N stretching and N-H in plane bending from amide linkages (Liu et al., 2007; Wang et al., 2008).

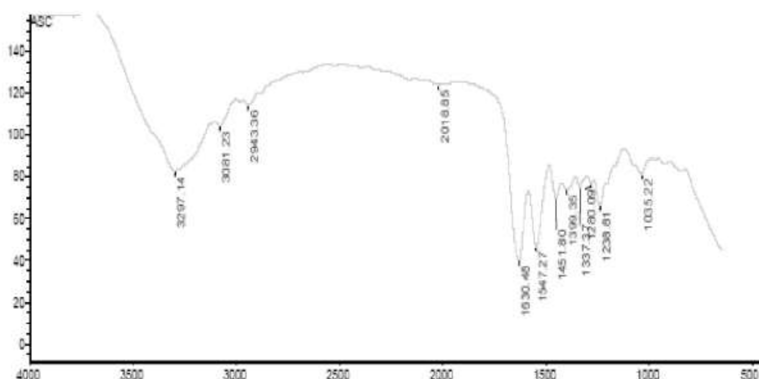


Fig. 2. FT-IR PSC from Indonesian local "Kacang" goat skin

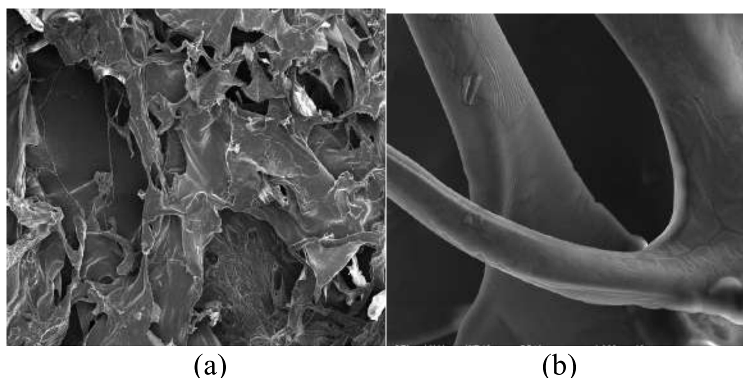
3.3 Electrophoretic pattern of PSC

Electrophoretic patterns during PSC extraction process from Indonesian local "Kacang" goat skin were measured by SDS-PAGE and data not shown. The protein pattern of each isolation process PSC had two α chain (α1 and α2), β, and γ-component. The α1 chain of each process has molecular weight range about 60 KDa and α2 chain about 30 KDa. PSC from Indonesian local "Kacang" goat skin has molecular weight lower than that of PSC from raw material others. PSC from Nothern pike (Esox lucius) had molecular weight about 118 KDa (α1) and 108 KDa (α2)

(Kozlowska, et al., 2015). . The similar study of PSC from grass carp (Ctenopharyngodon idella) had molecular weight approximately 120 KDa for α1 and 100 KDa for α2 (Liu et al. 2015). PSC in this study had smaller molecular weight because the extraction process at 38°C was higher than that extraction process of other raw materials. In low temperature, the collagen extract from goat skin become to a gel which difficult to filter process. PSC had significally lower proportions of crosslinked component and smaller molecular weight (Liu et al. 2015). Pepsin was remove of all the crosslinked region at the telopeptide of tropocollagen, β-, and γ-chain without the effecting structural integrity of super triple-helix chain (Kittiphattanabawon et al., 2010).

3.4 Scanning electron microscopy

The lyophilized of PSC from Indonesian local "Kacang" goat skin had a fibrous network with thinner collagen fibers and regular alveolate pores structures. Structure of PSC from Indonesian local "Kacang" goat skin can showed in this below Fig.4. In similar study of PSC from Spanish mackerel skin had also loose and endowed structure with uniform and the regular pores (Li et al. 2013). PSC from chicken skin had thinner collagen, pepsin enzyme in extraction of PSC is causes non-helical structure become to breakdown so thus the least fibril-like filament owing with large pore (Oechsle et al., 2016).



(a) (b)

Figure 3. SEM of PSC from Indonesian local "Kacang" goat skin.
(a) Scale bar = 100 μm x 100; (b) scale bar = 10 μm x 1000

4. CONCLUSION

The present study was the first to demonstrate that collagen extracted from goat skin using pepsin has characteristics comparable to other sources of collagen. FTIR data supported the conclusion that pepsin extractions may contain amide I, II and amide III and low MW collagen. Therefore, goat skin can be considered an alternative source of raw material for collagen extraction.

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Biotechnology & Food Technology **BFT-03**

The Effect of Organic Additives on Multiple Shoot Induction of Fig (*Ficus carica)* cv. Lisa

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ABSTRACT

Ficus carica is a plant native to the Middle East and Mediterranean regions, well known for the nutritious fruits that they produce. The Common Fig or "Tin" in Arabic, are popularly cultivated for figs rich in calcium, phenolic antioxidants and dietary fiber. The current study aims to establish an effective and reliable protocol for the micropropagation of *Ficus carica* cv. Lisa. Surface sterilization was performed on axillary shoot tip explants and optimized with the aseptic rate of 42.9%. Sterile axillary shoot tips were cultured in full MS medium supplemented with 2.0 mg/L Thidiazuron (TDZ) and 1g/L activated charcoal for maintenance as stock plant material. The shoot induction effect of organic additives coconut water and banana homogenate was evaluated. Stem segment explants were cultured in MS media supplemented with 2.0 mg/L TDZ and 0, 100 and 200 ml/L coconut water. Separately explants were also cultured in similar media supplemented but with 0, 20, 40 and 60 g/L of banana homogenate. After 6 weeks of culture, explants cultured in MS media without any supplementation of organic additive produced the highest induced shoot number and shoot length. In conclusion, MS media with 2.0 mg/L TDZ was identified as the optimal treatment for the micropropagation of *Ficus carica* cv. Lisa. Plantlets were successfully acclimatized after 12 weeks of culture with a survival rate of 70% for field adaptation.

Keywords: *Ficus carica axillary shoot tip; stem segment; organic additive; acclimatization*

1. INTRODUCTION

Fig plants or the Common fig have a scientific name of *Ficus carica*. Figs are not only well known for the fruits they produce but also for their religious significance. Figs are native to the Middle East and the Mediterranean region and have long been associated with the initiation of horticulture in the Mediterranean basin (Zohary & Hopf, 1988). *Ficus carica* are subdivided into four different groups where common figs are the only ones producing edible fresh figs. There are many cultivars of figs, one of which is the *Ficus carica* cv. Lisa which is native to Japan. They produce medium-sized amber toned figs that are mildly sweet. Currently, figs are still predominantly propagated through cuttings and air layering, producing low survival rate of only 20-30% due to poor rooting (Kumar *et al.*, 1998). Plant tissue culture is an efficient alternative in mass propagating plants at a higher rate under controlled conditions. Organic additives supplement plants with natural vitamins, phenols, fiber, hormones and proteins which boost plant growth (Gnasekaran *et al.*, 2010).There are many types of natural supplements used in plant tissue culture to increase the growth rate of plants such as coconut water and banana homogenate. The current study aims to evaluate the efficiency of the addition of organic additives in enhancing the induction of shoots for stem segment explant of *Ficus carica* cv. Lisa.

2. MATERIALS AND METHODS

2.1 Explant surface sterilization

Four protocols which were carried out to obtain aseptic axillary shoot tip explants. Axillary shoot tip explants were collected and rinsed with 2% Sunlight dishwashing liquid and Tween 20 under running tap water for 30 minutes. Explants were then agitated with different concentrations and exposure duration of Ethanol and Clorox© (NaOCl) followed by rinsing thrice with sterile distilled water. Explants were then dried on sterile filter paper, trimmed to approximately 1.5cm then established under *in vitro* condition in MS media containing 3g/L activated charcoal for a week under white fluorescent light with a photoperiod of 16 hours, light intensity of 2600 lm and at a temperature of 25±2°C.and were observed for contamination. Aseptic explants were then cultured in MS media with 2.0 mg/L TDZ and kept as stock for the subsequent experiments.

2.3 Multiple shoots induction via supplementation of banana homogenate in MS media

Stem segment explants obtained from stock plants were transferred into MS media containing 1 g/L activated charcoal, supplemented with 2.0 mg/L TDZ and homogenized banana at 0, 20, 40 and 60 g/L and cultured under similar conditions. Data were collected after six weeks and subjected to data analysis using one way variance (ANOVA) analysis and Duncan's Multiple Range Test at a significance of $p < 0.5$.

2.4 Acclimatization of plantlets

Explants in steps 2.2 and 2.3 were cultured for another 6 weeks before acclimatisation. The plantlets were removed from the semisolid media and roots were rinsed. Plantlets are grown in soil mixture of black soil and red soil at a ratio of 3:1. Acclimatized plants were transferred to the greenhouse.

3. RESULTS AND DISCUSSION

3.1 Establishment of aseptic cultures

The results for all four surface sterilisation protocols are shown in Table 1. In this study, it was observed that agitation in NaOCl twice was effective in reducing the rate of contamination as observed in protocol C but was ineffective when the concentration of NaOCl was increased

further. Therefore, it was clear that Protocol C was the best protocol to obtain the most sterile axillary shoot tip explants and was selected to be used to generate sterile explants for the subsequent experiments.

Table. The rate of survival of *Ficus carica* cv. Risa from different surface sterilization protocols.

Protocol		Rate of survival (%)
A	70% Ethanol (5 min), 30% NaOCl (10 min)	25.00
B	70% Ethanol (5 min), 50% NaOCl (10 min)	22.00
C	70% Ethanol (5 min), 30% NaOCl (10 min), 50% NaOCl (10 min)	42.86
D	70% Ethanol (5 min), 30% NaOCl (10 min), 60% NaOCl (10min)	14.29

3.2 Multiple shoots induction via supplementation of coconut water in MS media

The highest average number of shoots was produced by stem segment explants cultured in MS media without the supplementation of coconut water with a shoot number of 1.75 ± 0.25[a], significantly higher than explants in 200 ml/L coconut water (Table 2). Similarly, explants in coconut free media also gave the highest average shoot length of 0.87 ± 0.23[a] cm, significantly higher than explants in 200 ml/L coconut water with only 0.24 ± 0.02[b] cm (Table 2). In terms of callusing and rooting, explants in coconut free media has the highest callus induction rate while explants in 100 ml/L coconut water has the highest root induction rate.

These results however contradicts with Nasib *et al.,*(2008) whereby *Actinidia deliciosa* explants were found to give the highest shoot length, number of shoots and nodes in MS medium supplemented with 200 ml/L coconut water. The plantlets were also observed to be highly robust plants, increasing their survival in the green house conditions (Nasib *et al.*, 2008).

Table 2. The rate of shoot induction, average number of shoots and shoots length and percentage of callus & root induction of the stem segment explants treated with MS media with coconut water.

Coconut water (ml/L)	Rate of shoot induction (%)	Average number of shoots (N)	Average shoot length (cm)	Percentage of explants producing callus (%)	Percentage of explants producing roots (%)
0	100.00	1.75 ± 0.25[a]	0.87 ± 0.23[a]	50.00	25.00
100	100.00	1.33 ± 0.21[ab]	0.45 ± 0.22[ab]	16.67	50.00
200	36.00	1.00 ± 0.00[b]	0.24 ± 0.02[b]	0.00	0.00

*Columns with the same letter indicates that there was no significant difference between means (Duncan's test, p ≤ 0.05)

Figure 1. The induction of shoots from stem segment explants in MS media with different treatments of coconut water after six weeks of culture. **(A)** MS media supplemented with 2.0 mg/L TDZ, **(B)** MS media supplemented with 2.0 mg/L TDZ and 100 ml/L coconut water, **(C)** MS media supplemented with 2.0 mg/L TDZ and 200 ml/L coconut water. Scale bars represent 0.5cm.

3.3 The induction of multiple shoots from stem segment explants via banana homogenate supplementation of MS media.

The highest shoot induction rate was obtained by explants in MS media with 40 g/L banana homogenate while the lowest was explants in banana free MS media (Table 3). No significant difference observed in terms of average number of shoots between all treatments (Table 3). However, explants cultured in banana free media gave the highest average shoot length of 1.00 ± 0.08^a cm, significantly higher than all the other treatments (Table 3). In callus and root induction, explants in MS media with 20 g/L banana gave the highest rate of 80.0% and 40.0% respectively (Table 3).

Kaur & Bhutani (2012) reported that *Cymbidium pendulum* explants cultured in 50g/L banana homogenate produce the most optimal formation effect whereas in this experiment, 20g/L banana was found the most effective in inducing roots. It was also reported that high concentrations of banana homogenate can be detrimental for the survival of cultures (Kaur & Bhutani, 2012), as could be seen in this experiment where the number of shoots and shoot length reduced in cultures above 40 g/L banana.

Table 3. The rate of shoot induction, average number of shoots and shoots length and percentage of callus & root induction of the stem segment explants treated with MS media with banana homogenate.

Banana (g/L)	Rate of shoot induction (%)	Average number of shoots (N)	Average shoot length (cm)	Percentage of explants producing callus (%)	Percentage of explants producing roots (%)
0	72.73	1.50 ± 0.27^a	1.00 ± 0.08^a	63.64	9.10
20	80	1.00 ± 0.00^a	0.55 ± 0.09^{bc}	80.00	40.00
40	83.33	1.40 ± 0.24^a	0.68 ± 0.21^b	33.33	0.00
60	77.78	1.29 ± 0.18^a	0.29 ± 0.06^c	44.44	0.00

*Columns with the same letter indicates that there was no significant difference between means (Duncan's test, $p \leq 0.05$)

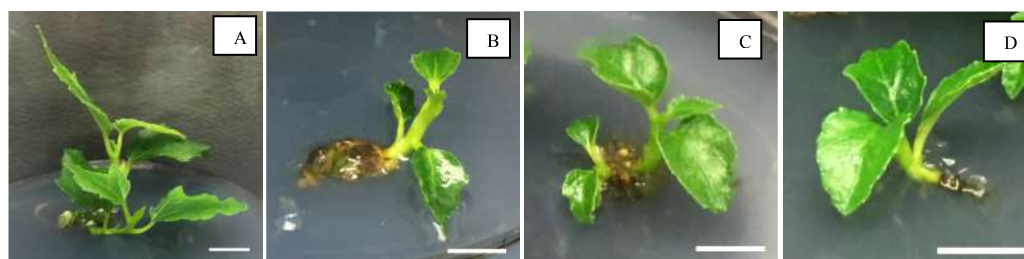

Figure 2. The induction of shoots from stem segment explants in MS media with different treatments of banana homogenate after six weeks of culture. **(A)** MS media supplemented with 2.0 mg/L TDZ, **(B)** MS media supplemented with 2.0 mg/L TDZ and 20 g/L banana, **(C)** MS media supplemented with 2.0 mg/L TDZ and 40 g/L banana & (D) MS media supplemented with 2.0 mg/L TDZ and 60 g/L banana. Scale bars represent 0.5cm.

3.4 Acclimatisation of *Ficus carica* cv. Lisa plantlets

Acclimatisation of *in vitro* plantlets was successfully carried out with a survival rate of 70.0%. It was observed that all acclimatized plants were capable of adapting to the greenhouse environment.

4. CONCLUSION

The best surface sterilization protocol optimized in this experiment was Protocol C with an explant survival rate of 42.9 %. Stem segment explants were successfully induced to produce multiple shoots in MS media supplemented with organic additives coconut water and banana. Based on data obtained, we concluded that MS media with 2.0 mg/LTDZ without organic additives produced the most optimal shoot number and length. Further studies include the evaluation of different cytokinins in increasing the rate of shoot induction.

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Biotechnology & Food Technology **BFT-04**

Induction of Multiple Shoots of Meyer Lemon (*Citrus X meyeri*)

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ABSTRACT

Meyer lemon or *Citrus x meyeri* is from the family of Rutaceae. It is a hybrid of true lemon and sweet orange (*Citrus limon* x *Citrus sinesis*) indigenous to China. This sweet flavored lemon is not only enriched with high amounts of vitamin C and organic acids, but also possess excellent source of antioxidants, anti-proliferative and anti-inflammatory properties. The current study aims to establish *in vitro* protocol to induce multiple shoots from shoot tip explants of Meyer lemon using different types of cytokinin. Surface sterilization was carried out on harvested shoot tip explants with agitation in 70% (v/v) of ethanol and in 18% (v/v) of commercial bleaching solution (Clorox®). Sterile shoot tips were inoculated on half strength Murashige and Skoog (MS) media supplemented with single treatments of cytokinins (Kinetin, Thidiazuron and Zeatin) at the concentrations of 0, 0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/L for 8 weeks. The optimal percentage of shoot induction and multiple shoot number was obtained on the single treatment of 0.1 mg/L of Zeatin with the values 83.3% and 1.60 ± 0.163, respectively. Future work involves combination treatments of plant growth regulators for shoot induction and rooting prior to acclimatization.

Keywords: *Citrus x meyeri, shoot tips, Kinetin, Thidiazuron, Zeatin, rooting*

1. INTRODUCTION

The Meyer lemon is one of the well known lemon hybrids from the Rutaceae family and genus *Citrus*. Meyer lemon or *Citrus x meyeri* surprisingly set itself apart from other lemon varieties and cultivars and gains very much popularity and recognition intercontinentally all due to its own exceptional traits. It is known to be a product from natural crossing between true lemon (*Citrus limon*) and sweet orange (*Citrus sinensis*) and also documented to be native from Beijing, China (Miyake *et al.* 2012). Morphologically, Meyer lemon appeared much more like oranges whereas the fruits are bigger sized, the skin and pulp are orange-yellow in colour, plentiful juice content due to bigger-sized pulp and thinner peel. Astonishingly, the juice and flesh of Meyer lemon are tasted considerably sweeter and less acidic which totally contrast to all lemons (Uckoo *et al.* 2015). Similarly to lemon fruits, Meyer lemon is also a great source of high amounts of organic acids (citric acid, ascorbic acid and malic acid) and numerous health-promoting phytochemicals such as amines, flavonoids and limonoids which play vital roles as antioxidant, anti-proliferative and anti-inflammatory agents against oxidative stress diseases for instance, cancer and cardiovascular diseases.

In Malaysia, lemons are usually imported from abroad and being sold at high price. Fortunately, Meyer lemon has been found to thrive in the local soils and is currently being propagated through grafting and cutting by the local growers. However, these conventional methods are not effective in mass producing of lemon plants for commercial scale planting. Plant tissue culture is an efficient alternative approach in mass propagating plants that are disease free and true-to-type within short period of time. The current study aims induce multiple shoots from

shoot tip explants of Meyer lemon using different types and concentrations of cytokinin namely Kinetin (Kn), Thidiazuron (TDZ) and Zeatin for micropropagation.

2. MATERIALS AND METHODS

2.1 Plant materials

Shoot tip explants of Meyer lemon were collected from Meyer lemon plants grown at the School of Biological Sciences, Universiti Sains Malaysia. The two-weeks-old shoot tips were harvested and thorns were removed prior to surface sterilization.

2.2 Surface sterilization

The shoot tips were immersed in 10% (v/v) of liquid detergent (Sunlight®) for 10-15 min with gentle brushing before washing under running tap water. The shoot tip explants were sterilized by agitation in 70% (v/v) of ethanol, followed by 18% (v/v) of commercial bleaching solution (Clorox® Regular-Bleach) for 6 min then rinsed with sterile, distilled water. Sterile explants were blot dry on filter paper and placed in media for multiple shoot regeneration.

2.3 Inoculation for multiple shoot induction

Shoot tip explants were excised into 2.5 cm in length and inoculated on half strength MS basal media (Murashige and Skoog, 1962) containing sucrose (20 g/L) supplemented with different concentrations of cytokinins such Kinetin (Kn), Thidiazuron (TDZ) and Zeatin (0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/L) for 8 weeks. Two explants were inoculated for each culture jars (80 mm x 90 mm) containing 30 mL of above mentioned MS media. The explants were maintained in culture room at 25±2 °C for 16h photoperiod and 8h dark regime and 50% of relative humidity.

2.4 Parameters and statistical analysis

After 8 weeks of culture, parameters such as percentage of shoot induction, average number of new shoots and average length of new shoots were evaluated. All data were subjected to One-Way Analysis of Variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) at the significance level of $p \leq 0.05$.

3. RESULTS AND DISCUSSION

In vitro culture or micropropagation was widely proven to be an effective modern technique to encourage mass propagation of various elite plants including ornamentals, timbers and fruit crops within shortened duration which able to reproduce genetically stable of the plantlets or offspring (Goswami *et al.* 2013). This approach is seen as ideal to overcome the limitation of most conventional propagation methods that are commonly time-consuming and restricted propagation for mass planting production (Pati *et al.* 2011).

After 8 weeks of inoculation, it was observed that 2.0 mg/L Zeatin produced the highest shoot induction percentage (91.67%), and followed by other Zeatin concentrations at the 0.1, 1.0 and 4.0 mg/L concentrations with the similar rates, 83.33% as indicated Figure 1. This study has proven that there was no significant difference between the control and both 0.1, 4.0 mg/L Zeatin concentrations concerning average number of new shoots with the values 1.63 ± 0.263 and 1.60 ± 0.163, respectively (Figure 2). However, control treatment exhibited less satisfactory shoot induction percentage as compared to the Zeatin concentrations after 8 weeks of culture. Therefore, 0.1 mg/L Zeatin was found the most optimal cytokinin concentration to induce multiple shoots from the shoot tip explants of Meyer lemon.

On the other hand, 0.5 mg/L Zeatin generated the highest length of new shoots, closely followed by 1.0 and 3.0 mg/L Zeatin with 1.25 ± 0.137 cm, 1.15 ± 0,157 cm and 1.00 ± 0.097 cm respectively (Figure 3). Based on the displayed figures below (Figures 1, 2 and 3), it can be concluded that Zeatin treatments exhibited better overall performance referring to all evaluated

parameters in comparison to Kinetin treatments. Highest percentage of root induction (47.4%) initiated from control treatments while no root formation was observed from the other cytokinin treatments.

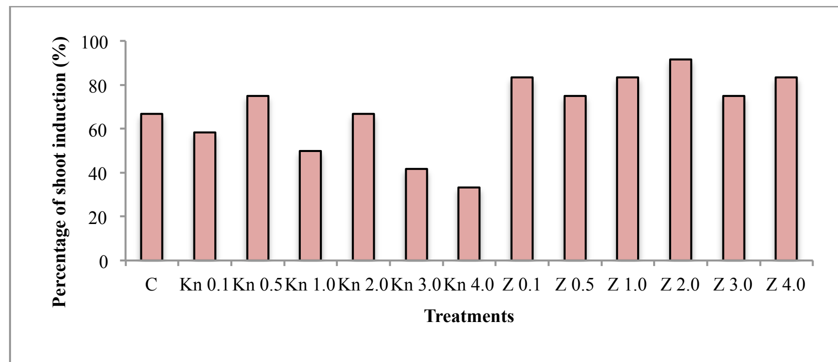

Figure 1. Percentage of Shoot Induction after 8 Weeks of Culture

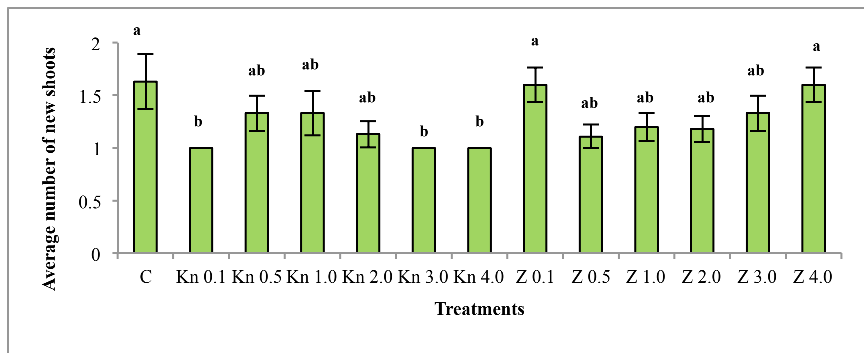

Figure 2. Average Number of New Shoots after 8 Weeks of Culture

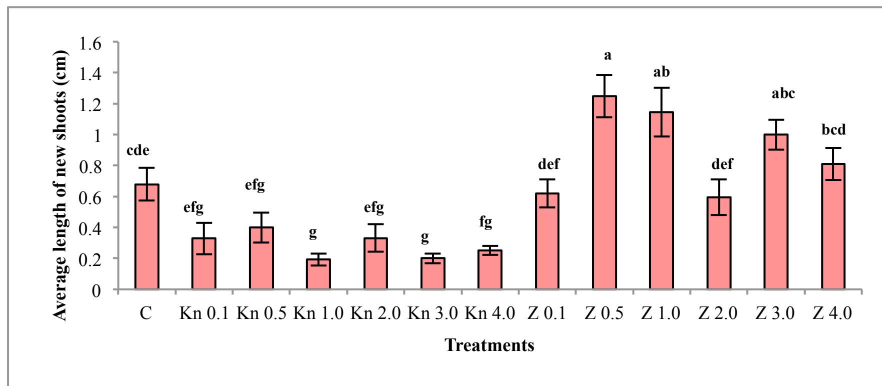

Figure 3. Average Length of New Shoots after 8 Weeks of Culture

The plants are able to grow, reproduce healthily and virus-free using the apical meristem. It is defined as new, undifferentiated tissue located at the microscopic shoot tip where the meristematic cells have yet to be assimilated with vascular system of the main plant, thus renders this part free from virus infection in diseased crops (Kleyn *et al.* 2013).

In contrast, Thidiazuron supplemented shoot tips cultures were noticed to form pale yellow, semi-friable, watery and sticky callus after 8 weeks inoculation. The callus was observed to be initiated specifically at the explant part which in contact with the media, as shown Figure 4. The degree of callus percentage was abundance as TDZ concentrations increased. Callus

response was greatly affected by both explant type and plant hormones uitilized (Osman *et al.* 2010).

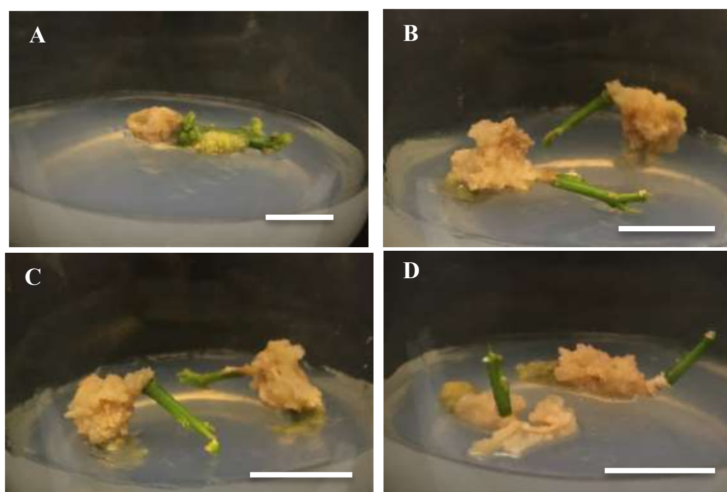

Figure 4. The Induction of Callus from Shoot Tip Explants of Meyer Lemon on Thidiazuron (TDZ) treatments. A) 1.0 mg/L B) 2.0 mg/L; C) 3.0 mg/L; D) 4.0 mg/L. (Scale bars represent 1.0 cm)

4. CONCLUSION

The results from data above have proved that supplementation of Zeatin is essential for optimal multiple shoot induction from shoot tips explants of Meyer lemon with the most optimal concentration at 0.1 mg/L. Future work involves the evaluation of combined treatments of cytokinin and auxin for better shoot induction rate followed by rooting media optimisation for further acclimatisation attempts.

ACKNOWLEDGEMENTS

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Biotechnology & Food Technology **BFT-05**

Entrapment of *Pseudomonas aeruginosa* USM-AR2 Cells Producing Rhamnolipid Cultivated in a Fluidized Bed Reactor

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ABSTRACT

A Gram negative bacterium, *Pseudomonas aeruginosa* USM-AR2 is capable of producing rhamnolipid, a biosurfactant that has found wide applications in many industries. Various bioprocess engineering approaches are conducted to enhance and sustain rhamnolipid production, including cell entrapment. Cell entrapment helps to improve the stability and reusability of the viable cells, thus increase cell tolerance to perturbations in fermentation conditions. This study aims to assess rhamnolipid production in *P. aeruginosa* USM-AR2 by entrapping the cells in polyvinyl alcohol-alginate hydrogel beads. The entrapment system was developed in shake flask cultures, agitated at 200 rpm by manipulating hydrogels composition and cell loading capacity. The ratio of 6:2:3 of polyvinyl alcohol: alginate: cell loading capacity is able to produce durable hydrogel beads by retaining the cell performance for 22 days in the shaken flask cultures. Rhamnolipid production by 1 g entrapped cells in repeated batch shaken flask culture was 3.0344 g/L. Further development was performed in a custom-designed fluidized bed reactor. Different fermentation modes such as repeated batch with and without broth recirculation and extended batch culture were carried out, and the best performance was achieved in repeated batch with broth recirculation at an aeration rate of 0.6 vvm producing 3.8352 g/L of rhamnolipid by 25 days.

Keywords: *Pseudomonas aeruginosa* USM-AR2, cell immobilization, repeated batch cultivation, fluidized bed reactor.

1. INTRODUCTION

Biosurfactants are amphiphilic substances produced by microorganisms, which are advantageous in industrial applications as they have significant structure diversity, antibiotic effects, and can be produced from renewable resources. Moreover, as described by Arrigoni-Martelli (1995), numerous of biosurfactants are unaffected by environmental factors such as ionic strength tolerance, pH and temperature. One of the mostly studied biosurfactant due to its wide industrial and environmental applications is rhamnolipid, which is predominantly produced by the opportunistic pathogenic bacterium, *Pseudomonas aeruginosa*. However, the cultivation of bacterial cells for rhamnolipid production has a drawback, that is heavy foaming. Foam fractionation may help in reducing heavy foam, but this results in a reduction of biomass concentration in the medium as cells are accumulated in the foam (Kosaric and Vardar-Sukan, 2015). Hence, this generates the importance of implementing an immobilized cells system, where cells are separated from the medium throughout the cultivation. Besides that, immobilization does not only provide physiological stability, but also reduce the product recovery cost, since the growth and product formation phase are separable (Desai and Banat 1997). A commonly applied techniques for cells immobilization is the cells entrapment, which encloses cells within a porous polymeric matrix that allows diffusion of substrates and products into and out respectively.

Therefore, in this study, an entrapment system for rhamnolipid production by *Pseudomonas aeruginosa* USM-AR2 is developed and applied in a custom designed fluidized bed reactor. Several parameters were studied, initially hydrogels composition, cell loading capacity, different cultivation mode and the reusability and stability of the entrapped cells for rhamnolipid production.

2. MATERIALS AND METHODS

2.1 Biomass Production in Shake Flask

Cells were precultured in nutrient broth overnight and inoculated into a complex medium containing 0.6% (w/v) yeast extract, 0.05% (w/v) $MgSO_4$ and 5% (v/v) waste cooking oil, cultivated for 90 h on a rotary shaker at 200 rpm at room temperature (25-28 °C) to reach stationary phase.

2.1 Cells Entrapment in Polyvinyl Alcohol-Alginate (PVA-Alginate) Hydrogels

Cells entrapment in PVA-Alginate was carried out following the method described by Van Pham and Bach (2014). Three parameters were screened for the rhamnolipid production by entrapped cells. 6-10% (w/v) polyvinyl alcohol was initially added to distilled water and heated to dissolve. Then, 0.5-2% (w/v) sodium alginate was added to the gel mixture. The solution was autoclaved at 121˚C for 15 min. Cells were harvested by centrifugation at 8000 x *g* for 10 min at room temperature, and 3-10% (w/w) cells were mixed with the gel solution. Hydrogels were formed through dropwise technique by using a peristaltic pump. Gel mixture was dropped into a saturated 7% (v/v) H_3BO_3 containing 2% (w/v) $CaCl_2$ to form hydrogels and immersed for an hour. Then, the hydrogels were rinsed with sterilized distilled water and immersed in 0.5M Na_2SO_4 solution for an hour.

2.2 Rhamnolipid Production in Shake Flask

Hydrogels were rinsed with sterilized distilled water prior to introduction to 100 mL minimal salt medium (MSM) containing 5.5% (w/v) $NaNO_3$, 0.5% (w/v) $MgSO_4.7H_2O$, 1.0% (w/v) KCl, 0.1% (v/v) trace elements, 0.1% (v/v) Tris-HCl, supplemented with 2.164% (v/v) waste cooking oil in 250 mL conical flasks. The entrapped cells were cultivated for 120 h, then rinsed thoroughly before introduced into a new fresh MSM. The steps were repeated until the entrapped cells showed a significant decrease in rhamnolipid productivity. The experiments were carried out in triplicates. Cultivation is terminated as cells in the hydrogels leaked into the medium. Cells leakage is determined by centrifugation of samples at 10 000 x g for 5 minutes to obtain cells pellet.

2.3 Design of Fluidized Bed Reactor

A glass column is utilized and connected with durable tubing to different components, which are air pump, flow meter, oxygen diffuser, peristaltic pump, sampling port and foam reservoir to work as a fluidized bed reactor.

2.4 Application of Entrapped Cells in Fluidized Bed Reactor

Cultivation of entrapped cells were carried out in fluidized bed reactor containing 100 mL MSM supplemented with 2.164% (v/v) waste cooking oil. Repeated batch with and without broth recirculation and extended batch cultivation modes were carried out and the performance of the entrapped cells in different cultivation modes were compared. Cultivation is terminated as cells in

the hydrogels leaked into the medium. Cells leakage is determined by centrifugation of samples at 10 000 x g for 5 minutes to obtain cells pellet.

2.5 Rhamnolipid Quantification: Orcinol Assay

Rhamnolipid production was quantified by performing orcinol assay, using rhamnose concentration as reference. 0.3 mL of diluted sample was added to 2.7 mL of 0.19% (w/v) orcinol in 53% H_2SO_4, incubated at 70˚C for 45 min, and cooled to room temperature. The optical density was measured at 421 nm using spectrophotometer (Hitachi UV-Vis Ratio Beam Spectrophotometer, Model U-1900). Rhamnolipid concentration was calculated and expressed as rhamnose equivalent (RE) in g/L.

2.6 Leftover Oil Quantification: Mass Loss Method

Waste cooking oil consumption was measured using mass loss method, which is gravimetric analysis. 2 mL of sample was mixed at ratio 1:1 with hexane and vortexed for 10 min to extract the oil. The oil layer was transferred onto a dried aluminium cups and incubated overnight in fume hood. The quantification was expressed in g/L and percentage.

3. RESULTS AND DISCUSSION

The formation of entrapped cells was performed with different concentrations of PVA and alginate, at differentcell loading capacities. The optimum hydrogels composition is important as it affects the mechanical strength of the hydrogels. Cell loading capacity refers to the wet cell weight per final hydrogels weight. Table 1 shows PVA concentration higher than 6% (w/v) resulted in non-uniform sizes of hydrogels, while alginate concentration below 2% (w/v) resulted in agglomeration of the hydrogels. Hydrogels composition for entrapped cells is 6% (w/v) PVA with 2% (w/v) alginate produced uniform sizes of hydrogels, and the optimal cell loading capacity was showed at 3% (w/w). A uniform hydrogels, stable and beneficial production of rhamnolipid by entrapped cells was achieved with these optimal concentrations. Therefore, further experiments apply this hydrogels composition.

Table 1. Screening of Hydrogels Composition for Cells Entrapment in PVA-Alginate

Polyvinyl Alcohol: Alginate (v/v)	Cell Loading Capacity (w/w)	Result
6: 0.5	-	Hydrogels agglomerated
6: 1	-	Hydrogels agglomerated
6: 2	3	Uniform sizes of hydrogels, cells leaked at 528 h of cultivation
6: 2	5	Uniform sizes of hydrogels, cells leaked at 48 h of cultivation
6: 2	10	Uniform sizes of hydrogels, cells leaked at 48 h of cultivation
8: 1	-	Hydrogels agglomerated
8: 2	3	Non-uniform sizes of hydrogels, cells leaked at 288 h of cultivation
8: 2	5	Hydrogels agglomerated
8: 2	10	Hydrogels agglomerated
10: 1	-	Hydrogels agglomerated
10: 2	3	Non-uniform sizes of hydrogels, clumped together at 48 h of cultivation

The entrapped cells were introduced into a fluidized bed reactor. As described by Dewi Rohayuh (2018), among different cultivation modes, which are repeated batch with and without broth recirculation, and extended batch cultivation, the highest rhamnolipid was produced in repeated batch with broth recirculation. Thus, the optimal entrapped cells applied the cultivation mode in fluidized bed reactor. The residence time for each cycle was determined based on the percentage of leftover oil. However, the leftover oil concentration remained stable after 5 days of cultivation, and after the third cycle, the result of leftover oil is no longer reliable as the values obtained fluctuated. This may be caused by the non-homogenize mixing of oil in the cultivation medium.

As shown in Figure 1, 1 g of entrapped *P. aeruginosa* USM-AR2 cells retained rhamnolipid production in shaken flasks for up to 528 h at 200 rpm, at 3.0344 g/L with productivity of 0.0057 g/L/h and the cells were eventually leaked at 528 h. Figure 2 showedRhamnolipid production by entrapped cells in fluidized bed reactor at 0.6 vvm aeration was 3.8352 g/L within 600 h with productivity of 0.0064 g/L/h. Based on these results, rhamnolipid productivity is slightly higher in fluidized bed reactor as compared to shaken flasks. In addition, the entrapped cells are more stable in fluidized bed reactor, where the cultivation period is longer, and no cells leakage was observed after 600 h of cultivation. Further repeated batch cultivations are fixed at 5 days of residence time for every cycle, due to unstable leftover oil measurement after this period.

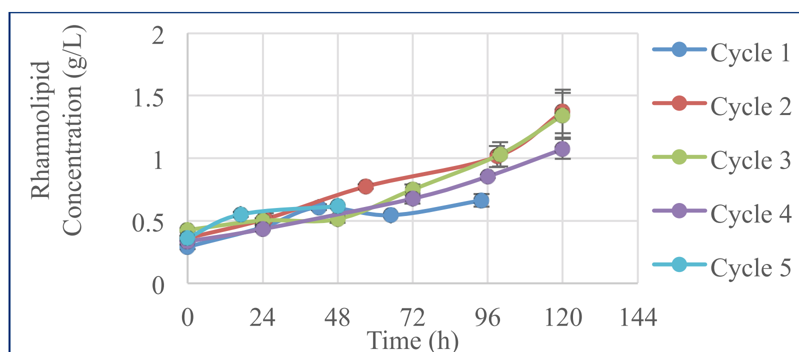

Figure 1. Rhamnolipid production by 3% (w/w) of *P. aeruginosa* USM-AR2 cells entrapped in 6% (w/v) PVA and 2% (w/v) alginate in shaken flasks

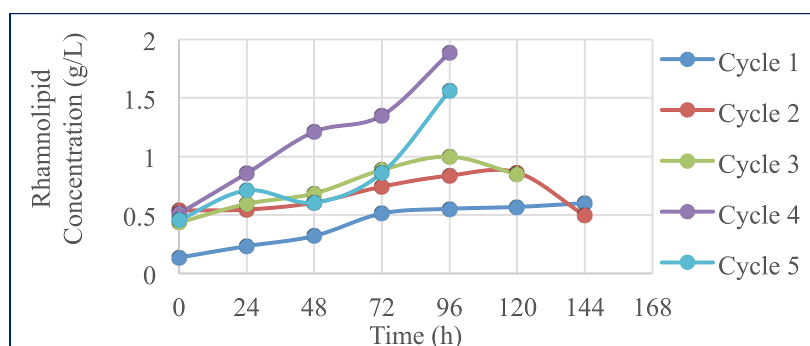

Figure 2. Rhamnolipid production by 3% (w/w) of *P. aeruginosa* USM-AR2 cells entrapped in 6% (w/v) PVA and 2% (w/v) alginate in fluidized bed reactor

4. CONCLUSION

The entrapment system for rhamnolipid production by *P. aeruginosa* USM-AR2 has been developed in a custom-designed fluidized bed reactor. The entrapped cells produced 3.8352 g/L of rhamnolipid with reusability of 5 cycles in 25 days in repeated batch with broth recirculation

mode. Work under progress include the determination of optimal beads to medium ratio, substrate characterization, continuous cultivation of entrapped cells and free fatty acid degradation by the entrapped cells.

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Agriculture & Agro-Industry **AAI-02**

Micropropagation of Fig (*Ficus Carica* L.cv. Violette De Solliès)

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ABSTRACT

The common fig or scientifically known as *Ficus carica* is a well-known source of nutrition that is beneficial to the human diet. It is mainly propagated via cuttings, grafting and air layering due to its non-viable seeds. However, these vegetative propagation methods have relatively low multiplication rates and are affected by plant diseases dissemination leading to loss in crop yield. The current study aims to mass propagate fig plantlets *Ficus carica* cv. Violette de Solliès using apical buds explants. Sterile explants were cultured on Murashige and Skoog medium (MS) supplemented with different concentrations of cytokinin, namely zeatin, kinetin and thidiazuron (1.0-6.0 mg/L). The highest shoot number (10.33±0.70) was observed on the MS medium supplemented with 6 mg/L zeatin. The overall results indicated that zeatin was more efficient than kinetin and thidiazuron in inducing new shoots, increasing shoot length, leaf number and plant height. Individual shoots were transferred to Woody Plant Medium (WPM) for rooting. After 6 weeks of cultivation, rooted plantlets obtained were acclimatized for further growth in local fields.

Keywords: growth regulators; shoot multiplication; zeatin; tissue culture

1. INTRODUCTION

Ficus carica, well known as common fig is one of the first cultivated plants in the world. It belongs to the Moraceae family and is native to Western Asia; which was then distributed throughout the Mediterranean basin through human migration (Crisosto *et al*. 2011). The fig fruit can be consumed either dried or fresh and contains higher nutrition in comparison to most of the common fruits, namely apples and oranges. Besides, it possess antioxidant, anticancer and antipyretic properties (Mawa *et al*. 2013). Conventional propagation of fig plants still rely on conventional methods such as cuttings, grafting and air layering which were less efficient in producing mass number of plant stocks . Plant tissue culture technology is an alternative in mass propagating plants in a consistent and efficient manner. Several studies have reported on the application of tissue culture technique on different fig cultivars namely Roxo de Valinhos (Fráguas *et al.* 2004), Sarilop (Hepaksoy & Aksoy 2006), and others but none of them reported on the cultivar of Violette de Solliès. The aim of the current study is to establish an efficient protocol for mass propagation of Violette de Solliès via shoot induction from apical bud explants.

2. MATERIALS AND METHODS

2.1 Surface sterilization

The young apical buds of *Ficus carica* cv. Violette de Solliès (VDS) were excised from mother plants grown at the School of Biological Sciences, Universiti Sains Malaysia. The

explants were washed with 2% Sunlight® dishwashing liquid, followed by running tap water for 1 hour. The explants were surface sterilized using 50% ethanol and 25% Clorox© containing three drops of Tween 20. The explants were then rinsed three times with sterile distilled water and dried on the sterile filter papers prior to culture.

2.1 Shoot Induction and Multiplication

Explants were excised into 2 cm long before cultured in the MS medium (Murashige and Skoog (1962) basal medium) supplemented with 4 mg/L BAP (6-Benzylaminopurine) for the establishment of *in vitro* explants. Newly induced shoots were separated and cultured in MS media with different concentrations of cytokinins, namely TDZ (thidiazuron), Kn (kinetin) and Zn (zeatin) at the concentration range from 1 to 6 mg/L (1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L, 5.0 mg/L and 6.0 mg/L). The average shoot number, length, and plant heights were collected and evaluated after 6 weeks of culture.

2.2 Culture conditions

All the media used containing 30 g/L sucrose and 8 g/L plant agar. The pH of all media was adjusted to 5.7 with 1M NaOH or 1M HCl and autoclaved at 121°C for 20 min. Cultures were maintained at a temperature of 25 ± 2ºC with 60-70 % relative humidity and provided a 16/8 h light/dark photoperiod under white LEDs (light-emitting diodes) light.

2.3 Rooting and Acclimatization

Individual shoots (2 - 3 cm long) were excised and transferred to WPM (Woody Plant Medium) for root induction. Rooted shoots were removed from the culture vessels and grown on BioChar soil for acclimatization. The plantlets were kept in the closed containers for 30 days and transferred to polybags containing a mixture of garden soil, coconut sheath and red soil in the ratio 3:1:1.

2.4 Statistical Analysis

All data for shoot induction, shoot length and plant height were analysed by one-way ANOVA using the SPSS Statistics 22, following by post-hoc test using Duncan's multiple range test (DMRT) ($P \leq 0.05$).

3. RESULTS AND DISCUSSION

Based on results shown in Table 1, explants grown in MS medium containing 6.0 mg/L zeatin scored the highest number of adventitious shoot (10.33±0.70) compared to control and other concentrations of cytokinins. Nevertheless, this was not significant to the treatments of 5.0 mg/L zeatin, 5.0 mg/L and 6.0 mg/L TDZ as those treatments also indicated high number of induced shoots. Treatments of zeatin also produced higher shoot elongation and adventitious shoot length as provided in Figure 1.

Results from this study indicated that zeatin was more effective on shoot development of VDS in comparison to kinetin and thidiazuron in terms of adventitious shoot number and length, plant height and leaf number. Similar findings were reported in other studies on other plant species such as *Drosera intermedia* (Rejthar *et al.* 2014). Studies also suggested the explants response to hormones provided could be influenced by concentration of PGRs, as well as the uptake, metabolism and transport of hormone inside the explant (Ling, *et al.* 2013). Individual shoots from all the treatments were rooted and acclimatized. The morphological characteristics of acclimatized plants were confirmed to be identical to the mother plant.

Table 1. Effect of Different Concentrations of Various Cytokinins on Shoot Development

Plant growth	Concentration	Adventitious	Adventitious	Plant height	Number

regulators (PGRs)	(mg/L)	shoot number	shoot length (cm)	(cm)	of leaves
Control	0.0	0.95±0.27^{a}	0.25±0.04^{a}	1.31±0.16^{a}	7.53±0.70^{a}
Kinetin	1.0	0.27±0.15^{a}	0.09±0.05^{a}	1.36±0.14^{a}	6.47±0.87^{a}
	2.0	0.67±0.40^{a}	0.15±0.07^{a}	1.57±0.30^{a}	7.73±1.56^{a}
	3.0	0.73±0.41^{a}	0.22±0.13^{ab}	1.28±0.16^{a}	8.07±1.49^{a}
	4.0	0.80±0.24^{a}	0.17±0.06^{a}	1.47±0.13^{a}	8.73±1.45^{a}
	5.0	8.20±1.12^{ef}	0.49±0.05^{bc}	2.91±1.37^{b}	19.47±2.75^{def}
	6.0	7.13±0.53^{de}	0.36±0.05^{bc}	1.32±0.05^{a}	16.27±1.63^{bcde}
Thidiazuron	1.0	3.53±0.50^{b}	0.31±0.03^{b}	1.39±0.10^{a}	11.27±1.62^{ab}
	2.0	4.53±0.58^{bc}	0.29±0.02^{ab}	1.47±0.12^{a}	12.40±1.55^{abc}
	3.0	4.87±0.44^{bc}	0.30±0.02^{ab}	1.67±0.10^{a}	13.00±1.48^{abcd}
	4.0	4.33±0.30^{bc}	0.31±0.03^{ab}	1.55±0.10^{a}	13.27±1.38^{abcd}
	5.0	9.33±0.87^{fg}	0.30±0.03^{ab}	1.37±0.08^{a}	7.53±0.89^{a}
	6.0	8.87±0.61^{efg}	0.24±0.02^{ab}	1.35±0.06^{a}	9.60±1.75^{ab}
Zeatin	1.0	3.60±0.32^{b}	1.53±0.21^{e}	2.23±0.23^{ab}	18.20±2.43^{cde}
	2.0	3.07±0.82^{b}	1.27±0.19^{de}	2.79±0.21^{b}	18.13±3.52^{cde}
	3.0	3.73±0.37^{b}	1.24±0.14^{d}	2.84±0.25^{b}	19.93±2.45^{ef}
	4.0	6.13±1.06^{cd}	1.47±0.11^{de}	2.93±0.22^{b}	32.87±4.13^{h}
	5.0	8.67±0.51^{efg}	0.64±0.05^{c}	1.53±0.12^{a}	24.73±1.61^{fg}
	6.0	10.33±0.70^{g}	0.66±0.06^{c}	1.57±0.10^{a}	28.80±2.38^{gh}

Mean followed by same letter in a block do not differ significantly at 5% level based on DMRT

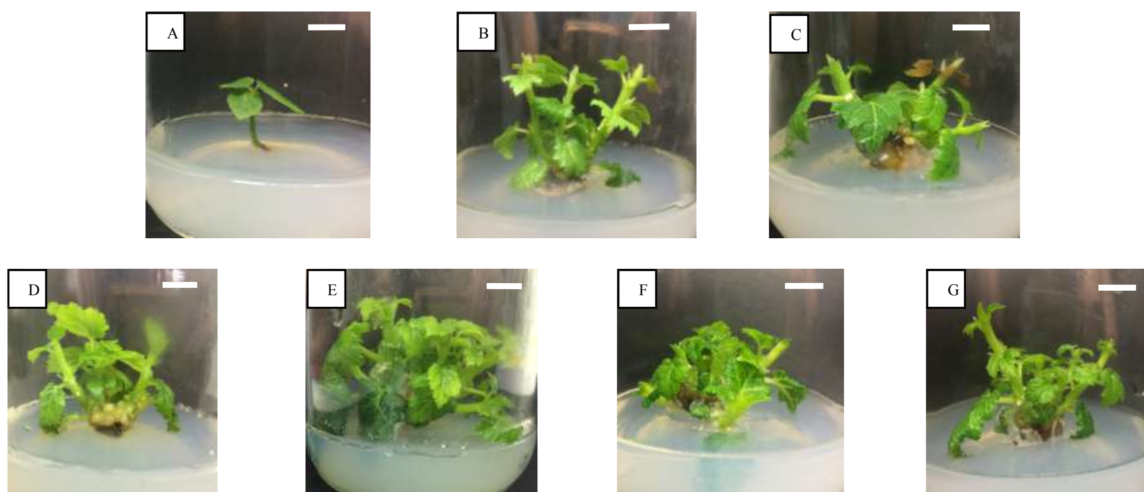

Figure 2. Shoot induction of Ficus carica cv. Violette de Solliès from apical bud explants cultured in MS media supplemented with different concentrations of zeatin (Zn) after 6 weeks of culture. A) control; B) 1.0 mg/L Zn; C) 2.0 mg/L Zn; D) 3.0 mg/L Zn; E) 4.0 mg/L Zn; F) 5.0 mg/L Zn; G) 6.0 mg/L Zn. (Scale bar = 1cm)

4. CONCLUSION

In conclusion, MS media supplemented with 6.0 mg/L of zeatin recorded the highest number of induced shoots from apical bud of *Ficus carica* cv. Violette de Solliès. In addition, overall results indicated zeatin was more efficient than kinetin and thidiazuron in the promoting shoot number and length, leaf number and plant height.

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Agriculture & Agro-Industry **AAI-03**

The Growth and Gonadal Development of Tropical Oyster, *Crassostrea iredalei* Cultured in Sungai Merbok, Kedah, Malaysia

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ABSTRACK

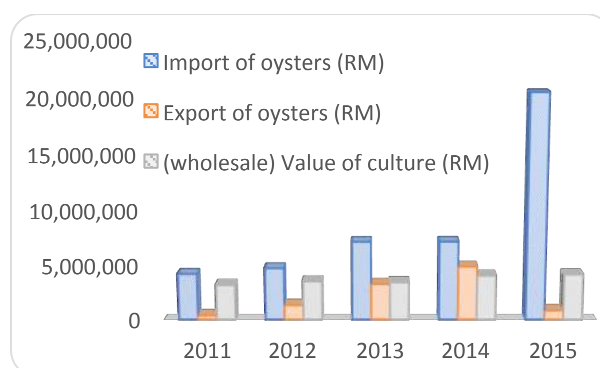
Oyster is a type of seafood that contains high levels of protein coupled with a great commercial value. Oyster aquaculture is a sustainable and green aquaculture. Therefore, most studies on oysters emphasize on its economic importance. The growth and gonadal development of tropical oyster, *Crassostrea iredalei* in the estuarine of Sungai Merbok located at the north of Peninsular Malaysia were studied. The shell length (SL), shell height (SH), shell thickness (ST), oyster weight (OW) and water parameters (pH, salinity, temperature, dissolved oxygen and chlorophyll a) were recorded twice a month. Gonadal development of *C. iredalei* was studied using histological method where samples were taken once a month. The growth increment of *C. iredalei* was 40.83mm (SL), 21.86mm (SH), 15.21mm (ST) and 41.83 (OW) with survival rate of 62% for 1 year. The first maturity (54%) was observed in September to October, around 5 months old spatwhile the peak spawning period can be observed during the period of October to December (35% developing, 59% spawning, 6% indeterminate). The growth of the oysters strongly correlated to the salinity and dissolved oxygen, while temperature, pH and chlorophyll *a* showed weak correlation.

Keywords: reproduction, survival, estuary, histology, water parameters

1. INTRODUCTION

In Malaysia, half shell oyster is considered luxury seafood which served in the fine dining restaurant. From table 1, It shows there is an increasing demand of oyster from the increasing import of oysters from 2011 to 2015. But, the oyster production in Malaysia remain low to meet the demand.

Table 1. Total Import, Export and Production Value of Oysters in Malaysia from 2011-2015



Reference: Annual Fisheries Statistics (2011-2015)

Among different species of oysters, *Crassostrea* species is one of the commercially important species in Malaysia, which known for its high potential for cultivation (Mohamad Yatim, 1993; Tan *et al*., 2014). Generally, oysters have high tolerance to fluctuating estuaries conditions and abundant spat fall, but oyster growth is sensitive to the salinity changes as it affects the oyster's feeding process (Taylor *et al*., 2004; Dickinson, 2012). This study aims to understand the growth and survival rate of *C. iredalei* in an estuarine environment to meet the increasing demand in the future.

2. MATERIALS AND METHODS

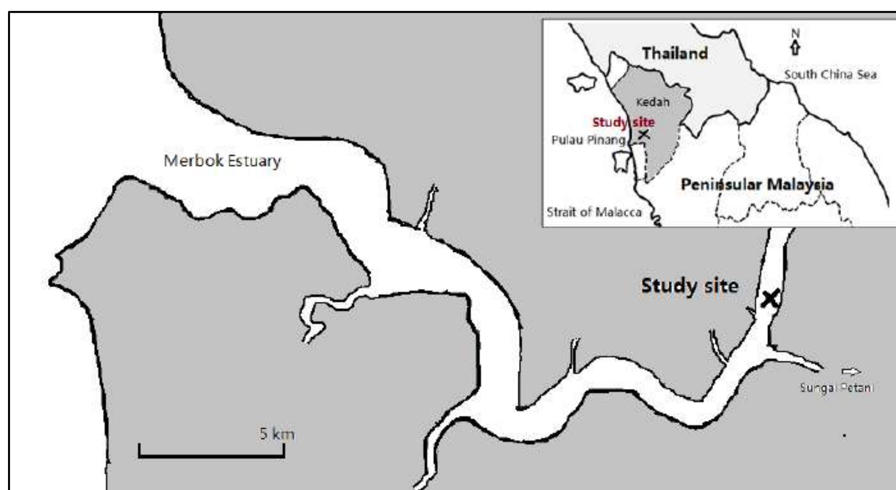


Figure 1. Location of Study Site

This ecological study was conducted at Sungai Merbok, an estuary in Kedah located in the north of Peninsular Malaysia (Figure 1) with longitude (E100°27'25. 2") and latitude (N 05°40'57.1"). The oyster farm at study site was using off bottom culture method, floating raft. The site is surrounded by mangrove forest and receive freshwater input from multiple small river from inland.

A total of 2000 oysters of *Crassostrea iredalei* were randomly chosen and purchased from the commercial oyster farm. Then, these oysters were transferred to the study site. Among the 1000 oysters, 800 of oysters were used for histology and 200 oysters for measurement of growth. The growth parameter was shell length (SL), shell height (SH), shell thickness (ST) and oyster weight (OW). While the remaining 1000 oysters is for monitoring the survival rate. The initial stocking density was 200 oysters spat per basket it was gradually reduce to 50 oysters per basket when the oyster spat grown. The initial spat used were 3-4 months old with initial average size of *C. iredalei* is 26.17±4.03mm (SL), 21.14±3.46mm (SH), 7.69±1.68mm (ST) and 1.77±0.61g (SW). In addition, water parameters included pH, salinity(ppt), temperature(°C), dissolved oxygen(mg/L) and chlorophyll *a*(mg/m^3). Growth and water parameters were recorded every two weeks. While, gonadal development of *C. iredalei* was studied using histological method where samples were taken once a month. Water parameters such as salinity (ppt), dissolved oxygen (mg/L), temperature (°C) and pH were recorded every two weeks. The study was conducted about 1-year duration (September 2014- September 2015).

3. RESULTS AND DISCUSSION

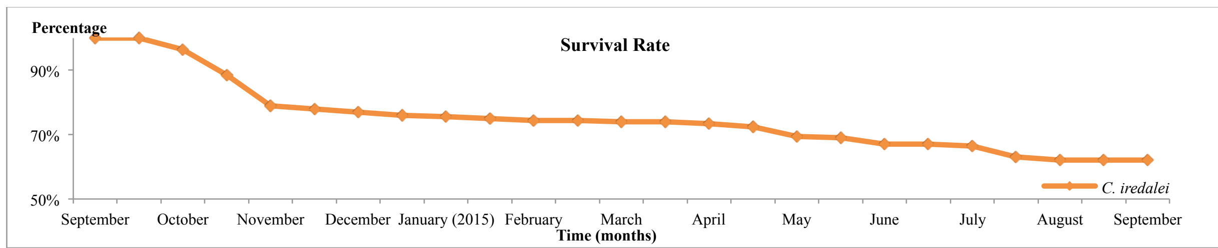


Figure 2. Survival Rate of *Crassostrea iredalei* Cultured at Sungai Merbok.

From the Figure 2, the result shown at early cultivation *C. iredalei* have a sharp drop of survival rate between first October (96.50%) and November (79.00%). While, the in-situ observation shown that the left-over death oyster shell is very thin, fragile and broken. After that, the survival rate of the oyster is remained fairly stable throughout the years. The final survival rate of *C. iredalei* is 62.00%. The environment and predator-prey could be the factors which cause the mortality of oyster. During that period, it is raining season and according to Mayowa *et al.,* (2015) maximum rainfall at that region occurred during the south west monsoon and the transitional period (June to November). In addition, the oyster's predator the crab is found in the basket mostly together with the empty oyster shell.

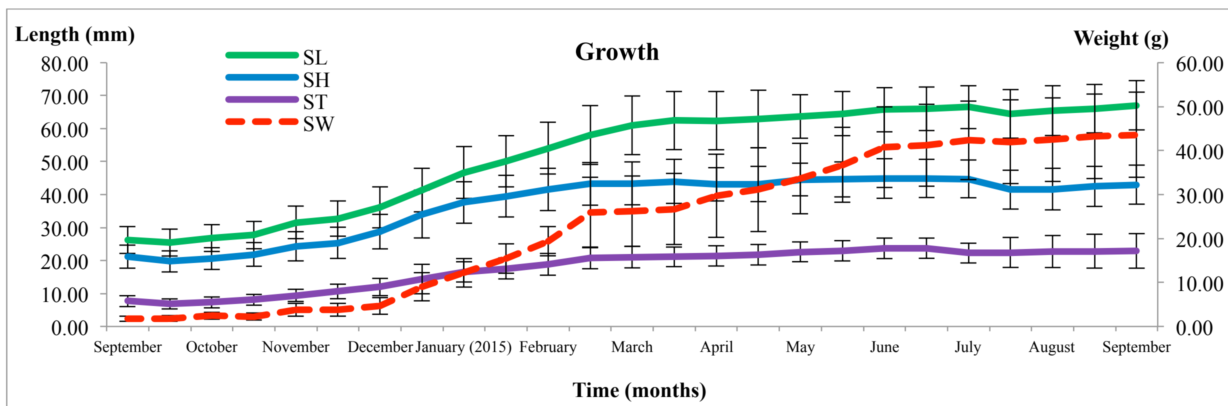


Figure 3. The Growth Morphology of *Crassostrea iredalei* Cultured in Merbok estuary

Figure 3 show there are 3 different stage of growth, which is slow growth during September 2014 to December 2014. Then, rapid growth from December 2014 to June 2015. Finally, slow growth from June 2015 to September 2015. Among the growth parameters, Oyster weight had dramatically increase around ten times between December 2014 to June 2015 (4.64g to 40.8g). the oyster weight increases steadily compare to shell length, shell height and shell thickness. It could be the energy been used more to develop the inner part of oyster thus increase more oyster weight (Dridi *et al.,* 2007). The final measurement at the end of study duration were 40.83mm (SL), 21.86mm (SH), 15.21mm (ST) and 41.83mm (OW).

Table 2. Correlation (r^2) between Environmental Parameters and Growth Parameters of *Crassostrea iredalei*

C. iredalei	Salinity	pH	Temperature	DO	Chlorophyll a
SL	0.66	-0.10	0.04	0.53	0.04
SH	0.43	-0.10	-0.02	0.68	-0.18
ST	0.50	0.18	-0.15	0.46	-0.09
OW	0.44	0.16	-0.13	0.30	-0.14

In this study, salinity showed the strongest positive correlation to oyster growth (r^2= 0.43-0.66), followed by DO (r^2= 0.30-0.68). While pH, temperature and chlorophyll a showed the least

correlation to oyster growth (Table 2). A study by Gosling (2003) shown the bivalve feeding rates reduced when exposed to changes in salinity, thus changing the growth rate which supports the results of this study.

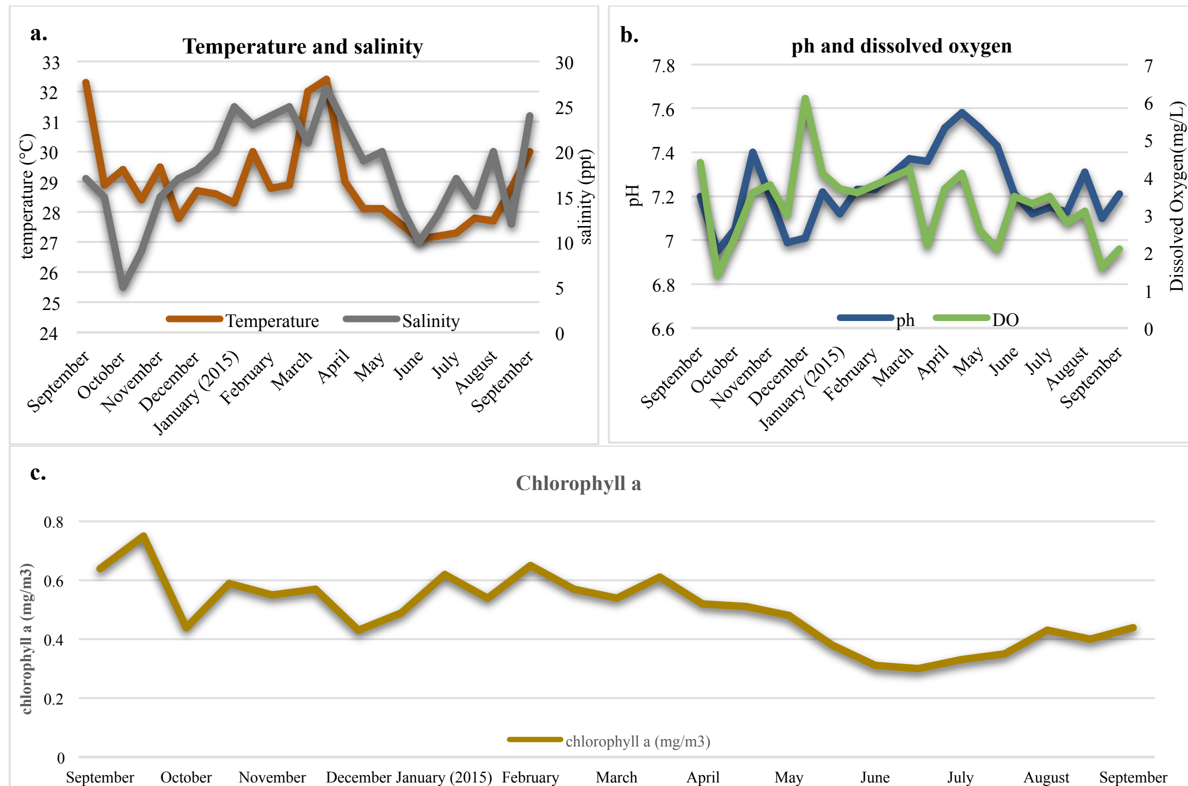


Figure 4. Water quality parameters at Sungai Merbok a. Temperature and salinity, b. ph and dissolved oxygen, c. Chlorophyll a.

Salinity of Sungai Merbok was having some fluctuation with minimum 5.00 ppt to maximum 27 ppt. On the other hand, the temperature value in Sungai Merbok stable and has little changes. It's ranged from 27.10 °C to 32.30 °C, with an average 29.70 °C. Besides that, pH at Sungai Merbok was ranged from 6.95 to 7.58 throughout the sampling. Dissolved oxygen value in Sungai Merbok ranged from 1.40 mg/L to 6.10 mg/L. The average DO in Sungai Merbok was 3.75 mg/L (Figure 4).

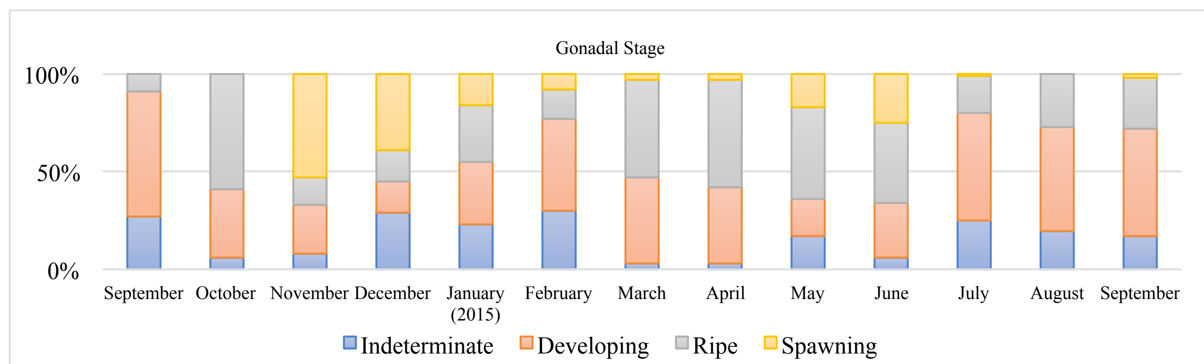


Figure 5. Distribution of Gonadal Stage in *Crassostrea iredalei*

The peak spawning period was observed during November to December (39%-53% spawning) which coincided with wet season in Peninsular Malaysia. In general, high mortality of oysters can be correlated to energy expenditure during spawning as well as the result of multiple

stressors like low salinity, low dissolved oxygen or pathogens which create physiological pressure (Patrick *et al.,* 2006).

4. CONCLUSION

C. iredalei have survival rate (62.00 %) and the final measurement at the end of study duration were 40.83mm (SL), 21.86mm (SH), 15.21mm (ST) and 41.83mm (OW). The marketable size for half shell oyster is around 80-100mm, it would need around 1.5 years to reach marketable size by this rate. In addition, the growth and survival rate can be greatly improving if avoid put small oysters spat during raining season. The results of this study show that at Sungai Merbok the main factors attributed to oyster growth were salinity followed by pH, temperature.

ACKNOWLEDGEMENT

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Appendix 1: The study site, located in Sungai Merbok, Kedah

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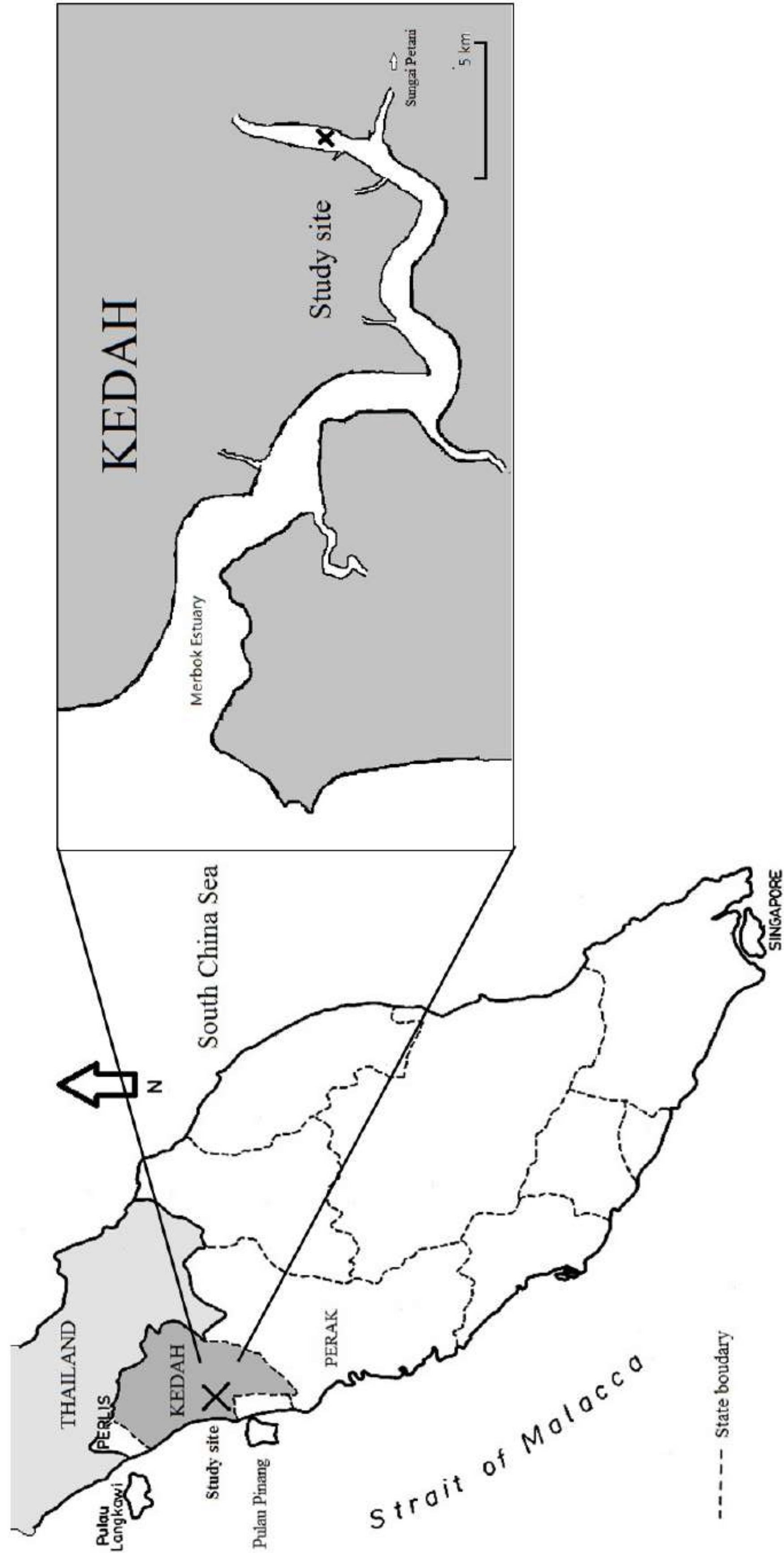

Agriculture & Agro-Industry **AAI-04**

Assesment of Responsiveness level of Ground Cover Plants Towards Arbuscular Mycorrhizal Fungi Colonizing Activity Through Fungi Infection Ability and Frequency of Plant Occurence

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) play an important role in supporting plant growth system through exploration and nutrient uptake especially phosphorus from soil solution to plant roots. The fungi have low plant specificity where most of plant families can be associated with AMF. However, previous study showed that the fungi colonizing activity was influenced by its preference on host plants. Therefore, this study was aimed to determine the responsiveness level of ground cover plants towards AMF through percentage of infection and frequency of occurrence of plant. The ground cover plants were collected across four sampling sites within USM main campus including PALAPES training area, Tasik Harapan, School of Biological Sciences and Minden field. Each sampling site was comprised of six quadrates laid in every 10 m within 50 m of line transect. The results revealed all 42 collected species of ground cover plants were colonized with AMF. Eleven shortlisted species had low and average responsiveness level. Four species of ground cover plants; *Asystasia gangetica, Mimosa pudica, Chrysopogon aciculatus* and *Axonopus compressus* had no significant differences of AMF infection. The finding of this study can provide advantages in selecting a preference AMF host plant especially in mass propagating AMF inoculums by functioning as AMF trap plant.

Keywords: Arbuscular mycorrhizal fungi; ground cover plant; responsiveness level, infection percentage; frequency of occurrence; trap plant

1. INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) were classified under the phylum Glomeromycota. The fungi are ubiquitous even in soil with poor condition (Entry *et al.* 2002) yet could form an association with plants (Gaur & Adholeya 2004). This obligate symbiont has low plant specificity where more than 80% angiosperm plant (Harrison 2005) and 92% plant families (Smith & Read 2008)

can be associated with AMF. This mutual symbiosis relationship representedthrough the bi-direction exchange of nutrients especially phosphorus (Smith *et al.* 2011) taken up by AMF and channelled into plant body whereas in return the fungi received carbon source from the host to proliferate and sustain its living.

A broad range of host plants lead to high possibility for mutual relationship to be established between AMF and plants. Certainly, both abiotic and biotic factors could influence AMF favourable environmental conditions in sustaining its living including host plant (Khara & Khakpour 2012). A study carried out by Torrecillas *et al*. (2012) showed that AMF colonizing activity on the roots of different annual herbaceous species was diverse with the presence of different AMF communities to the respected plants. In this study, assessment of wide range of host plant was done on ground cover plant. Ground cover plant is plant growing over an area of ground including grass and weed. Interestingly, many of ground cover plants were angiosperm and in Malaysia, 15 000 species flowering plants have been identified (NRE 2010). This study was designed to list the range of AMF host plant through collection of ground cover plants and to determine the responsiveness level of each plant through AMF infection and frequency of plant occurrence.

2. MATERIALS AND METHODS

2.1 Study Area and Sample Collection

The study area covering USM main campus situated on the Eastern Coast of Penang Island and 7 km from the city centre, Georgetown. The campus location is at latitude 5°21'14.99" N and longitude 100°18'3.00" E covering a total of 236 ha (Lee *et al.*, 2002). Sampling was done from May to November 2017. The ground cover plants were collected across four sampling sites including PALAPES training area, Tasik Harapan, School of Biological Sciences and Minden field. Each sampling site was comprised of six quadrates laid in every 10 m within 50 m of line transect. All plants species presented in the quadrate were uprooted and proceeded to lab for further root analysis.

2.2 Root Infection Percentage

Infection percentage of AMF was determined by gridline intersect method (McGonigle *et al*. 1990). Plant roots were cleaned using distilled water, cut into approximately 1 cm fragments and cleared with 10% potassium hydroxide (KOH) for 24 hours at room temperature. Extra measure was taken for thick and dark roots by increasing the oven temperature up to 60°C for 6 hours. The bleaching procedure was repeated twice. The KOH solution was discarded and the roots were neutralized with 1% hydrochloric acid (HCl) for 30 minutes and rinsed off with distilled water. Root samples were then stained with 0.05% Tryphan Blue for 24 hours prior rinsing and storing in lactoglycerol. A

total of 30 root fragments were laid on petri dish attached with gridline paper and observed under dissecting
microscope. Blue-stained of the fungi structures including hyphae, vesicle and spore presence at the gridline 1 cm × 1 cm was recorded as positive and otherwise. Root infection percentage was calculated by following formula:

$$\text{Root infection percentage (\%)} = \frac{\text{No. of gridline with positive root of AMF}}{\text{Total of gridline intersect with roots}} \times 100\%$$

2.3 Statistical Analysis

Statistical analysis was done using IBM SPSS Statistic 21 to determine the differences between the treatments by analysis of variance (ANOVA) and post hoc analysis was done using Tukey's Test at P<0.05.

3. RESULTS AND DISCUSSION

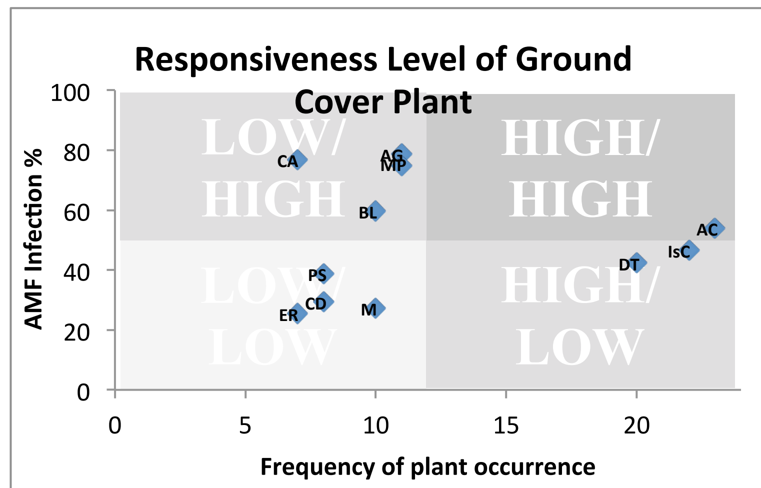

Figure 1. Responsiveness level of eleven ground cover plants accessed through AMF infection percentage and frequency of plant occurrence.

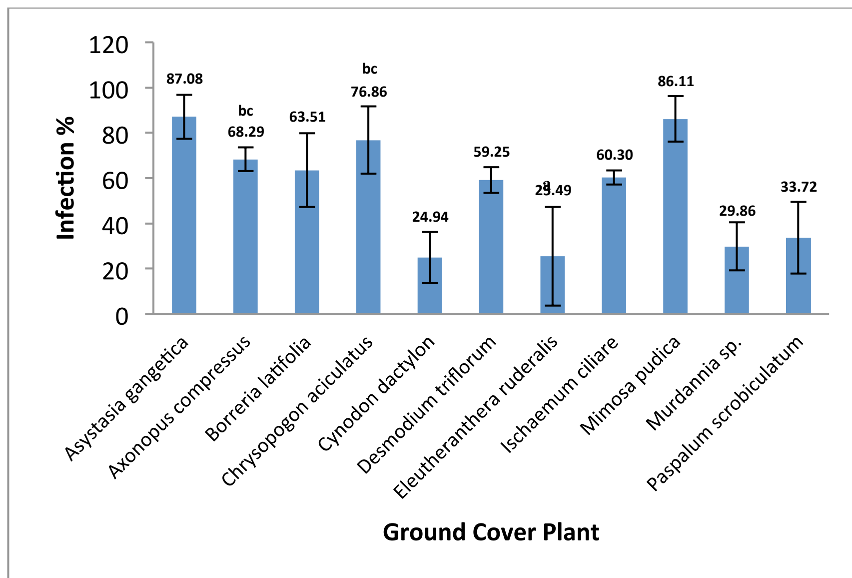

Figure 2. AMF Infection percentage of eleven ground cover plants from all sampling sites with synchronized sample size, n= 7. Bars represent mean ± SD, bar with different letter is significantly different at P<0.05.

Overall, 42 ground cover plant species were successfully collected across 4 sampling sites and all of the species were colonized with AMF (data not shown). Further, eleven species were shortlisted through elimination of species with low frequency of occurrence (less than seven) for the assessment of responsiveness level. Figure 1 shows the distribution of eleven ground cover plants species in responsiveness level graph accessed from their frequency of occurrence and percentage of AMF infection. Four species (ER= *Eleutheranthera ruderalis*, CD= *Cynodon dactylon*, PS= *Paspalum scrobiculatum* and M= *Murdannia sp.*) were classified in low level of responsiveness while the rest of the species were in average level.

Three species of ground cover plant; *Desmodium triflorum, Ischaemum ciliare* and *Axonopus compressuss*, were dominant in all sampling sites due to high frequency of plant occurrence. Despite being dominant, AMF infection percentage was average and this lead to average level of responsiveness. On the contrary, similar average level of responsiveness showed by *Asystasia gangetica, Mimosa pudica* and *Chrysopogon aciculatus* was due to high AMF infection albeit frequency of plant occurrence. As preference of host plant by AMF can be represented by high colonizing activity of that specific plants living in natural conditions, *Asystasia gangetica, Mimosa pudica* and *Chrysopogon aciculatus* can be attributed as preferred ground cover plants to be associated by AMF.

The AMF infection ranged from 24.94% to 87.08% where the lowest value was observed in *Cynodon dactylon* and the highest value represented by

Asystasia gangetica. Despite high AMF infection in *Asystasia gangetica,* the infection was not significantly different with *Mimosa pudica, Chrysopogon aciculatus* and *Axonopus compressus*. Therefore, these four species of ground cover plants can potentially be used as trapping plant for AMF propagation purpose.

4. CONCLUSION

In this study all 42 collected ground cover plants species were associated with AMF which can be reflected to the fungi broad range of host plant. Assessment of plant's responsiveness level revealed the shortlisted plant species was only average and low level of responsiveness towards AMF. However, amongst eleven selected plant species, four species had greater potential to be used as only efficient AMF trapping plant. In future work, the efficiency of the proposed ground cover plants for trapping and propagating AMF can be tested particularly in mass propagating AMF inoculums.

5. ACKNOWLEDGEMENT

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Agriculture & Agro Industry **AAI-05**

Preliminary Study on the Role of Soluble Silicon to Control *Rigidoporus microporus* in Rubber (*Hevea brasiliensis*)

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ABSTRACT

Currently, the rubber growers in Malaysia rely almost solely on soil drenching with Propiconazole (Tilt) fungicide to control *R. microporus* root rot. Alternative chemicals must be sought to prevent any potential resistance from emerging. The present study investigated the use of soluble sodium silicate, sodium metasilicate and calcium silicate for activity against *R. microporus* from Ayer Molek strain which caused the incidence of white root disease of rubber (*Hevea brasiliensis*). In-vitro dose-responses towards soluble sodium silicate, sodium metasilicate and calcium silicate were determined for *R. microporus*. The Poison Agar Technique was used for these tests. The initial pH of unamended agar was recorded at 5.86. Inhibition of mycelial growth was dose-dependant with 94.12 % inhibition at 7 ml (pH 10.58) and 10 ml (pH 10.90) Sodium silicate per litre of agar whereas 94.12 % inhibition at 7 ml (pH 10.94) and 10 g (pH 11.29) Sodium metasilicate per litre of agar for *R. microporus* tested with the exception of Calcium silicate which only inhibited 31.76 % of mycelial growth at 10 g (pH 8.30) per liter agar. Subsequent investigations into the effect of higher pH at 7, 8, 9, 10 and 11 of agar in the absence of soluble silicon, showed that *R. microporus* (Ayer Molek strain) was growing at full capacity (85.00 mm) in all of agar plates tested after 10 days incubated. Clearly, soluble silicon had an inhibitory effect on fungal growth in vitro and this was mostly fungicidal.

Keywords: *Hevea brasiliensis*, *R. microporus*, Silicon, Inhibition

1. INTRODUCTION

According to Jayasuriya and Thennakoon (2007) white root disease is the most devastating root disease of *Hevea brasiliensis* (Willd. Ex Adr. & Juss) Mull. Arg in most of the rubber producing countries. In Malaysia, survey of rubber diseases conducted in 2012 revealed that the incidence of white root disease was occurred in 10-15% of area in Peninsular Malaysia, 20 – 30% of area in Sabah and 9 – 20% of area in Sarawak out of the total planting rubber area in Malaysia amounting of 1,065,630 hectares (Atan, 2015). Rubber industries worldwide have confronted a significant reduction in economic return since the infection of white root disease has killed rubber trees irrespective of age (Soytong & Kaewchai, 2014). Generally, white root disease poses a threat problem for the first few years after planting (Omorusi *et al.* 2014). Moreover, white root disease has caused more tree losses compared to either red root disease or brown root disease especially from the first to the fourth year after planting. The application of Sulphur as one of the common preventive methods of white root disease during the early stage of rubber cultivation has been adopted widely in rubber growing countries (Vimaladevi Satchuthananthavale, 1971). In addition, drenching with Propiconazole (Tilt) fungicide was claimed as an effective method to control *R. microporus* based on the simple and fast procedures required (Hashim & Chew, 1997). However, the incidence of white root disease still occurs massively and further investigation needs to be carried out. Apparently, there has not been done any research on the ability of Silicon (Si) to inhibit *R. microporus* in vitro as well as enhance resistance in *Hevea brasiliensis* in vivo. Indeed, Silicon (Si) treatment has shown a great impact in controlling plant diseases (Tubana, Babu, & Datnoff, 2016). According to Carneiro-carvalho *et al.* (2017), Silicon (Si) can improve resistance to diseases and insects by increasing cell 'toughness' and makes it more difficult for fungi and insects to puncture plant cells. Besides that, in vitro test had revealed the effectiveness of silicon (liquid Potassium silicate) in reducing hyphal growth of rice blast fungus on agar plates (Maekawa *et al*. 2003). This result was supported by Kaiser *et al.* (2005) who reported that 100 percent inhibition of mycelial growth occurred on several types of phytopathogenic fungi at 40 ml and 80 ml of soluble silicon (20.7% silicon dioxide) per liter of agar media. Thus, if the Silicon (Si) supplementation proves to be a viable alternative treatment for *R. microporus*, Silicon (Si) must be studied further to identify the multiple modes of its action and potential in order to solve the problem of white root disease. Therefore, the present study has been designed to examine the ability of Silicon (Si) to suppress white root disease of rubber, *R. microporus* in vitro.

2. MATERIALS AND METHODS

2.1Strain

Strain of *R. microporus* used in this study was Ayer Molek (AM) and the locality of collection was from Ayer Molek, Melaka.

2.2 Agar Preparation

Three types of soluble silicon were used in this study known as Sodium silicate, Sodium metasilicate and Calcium silicate. Each of soluble silicon was screened against *R. microporus* using Poison Agar Technique. Soluble silicon were incorporated into potato dextrose agar (PDA) to achieve the desired concentration of 500, 1000, 2000, 4000, 7000 and 10000 ppm. Agar solutions were mixed with magnetic stirrers to ensure even distribution of silicon and then autoclaved. PDA without soluble silicon served as a negative control. pH of the prepared medium were recorded. Then, the agar solutions were decanted into Petri dishes and incubated for two days to ensure no contamination had taken place.

2.3 Antifungal Activity Assay

Five mm diametric mycelial plug of 7 days old culture of *R. microporus* was placed at the centre of the plate and incubated at 25 °C for 7 days. Inhibition percentage of radial growth (PIRG) was calculated using the following formula:

PIRG (%) = (1- (fungal growth/control growth)) x 100%

Percentage inhibition was calculated and an analysis of variance for the different treatments was conducted using XLAT (Table 1 and Figure 1).

3. RESULTS AND DISCUSSION

At the concentrations of 7 ml/g and 10 ml/g.l $^{-1}$ PDA, soluble Sodium silicate and Sodium metasilicate had inhibited 94.12 % of *R. microporus* (Tables 1 & Figure 1), with the exception of Calcium silicate (31.76% inhibition) at the concentration of 10 g.l^{-1} PDA (Experiment 1) (Tables 2 & 3). The result showed the ability of soluble Sodium silicate and Sodium metasilicate in inhibiting almost 100% growth of *R. microporus* at the concentration more than 7 ml/g.l^{-1} whereas Calcium silicate was less effective in suppressing the growth of *R. microporus* albeit at the higher concentrations rate. Presumably, silicon incorporated into the PDA had increased the pH which could be one of the inhibition factors of *R. microporus*. Thus, second experiment (Experiment 2) had been carried out to investigate the growth of *R. microporus* at different pH (7, 8, 9, 10) in ameliorated PDA while pH 5.88 served as a negative control. The results obtained revealing that higher pH of PDA did not affect the growth of *R.*

microporus where the strain used in this test had growth 100% (85.00 mm) in all agar plates (Table 2). Based on these results, early prediction can be made where soluble silicon had an inhibitory effect on fungal growth in vitro and this was mostly fungicidal.

Table 1. Mean Inhibition Of *R. microporus* At Different Silicon Types And Concentrations (Experiment 1) In Ameliorated Potato Dextrose Agar.

Silicate* (g/l) or (ml/l)	Sodium silicate (% Inhibition)	pH	Sodium metasilicate (% Inhibition)	pH	Calcium silicate (% Inhibition)	pH
10	94.118 a	10.90	94.118 a	11.29	31.762 a	8.30
7	94.118 a	10.58	94.118 a	10.94	0.000 b	8.16
4	89.362 b	10.24	82.274 b	10.35	0.000 b	7.77
2	20.322 c	9.58	10.275 c	9.43	0.000 b	7.58
0	0.000 d	5.86	0.000 d	5.86	0.000 b	5.86
0.5	0.000 d	8.19	0.000 d	7.61	0.000 b	6.53
1	0.000 d	8.94	0.000 d	8.47	0.000 b	7.18
Pr > F(Model)	< 0.0001		< 0.0001		< 0.0001	
Significant	Yes		Yes		Yes	

* The SI unit of amount of Sodium silicate is in ml/l whereas Sodium metasilicate and Calcium silicate is in g/l.

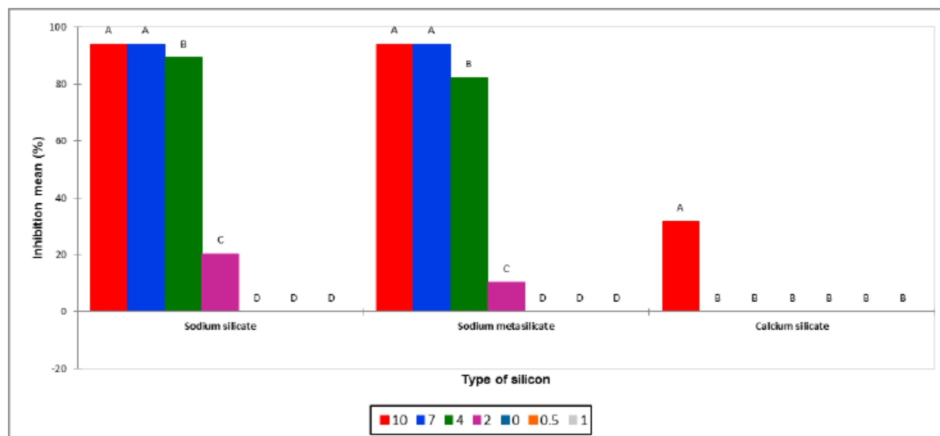

Figure 1. Inhibition Mean Analysis % of Soluble Silicon Inhibition (Experiment 1)

Table 2. Mean Growth (mm) Of *R. microporus* At Different pH (Experiment 2) In Ameliorated Potato Dextrose Agar.

Strain	**pH of medium**	**Mean Growth of *R. microporus* (mm)**						
		1	**2**	**3**	**4**	**5**	**6**	**Average**
AM	Control (5.88)	85.00	85.00	85.00	85.00	85.00	85.00	85.00
	7	85.00	85.00	85.00	85.00	85.00	85.00	85.00
	8	85.00	85.00	85.00	85.00	85.00	85.00	85.00
	9	85.00	85.00	85.00	85.00	85.00	85.00	85.00
	10	85.00	85.00	85.00	85.00	85.00	85.00	85.00
	11	85.00	85.00	85.00	85.00	85.00	85.00	85.00

4. CONCLUSION

Clearly, soluble silicon is expected to have fungicidal activity. The concentration at which complete suppression of a particular fungus occurs is varied and must be determined in vitro before in vivo investigations are initiated. Pot and field trials will be carried out in the future to confirm the efficacy of silicon against *R. microporus* in vivo.

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Agriculture & Agro-Industry **AAI-06**

The Potential Usage of Arbuscular Mycorrhizal Fungi as Biofertiliser to Promote the Chilli Plant (*Capsicum* sp.) Growth

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ABSTRACT

Biofertiliser can be define as a substance that contained living microorganisms, which when applies to plant for example to seeds, roots, plant surface or soils, it will colonise the rhizosphere area or the plant roots, and promote the plant growth by helping the plant to obtain more nutrient from the environment. Biofertiliser has the capability to increase the nutrient availability in the soil. Mycorrhiza fungi are known to help the plant to uptake nutrient from soil, which in return, the fungi will receive photosynthate (carbon) from the plant. This mutualistic symbiosis showed that mycorrhizal has a potential to be used as biofertiliser. Arbuscular mycorrhizal (AM) fungi is type of mycorrhizal fungi and plays importance role in soil and plant health. The main objective of this study is to observe how arbuscular mycorrhizal can be combined with commercial fertiliser in conjunction to reduce the consumption of the commercial fertilisers. In this study, different concentration of mineral and slow release fertilisers (100%, 50% and 25%) were mixed with arbuscular mycorrhizal fungi and were introduced to the chilli plants. The growth of the chili plants were recorded after transplantation, before and after fertilisation as per harvesting plans after the fertilisation. The spores were identified as *Glomus* sp, *Scutellospora* sp., and *Acaulospora* sp. The present of AM structures (vesicle, arbuscules, and hyphae) indicate the successful colonisation of mycorrhizal in the plants roots. From the plant growth, it showed that the usage of mineral fertiliser can be reduced to 50% with the presence of arbuscular mycorrhizal fungi associated with the chilli plant roots. This suggests that for a better agricultural practice, farmers could potentially mix their fertiliser with arbuscular mycorrhizal fungi at the correct ratio as biofertiliser to reduce their cost and maintain the soil health.

Keywords: Mycorrhiza; fungi; symbiosis; fertiliser; nutrient.

1. INTRODUCTION

Arbuscular mycorrhizal (AM) fungi from phylum Glomeromycota is a sister group of phylum Basidiomycota and phylum Ascomycota (Schüβler et al., 2001) and have characteristic finely branched hyphal structure known as arbuscules inside the cortical cells of plant roots (Douds et al., 1999; Mukerji et al., 2012). The AM fungi play crucial role in soil health and fertility. They help in soil aggregation and most importantly, the AM fungi helps in nutrient transfer from soil to plant, and in return the fungi will receive carbon from plant as source of energy. This symbiosis has made the AM fungi potentially to be used as biofertilizer, as an alternative to expensive chemical fertilisers used to improve plant productivities. The use of AM fungi as biofertiliser technology will be interesting way to manage the native plant and restoration of natural habitat by using minimal chemical input (Bencherif et al., 2015). Biofertiliser are the products containing living cells of different type of microorganisms, that have an ability to convert nutrition (Hedge et al 1999; Vessy, 2003) and have emerged as a vital component of the integrated nutrient supply system and also hold great promise to improve for a better crop production by better nutrient supply (Wu et al., 2005).The ability of the AM fungi contribute to rise availability and uptake of phosphate and other micronutrient are well documented (e.g Bolan, 1991; Bucher, 2007; Abbott & Robson, 2018; Watts-Williams etal., 2018). The current practise of applying chemical fertiliser to chili plants by grower is very costly and this study is conducted to find alternative ways to potentially reduce the fertiliser cost. With that respect, this study is conducted with the following objectives: a) to investigate the effect of fertiliser on the AM association with the chili plant roots and, b) to suggest a recommendation ratio between the AM fungi and fertiliser to be used by farmer. It is hypothesised the chili plant with present of the AM fungi will perform better or potentially equal in comparison to the plant fertilised with 100% chemical fertiliser with no present of the AM fungi.

2. MATERIALS AND METHODS

2.1 Propagation of Arbuscular Mycorrhiza

Approximately 100g of AM inoculum soil were introduced to 10L pot with mixture of clay and organic matter (2:1), and *Setaria anceps* as host plant was used as a host plant to propagate the AM spores. The host plants were watered twice a day, and left for 30 days for the AM spores to multiply and ready to be used in the next step.

2.2 Isolation AM using wet-sieving method.

100g of soil was weighted and mixed with water and then sieved through four different sizes of sieves;1000µm, 250µm, 75µm and 45µm. The

soil samples accumulated on each sieve were collected into 50ml centrifugation tubes for centrifugation process. Method by Brundrett et al. (1996) were followed to isolate the AM spores from soil. Approximately 40 ml of distilled water was added to the samples. The samples were then centrifuged for five minutes at 2000 rotation per minute (RPM) to separate the spore from the soil sample. The debris with supernatant was discarded and the pellets were kept for the second centrifugation. The pellet was re-suspended in 50% sucrose and was centrifuged for one minute at 2000 rotation per minutes (RPM). The supernatant was filtered using vacuum pump containing filter paper. The collected spores were observed under the dissecting microscope. The physical morphology of the spores; size, colour, spore arrangement and shapes were recorded accordingly.

2.3 Germination of Chilli seeds, transplantation and inoculation of arbuscular mycorrhizal

The chilli seeds were germinated using seed trays for one month using peat-organic soil as substrate and watered twice a day. The seeds germinated after one week. After one month, the chilli seedlings were transferred into a bigger polybags containing mixture of clay, sand and organic soil in ratio of 1:1:1 (clay: sand: organic + peat) and 50g of AM inoculum was added into each polybag of chilli seedling.

2.4 Fertilisation

After one month of transplantation, the seedlings were introduced with fertilisers; mineral (M) and slow release fertilisers (SLR) at different percentage which were 100% M, 50M%, 25M %, 100 SLR%, 50 SLR%, and 25 SLR%. The plant height of every chilli plant was conducted during the transplantation, before the fertilisers were introduced and after the fertiliser were introduced as per harvesting plan.

2.5 Staining of arbuscular mycorrhizal roots.

After harvesting, the chili plant roots was immersed in a bucket of water and were gently agitated to remove the soil. The roots were preserved in 50% ethanol prior staining. The roots were immersed in 10% KOH at 60°C for 2-4 hours in the water bath. The roots were then taken out from 10% KOH washed and immersed in 2% HCl for a few minutes. This process will neutralize the roots. The roots were then immersed in 0.05% trypan blue with lactoglycerol (TBLG)

2.6 Observation of AM

The stained roots were observed under the dissecting microscope. The root that suspected colonised by the AM. The cutting part of root were observe under the light microscope. The picture of these structure were taken using

camera Olympus BX41 with magnification 10 x 10, 20 x 10, 40 x 10 at software of Cell A attached to compound microscope at Electron Microscope Unit, School of Biological Sciences, Universiti Sains Malaysia.

3. RESULTS AND DISCUSSION

Acaulospora sp. showed 40:60: 80 (Cyan: Yellow: Magenta; CYM) (Figure 1a&b), *Scutellospora* sp. showed 40: 60:80 CYM (Figure 1e&f) and *Glomus* sp. showed colour of 20:60:80 CYM (Figure 1c&d) as referred to Morton (1990) for colour description. These AM fungi are commonly distributed in Malaysia and they are not host specific, which mean they are able to form AM association with almost plants. All these spore are globose (spherical) and only *Acaulospora* has no subtending hyphae. Meanwhile, *Glomus* sp. has simpler subtending hyphae compared to *Scutellospora* sp. that form swollen subtending hyphae (Figure 1c-f). *Scutellospora* spores develop various inner wall layers and have a germination shield (Figure 1d&f)

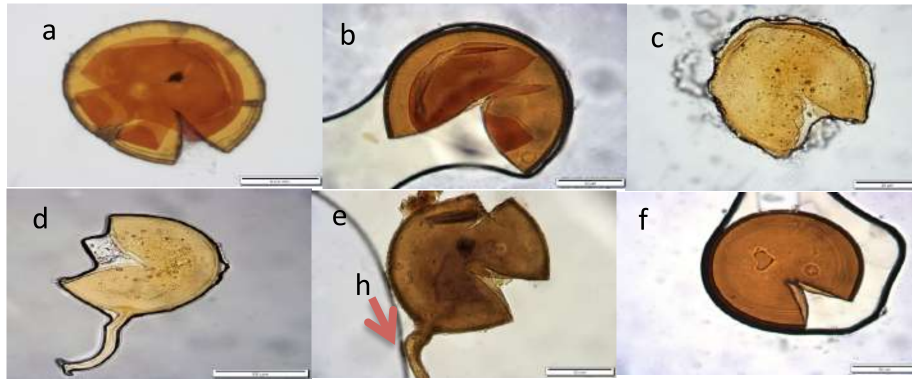

Figure 1 Structure of AM spore isolated from soil samples of chilli plants; a&b) *Acaulospora* sp. spores; c&d) *Glomus* sp. spores and e&f) *Scutellospora* sp. spores. h: hyphae, arrow: swollen subtending hyphae.

Figure 2 shows morphology of AM association in chilli plant roots from different treatments. There is no AM association found in chilli plant roots with 100% mineral and slow release fertiliser without AM (Figure 2a-d). These are the control polybags for this study, and it shows that the soil used for the study are free from AM, and any AM association that formed in other treatments came from the AM inoculum that was introduced to the systems. From the physical morphology, it is found that the AM fungi has managed to form association with the plant roots. The present of vesicles and hyphae in the plant roots has confirmed that the AM association are successfully formed in other treatments (Figure 2e-n). The vesicle plays an important function for nutrient storage while waiting to be transferred to the host plants.

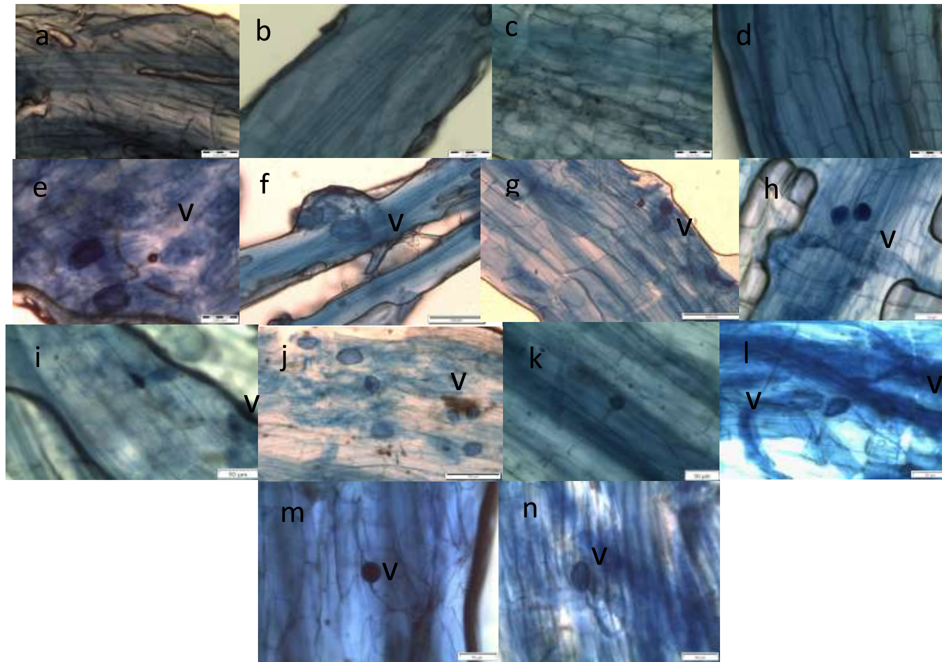

Figure 2. Morphology of arbuscular mycorrhizal (AM) fungi association in chilli plant roots. a-d) The cleared structure of root without AM fungi colonisation. a&b) T1; treatment 1(100% of mineral fertilizer), c&d) T2; treatment 2 (100% slow release fertiliser). e&f) T3; treatment 3 (AM fungi with 50% mineral fertilizer), g&h) T4; treatment 4 (AM fungi with 25% mineral fertilizer) i&j) T5; treatment 5 (AM fungi with 50% slow release fertiliser), k&l) T6; treatment 6 (AM fungi 25% slow release fertiliser), m&n) T7; treatment 7 (AM fungi only). The roots are stained with 0.05% trypan blue in lactoglycerol (Brundrett et. al, 1996). v: vesicle.

Figure 3 shows mean of plant height from each treatment is significantly different (P-value <0.05, F-value: 25.37). The mean plant height in treatment 3 (AM with 50% mineral fertiliser) showed no significant different compared to mean plant height in treatment 1 that used 100% mineral fertiliser, but significantly higher compared to mean plant height in treatment 7 that used mycorrhiza only. As for treatment 6 (AM with 25% slow release fertiliser) also showed no significantly different in mean plant height compared to treatment 2 that used 100% slow release fertiliser, but it showed significantly higher in mean plant height compared to treatment 7. These patterns showed that with the usage of AM and fertiliser together, it could potentially produce the same plant height with the usage of 100% fertiliser. This showed that quantity of fertiliser used can be cut down to almost 50% with the combination of AM fungi. This is because the AM fungi are able to unlock the nutrient in the soil when the plant needs them. As such, the AM fungi can be a good source to be used together with chemical fertiliser because it can help to sustain the nutrient availability in the soil and can also help to maintain the soil health.

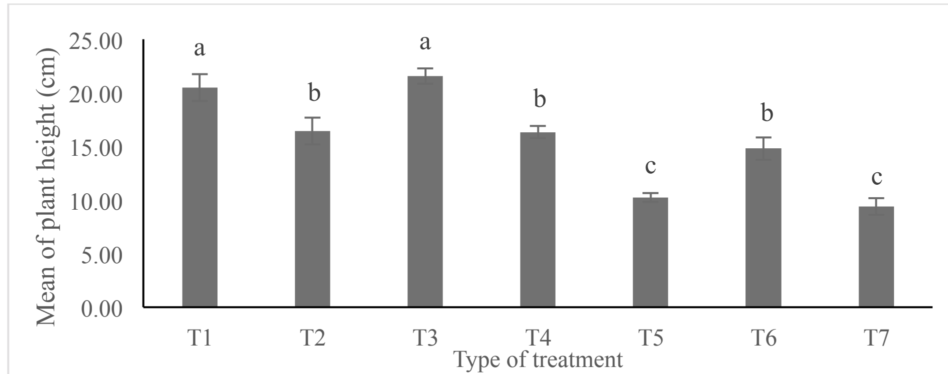

Figure 3. Mean of plant height measured from different types of treatments. T1; treatment 1(100% of mineral fertilizer), T2; treatment 2 (100% slow release fertiliser), T3; treatment 3 (AM fungi with 50% mineral fertilizer), T4; treatment 4 (AM fungi with 25% mineral fertilizer), T5; treatment 5 (AM fungi with 50% slow release fertiliser), T6; treatment 6 (AM fungi 25% slow release fertiliser), T7; treatment 7 (AM fungi only). The bar sharing the same alphabet indicate there is no significant different between them.

4. CONCLUSION

From the study, the AM fungi may be used as biofertilizer in combination with chemical fertiliser to reduced usage of chemical fertiliser as per current practice. The reduce of chemical fertilisers quantity in plantation will indirectly help the farmers to reduce their operation cost. Furthermore, it will also help to maintain the soil health.

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Agriculture & Agro-Industry **AAI-07**

Effects of Storage Temperature and Cooking Treatment on Fatty Acid Profiles in Chicken Eggs

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ABSTRACT

Chicken eggs are a nutritious source of food comprising high level of polyunsaturated fatty acids (PUFA) that are essential in human diet. The poultry industry actively modify the egg's lipids content in order to provide consumer with maximum values of PUFA, thus there are many types of enriched eggs available in the local market. Heating could cause physicochemical changes especially on PUFA, which may happen during storage and cooking of the eggs. The aim of this study is to determine the differences of chicken egg yolk fatty acid profiles in conventional eggs and enriched eggs. The impact of room temperature and refrigeration in storage of the eggs, as well as fried and hard-boiled cooking treatment on the fatty acid profiles of the conventional egg yolks and enriched egg yolks were determined and compared through gas chromatography. Enriched eggs contain higher total omega-3 and docosahexaenoic acid (DHA). Raw and hard-boiled egg yolks showed similar fatty acid profiles. However, total omega-3 content were lower than hard-boiled egg yolks. This study demonstrated that differences of storage temperature and the method of cooking treatment contributed to the significant changes of egg fatty acid profiles. Hard-boiled enriched eggs revealed the most favourable fatty acids profiles in terms of omega-3 content which is an excellent choice for the consumer.

Keywords: fatty acids, polyunsaturated fatty acids, omega-3, chicken eggs

1.0 INTRODUCTION

The chicken egg is one of the most inexpensive and important source of animal lipids for humans. Eggs contain saturated, monounsaturated and polyunsaturated fatty acids (PUFA) which is essentially important in human diet (Simčič et al., 2011). Fatty acids play major roles in human health; are sources of energy and are the building blocks of cell membranes. They are capable of influencing health, well-being and cardiovascular-related diseases. PUFA contain many important compounds which are necessary for humans, thus they are the primary element for the evaluation of various fats (Collins et al., 2007). Linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) are PUFA which are termed as essential fatty acids for humans as they cannot be synthesized by the mammalian body and therefore must be ingested from dietary intake (Tvrzicka et al., 2011). LA and ALA are essential as they are the beginning point of the omega-3 and omega-6 long-chain polyunsaturated fatty acid (LC-PUFA) synthesis pathways. Important omega-3 such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), and omega-6 such as arachidonic acid (ARA, 20:4n-6) gets synthesized from ALA and LA with the assistance of desaturation and elongation enzymes along the pathway. ALA is an omega-3 PUFA commonly found in vegetable oils and nut oils. LC-PUFA such as EPA and DHA are found naturally in high amounts in fish such as salmon, mackerels and tuna. EPA and DHA are related to the importance in early cognitive and growth development, as well as benefits in the area of behavioural functions, adult psychiatric disorder and neurological decline with ageing (Childs et al., 2008, Ruxton et al., 2005). As fish are a high source of LC-PUFA for humans, there is a current trend which is observed in the commercial market. There are existence of omega-3 enriched or enhanced food available and advertised in line with improving human health. One of these products are omega-3 enriched eggs. This study was conducted to compare the fatty acid composition in conventional and omega-3 enriched chicken eggs. Eggs were stored in room temperature and cold temperature prior to being processed in various ways. It will be interesting to map out the differences in fatty acid patterns when the eggs were in raw, fried or hard boiled. Essential fatty acid content can be unstable because of the presence of two or more double bonds in their structure, whereby these bonds may be loss during food processing and storage (Yasodhara et al., 2009). There are also chances of changes in fatty acid content due to oxidation as well. Hence, it is necessary to investigate the impact of storage temperature and cooking methods on the fatty acid profiles in chicken eggs.

2.0 MATERIALS AND METHODS

One batch of conventional chicken eggs (A) and two brands of local omega-3 enriched chicken eggs (B & C) were purchased from the local stores on the same date. All eggs were grade A, large size (50-65g) with similar expiry dates were chosen. A total of 54 eggs (three brands) were used in this study, whereby 6 eggs

from the same manufacturer were used in each cooking treatment (raw, fried, hard boiled).

Eggs were labelled accordingly and stored in room temperature (25-27°C) and refrigeration (2°C) for a period of 5 days. A triplicate of egg from each manufacturer was used in each cooking treatments. The egg yolks were carefully separated from the albumin and subjected to frying (without addition of oil) in a non-stick frying pan (80°C) (Ren et al., 2013). A pot of boiling water was set to 100°C and the eggs were boiled for 10 minutes.

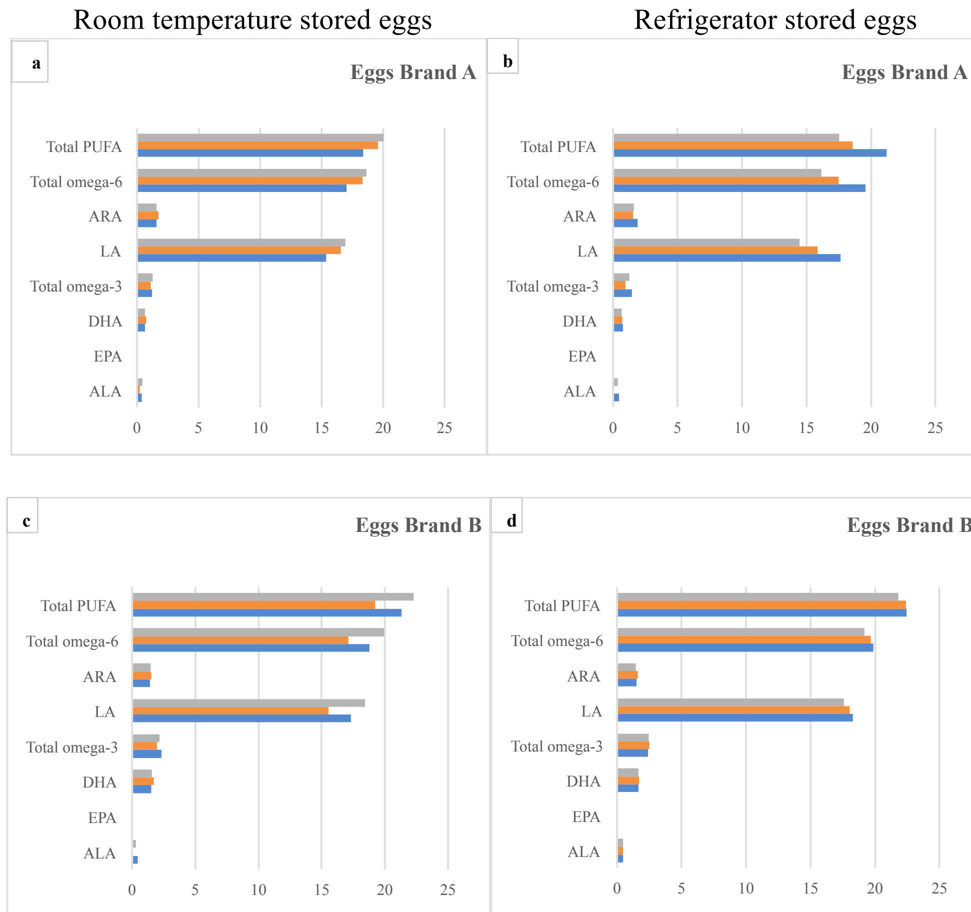
All samples were subjected to total lipid extraction (Folch et al., 1957). The extracted lipids were then converted to fatty acid methyl esters (FAME) according to the AOAC (1997) method. FAME were separated and analysed using a gas chromatograph (GC-2010, Shimadzu, Japan) equipped with a flame ionization detector and a fused silica highly polar cyanosiloxane column SP-2380 (30m x 0.25mm x 0.20µm; Supelco, USA). The fatty acids were compared with commercially available standard references (37 Component FAME Mix and PUFA no. 3 from Menhaden Oil, Supelco, USA). All data were analysed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test at 5% significance level using the SPSS 15.0 software.

3.0 RESULTS AND DISCUSSION

The conventional egg yolks (A) had a lighter shade of yellow compared to the omega-3 enhanced eggs which displayed a deeper darker shade of yellow-orange in both brands (B and C). The fatty acid composition of raw egg yolks were used as a control in measuring the fatty acids in the variant cooking methods. Fatty acid analysis of conventional egg yolks revealed that they do not contain any EPA and relatively low DHA (Figure 1a,b). There was no significant differences between the DHA content of room temperature and refrigerated eggs regardless of the cooking method.

An interesting find is the increase in LA detected for refrigerated raw eggs A compared to the room temperature stored fried and hard boiled eggs. This contributed to the raised level of the total omega-6 and PUFA collectively for refrigerated raw eggs A. In previous studies, it was reported that LA and ALA were quite stable for approximately 49 days at 4°C (Ren et al., 2013). Docosapentaenoic acid (DPA, 22:5n-3), DHA and total omega-3 were among the fatty acids which were inconsistent and prone to loss through lipid oxidation. In this study, after 5 days of storage in the refrigerator, raw eggs from all brands had a slightly higher amount of DHA and total omega-3 compared to the raw eggs kept in room temperature. This could be because the eggs in the refrigerator was protected from heat, light and circulating oxygen which preserved them from oxidation damage compared to the eggs stored in room temperature (Jacob et al., 2000).

For both brands of omega-3 enriched eggs (B and C), EPA was detected, indicating the difference compared to the conventional eggs which was totally void of this fatty acid. However, the amount detected was minimal, and lower than 0.1% of total fatty acids. Within brand B, results showed that the DHA content in fried eggs contained higher DHA regardless of being stored in room temperature or refrigerator.



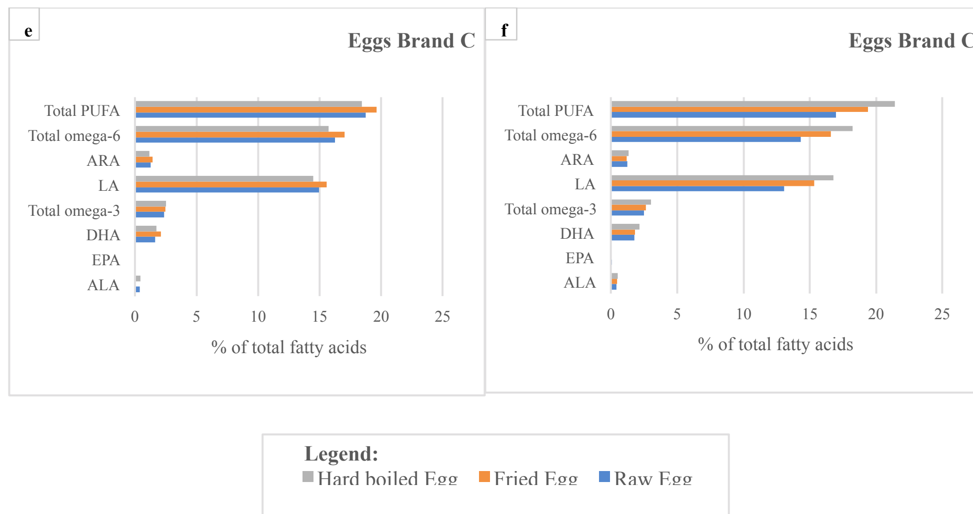


Figure 1. Comparison of selected fatty acids (% of total fatty acids) in yolks of conventional eggs (brand A) and omega-3 enriched eggs (brands B and C) while stored in room temperature or refrigerator. Each brand of eggs were subjected to different cooking methods, namely hard boiled or fried and compared with uncooked raw egg form. Data is a representation of the mean from triplicates. refrigerated. Raw and hard boiled eggs of brand B showed similar levels of DHA amongst each other, but slightly lowered DHA contents compared to fried eggs. In brand C refrigerated eggs, total PUFA was highly detected in the hard boiled yolks, followed by the fried eggs then the raw eggs. This pattern was not reflected in room temperature eggs C, as the total PUFA was lowest compared to the raw and fried eggs. The highest level of DHA was detected in refrigerated hard boiled eggs of brand C if compared to all brands, cooking methods and storage temperatures.

4. CONCLUSION

The outcome of this study showed that there were differences in fatty acid profiles of conventional eggs and omega-3 enriched eggs, with the detection of EPA and slight increase in DHA. Short duration of different storage temperatures did not show significant differences in fatty acid profiles. However, fried eggs have a variation in fatty acid patterns compared to raw and hard boiled eggs. This could be due to the exposure of cooking method to oxygen, light and heat as the egg yolks were fried without the protection of the egg shells from these elements. The differences in fatty acid profiles confirm that different cooking methods do make an impact on the fatty acid contents in the eggs. Hard boiled egg yolks from brand C which were stored in the refrigerator revealed the highest DHA content compared to the others.

ACKNOWLEDGEMENT

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Agriculture & Agro-Industry **AAI-12**
Presentation Type: Poster

Simple Micropropagation of *Typhonium flagelliforme*

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ABSTRACT

Rodent tuber (*Typhonium flagelliforme*) is a medicinal plant from the Araceae family. Rodent tubers contain several bioactive compounds. Theyhave been found to be successful in inducing apoptosis of breast cancer cells, cure dermatitis and can be used for former drug users to neutralize their body from toxins caused by addictive substances drugs. However, this plant has a low reproduction rate and is very sensitive to environmental factors. Plant tissue culture is an effective way to increase the number of plants in a short time and to have consistency of plants. *In vitro*rodent tuber plantlets were induced through direct shoot culture. The results showed that MS containing 0.5 mg/L of BAP combined with 0.5 mg/L of NAA gave the best shoot induction at 2.6 shoots/explant and shoot height at 5.92 cm. For shoot multiplication, results showed that MS medium supplemented with 1.0 or 1.5 mg/L of BAP combined with 0.5 mg/L of NAA was effective in shoot multiplication at 2.56 shoots/explant. Finally, *in vitro* root induction of rodent tuber was achieved on ¼MS medium (8.83 roots/shoot). Plantlets were successfully acclimatized with the survival rate of 100 % after 2 weeks.

Keywords: rodent tuber, organogenesis, *in vitro*

INTRODUCTION (Title Times New Roman, 12 font size, bold)

Rodent tuber or Wan paya-horkhuck (*Typhonium flagelliforme* Lodd.) is a potential specie of the Araceae family (Surachman 2009). This medicinal plant can be found in several countries such as India, Indonesia, Malaysia, Sri Lanka and

South of Thailand. Corm of rodent tuber contains several bioactive compounds such as alkaloids, flavonoids, terpenoids, steroids (Surachman 2009). Moreover, Chan *et al*. (2005) reported that this plant could be used as source for the treatment of human lung and breast cancer. So, the plant is highly demanded for cancer treatment.

Normally, rodent tuber has a slow propagation rate by vegetative method that had low genetic diversity, and is very sensitive to environmental factors. Vegetative propagation in *in vitro* culture is an appropriate method for overcoming this problem of low numbers because this method is able to produce many plants in a short period of time (Tiwar & Arnold 2011). The achievement of propagation in tissue culture is influenced by several factors i.e. plant growth regulator, strength and type of media and genotype of mother plant. So, the objective of this present work were to study the effects of PGRs and strength of media on *in vitro* plantlet regeneration of rodent tuber

MATERIALS AND METHODS

2.1 Surface sterilization and shoot induction

Spouting corms of Rodent tuber (*Typhonium flagelliforme)* were collected form Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Songkhla province in Thailand (Figure 1a). The explants were washed in running tap water for 30 min, surface sterilized with 70% ethanol solution for 1 min followed by soaking in 0.1% mercuric chloride for 15 min in a laminar hood. After that explants were washed with sterile distilled water for 5 times, cut into small pieces at 0.5-1.0 cm in length and cultured on MS (Murashige and Skoog 1962) basal medium supplemented with different concentration of BAP and 0.5 mg/L BAP combination with 0.5 mg/L NAA, 3% sucrose, solidified with 0.75% agar and adjusted pH to 5.7. Cultures were maintained at 25±2˚C and 10-h photoperiod with a light intensity of 15 μmol/m^2/s provided by fluorescent lamps. After culturing for 4 weeks, the data was documented on percentage of shoot induction, number of shoot per explant and shoot height (cm).

2.2 Shoot multiplication

Shoot from previous study were cut and transferred to MS medium supplemented with various concentration of BAP (0.5, 1.0, 1.5 and 2.0 mg/L) alone or combination with 0.5 mg/L NAA, 3% sucrose, solidified with 0.75% agar and adjusted pH to 5.7. Cultures were maintained at 25±2˚C and 10-h photoperiod with a light intensity of 15 μmol/m^2/s provided by fluorescent lamps. After culturing for 4 weeks, the data was documented on number of shoot per explant and shoot height (cm).

2.3 *In vitro* of rooting and acclimatization

Single shoot was separated and transferred to (full, ½ and 1/4) MS strength medium without plant growth regulator containing 3% sucrose, solidified with 0.75% agar

and adjusted pH to 5.7. After culturing for 4 weeks, Root induction, root number and root length were recorded. For hardening, complete plantlets, especially their roots, were washed with water, and transferred to a medium composed of soil and copra meal at ratio of 1:1 in plastic pot. These plantlets were maintained in a greenhouse for 2 weeks.

2.4 Statistical analysis

Each treatment consisted of three replicates. Mean value of various treatments was subjected to analysis of variance (ANOVA) and significant difference was separated using Duncan's Multiple Range Test (DMRT). The R Statistical software was used to determine significant difference at $P \leq 0.01$.

RESULTS AND DISCUSSION

Effect of plant growth regulators on shoot induction and proliferation after 4 weeks of culture

The result showed that all treatment gave 100 % of shoot induction. However, sterilized spouting corm cultured on MS medium supplemented with 0.5 mg/L BAP and NAA gave the highest number of shoot at 2.60 shoots/explant and shoot length at 5.92 cm significant difference ($P \leq 0.01$) with BAP alone (0.5 mg/L) (Table 1; Figure 1b). For multiplication, the result revealed that 1.0 or 1.5 mg/L BAP combined with 0.5 mg/L NAA gave the highest number of shoot at 2.56 shoots/explant (Table 2; Figure 1c). Thus, same concentration of PGRs (0.5 mg/L BAP and NAA) gave the highest shoot height at 4.54 cm but not significant different with another treatment after transferring for 4 weeks. So, adding PGRs between auxin and cytokynin in culture media can help to stimulate axillary bud in this plant. While, Sianipar *et al*. (2015) reported that 2.0 mg/L BAP in combination with 0.5 mg/L NAA gave the best result in shoot induction.

Table 1. Effect of plant growth regulators on shoot induction of rodent tuber after 4 weeks of culture

PGRs	Shoot induction (%)	Number of shoot (shoots/explant)	Shoot height (cm)
0.5 mg/L BAP	100	1.00 ^{ns}	1.78^{b}
1.0 mg/L BAP	100	1.80	2.06^{a}
2.0 mg/L BAP	100	2.40	2.08^{a}
0.5 mg/L BAP/0.5 mg/L NAA	100	2.60	5.92^{a}

ns: non-significant differences

Means followed by the same letter within column are not significantly different at $P \leq 0.01$ using DMRT.

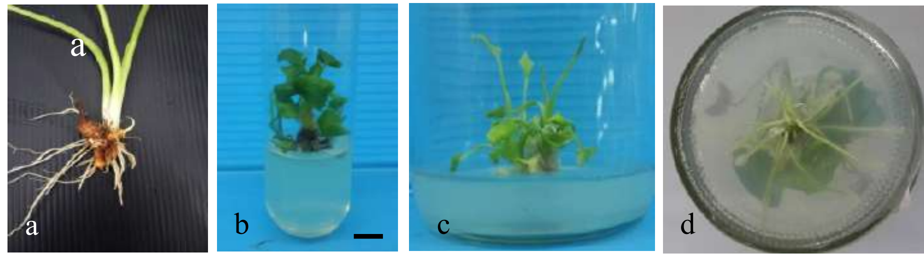

Figure 1. *In vitro* plantlet regeneration of rodent tuber a) spouting corm, b) shoot induction in 0.5 mg/L of BAP and NAA added in MS medium for 4 weeks, c) shoot multiplication after culturing on MS medium containing in 1.5 mg/L BAP with 0.5 mg/L NAA for 4 weeks, d) rooting on ¼ MS medium without PGRs for 4 weeks of culture (Bar = 1 cm)

Table 2. Effect of plant growth regulators on shoot multiplication after 4 weeks of culture

Concentration (mg/L)		Number of shoot (shoots/explant)	Shoot height (cm)
BAP	NAA		
0.0	0.0	1.00^{c}	0.69^{c}
0.5	0.0	1.22^{c}	0.89^{b}
1.0	0.0	1.56^{c}	1.01^{b}
1.5	0.0	1.11^{bc}	0.77^{b}
2.0	0.0	1.00^{ab}	1.12^{a}
0.5	0.5	2.00^{a}	4.54^{a}
1.0	0.5	2.56^{a}	2.76^{a}
1.5	0.5	2.56^{a}	3.19^{a}
2.0	0.5	2.33^{a}	3.34^{a}

Means followed by the same letter within column are not significantly different at $p \leq 0.01$ using DMRT.

3.2 Effect of strength of medium on root induction

For root induction, ¼ MS strength medium gave the best result in number of roots (8.89 roots/shoot) and root length (2.86 roots/shoot) not significant different with 1/2 MS medium (Table 3; Figure 1d). Rodent tuber is so easy to induce root, and no need PGRs. Because of nutrient deficits, the plant was powerful stimulants for

rhizogenesis of plant depended on species. Well developed *in vitro* rooted healthy plantlets with 2-4 leaves were transferred to pots containing soil and copra meal at ratio of 1:1 in plastic pot for 2 weeks. The plantlets were a high survival rate of 100% (Figure 2). No abnormal morphological in growth characteristic in regenerated plantlets as compared to mother plant.

Table 3. Effect of plant growth regulators on roots induced during two weeks

Strength of MS medium	Number of roots (roots/explant)	Root length (cm)
MS	5.41^{b}	1.43^{b}
½MS	7.08^{ab}	1.94^{a}
¼MS	8.89^{a}	2.86^{a}

Means followed by the same letter within column are not significantly different at $p \leq 0.01$ using DMRT.

Figure 2. Acclimatization of plantlets for 2 weeks in greenhouse

4. CONCLUSION

For simple micropropagation of rodent tuber, the result revealed that MS medium supplemented with 0.5 mg/L BA and 0.5 mg/L NAA gave the suitable for shoot induction from sprouting corm for 4 weeks. When increasing concentration (1.0 or 1.5 mg/L BAP) and combination with 0.5 mg/L NAA gave the best result in shoot multiplication. Rooting in ¼ MS strength gave the highest root number and root length. Complete plantlets were successfully 100 % survival rate after acclimatization for 2 weeks.

ACKNOWLEDGEMENT

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Agriculture & Agro-Industry **AAI-13**

Effects of Agar Andforchlorfenuron on Somatic Embryo Induction of Oil Palm SUP-PSU

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ABSTRACT

Clonal propagation of high yield oil palm is possible through somatic embryogenesis. The objective of this study was to evaluate effects of agar and forchlorfenuron (CPPU) on somatic embryo (SE) induction. The embryogenic callus (EC) was chopped then transferred to oil palm culture medium (OPCM) with various concentrations of agar. The results revealed that 0.7% agar gave the highest callus fresh weight at 587.50 mg/tube and SE induction at 25%. For number of SEs, the highest number of SEs at 1.4 embryos/tube was obtained on 0.6% agar containing OPCM after 4 weeks of culture. When EC was chopped and cultured on solidified medium with different concentrations of CPPU, it was found that CPPU-free OPCM gave the highest callus fresh weight at 612 mg/tube. In case of SE induction, the results showed that 0.2 mg/l CPPU gave the highest SE induction at 100% and number of SEs at 2.5 embryos/tube. Therefore, the results can be concluded that chopped EC cultured on 0.2 mg/l CPPU and solidified with 0.6% agar was suitable for SE induction of oil palm SUP-PSU.

Keywords: Oil palm, clonal propagation, CPPU, somatic embryo

1.0 INTRODUCTION

Oil palm is a cross-pollinated crop which is an economically importance for consumption and biofuel. Oil palm SUP-PSU is an elite genotype improved for increasing in yield and can be tolerated to drought condition. Propagation by conventional method can be carried out using seed, a fact that makes it practically impossible to obtain uniform plant. Tissue culture technique through somatic embryogenesis is key method for multiplication of oil palm elite genotypes (Thuzar

et al., 2011). It is provide high multiplication within a short period. Moreover, production of highly uniform plants is feasible. This process is affected by explant, culture medium, agar and plant growth regulators etc. Agar is the most commonly used as gelling agent in media for plant tissue culture (Kaçar *et al*., 2010). The properties of agar are stability, high clarity and resistance to metabolism during culture (Jain and Babbar, 2002). Plant growth regulators such as N^6-benzyladenine (BA), α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) is a critical factors for somatic embryogenesis in date palm (Kurup *et al*., 2014; AL-Mayahi, 2015). N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) commonly known as forchlorfenuron that is one of cytokinins in form of phenylurea that stimulates high frequency of cell division in tobacco callus cultures (Takahashi *et al.,* 1978) and is 10-fold more active than BA in inducing shoot formation (Okamoto *et al.,* 1978). Moreover, CPPU improves somatic embryogenesis in peanut (Murthy and Saxena, 1994). However, there are no reports of CPPU on tissue culture of oil palm. Therefore, the aim of this research was to study effects of agar and CPPU on SE induction of oil palm SUP-PSU.

2.0 MATERIALS AND METHODS

Plant material

Embryogenic callus (EC) of oil palm SUP-PSU was achieved at Crop Biotechnology Laboratory, Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University. It was used as starting plant material for EC proliferation by culturing on oil palm culture medium (OPCM) supplemented with 0.1 mg/l dicamba and 200 mg/l ascorbic acid. The culture was maintained at 26±2°C under 14 h photoperiod (15 µmol/m^2/s) for 4 weeks.

Effects of concentrations of agar and CPPU on somatic embryo (SE) formation

One gram fresh weight (FW) of EC was chopped at frequency of 100 times. Then, 0.1 gFW of chopped EC was transferred to OPCM with 0.1 mg/l dicamba and different concentrations of agar or CPPU alone. All culture media were supplemented with 200 mg/l ascorbic acid, 3% sucrose and adjusted to pH 5.7 prior to autoclaving at 1.05 kg/cm^2, 121°C for 15 min. The cultures were placed at 26±2°C under 14 h photoperiod provided by cool-white fluorescent lamps. After 4 weeks of culture, callus fresh weight, SE induction, number of SEs, size of SE and color of callus were recorded.

3.0 RESULTS AND DISCUSSION

Effect of agar concentrations on SE formation

After 4 weeks of culture, 0.7% agar solidified OPCM gave the highest results in callus fresh weight at 587.50 mg/tube. For SE induction, few SEs were formed on the callus. The results showed that the culture medium solidified with 0.65 and 0.7% agar gave the highest results in SE formation at 25%. In case of number of SEs and size of SE, the highest results at 1.40 embryos/tube and 0.29 cm were obtained on 0.6% agar containing medium (Table 1). The characteristic of

callus was yellowish friable in all treatments as shown in Fig. 1. Similar result was also obtained in direct somatic embryogenesis in coffee (Almeida *et al*., 2007). However, the effective concentration of agar in coffee was 0.3% which far lower than that used in oil palm in this present study. In contrast, percentage of explants forming shoots and hyperhydric shoots increased with decreasing agar concentration in sunflower (Abdoli *et al*., 2007).

Table 1 Effect of agar concentrations on SE formation of oil palm SUP-PSU cultured on OPCM with 0.1 mg/l dicamba and 200 mg/l ascorbic acid for 4 weeks

Conc. of agar (%)	Callus FW (mg/tube)	SE induction (%)	No. of SEs (embryos/tube)	Size of SE (cm)
0.55	472.50c	16.67b	1.00	0.27
0.60	517.00b	21.67ab	1.40	0.29
0.65	532.80b	25.00a	1.17	0.23
0.70	587.50a	25.00a	1.33	0.27
0.75	544.00b	20.84ab	1.40	0.29
F-test	**	*	ns	ns
C.V. (%)	3.08	15.39	36.74	35.19

ns= not significantly different
* significantly different (p<0.05), ** significantly different (p<0.01)
Mean values followed by the same letter within column are not significantly different according to duncan's multiple range test (DMRT).

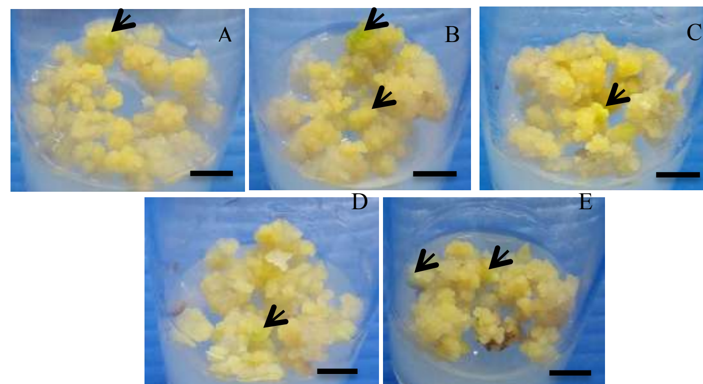

Fig. 1 Characteristic of EC and SE (arrows) of oil palm SUP-PSU cultured on OPCM with 0.1 mg/l dicamba and 200 mg/L ascorbic acid for 4 weeks
A. 0.55% agar B. 0.60% agar C. 0.65% agar D. 0.70% agar E. 0.75% agar

Effect of CPPU concentrations on SE induction

Increasing in concentrations of CPPU resulted in decrement in callus fresh weight. The highest callus induction at 612 mg/tube was obtained from medium without CPPU. For SE induction, CPPU promoted somatic embryogenesis in peanut (Murthy and Saxena, 1994). SE was developed on CPPU containing medium after 4

weeks of culture. The highest SE induction at 100% was obtained on 0.1-0.3 mg/l CPPU containing medium. However, 0.2 mg/l CPPU gave the highest number of SEs at 2.05 embryos/tube (Table 2). The friable callus was occurred in all treatments. Low concentrations of CPPU (0-0.2 mg/l) provided yellowish friable callus. Whereas CPPU at concentrations of higher than 0.2 mg/l promoted browning callus as shown in Table 2 and Fig. 2. Matsuta and Hirabayashi (1989) reported that CPPU was more effective than BAP for somatic embryogenesis in grape. Somatic embryogenesis can be improved by combination of cytokinins and auxins. CPPU at concentration of 2 mg/l in combination with 0.2 mg/l NAA gave the highest SE induction at 90-97% from leaf, petiole and stem in Golden Pothos (Zhang *et al*., 2005). In addition, the regenerated shoots of apple were successfully occurred from callus on 4 mg/l CPPU containing medium (Liu *et al*., 1994). However, high concentrations of CPPU resulted in increment of explant browning in oil palm in this study. Moreover, hyperhydricity was affected by CPPU in pear (Kadota and Niimi, 2003)

Table 2 Effect of concentrations of CPPU on SE induction on OPCM with 200 mg/l ascorbic acid for 4 weeks

Concentrations of CPPU (mg/l)	Callus FW (mg/tube)	SE induction (%)	No. of SEs (embryos/tube)	Color of callus
0	612a	60c	1.00±0.00c	Yellow
0.1	485[b]	100a	1.46±0.16bc	Yellow
0.2	468b	100a	2.05±0.23a	Yellow
0.3	464bc	100a	1.83±0.07ab	Brown
0.4	412d	80b	1.39±0.16bc	Brown
0.5	440c	80b	1.00±0.00c	Brown
F-test	**	**	**	
C.V. (%)	2.95	3.33	21.06	

** significantly different ($p<0.01$)

Mean values followed by the same letter within column are not significantly different according to DMRT.

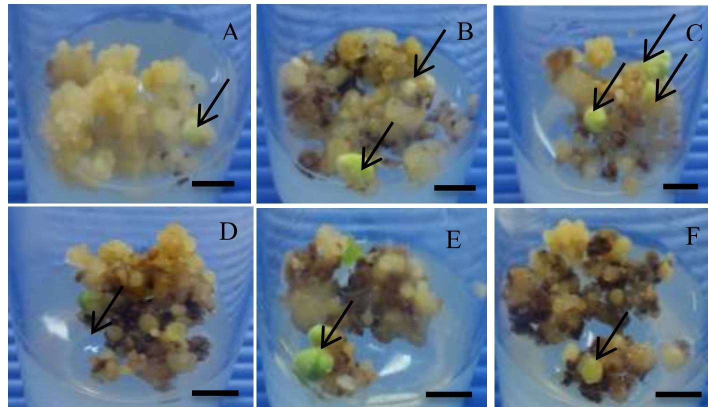

Figure 2 Characteristic of callus and SE (arrows) on OPCM with various concentrations of CPPU in combination with 200 mg/l ascorbic acid for 4 weeks (bar=0.5 cm)
A. CPPU-free medium B. 0.1 mg/l CPPU C. 0.2 mg/l CPPU
D. 0.3 mg/l CPPU E. 0.4 mg/l CPPU F. 0.5 mg/l CPPU

CONCLUSION

The result can be concluded that somatic embryogenesis was successfully obtained on 0.2 mg/l CPPU containing OPCM medium solidified with 0.6% agar. The highest results in somatic embryo induction at 100% and number of SEs at 2.05 embryos/tube were obtained after 4 weeks of culture.

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Agriculture & Agro-Industry **AAI-15**

Effect of Sodium Chloride on Growth and Proliferation of Cell Suspension Culture of Oil Palm SUP-PSU

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ABSTRACT

To study effect of sodium chloride (NaCl) on growth and proliferation of cell suspension culture of oil palm SUP-PSU, young leaf-derived callus of oil palm was chopped and cultured on oil palm culture medium (OPCM) or Eeuwen's Y_3 (Y_3) supplemented with 0.1 mg/L dicamba for 4 weeks. The results showed that OPCM gave the better results than Y_3 medium in term of callus proliferation at 100% and sizes of callus at 1.31 cm. For cell suspension culture, callus at 12 days of culture was transferred to liquidified OPCM supplemented with 0.1 mg/L dicamba and different concentrations of NaCl (0, 50, 100, 150 and 200 mM). The results showed that 150 mM NaCl containing OPCM gave the highest results in pack cell volume at 36 cell clumps. However, 200 mM NaCl gave the largest cell clump at 0.405 cm after 4 weeks of culture. Thus, callus cultured in OPCM with 0.1 mg/L dicamba and 150 mM NaCl was suit for growth and proliferation of cell suspension culture of oil palm SUP-PSU.

Keywords: Oil palm, NaCl, growth, proliferation, cell suspension

1.0 INTRODUCTION

Salt stress is a factor that limits plant growth from major environmental factors. Salt stress affect important processes in plant cell such as growth, photosynthesis, protein synthesis and metabolisms of the cell because salt stress causes oxidative damage to membrane lipid, nucleic acids, and proteins (Kaewneramit and Wutipraditkul, 2014). It also inhibits transportation of potassium, calcium, phosphorus, and magnesium (Hanson *et al*., 1994). However, many studies showed that salt stress played either negative or positive result to fresh and dry weights depend on salinity concentrations, types of salt presence and plant species (Qados, 2011).

Oil palm SUP-PSU is an economically importance for consumption and biofuel. It is can be tolerated to drought condition and elite genotype improved for increasing oil yield. Propagation and proliferation can be carried out by suspension

culture instead of using seed because it provides high multiplication rate within a short period and true to type more than seed. Oil palm has been grown in southern part of Thailand both east and west coastal areas. However, salinity tolerance of oil palm was unknown. Thus, the objective of this study was to describe the response of cell suspension culture of oil palm SUP-PSU to sodium chloride (NaCl).

2.0 MATERIALS AND METHODS

Plant material

Callus was obtained from young leaf culture of oil palm SUP-PSU at Crop Biotechnology Laboratory, Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University. It was chopped and cultured on oil palm culture medium (OPCM) supplemented with 0.1 mg/L dicamba and 200 mg/L ascorbic acid. It was used as plant material for callus proliferation. The culture was maintained at 26±2 °C under 10 h photoperiod (15 µmol/m^2/s) for 4 weeks.

Effect of culture media on callus proliferation

Callus was transferred to OPCM or Y3 supplemented with 0.1 mg/L dicamba and 200 mg/L ascorbic acid, 3% sucrose and adjusted to pH 5.7 prior to autoclaving at 1.05 kg/cm^2, 121°C for 15 min. The cultures were placed at 26±2 °C under 10 h photoperiod provided by cool-white fluorescent lamps. After 4 weeks of culture, callus proliferation, and size of callus were recorded.

Effect of sodium chloride on growth and proliferation of cell suspension culture

Callus cultured on OPCM with 0.1 mg/L dicamba for 12 days of culture was transferred to liquidified OPCM supplemented with 0.1 mg/L dicamba and different concentrations of NaCl (0, 50, 100, 150 and 200 mM), 3% sucrose and adjusted to pH 5.7. Media were autoclaved at 1.05 kg/cm^2, 121°C for 15 min. The cultures were placed at 26±2 °C under 14 h photoperiod provided by cool-white fluorescent lamps. After 4 weeks of culture, pack cell volume and size of cell clumps were recorded.

3.0 RESULTS AND DISCUSSION

Effect of culture media on callus proliferation

The results showed that OPCM gave the better results than Y_3 medium in term of callus proliferation. Proliferation rate of callus on OPCM medium supplemented with 0.1 mg/L dicamba was obtained at 100% and sizes of callus at 1.31 cm after 4 weeks of culture (Table 1). Thongpae *et al*. (2015) reported that OPCM with 0.1 mg/L dicamba gave the best results in proliferation of nodular callus. Similar result was also obtained from Kerdsuwan and Te-chato (2013) who

reported that OPCM with 0.1 mg/L dicamba gave increment in fresh weight and prolong of culture period.

Table 1 Effect of culture media on callus proliferation of oil palm cultured on medium with 0.1 mg/L dicamba for 4 weeks

Culture media	Callus proliferation (%)	Sizes of callus (cm)
OPCM	100	1.312
Y3	100	1.225
F-test		ns
C.V. (%)		11.61

ns= not significantly different

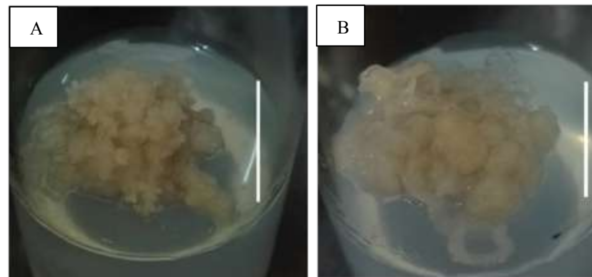

Fig.1 Characteristic of callus of oil palm SUP-PSU cultured on different culture media with 0.1 mg/L dicamba for 4 weeks (bar=1 cm)
A. Y_3 medium
B. OPCM

Effect of sodium chloride on growth and proliferation of cell suspension culture

The results showed that 150 mM NaCl containing OPCM gave the highest results in number of cell clumps or aggregates at 36 clumps (Table 2). However, 200 mM NaCl gave the largest size of clump at diameter of 0.405 cm after 4 weeks of culture as shown in Table 2 and Fig. 2.

Normally, salt stress is harmful to plant because it decreases availability of high-quality irrigation water (Lee and Iersel, 2008), toxic effects of salt ions and nutritional imbalance (Beltagi *et al.*, 2006; Keokene and Pattanagul, 2006). Some studies showed a negative relationship between growth and level of salt. The decrease in plant growth was found when concentration of sodium chloride increase (Qados, 2011). Some studies showed that the fresh and dry weight of the shoot affected, either negatively or positively by changes in salinity concentrations, types of salt presence or plant species. Some species, especially halophytes and some plants with the CAM or C4-photosynthetic pathway, grow better in the presence of Na^+ (Lee and Iersel, 2008). Therefore, callus of oil palm SUP-PSU may tolerance to all salt stress levels treated in a short period of time. Thus, further observation on

growth of cell in suspension culture must be carried out to elucidate the long term effect of NaCl on their growth inhibition.

Table 2 Effect of sodium chloride on growth and proliferation of cell suspension culture on OPCM with 0.1 mg/L dicamba for 4 weeks

Concentrations of sodium chloride (mM)	Number of cell clumps (cell/flask)	Size of cell (cm)
0	26.6^{b}	0.3578
50	26.0^{b}	0.3685
100	32.4^{ab}	0.2695
150	36.0^{a}	0.3143
200	25.8^{b}	0.4052
F-test	*	ns
C.V. (%)	21.42	17.19

ns= not significantly different
* significantly different (p<0.05)
Mean values followed by the same letter within column are not significantly different according to duncan multiple range test (DMRT).

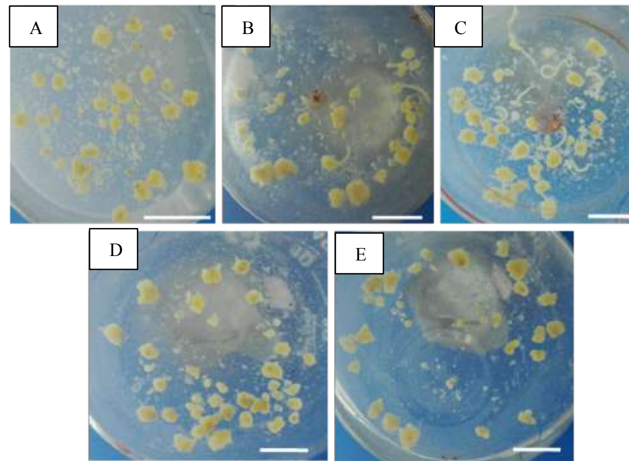

Fig.2 Characteristic of cell clump of oil palm SUP-PSU cultured in liquidified OPCM with 0.1 mg/l dicamba and different concentrations of NaCl for 4 weeks. (bar=1 cm)
A. Without NaCl B. 50 mM NaCl C. 100 mM NaCl
D. 150 mM NaCl E. 200 mM NaCl

CONCLUSIONS

The results could be concluded that callus proliferation at 100% and sizes of callus at 1.31 cm were successfully obtained on OPCM medium with 0.1 mg/L dicamba after 4 weeks of culture. Upon transferring12-day-old callus to liquidified OPCM with 0.1 mg/L dicamba and 150 mM NaCl, the highest number of cell clumps at 36 clumps was obtained after 4 weeks of culture.

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Agriculture & Agro-Industry **AAI-16**

Effect of Plant Growth Regulators on Shoot Multiplication and Root Induction from Culturing Shoot Tips of *Sang Yod Muang Phatthalung* Rice

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ABSTRACT

To study the effect of PGRs on shoot multiplication and root induction, shoot tips of *Sang Yod Muang Phatthalung* rice were cultured on PGR-free liquidified oil palm culture medium (OPCM) or OPCM medium with different concentrations of 6-benzyladenine (BA) (0, 0.5, 1, 1.5 mg/L) or α-naphthaleneacetic acid (NAA) (0 and 0.5 mg/L). The results showed that OPCM medium supplemented with 1 mg/L BA and 0.5 mg/L NAA gave the best results in a number of shoots at 16.25 shoots/single shoot, leaf length at 17.10 cm, number of roots at 92.50 roots/single shoot and root length at 8.18 cm after culture for 30 days, significantly different ($p<0.01$) with another concentrations of BA and NAA. Therefore, shoot tips cultured on OPCM medium with 1 mg/L BA and 0.5 mg/L NAA were suit for shoot multiplication and root induction of *Sang Yod Muang Phatthalung* rice.

Keywords: *Sang Yod Muang Phatthalung* rice, *in vitro*, shoot induction, root induction

1.0 INTRODUCTION

Rice (*Oryza sativa* L.) belongs to family Poaceae. It is a major food and one of the most important crops in the worldwide (Yinxia and Te-chato, 2013) and feeds over half of the global population. Global rice demand is projected to rise 26 % in the next 25 years and achieve nearly 555 million tons in 2035 (Ho *et al*., 2017a). However, increase in population, reduction of arable land and rice yield affected by disease and insect pest, environmental stresses e.g. drought, chilling temperature, saline and acid soil (Rattana *et al*., 2012) cause rising demand of rice production.

Sang Yod Muang Phatthalung rice get Geographical Indications in first cultivar of Thailand. It is grown in Patthalung province for hundreds of years and

one special rice variety with a dark-red color dehusk seed, soft and aromatic when cooked. Red rice had more 14-15 percent of amylose content, 8.6 gram of protein per 100 gram fresh weight, 82.01 mg in gallic acid per 100 gram fresh weight of polyphenol, 15.14 mg in cyanidin-3-glucoside of anthocyanin (Yodmanee *et al*., 2011) which were higher than those of white rice. Pigmented rice also contains high antioxidant activity that helps reduction in the risk of some chronic diseases for people such as diabetes, cancer and cardiovascular diseases (Ho *et al*., 2017a). Recently, the demand for healthier rice product are increasing globally. If special rice variety for market is produced it will increase economic profits to farmers and nutritional benefits to consumers. Thus, research and development of efficient mass propagation for *Sang Yod Muang Phatthalung* rice in the future is required (Ho *et al*., 2017a). However, the transformation of *indica* rice is still difficult due to a low frequency of plantlet regeneration, difficult to culture and require a longer period in contrast to *japonica* varieties.

Furthermore, there are many reports are studied on increasing of regeneration under *in vitro* culture in rice. The successful regeneration *in vitro* culture in rice normally depends on genotypes, culture media, PGRs, carbon sources, culture conditions and types of explants (Saharan *et al*., 2004; Hoque and Mansfield, 2004; Lin *et al*., 2005; Ge *et al*., 2006; Wani *et al*., 2010; Feng *et al*., 2011). In rice, plant regeneration has been obtained from different types of explants such as immature seeds (Hiei and Komari, 2008), mature seed (Zinnah *et al*., 2013; Zuraida et al., 2010), anther (Raina et al, 1987; Xa and Lang, 2011), microspore (Datta *et al*., 1990), leaf (Karthikeyan *et al*., 2011; Abiri *et al*., 2017) and root (Abiri *et al*., 2017). Generally, mature seeds are applied for callus formation and plantlet regeneration of *in vitro* culture and best explant compare with other explants. Oil palm culture medium (OPCM) medium is best medium used for propagation of rice cv. Sangyod (Ho *et al*., 2017b). Application of different types and concentrations of PGRs is extensively used to enhance of plantlet regeneration and improvement pathway of plant cells in culture media. Cytokinin was used for stimulation cell division and formation and growth of axially and adventitious shoots. Addition of BA in regeneration medium are practical for shoot regeneration in rice (Chuenboonngarm *et al*., 2001). Cytokinin in combination with auxin was also reported to encourage plantlet regeneration in rice (Rueb *et al*., 1994; Ho *et al*., 2017a)

The objectives of this study were to determine the optimum concentrations of BA or NAA for plantlet regeneration from shoot tips derived from mature seeds of *Sang Yod Muang Phatthalung* rice.

2.0 MATERIALS AND METHODS

Plant material and sterilization

Mature seeds of *Sang Yod Muang Phatthalung* rice were kindly provided by Phatthalung Rice Research Center and used as an explant source. The seeds were dehusked, washed with running tap water for 20 min, surface sterilized with 70 % (v/v) ethanol for 2 min and immersed in 20 % (v/v) Clorox (commercial bleach) containing 0.05 - 0.1 ml of a wetting agent “Tween-20” on an orbital shaker at 100 rpm for 10 min. Finally, the seeds were rinsed with sterile distilled water for 5 times

in a laminar air flow hood before blotting dry on autoclaved sterile tissue paper. Sterile seeds were then cultured on callus induction medium (CIM) as described by Ho *et al*. (2017b).

Explant preparation

Disinfected seeds were cultured on OPCM medium supplemented with 1 mg/L of dicamba. Medium was composed of 3 % (w/v) sucrose and solidified with 0.7 % (w/v) agar. The pH of the culture medium was adjusted to 5.7 before autoclaving at 121 °C, 1.07 kg/ cm^2 for 20 min. All cultures were carried out in Petri-plate (Ø 9 cm), sealed by Parafilm and maintained at 26 ± 2 °C in the culture room under 14 h photoperiod with irradiance of 25 µmol/m^2/s provided by cool white fluorescent tubes.

Experiment 1 Shoot multiplication and root induction

Shoot tips at approximately 5 mm in length were transferred to liquidified regeneration medium (RM) with different concentrations of BA (0, 0.5, 1, 1.5 mg/L) and NAA (0 and 0.5 mg/L). All PGRs containing medium were supplemented with 30 g/L sucrose, adjusted to pH 5.7 prior to autoclaving at 121 °C, 1.07 kg/ cm^2 for 20 min. Shoot tips were cultured in Erlenmyer flasks containing 25 ml of RM and incubated on a rotary shaker at 100 rpm under 14 h photoperiod in the culture room in order to optimize plantlet regeneration. Mean number of shoots per culture shoot tip, leaf length and mean number of roots and root length were recorded after 30 days of culture.

Acclimatization

Complete plantlets were transferred to clay soil containing in 8 inch plastic pots covered with plastic bottle and acclimatization in the greenhouse at 28-30 °C supplied by natural light conditions.

Statistical analysis

All the tissue culture experiments of shoot multiplication and root induction were analyzed using completely randomized design (CRD) with 5 replicates per treatment (2 explants per replication). Data were tested by using one-way analysis of variance (ANOVA) and the significant differences among means were separated by Duncan's multiple range test (DMRT) ($p \leq 0.01$) using the program R statistical package version 2.14.2.

3.0 RESULTS AND DISCUSSION

Effects of BA and NAA on shoot multiplication and root induction

In rice, plantlet regeneration is affected by many factors for example genotypes, physiological status of the explants, PGRs and culture environments. Shoot tips are necessary for nice explants and indicate strong dividing of the meristematic cells that might easily maintain *in vitro* regeneration (Ho *et al*., 2017a). The ratio of cytokinin (BA) to auxin (NAA) is important for in vitro regeneration efficiency of *Sang Yod Muang Phatthalung* rice due to it controls

many growth processes including organ regeneration, promote shoot multiplication and root induction from shoot tips. Base on the results in **Table 1**, shoot and root were observed to induce from all treatments. The greatest mean number of shoot/cultures shoot tip at 16.25 shoots, leaf length at 17.10 cm, number of roots at 92.50 roots and root length at 8.18 cm was observed in liquidified OPCM medium supplemented with 1 mg/L BA and 0.5 mg/L NAA after culture for 30 days **(Figure 1)**. However, 1 mg/L BA alone containing the medium cannot produce shoot and root because of browning of shoot tip. While low concentration (0.5 mg/l) or higher concentrations in combination with NAA could promote multiple shoot formation. Yusoh (2014) reported that shoot tips of *Hom Kra-Dang-Nga* rice culture in liquidified MS medium supplemented with 1 mg/L BA, 0.5 mg/L NAA and 3% sucrose gave the greatest survival rate of shoot, number of multiple shoots per flask and number of leaf per flask. However, the previous study by Ho *et al*. (2017a) found that addition of Kn at concentration of 0.5 mg/L together with BA and NAA to liquidified MS medium gave the greatest mean number of shoots/explant and root formation percentage. It is possible that OPCM medium contains lower concentration of NH4NO3, thus, improve the greater formation number of shoots.

Table 1 Effects of BA and NAA containing liquidified OPCM medium on shoot

BA (mg/L)	NAA (mg/L)	number of shoots/single shoot	leaf length (cm)	number of roots /single shoot	root length (cm)
0	0	4.50^{bc}	5.45^{b}	17.75^{d}	2.93^{ab}
0.5	0	9.00^{b}	14.88^{a}	24.50^{c}	4.45^{ab}
1	0	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
1.5	0	7.00^{b}	13.50^{a}	88.25^{a}	5.23^{ab}
0	0.5	5.75^{bc}	5.03^{b}	13.75^{d}	5.45^{ab}
0.5	0.5	6.75^{bc}	5.4^{b}	14.5^{d}	5.53^{ab}
1	0.5	16.25^{a}	17.10^{a}	92.50^{a}	8.18^{a}

multiplication and root induction from single shoot-derived OPCM medium with 1 mg/L dicamba of *Sang Yod Muang Phatthalung* rice after culture for 30 days

1.5	0.5	9.75^{b}	16.92^{a}	58.75^{b}	6.53^{a}
F-test		**	**	**	**
C.V. (%)		16.23	1.72	1.82	15.20

** Significantly different (p<0.01)
Means of those parameters with the same letter within column are not significantly different using DMRT

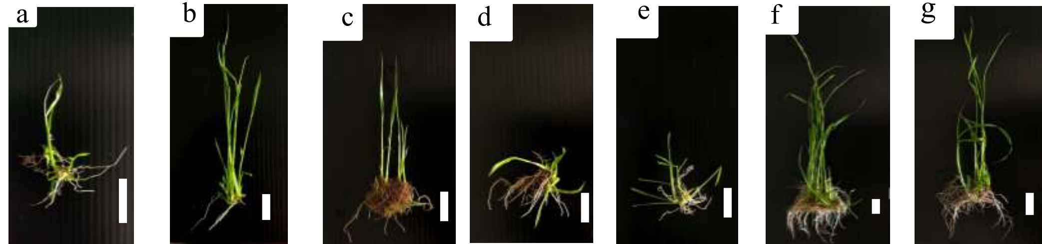

Figure 1 Morphological of shoots and roots of Sang Yod Muang Phatthalung rice culture in liquidified OPCM medium supplemented with different concentrations of BA and NAA after culture for 30 days. (a) PGR-free (b) 0.5 mg/L BA (c) 1.5 mg/L BA (d) 0.5 mg/L NAA (e) 0.5 mg/L BA and 0.5 mg/L NAA (f) 1 mg/L BA and 0.5 mg/L NAA (g) 1.5 mg/L BA and 0.5mg/L NAA (bars = 2 cm)

CONCLUSION

In the present study, an efficient plantlet regeneration protocol for *Sang Yod Muang Phatthalung* rice was established from shoot tip derived from mature seeds. The results revealed that PGRs were key factors in promoting plantlet regeneration. The successful optimum medium for shoot multiplication and root induction of *Sang Yod Muang Phatthalung* rice was liquidified OPCM medium supplemented with 1 mg/L BA and 0.5 mg/L NAA.

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Agriculture & Agro-Industry **AAI-17**

Insoluble Carbohydrate Distribution during Pollen Abortion in Mangosteen (*Garcinia mangostana* L.) Compared with Pollen Development in Seashore Mangosteen (*Garcinia celebica* L.)

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ABSTRACT

Pollen abortion in mangosteen (*Garcinia mangostana* L.), an important economical fruit of many tropical countries, was hypothesized to be related to the alteration of insoluble carbohydrate distribution. Ultrastructural and histochemical analyses of insoluble carbohydrate (starch) during pollen abortion were therefore needed, as well as a comparison with seashore mangosteen (*G. celebica* L.), the close relative species having normal pollen development. The starch accumulations studied by transmission electron microscopy and the periodic acid schiff polysaccharide specific reactions of both species were clearly different. *Garcinia celebica* exhibited a typical starch accumulation which first appeared at microspore mother cell stage and peaked in unicellular microspore stage. Meanwhile, in *G. mangostana*, starch slightly accumulated only in degenerated MMC, and no starch granule found from the microspore tetrad stage until microspore degeneration. The lower accumulation of starch granules in *G. mangostana* strongly proposed its cellular starvation leading to pollen abortion in *G. mangostana*. This study provides remarkable data which will broaden the current knowledge of mangosteen male sterility and will be useful for further study on mangosteen breeding system.

Keywords: insoluble carbohydrate, mangosteen, pollen abortion, seashore mangosteen

1.0 INTRODUCTION

Mangosteen (*Garcinia mangostana* L.), an economically important tropical fruit, has been reported as an apomictic plant providing sterile anthers with pollen abortion (Sutthinon *et al*. 2013). According to the roles of carbohydrate in providing energy source and structural component, an abnormality of polysaccharides distribution through the sequential pollen development has been claimed to result in pollen abortion (Tütüncü Konya 2018) in several male sterile species such as maize (Datta *et al.* 2002), chinese cabbage (*Brassica campestris*) (Xie *et al*. 2005), and sorghum (Jain *et al*. 2007). To gain the more insight into the relative arrangement of carbohydrate during pollen abortion in mangosteen, ultrastructural and histochemical analyses of carbohydrate were therefore needed, as well as a comparison with seashore mangosteen (*G. celebica* L.), the close relative species having fertile anther (Sutthinon *et al*. 2018).

2.0 MATERIALS AND METHODS

Plant materials

The fresh flower buds of *G. celebica* and *G. mangostana* at different developmental stages ranging by their flower diameters (Table 1) were collected from Kho Hong hill (March-April) and the mangosteen orchards in Hat Yai, Songkhla Province, Thailand (April-July), respectively. The developmental stages were determined with regard to the preliminary study on histological observation by paraffin technique.

TEM observation

Anthers at all stages were carefully isolated and pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer solution (PBS) (pH 7.2) for 3 h. These samples were washed with 0.1 M PBS 3 times for 5 min each. The pre-fixed samples were then fixed in 1% osmium tetroxide for 1-2 h, washed with distilled water 5 min for 3 times and stained with uranyl acetate for 20 min. The post-fixed samples were dehydrated using a graded ethyl alcohol (ethanol) series (70 %, 80%, 90% and 100%) for 15 min each. Embedding process was done in a hardening epoxy resin. The ultrathin sections (100 nm) were cut with a diamond knife (DiATOME Ltd., Nidau, Switzerland) followed by double staining with 5% uranyl acetate and lead citrate for 10 min and 5 min, respectively. After rinsing with distilled water, grids were allowed to dry, and imaged using a JEM-2010 TEM (JEOL Ltd., Tokyo, Japan).

The periodic acid Schiff (PAS) polysaccharide specific reaction

PAS reaction was conducted by following Varnier *et al* (2005). The semi-thin sections (1000 nm) were kept on glass slides. Slides were firstly immersed in 1% (w/ v) periodic acid for 4 h, Shiff's reagent for 16 h, and in 5% (w/v) sodium metabisulfite for 20 min. Sections were then rinsed in distilled water, and allowed to dry. Insoluble carbohydrates were stained pink to purple.

3.0 RESULTS AND DISCUSSION

The previous studies on histological examination by paraffin technique revealed that *G. celebica* had normal pollen development (Sutthinon *et al*. 2018). Meanwhile, *G. mangostana* showed the gradual degeneration of microspore leading to the absent of pollen at anthesis (Sutthinon *et al*. 2013). The insoluble carbohydrate accumulations studied by TEM and PAS reaction of both species were also clearly different. In *G. celebica*, starch granules first appeared in MMC **(Figure 1A-B, arrowheads)**, and microspore tetrad **(Figure 1C, arrowheads)**. Its accumulation peaked in the cytoplasm of unicellular microspore **(Figure 1D-F)**. These granules gradually declined at the vacuolated microspore stage **(Figure 1G-H)** and eventually absent at anther dehiscent phase providing starchless pollen **(Figure 1I)**. This event, often called amylolysis, has been shown in some tomato varieties (Carrizo Garcia *et al*. 2016).

Pacini *et al* (2006) claimed that mature pollen can generally be either starchy or starchless pollen depended on species. The amylolysis has been known to provide energy for cell metabolism, viability maintenance, and preparing for pollen disposal (Pacini *et al*. 2006; Tütüncü Konyar 2016). Vice versa, the gradual process of amylogenesis providing starchy pollen in some plants (e.g., sorghum and maize) has been suggested to be in conjunction with pollination mechanism (Carrizo Garcia *et al*. 2016). Meanwhile, male sterile line of those species failed in starch accumulation (Carrizo Garcia *et al*. 2016). This is also correlated to our results on male sterile *G. mangostana*, in which its starch slightly accumulated only in degenerated MMC **(Figure 1J-K, arrowheads)**. There was no starch granule found from the microspore tetrad stage **(Figure 1L)** until microspore degeneration stage (data not shown). The lower accumulation of starch granule in *G. mangostana* strongly proposed its cellular starvation leading to pollen abortion in *G. mangostana* as shown in male sterile line of maize (Datta *et al.* 2002), chinese cabbage (*Brassica campestris*) (Xie *et al*. 2005), and sorghum (Jain *et al*. 2007).

Table 1. Correlation between flower bud sizes and pollen developmental stages in *G. celebica* and *G. mangostana*

G. celebica		***G. mangostana***	
Developmental stages	**Flower bud sizes (cm)**	**Developmental stages**	**Flower bud sizes (cm)**
Microspore mother cell (MMC)	0.2-0.3	Microspore mother cell (MMC)	1.1-1.2
Microspore tetrads	0.4-0.5	Microspore tetrads	1.3-1.6
Unicellular microspore	0.6-0.7	Microspore and pollen	≥1.7
Vacuolated microspore	0.8-1.0	degeneration	
Pollen	Flowering		

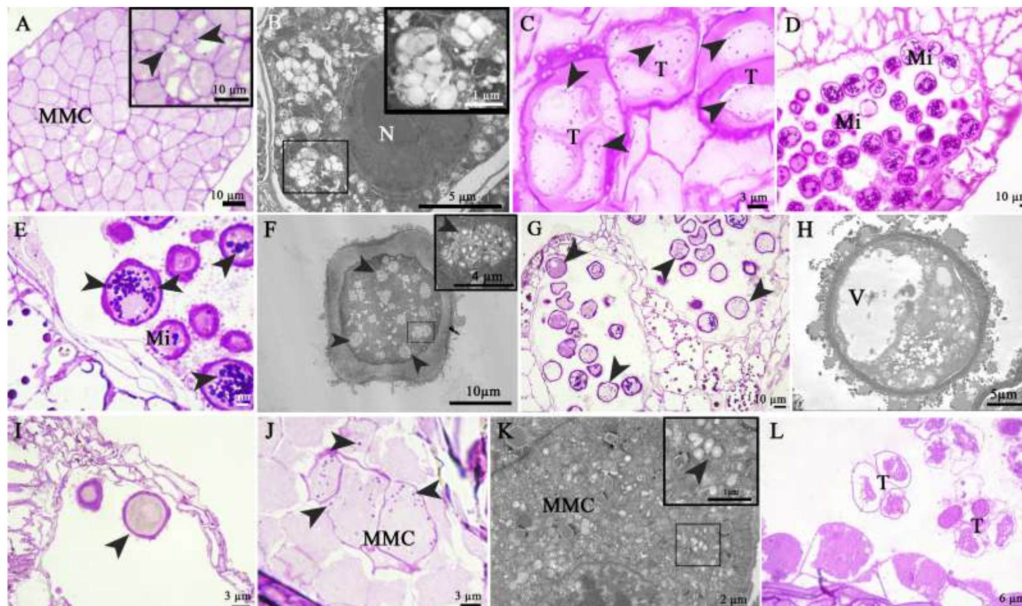

Figure 1. Insoluble carbohydrate distribution during pollen development in *G. celebica* (A-I) and *G. mangostana* (J-L) investigated by PAS reaction and TEM analysis **A**: Starch (arrowheads) in MMC and **B**: ultrastructure of starch granules in MMC **C**: Starch distribution (arrowheads) in microspore tetrad and **D**: in unicellular microspore **E**: Magnified view of uninucleate microspore showing high starch accumulation (arrowheads). **F**: Ultrastructure of starch granules (arrowheads) in cytoplasm of unicellular microspore. **G:** Vacuolated microspores (arrowheads) and **H**: ultrastructure of vacuolated microspore with lower starch accumulation. **I**: The starchless pollen (arrowhead) at anthesis stage. **J**: Starch

contents (arrowheads) and **K**: ultrastructure of starch granules (arrowhead) in MMC **L:** The degenerating microspore tetrad with no insoluble carbohydrate accumulation (*Mi* microspore, *MMC* microspore mother cell, *N* nucleus, *T* microspore tetrad, *V* vacuole)

4. CONCLUSION

Pollen abortion in mangosteen is related to the low accumulation of insoluble carbohydrate leading to the defects in nutrient preservation. Further study on molecular mechanism controlling mangosteen male sterile system is, therefore, needed to be clarified.

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Biomedical Science & Engineering **BSE-01**

Effect of Methylcellulose and Xyloglucan Ongelation Temperature and Cytotoxicity enhancement of Pluronic F127 Injectable hydrogel Fordrug Delivery Systems

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ABSTRACT

Novel hydrogels with required gelation temperature and biocompatibility were successfully prepared by blending various concentration of pluronic F127 (PF) with 4 wt% methylcellulose (MC) and 0.1 wt% tamarind seed xyloglucan (TSX). These hydrogels were prepared by cold method. Gelation temperature was investigated by test tube inversion method (TIM). Biocompatibility of hydrogels was determined by MTT method on osteoblasts cell line (MC3T3-E1). 16PF/MC, 18PF/MC and 20PF/MC hydrogel were found as gel at 25, 24 and 23°C. 18PF/TSX and 20PF/TSX hydrogel turned from solution to gel at 29 and 26°C, respectively, while the 16PF/TSX was not form gel. The biocompatibility results were found that all the blended hydrogels non-toxic to the cell. These hydrogels can be used as injectable drug delivery system in various route of drug administration.

Keywords: Pluronic F127, methylcellulose, xyloglucan, hydrogel, cytotoxicity

1. INTRODUCTION

Pluronic F127 (PF) (PEO_{99}–PPO_{65}–PEO_{99}) is a triblock copolymer consisting of hydrophilic (PEO) and hydrophobic (PPO) units which are most commonly used as thermo-responsive hydrogels for drug delivery systems. As temperature increases, PPO chains are dehydrated and become insoluble. This induces the formation of the micellar structure and aggregation and gelation (He, Kim, and Lee 2008). Generally, gelation of various types of pluronics depends on temperature and concentration of pluronics. Upon cooling or lowering the systems, the polymers are hydrated and relatively soluble in water and the system turns into sol (Bercea *et al*. 2011). As the concentration of pluronic decreases, sol to gel transition temperature increases (Dumortier *et al.* 2006). Recently, PF based hydrogel has been widely investigated due to its ability to increase bioavailability (drug solubility and drug absorption), to promote drug stability and to control drug

release (Dumortier *et al*. 2006). However PF has some disadvantages including poor mechanical strength, short residence time, high permeability and limitation of molecular weight (He, Kim, and Lee 2008). PF may be able to form gel at a concentration ≥ 15 wt% (El-Kamel 2002). However, PF at high concentrations (20 wt%) was reported to be toxic for osteoblasts (MC3T3-E1) and myoblasts cell line (C2C12) (Rangabhatla *et al*. 2015). Methylcellulose (MC) has been able to reduce toxic effects in normal cells (Kim *et al*. 2012). A limitation of methylcellulsoe hydrogel is that its sol-to-gel transition is at temperatures higher than the body temperature (50-70°C). Tamarind seed xyloglucan (TSX) is widely used as a food additive and in pharmaceutical applications; it is non-toxic, biodegradable and biocompatible (Chen *et al*. 2012).

The aim of this work was to modify PF hydrogel in term of gelation temperature and biocompatibility by blending PF with methylcellulose and tamarind seed xyloglucan.

2. MATERIALS AND METHODS

2.1. Materials

PF (PEO_{99}–PPO_{65}–PEO_{99}), MC (27.5-31.5% methoxyl basis; viscosity 10-25 mPa.s; 2 % in H_2O at 20°C), PBS (pH 7.4), MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) reagent and sodium bicarbonate (for cell culture) were purchased from Sigma (St. Louis, MO, USA). TSX was purchased from Megazyme International Ireland Ltd (Wicklow, Ireland). A mouse osteoblastic cell (MC3T3-E1) Subclone 4 (ATCC® CRL-2593™) was obtained from the ATCC (Manassas, VA, USA). Alpha Minimum Essential Medium (α-MEM), Fetal bovine serum (FBS) and antibiotic were purchased from Gibco™, Thermo Fisher Scientific Inc (Waltham, MA, USA). All other chemicals and reagents used were of analytical grade.

2.2. Polymer solution and hydrogel blends preparation

2.2.1. PF solution preparation

Various concentrations of PF (16, 18 and 20 wt%, referred to as 16PF, 18PF and 20PF) were prepared using a cold method (Bercea *et al*. 2011). Briefly, the required amount of PF was dispersed in cold Mill-Q water on an ice bath with continuous stirring until a clear solution appeared. The PF solutions were refrigerated overnight to ensure that the polymer was thoroughly dissolved.

2.2.2. MC solution preparation

MC powder was dried at 55°C for 24 h to remove moisture before use. 4 wt% MC was prepared by dispersing the required amount of MC powder in hot water (70°C). After complete dispersion, the temperature of the mixture was reduced on an ice bath with continuous stirring until a clear solution appeared. Clear MC solution was kept in the refrigerator (4°C) at least 24 hours to ensure that it was completely homogenous (Klouda 2015).

2.2.3. TSX solution preparation

0.1 wt% of TSX solution was prepared by dispersing the required amount of TSX in Milli-Q water followed by magnetic stirring for 4 h at 50°C. The TSX solution was refrigerated overnight before use.

2.2.4. PF/MC solution preparation

PF/MC blends were prepared by dispersing the accurate amount of PF powder in cold MC solution on an ice bath under the stirring condition for 30 min. PF/MC solutions at different PF concentration (16PF/MC, 18PF/MC, and 20PF/MC) were kept in a refrigerator for further experiments.

2.2.5. PF/TSX solution preparation

PF/TSX blends were prepared by dispersing the accurate amount of PF powder in cold TSX solution on an ice bath under the stirring condition for 30 min. PF/TSX blends at different concentration (16PF/TSX, 18PF/TSX, and 20PF/TSX) were kept in a refrigerator for further experiments.

2.3. Test tube inversion method

The sol-gel transition temperature of hydrogels was observed by test tube inversion method (TIM). One ml of hydrogel in a test tube was placed on a controlling temperature water bath. The temperature was increased by 1°C increments and the samples were equilibrated for 5 min by increasing temperature from 20 to 40°C. After inversion of the test tube, temperature without flow was indicated as transition temperature from solution (sol) to gel (Rangabhatla *et al*. 2017).

2.4. Cytotoxicity Assay

2.4.1. Cell culture

MC3T3-E1 was cultured in α-MEM with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine and 1 mM sodium pyruvate, 10 % FBS and 1% antibiotics. As routine culture for osteoblast differentiation, complete growth medium was supplemented with 3 mM ascorbic acid. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. For cell passage, cells were rinsed with PBS twice and then harvested by treatment with 0.25% Trypsin/EDTA. Cells were resuspended in a complete growth medium at a desired volume before testing.

2.4.2. Preparation of hydrogel extracted for cell culture

Hydrogels solution (500 μl/well) were added to a 24-well plate. After the samples formed gel at 37°C for 1 h, 1 ml PBS was added to each well. The plate was incubated for 24 hrs and the PBS extract was sterilized by filter through the 0.2μm

syringe filter. PBS extract solutions were diluted with culture medium then 100 μl was added to each well for the cytotoxicity test.

2.4.3. *In vitro* cytotoxicity test

Cytotoxicity was determined by using the MTT method. Briefly, cell density around $2x10^4$ cells/ml was seed in 96-well plates (100 μl/well). The cells were incubated at 37ºC to allow cells attach for 24 hours. After incubation, the medium was removed and the fresh medium was added at 100 μl/well. Cells were incubated in 5% CO_2 at 37ºC for 24 hours. After incubation, the medium was removed and the cells were washed with 100 μl of PBS twice. Then, 90 μl of PBS and 10 μl MTT (5 mg/ml) were added and incubated at 37ºC for 4 hours. MTT solution was removed and 100 μl of DMSO was added instead. The formazan precipitates were quantitated by measuring the absorbance at 570 nm using the microplate reader (Beckman coulter; DTX 880 multimode detector, USA). Cytotoxicity of the sample was compared with those of 100 ppm zinc acetate and cell culture medium as a positive and negative control, respectively. Cytotoxicity was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{absorbance of sample treated cells}}{\text{absorbance of control}} x100$$

3. RESULTS AND DISCUSSION

3.1 Sol-gel transition temperature

The gelation temperatures of PF alone and the blend of PF/MC or PF/TSX were determined by the TIM method. The result is shown in Table 1. PF exhibits thermo-reversible behaviour depending on its concentration and temperature. 16PF, 18PF and 20PF solutions became clear gel at 29, 28 and 25°C, respectively. Therefore, increasing its concentration could decrease the gelation temperature. MC alone (4 wt%) was found to be a turbid gel at 52°C which is higher than body temperature. The blends of 16PF/MC, 18PF/MC and 20PF/MC solutions were found to be a turbid gel at 25, 24 and 23°C, respectively. Generally, unmodified TSX solutions cannot form gel. This study tried to use the lowest concentration (0.1 wt%) of xyloglucan solution to investigate its effect on gelation temperature of PF. Blending TSX with PF solutions slightly increased the gelation temperature of PF, 18PF/TSX and 20PF/TSX solutions formed gel at 29 and 26°C, respectively. However, the 16PF/TSX was not form into gel after increasing temperature to 40°C. As previously recorded, 16 wt% is the lowest concentration that can turn to gel of PF solutions (Sun and Raghavan 2010). The mixing of 0.1 wt% TSX to PF solution may interrupt the ordered structure of the PF phase and formation of the gel network structure during heating. However, 0.1 wt% TSX has less effect on gelation of higher PF solution concentrations.

Table 1. Gelation temperature of PF solution and their blends

Samples	MC	16PF	18PF	20PF	16PF/MC	18PF/MC	20PF/MC	16PF/TSX	18PF/TSX	20PF/TSX
Gelation temperature (°C)	52	29	28	25	25	24	23	Sol*	29	26

*Still remain as a viscous liquid at 40 °C

3.2 Cytoxicity study

To investigate whether the prepared hydrogels were non-toxic at the targeted organs after application, the cytotoxicity was then determined. The cytotoxicity of extractable hydrogels was determined using MTT assay on MC3T3-E1 cell lines. As shown in Figure 1, 16PF, 18PF and 20PF were toxic to the cells with cell viability of 66, 65, and 61%, respectively as compared to negative control. PF gels reduced cell viability in a dose-dependent manner. The blends of 16PF/MC, 18PF/MC, and 20PF/MC were found not only non-toxic to the cell but also enhancing cell proliferation higher than 100%. These results indicated that MC can reduce the toxicity of PF with good compatibility to MC3T3-E1 cell lines. 16PF/TSX was not form gel at 37°C, so the cytotoxicity result was not performed. 18PF/MC and 20PF/MC exhibited cell viability about 93 and 92%, respectively. TSX is one of biopolymers has biocompatibility and mucoadhesive properties which is widely used for biomedical applications such as tissue engineering and extracellular matrix, especially in drug delivery system (Joshny Joseph *et al*. 2012).

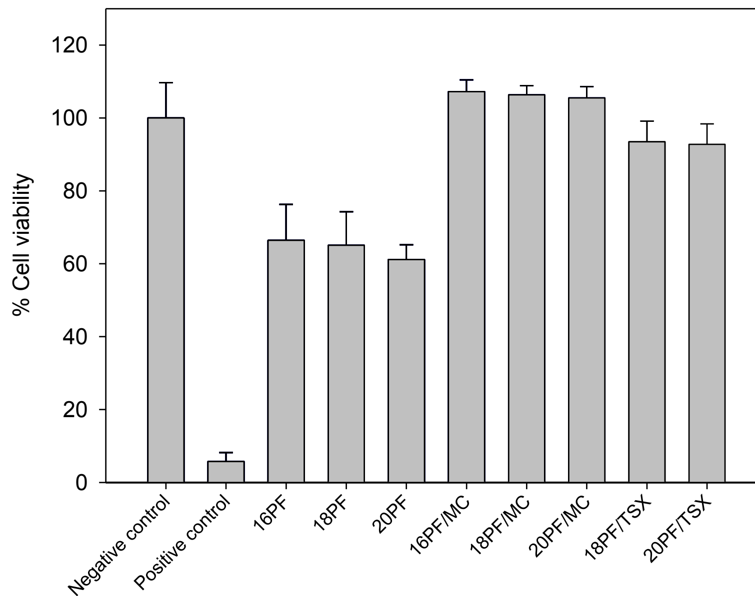

Figure 1. Cell viability of hydrogels extractable on MC3T3-E1 cells (n=8; mean±SD)

4. CONCLUSION

Gelation temperature of PF hydrogels has been altered to close the body temperature (37°C) by blending PF with MC and TSX. PF/MC and PF/TSX hydrogels were proved to be safe on MC3T3-E1. In addition, these blends, especially PF/MC were cytocompatible to the cells. Limitation of using higher concentrations of PF127 was improved by blending with these biopolymers.

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Environment & Resource Management **ERM-01**

Coastal Zone Management in Penang, Malaysia: Governance, Challenges and Recommendations

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ABSTRACT

The goal of this study is to implement an investigation of the status of coastal zone management in the state of Penang along with some discussions on the Importance of introducing Marine Spatial Planning (MSP) management approach. A contextual overview of boundaries and governance and challenges in implementing Integrated Coastal Zone Management (ICZM) in Penang Malaysia, has been performed. ICZM in Penang has been developed according to the needs at local, national and international levels. However, the centralism by the Federal government and the unwillingness to grant decentralization to states are the main obstacles to implement Penang ICZM plan of 1996 and enacting the ICZM policy draft of 2004 in Malaysia. The regulation system in Malaysia if it can transcend the shoreline in both directions will create a management system that can address maritime and coastal areas in a comprehensive and integrated approach, which in turn will motivate the Federal government to decentralize its coastal management. Based on that, this study recommends integrating coastal zone management with MSP concept for Penang state, hence, overcoming coastal zone management challenges.

Keywords: Integrated Coastal Zone Management (ICZM), Marine Spatial Planning (MSP)

1. INTRODUCTION

Coastal areas have always been a hub of resources and a target for infrastructure development (Mokhtar and Aziz, 2003). Coasts often faces dilemmas of urbanization and the worsening of environment for the living organisms, and the absence of an integrated approach in handling its issues (Prem, 2010). Therefore, Agenda 21 of the Earth Summit UN Conference on Environment and Development (UNCED) held in Rio de Janeiro, Brazil, 1992, introduced Integrated Coastal Zone Management (ICZM) as an integrated approach to managing coasts in sustainable most effective manner (Cicin-Sain et

al., 1998; Tiller et al., 2012). By adopting ICZM, the open access nature of the coastal areas is respected (Tiller et al., 2012) where all citizens have the right to access its various features. This can be achieved by getting many organizations involved and active in its management (Cooper, 2011).

Situated at the west coast of Malaysia, Penang state shoreline length is 152 km. However, this figure is in continuous change due to the loss of complexity in shoreline configuration, land reclamation and the artificial island projects (Chee et al., 2017). In 1996 the Malaysian government in a joint project with Danish Co-operation for Environment and Development (DANCED) sponsored ICZM project in Penang state. In addition, in 2007, Integrated Shoreline Management Plan (ISMP) study was initiated by Department of Irrigation and Drainage (DID) in Penang. Though shoreline management plans are more of engineering plan as they generally focus on coastal erosion and defenses as their primary objective, yet, it has demonstrated valuable aspects related to ICZM such as: state's rapid economic growth, pollution, loss of mangroves, overexploitation of coastal fisheries and worsening the water quality (NRE and PKSD, 2010). Thus, by reviewing the adapting governance patterns for coastal zone, and challenges in implementing it, the goal of this study is to implement an investigation of the status of coastal zone management in the state of Penang along with some discussions on the Importance of introducing Marine Spatial Planning (MSP) in enhancing coastal zone management in Penang state.

2. BOUNDARIES AND GOVERNANCE OF COASTAL ZONE IN PENANG, MALAYSIA

The ratification of the United Nations Conference on the Law of the Sea (UNCLOS) 1958 and its amendments in 1982 by Malaysian government came into force in 1996 (Territorial Sea Act, 2012), and subsequently its maritime acts and legislation were developed in accordance to the convention, this was first covered by the National Land Code Act 1965. The bill defined new terminologies such as *state land* as all land in a state (including much of the bed any river, and of the foreshore and bed of the sea, within the territories of the state or the limits of territorial waters); *shoreline* as the high-water-mark of ordinary spring tides, and *foreshore* as all land lying between the shoreline and the low-water mark (baseline) of ordinary spring tides (National Land Code Act,1965). (Figure 1) shows zones of Malaysia maritime zones and coastal area. In addition, Emergency (Essential Powers) Ordinance, No.7 of 1969 defined *the Territorial Sea* in West Malaysia as being within 12 NM from the shoreline. The ordinance described *Malaysian Maritime Zone* as consisting of territorial seas, continental shelf, the Exclusive Economic Zone (EEZ) as well as the airspace over EEZ. In 1984, the Exclusive Economic Act came into force where it sets out the *EEZ* as the area beyond and adjacent to the territorial sea extending to 200 NM from the baseline. As a follow-up on the series of Acts defining the Malaysia Maritime Zone came the Maritime Zones Act 2006, providing a declaration of geographical coordinates of base points, for determining the

baselines of Malaysia and for other matters connected therewith such as *low-water line, baselines, low-tide elevation; territorial sea, continental shelf, EEZ* and *maritime zones* (NRE and PKSD, 2010). Finally, the Territorial Sea Act 2012, focused on the Malaysian territorial waters, with elaboration on some definitions such as the *low-water* as a line on a low-tide elevation that is situated, wholly or partly, at a distance not exceeding the breadth of the territorial sea from the mainland or an island (Territorial Sea Act, 2012).

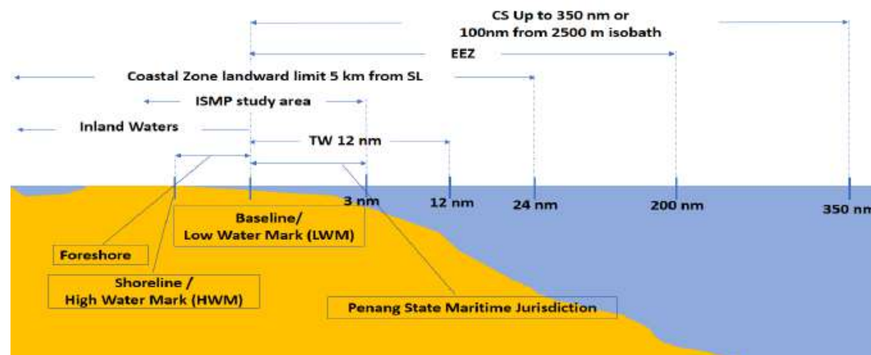

Figure 1. shows zones of Malaysia maritime zone and coastal area (applicable to Penang), (source: based on related Malaysian Acts)

The Executive Council in Penang is the highest administrative body in the State, however, the decisions of four types of institutions affect the CZM in Penang: they are the federal, state, local (de Oliveira, 2016), also, statutory bodies (Town and Country Planning Act, 1976); at the national level, ministries are represented by departments such as the DID and the Department of Environment (DOE) at the state level, (DID provide assistance to DOE in the form of comments on EIA submissions for projects affecting rivers, coast and water resources. Furthermore, due to the absence of a sole agency with overall coastal management responsibility, the EPU of the Prime Minister's Department plays an essential role in coordinating development between federal and state levels in the coastal areas (Siry, 2006). At the *state level*, the Town and Country planning department (TCPD) of Penang is entrusted with the responsibilities of ensuring the proper planning, development, and conservation of land in Penang, including coastal areas up to 3 nm (Town and Country Planning Act, 1976). At the local level, authorities not under the Federal government also come under the purview of the state government (de Oliveira, 2016). Finally, the statutory bodies, such as the *Penang Development Corporation* (PDC), is also under the state government control. PDC's primary aim is to explore new development opportunities, identify and execute social projects for the residents of Penang, assist the State government in formulating the State policies, and provide advice to other agencies toward making Penang a developed State (NRE and PKSD, 2010).

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Environment & Resource Management **ERM-02**

Accumulation Heavy Metal in Fruits/Vegetables and Animals in Loei Province, Thailand

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ABSTRACT

The objective was to examine the levels of As, Cu and Zn in fruits/vegetables and animals collected from wetlands and arable lands surrounding the gold mining region in Wang Saphung district, Loei province, Thailand. Sampling was performed between May 2016 and March 2017 to allow the detection of variations in the levels of the studied metals in both the rainy and dry season. As indicated by ICP-OES analysis, it was observed the average As contents detected in both fruits/vegetables and animals were found to exceed the maximum allowable concentrations. All the analyzed animals and almost all of the studied fruits/vegetables accumulated high contents of Zn, which were higher than the maximum allowable concentration. Almost all of the analyzed animals also contained high levels of Zn that exceeded the maximum allowable concentration. The accumulated low levels of Cu, which were within the maximum concentration, while most of animals contained Cu above the maximum allowable concentration. In most cases, the levels of heavy metals appeared to be more concentrated in fruits/vegetables and animals in the dry season than in the wet season. Moreover, animals were likely to contain higher levels of Cu and Zn than fruits/animals, whereas the greater levels of As was observed in fruits/vegetables.

***Keywords**:* Gold mining, Heavy metals, ICP-OES, Wang Saphung

1. INTRODUCTION

Heavy metals occur naturally in the environment with variations in concentration and can be released to the environment from a range of natural

sources and also by anthropogenic activities (Tchounwou et al., 2012). Even though adverse health effects of heavy metals have been known for a long time, exposure to heavy metals continues and is even increasing in some areas (Järup, 2003). For instance, Hg is still used in gold mining in many parts of the world (Balzino et al., 2015; Nakazawa et al., 2016; Pinedo-Hernández et al., 2015; Sippl, 2015) and in hair dye and tattoo pigments (Omolaoye et al., 2010). Ni and other transition metals including Fe, Co and Cu are still common in the production of carbon nanotubes and hydrogen (Acomb et al., 2016), and Li-ion batteries are receiving popularity in modern societies (Xin et al., 2016).

Emissions of heavy metals to the environment occur through a wide range of processes and pathways including vehicle emissions, stormwater runoffs, industrial discharges and other household activities (Djukić et al., 2016; Hu et al., 2009; Iqbal and Kim, 2016; Islam et al., 2016; Pulles et al., 2012; Tamim et al., 2016). With rapid urbanization and industrialization, urban areas are responsible for a major proportion of heavy metals discharged into the environment (Luo et al., 2012; Ma et al., 2016; Raffo et al., 2016). Meanwhile, large amounts of heavy metals and dissolved organic matters discharged into rural environment originate from agricultural practice and mining industry (Babin et al., 2016; Kapusta and Sobczyk, 2015), and mining industry is responsible for a large proportion of metal loading.

Even though mining is known to provide a variety of socio-economic benefits (Chuhan-Pole et al., 2015; Hajkowicz et al., 2011; Kitula, 2006), it is recognized as the single largest source of pollution after agriculture (Peng et al., 2016; US EPA, 2016; Wang et al., 2015). In general, gold mining activities result in considerable quantities of mine wastes, tailings, and effluents with large amounts of potentially harmful elements, which have raised a health and environmental concern in many countries of the world (Ngure et al., 2014). As heavy metals get accumulated by aquatic life to certain concentrations, they can cause toxic effects, which include genotoxicity and histopathological alteration, oxidative stress and apoptosis (Fatima et al., 2015; Morcillo et al., 2016). Also, heavy metals can affect diverse systems and organs resulting in both acute and chronic effects on children's health, ranging from minor upper respiratory irritation to chronic respiratory, cardiovascular, nervous, urinary and reproductive disease, as well as aggravation of pre-existing symptoms and disease (Zeng et al., 2016).

Thailand has at least ten gold mines currently in operation, in three major regions (North, Northeast and South) of the country. In Northeast Thailand, small-scale gold mining covers six areas in Wang Saphung district of Loei province with a total of approximately 2.066 km2, each of which lies within the Loei/Phetchabun/Prachin Buri Gold Belt. This gold mining operation is under the Phu Thab Pha Project underpinned by a lifetime concession granted to Thung Kham Co., Ltd in September 2002. The gold mines in Wang Saphung are surrounded by wetlands and arable lands in which the villagers earn their living by crop production such as rice, papaya and soybean, and by catching

fishes and many other types of aquatic life. The occurrence of soil and water contamination by heavy metals in these areas can inevitably lead to bioaccumulation of heavy metals in tissues of plants and animal species, and can be easily transferred to humans through consumption of the affected plants and animals (Liu et al., 2009). With this regard, determination of levels of heavy metals accumulated into plants and animals in wetlands and arable lands surrounding the gold mines in Wang Saphung is needed.

For this purpose, contamination levels of As, Cu and Zn were examined in fruits/vegetables and animals collected from wetlands and arable lands surrounding the gold minding area in Wang Saphung district, Loei province, Thailand (Fig. 1). Inductively coupled plasma optical emission spectrometry (ICP-OES) was employed for elemental analysis in this study.

2. MATERIALS AND METHODS

2.1. Gold mining in Wang Saphung and site description

Gold mining in Wang Saphung was commenced in September 2002 under the Phu Thab Pha Project underpinned by a lifetime concession granted to Thung Kham Co., Ltd., which is settled down in Khao Luang subdistrict, Wang Saphung district, Loei province, Thailand. The gold mines cover six areas of the district with a total of approximately 2.066 km2, each of which lies within the Loei/Phetchabun/Prachin Buri Gold Belt and is surrounded by wetlands and arable land in which the villagers earn their living by crop production and catching fishes and many other types of aquatic life.

2.2. Sampling sites and regime, and sample preparation

Sampling was conducted randomly from 10 zones across all wetlands and arable lands surrounding the gold mining region in Wang Saphung (Fig. 1), with timing of data collection performed between May 2013 and March 2014 to allow the detection of variations in the levels of the studied metals during rainy and dry seasons. A total of 16 samples of fruits/vegetables and animals, including seven different naturally growing fruits/vegetables [Colocasia esculenta var. aquatilis (elephant ear), Diplazium esculentum (vegetable fern), Limnocharis flava (yellow velvetleaf), Lasia spinosa (Phak Naam), Ipomoea aquatica (water morning glory), Bambusa bambos (bamboo) and Ficus spp. (fig)], five crop plant species [Cymbopogon citratus (lemon grass), Alpinia galanga (galangal), Zingiber montanum (Cassumunar ginger), Carica papaya (papaya) and Glycine max (soybean)] and four animal speices [Macrobrachium lanchesteri (Lanchester's freshwater prawn), Filopaludina martensi (river snail), Esanthelphusa dugasti (rice field crab) and Rasbora tornieri (yellowtail rasbora)] were collected for heavy metal analysis. From each sampling sites, a composite of at least 10 samples for each item was prepared. The collected samples were placed in plastic bags, labeled, packed in ice and transported to the laboratory.

For sample preparation, the samples were thoroughly cleaned, sliced or smashed into small pieces, sun-dried for 2 weeks, oven-dried at 70 ºC for 1 h, and ground to a find powder.

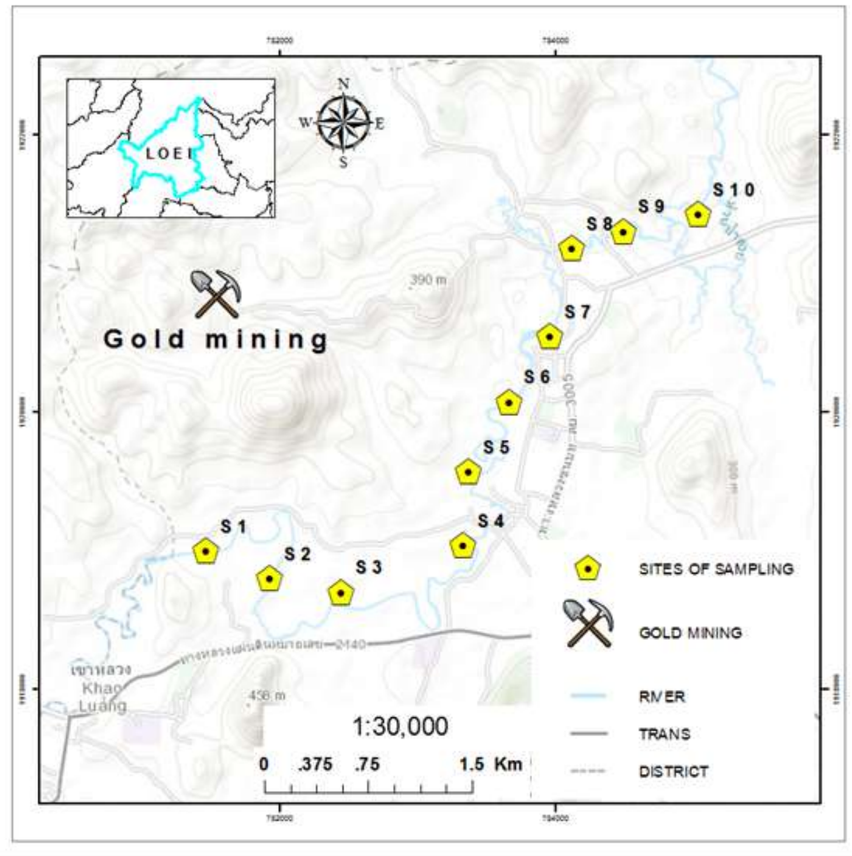

Figure 1. Located of Wang Saphung Loei Thailand

2.3. ICP-OES of metal ions

Before metal determination, 1 g of each plant sample was precisely weighed and digested with HNO3/HClO4 2:1 v/v, while 3 g of each animal sample were required for metal digestion in a mixture of 33% HNO3/36% HCl/ 72% HClO4 (10:3:3, v/v/v). The digestion process was carried out at150 ºC for 4 h and the extract was then filtered through a No. 42 filter paper and adjusted to 50 mL with 1% HNO3 and subjected to elemental analysis.

Elemental analysis was carried out using an Optima 8000 ICP-OES (Perkin Elmer, USA) axial spectrometer fitted with a Seaspray nebulizer and SPS3autosampler under the following conditions: RF generator power of 1.30 kW, plasma gas flow rate of 15 L/min, auxiliary gas flow rate of 0.2 L/min, and

pump rate of 1.5 mL/min. The analytical lines (nm) were 188.979 (As), 324.752 (Cu) and 213.857 (Zn).

2.4. Data analysis

Data analysis was performed using SPSS for Windows version 17.0 (SPSS Inc.). The statistical significance was set at $p < 0.05$. Comparison of metal concentrations in the samples collected from different stations within the study area was done by tests that employ 'means' and 'standard deviations' functions.

3. RESULTS AND DISCUSSION

3.1. Metal contents in fruits/vegetables and animals

In this study, concentrations of As , Cu and Zn were determined in the composite samples of seven wild fruits/vegetables, including fig (Ficus spp.), elephant ear plant (Colocasia esculenta var. aquatilis), vegetable fern (Diplazium esculentum), yellow velvetleaf (Limnocharis flava), Phak Naam (Lasia spinosa), water morning glory (Ipomoea aquatica) and bamboo (Bambusa bambos), five grown herbs/fruits, including lemon grass (Cymbopogon citratus), galangal (Alpinia galanga), Cassumunar ginger (Zingiber montanum), papaya (Carica papaya) and soybean (Glycine max) and four animals, including Lanchester's freshwater prawn (Macrobrachium lanchesteri), river snail (Filopaludina martensi), rice field crab (Esanthelphusa dugasti) and yellowtail rasbora (Rasbora tornieri). The levels of heavy metals in the analyzed samples were presented in Tables 1 and 2. All metal contents were expressed in dw basis. As shown in Table 1 which presents the levels of As, Cu and Zn in fruits/vegetables collected from wetlands and arable lands in the vicinity of the gold mines in Wang Saphung, the lowest and highest As contents detected in wild fruits/vegetables were found in elephant ear plant (10.94 µg/g dw) and bamboo (39.83 µg/g dw), respectively. On the other hand, as observed for grown herbs/fruits the As contents were least abundant in Cassumunar ginger (29.18 µg/g dw) and were most plentiful in galangal (60.43 µg/g dw). The average As contents of both the wild and grown fruits/vegetables were found to exceed the maximum allowable concentration (MAC) of 2 µg/g dw. The lowest and highest Cu contents in wild fruits/vegetables were detected in bamboo (7.61 µg/g dw) and water morning glory (19.48 µg/g dw), respectively. In grown herbs/fruits, papaya accumulated the lowest content of Cu (1.41 µg/g dw) while Cassumunar ginger contained the highest Cu content (7.66 µg/g dw). The average Cu contents detected in both the wild fruits/vegetables and grown herbs/fruits were within the MAC of 20 µg/g dw. Zn measured in wild fruits/vegetables was found at the lowest content in fig (73.42 µg/g dw) and at the highest content in yellow velvetleaf (212.90 µg/g dw). In grown herbs/fruits, Zn was least abundant in Cassumunar ginger (68.37 µg/g dw) and most

abundant in lemon grass (192.69 µg/g dw). The average Zn contents measured in most of the wild fruits/vegetables and grown herbs/fruits were greater than the MAC of 100 µg/g dw. The As contents detected in fruits/vegetables in this study were much higher than that measured in a variety of plant species in the Gatumba tin–tantalum mining district, Rwanda, while the Cu contents were comparable (Nieder et al., 2014). Food crops and vegetables grown in Enyigba community, located in Ebonyi State, Southeastern Nigeria, which is prominent for lead-zinc mining for decades, were also observed to accumulate Cu (Obiora et al., 2016) at the same range as detected in the present study.

As given in Table 2, which shows the contents of As, Cu and Zn in animals caught in the vicinity of the gold mining areas in Wang Saphung, it was found that the lowest and highest As contents in animals was found in yellowtail rasbora (4.86 µg/g dw) and rive snail (12.59 µg/g dw), respectively. The average As contents detected in all animals were higher than the MAC of 2 µg/g dw. Again, yellowtail rasbora was found to accumulate the lowest content of Cu (19.36 µg/g dw) while rive snail contained the highest Cu content (48.60 µg/g dw). The average Cu contents measured in most of the studied animals were also greater than the MAC of 20 µg/g dw. In the case of Zn, the lowest content was detected in Lanchester's freshwater prawn (152.78 µg/g dw) while the highest content was found in river snail (348.83 µg/g dw). Again, the average Zn contents detected in all animals were greater than the MAC of 100 µg/g dw. The levels of As accumulation in the animals studied in the present study were much greater than those in wild Arctic hares (Lepus arcticus) inhabiting a former lead-zinc mine in the Canadian high Arctic (Amuno et al., 2016).

The levels of As, Cu and Zn accumulated in fruits/vegetables and animals were also compared and the results are given in Fig. 2. As shown, it was found that fruits/vegetables accumulated As at higher levels than animals, while Cu and Zn were detected at greater levels in animals than in fruits/vegetables.

Table 1. Mean concentrations (µg/g) and standard deviations (means ± SD) of As, Cu and Zn in the composite samples of naturally growing fruits/vegetables and crop plant species collected from wetlands and arable lands surrounding the gold mine in Wang Saphung.

Tree/plants	Heavy metals (µg/g dw)		
	As	Cu	Zn
Naturally growing			
tree/plants	10.94 ± 10.81	10.62 ± 3.98	105.47 ± 54.55
Elephant ear	16.60 ± 16.44	11.18 ± 2.63	100.54 ± 30.41
Vegetable fern	33.36 ± 1.38	17.06 ± 1.03	212.90 ± 34.94
Yellow velvetleaf	25.57 ± 32.13	10.41 ± 3.17	126.46 ± 76.99
Phak Naam	33.11 ± 1.68	19.48 ± 15.16	112.81 ± 2.80
Water morning glory	39.83 ± 1.53	7.61 ± 1.48	108.97 ± 5.76
Bamboo	34.80 ± 7.51	17.47 ± 3.41	73.42 ± 11.89
Fig			
Crop plants	33.04 ± 4.87	2.95 ± 6.47	192.69 ± 9.13
Lemon grass	60.43 ± 4.32	7.18 ± 1.16	96.10 ± 4.87
Galangal	29.18 ± 4.58	7.66 ± 7.51	68.37 ± 9.17
Cassumunar ginger	29.46 ± 2.95	1.41 ± 0.04	75.80 ± 4.39
Papaya	44.33 ± 2.37	6.11 ± 0.59	71.12 ± 3.99
Soybean	2	20	100
MAC			

Table 2. Mean concentrations (µg/g) and standard deviations (means ± SD) of As, Cu and Zn in the composite samples of animal species collected from wetlands surrounding the gold mine in Wang Saphung.

Tree/plants	Heavy metals (µg/g dw)		
	As	Cu	Zn
Lanchester's	11.95 ± 5.65	28.74 ± 19.54	152.78 ± 93.72
freshwater prawn	12.59 ± 5.41	48.60 ± 23.21	348.83 ± 191.09
River snail	5.69 ± 3.07	47.74 ± 17.18	280.49 ± 87.57
Rice field crab	4.86 ± 4.09	19.36 ± 10.25	167.55 ± 85.47
Yellowtail rasbora	2	20	100
MAC			

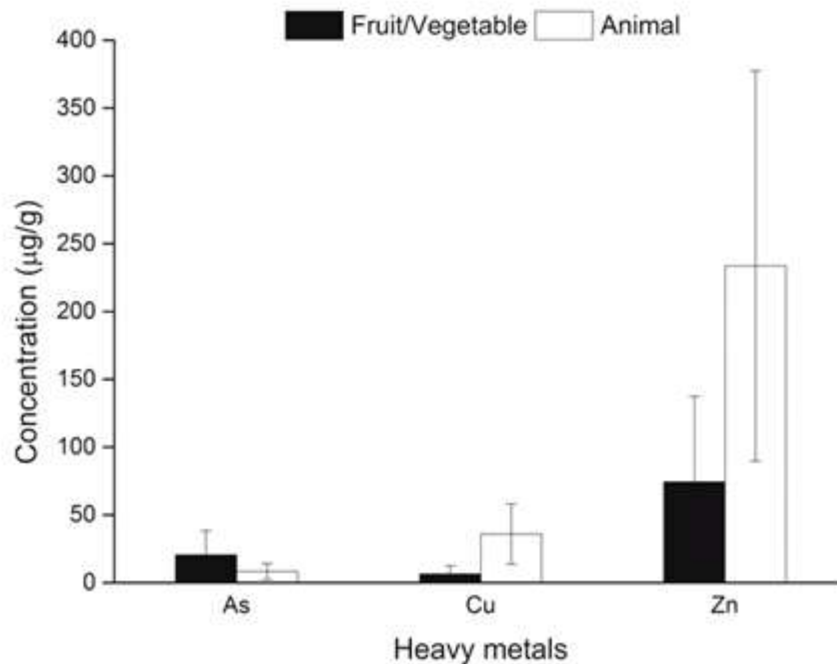

Figure 2. The Concentration of heavy metal in Fruit vegetable and animal

3.2. Seasonal distribution of heavy metals in fruit/vegetables and animals

The seasonal distribution of heavy metals in fruits/vegetables and animals is shown in Figs. 3 and 4. It was found that the distribution of As, Cu and Zn in fruits/vegetables and animals varied across the studied species. In the dry season, the lowest and highest contents of As measured in fruits/vegetables were found in water morning glory (31.267 µg/g dw) and galangal (62.317 µg/g dw), respectively. Meanwhile, the As contents were lowest (4.606 µg/g dw) in elephant ear plant and highest (39.830 µg/g dw) in bamboo in the rainy season (Fig. 3a). Again, water morning glory collected in the dry season was observed to accumulate the lowest Cu content (1.996 µg/g dw) while vegetable fern was found to contain the highest Cu content (12.904 µg/g dw). On the other hand, Cu measured in the wet season was lowest (1.386 µg/g dw) in papaya and was highest (28.221 µg/g dw) in water morning glory, as shown in Fig. 3b. The lowest Zn content detected in the dry season was found again in water morning glory (109.967 µg/g dw) while lemon grass was observed to contain the highest level of Zn (192.693 µg/g dw). On the other hand, Zn detected in the rainy season was least abundant in galangal (91.588 µg/g dw) and was most abundant in yellow velvetleaf (212.904 µg/g dw), as depicted in Fig. 3c. In the dry season, the lowest and highest contents of As was found in yellowtail rasbora (5.872

µg/g dw) and Lanchester's freshwater prawn (13.863 µg/g dw), respectively. Again, As was least abundant in yellowtail rasbora (3.241 µg/g dw) caught in the wet season while river snail accumulated the highest As content (9.984 µg/g dw), as illustrated in Fig. 4a. The lowest Cu content was also detected in yellowtail rasbora caught in both the dry (21.113 µg/g dw) and wet (15.192 µg/g dw) season. Meanwhile, river snail was observed to accumulate Cu at the highest level (53.001 µg/g dw) in the dry season and rice field crab was found to contain the highest Cu content (43.533 µg/g dw) in the rainy season, as presented in Fig. 4b. Zn was least abundant in Lanchester's freshwater prawn (184.212 µg/g dw) and most plentiful in river snail (385.287 µg/g dw) caught in the dry season. On the other hand, the lowest and highest Zn contents were was found in yellowtail rasbora (122.190 µg/g dw) and river snail (291.670 µg/g dw) caught in the rainy season, respectively (Fig. 4c).

It is interesting to note that the studied heavy metals detected in fruits/vegetables and animals were more abundant in the dry season than in the wet season. This occurrence might be due to the fact that the dry weather's combined effects of increased evaporation and decreased rainfall may result in elevated metal concentrations in water, sediments and soils as reported elsewhere (Duman and Kar, 2012; Tekin-Özan, 2008; Tekin-Özan and Kir, 2008), thereby increasing the metal accumulation in plants and animals. By contrast, the decrease in the metal accumulation in plants and animals in the wet season might be due to rainfall effects, which could increase the lixiviation process and contribute to the dilution of heavy metals (Khattabi et al., 2007). Moreover, changes in meteorological conditions may alter soil/water redox and pH conditions, which are responsible for the availability of heavy metals in water and soil matrices (Liang and Wong, 2003).

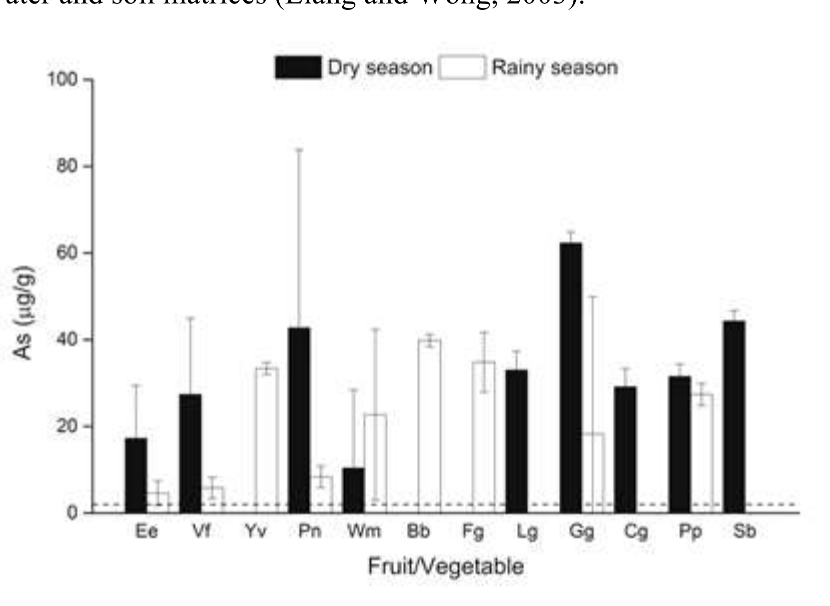

Figure 3. (a) The concentration of As in fruit/vegetable in seasons

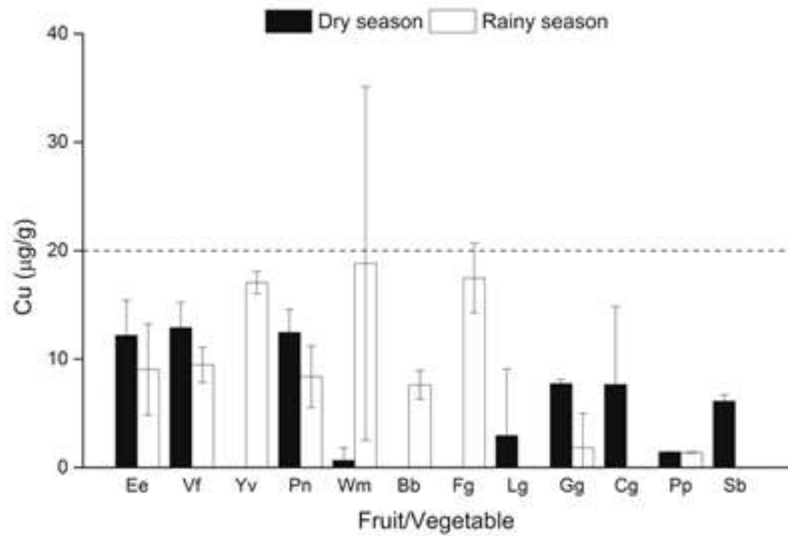

Figure 3. (b) The concentration of Cu in fruit/vegetable in seasons

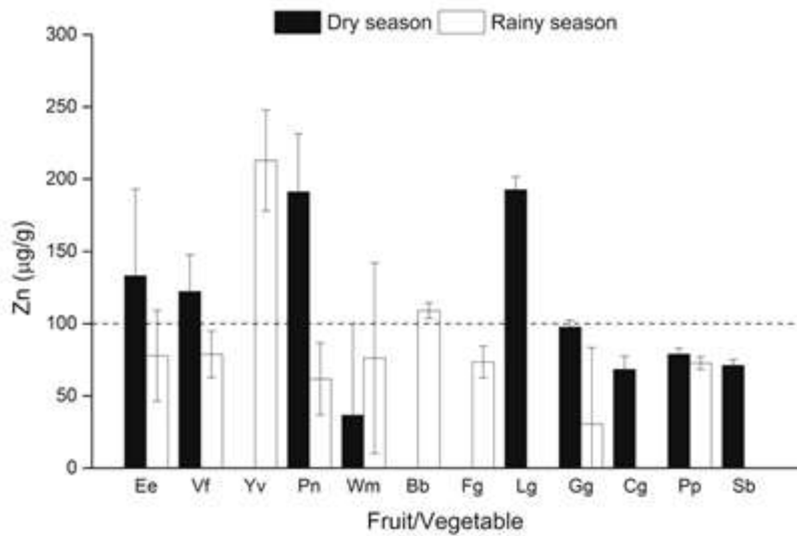

Figure 3. (c) The concentration of Zn in fruit/vegetable in seasons

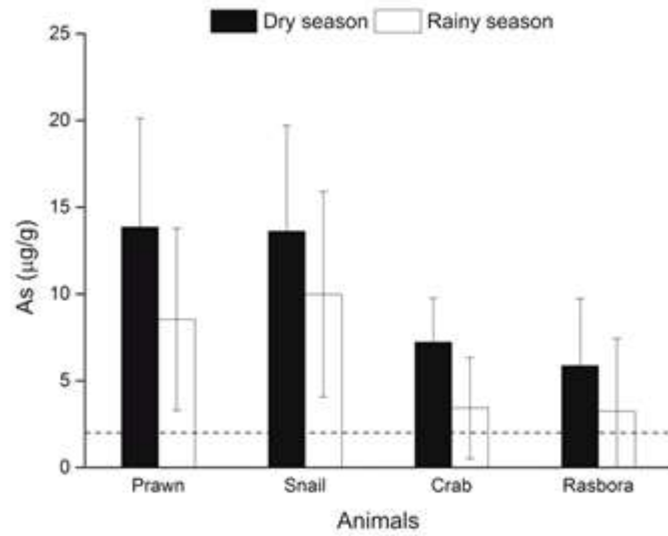

Figure 4. (a) The concentration of As in animals in seasons

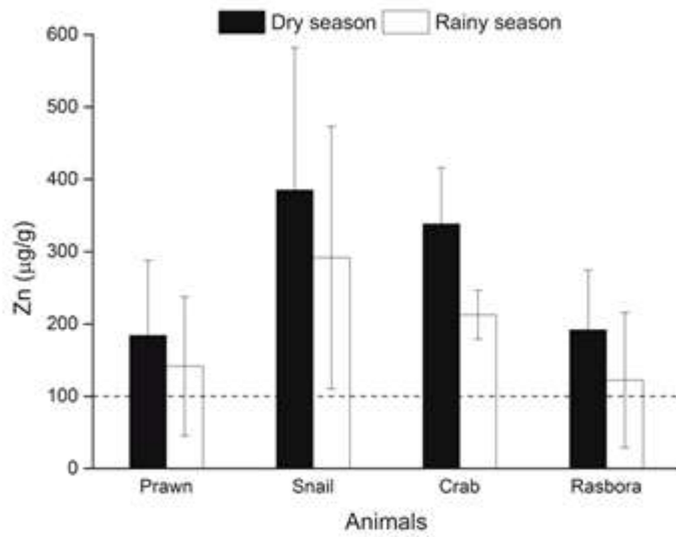

Figure 4. (b) The concentration of Zn in animals in seasons

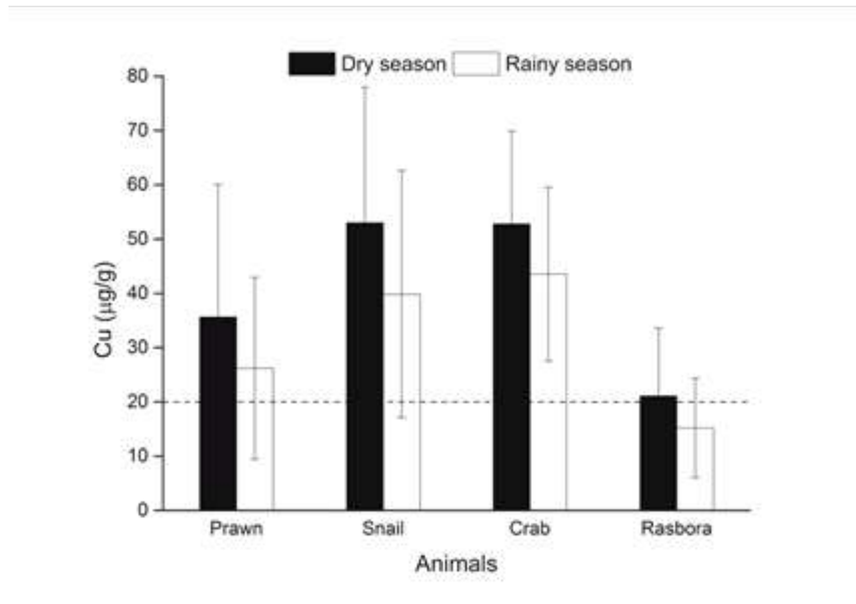

Figure 4. (c) The concentration of Cu in animals in seasons

4. CONCLUSION

This research has highlighted the occurrence of heavy metal (As, Cu and Zn) accumulation in fruits/vegetables and animals collected from wetlands and arable lands surrounding the gold mining region in Wang Saphung district, Loei province, Thailand. The results showed that the average As contents detected in all the studied samples were found to exceed the maximum allowable concentrations. All the analyzed animals and almost all of the studied fruits/vegetables accumulated high contents of Zn, which were higher than the maximum allowable concentration. Similarly, almost all of the analyzed animals were observed to accumulate high levels of Zn that exceeded the maximum allowable concentration. On the other hand, all the studied fruits/vegetables accumulated low levels of Cu, which were within the maximum concentration, while most of the analyzed animals contained Cu above the maximum allowable concentration. In most cases, the levels of heavy metals appeared to be more concentrated in both fruits/vegetables and animals in the dry season than in the wet season. Moreover, animals were likely to contain higher levels of Cu and Zn than fruits/animals, whereas the greater levels of As was observed in fruits/vegetables.

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Spatial Water Quality Assessment of Selected River Basins Using Environmetric Techniques

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ABSTRACT

This study investigates spatial water quality assessment of selected river basins in the three different states in Malaysia. Environmetric techniques namely, cluster analysis (CA), principal component analysis (PCA), and discriminant analysis (DA), were applied to study the spatial variations of the most significant water quality variables in order to determine the origin of pollution sources on water quality data of Juru River Basin, Kuantan River Basin and Johor River Basin. 13 water quality parameters were initially selected and analyzed. Three spatial clusters were formed based on CA, and these clusters were designated as high pollution source (HPS), medium pollution source (MPS) and low pollution source (LPS) at the three river basins, respectively. Forward and backward stepwise DA managed to discriminate water quality variables, respectively from the original 13 variables. The result of this spatial analysis assessment is supported by PCA (varimax functionality) that was used to investigate the origin of each water quality variable due to land use activities. Thus, this analysis makes it possible to observe the significance of the pollutant sources, which contribute to river pollution. Five principal components (PCs) were obtained for all HPS, MPS and LPS regions of all the three river basins, respectively. Finally, the environmetric techniques analysis manage to provide convincing result on the spatial variation of water quality in all the three studied river basins and this eventually will allow more effective and efficient river quality management activities.

Keywords: cluster analysis; principal component analysis; discriminant analysis; water quality

1. INTRODUCTION

Many rivers are experiencing from deterioration quality of its characteristic condition, which in turn affects people's health, economy and as well as the environment (Department of Environment, DOE, 2003). Surface water is one of the environmental components that are most vulnerable to pollution impact because this surface river water is the place that received all of the waste that are being disposed into the river by anthropogenic activities (Hamirdin, 2000). In Malaysia, river is the main source of drinking water supplies. The contaminated river will brings into a limited quantity of clean water and thus will eventually increase the water treatment cost. Spatial analysis is one of the methods that usually performed for the purpose of evaluating and identifying the most significant water quality parameters that supposed to be concerned due to the land use activities that affect river ecosystem (Griffith, 2002; Buck *et al*., 2004). Spatial analysis can be conducted by using environmetric technique or also known as chemometric which is one of the environmental analytical chemistry fields that utilize multivariate statistical approach for the data analysis (Simeonav *et al.,* 2000). It can be considered to be the most appropriate analysis performance in order to prevent misinterpretation upon analyzing a large environmental data set (Simeonav *et al*., 2002). Three common environmetric analysis that usually perform in order to classify wide range of data into groups are the hierarchical agglomorative cluster analysis (HACA) and principal component analysis (PCA) which are then furthered by pattern recognition analysis namely, discriminant analysis (DA) (Adam, 1998). The objectives of this study are (i) to evaluate spatial variations in the river water quality data of Juru, Kuantan and Johor river basins using environmetric techniques and (ii) to identify the pollution loadings variations due to land use and anthropogenic activities in the three studied river basins.

2. MATERIALS AND METHODS

2.1 Site description

Three river basins namely, Juru River Basin, Kuantan River Basin and Johor River Basin have been selected in this study.

2.2 The Data

Data of river water quality from three river basins which consist of a number of monitoring stations were obtained from Department of Environment (DOE) Malaysia. All the water quality data from each selected stations in this study were based on the available data that had been recorded from 2003-2007. Referring to the sample site, 5 sites represent the Juru sub-basin, 8 sites represent Kuantan sub-basin and 21 sites represent the Johor sub-basin. Due to

the fact that some monitoring stations in these three studied river basins have missing data, only 13 consistent parameters were analysed and examined among all the 30 river water quality data available. A total of 205 samples in Juru River Basins, 275 samples in Kuantan River Basins and 865 samples in Johor River Basins were used for the analyses. For this study, all the data obtained with 13 water quality parameters ; dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD), suspended solid (SS), pH, ammoniacal nitrogen (NH_3-N), dissolved solid (DS), total solid (TS), nitrate (NO_3^-), chloride (Cl^-), phosphate (PO_4^{3-}), *Escherichia coli*, and coliform were subjected for the environmetric techniques analysis by using XLSTAT2012 software.

2.3 Methods of analysis

2.3.1 Cluster Analysis

CA operates on data sets and forms well-defined groups that actually do not exist, but are assign to be clustered together due to the similar level characteristic that occupied by them. In this paper, hierarchical agglomerative cluster analysis (HACA) was employed in order to determine the classification of sampling sites (spatial) in the three river basins; Juru River Basin, Kuantan River Basin and Johor River Basin into groups. Cluster analysis was performed to all of the data sets in the three river basins in order to specify each monitoring stations according to the level of their homogenous characteristics.

2.3.2 Discriminant Analysis

Discriminant analysis specifies and examines variables that are dominant or well-discriminated among certain data groups.In this paper, discriminant analysis was performed in order to examine whether group differ with regards to the mean of variable in predicting the group membership. In order to conduct this analysis, data from the three assigned region groups in each of the three river basins which had been obtained from CA were selected. The discriminant analysis was performed by using standard, forward stepwise and backward stepwise modes. These three modes were performed in order to determine water quality variables that have high variations according to their spatial distribution among the three studied river basins.

2.3.3 Principal Component Analysis

PCA gives information upon the most significant variables according to the spatial and temporal variations which distinguish the whole data set by excluding the less significant parameters with minimum loss of original information (Singh et al., 2004, 2005 : Kannel et al., 2007).PCA was performed on the data set for the purpose of identifying the source of pollutant loading in each clustered region among the three river basins In this study, only the VF coefficient that have strong loadings (greater than 0.7) were being considered.

Source identification of different pollutants was made on the basis of different activities in the catchment area in light of previous literatures. In this paper, PCA was applied to the data set consist of 13 parameters for each region (HPS, MPS and LPS) of the three studied river basins. Calculations of input data matrices (variables × cases) for this PCA were 13 × 224 for HPS region, 13 × 312 for MPS region and 13 × 807 for LPS.

3. RESULTS AND DISCUSSION

3.1 Determination of the sampling station groups

3.1.1 Cluster analysis

The cluster analysis resulted into three cluster of sampling stations in Juru, Kuantan and Johor River Basins, respectively (Figure 1). The cluster procedure formed three clusters or groups in a very convincing way as the sites in each group have similar characteristics and natural backgrounds. The three clusters with three different regions are generated in each river basin which is high pollution sources (HPS) region, moderate pollution sources (MPS) region and low pollution sources (LPS) region (Table 1). Result of HACA technique implies that rapid assessment of water quality for the whole stations can be done by monitoring only one station in each cluster that had been assigned by CA. This is because only one monitoring station is already enough to represent the water quality data for the whole group members in each cluster group as every group have their own similar level of data quality characteristics. The result of this analysis proved that cluster analysis (CA) technique is functional upon the classification of river water quality data for optimum future sampling and monitoring strategies.

Table 1. Sampling stations that have been grouped into regions

Regions	River basins /sampling stations		
	Juru River Basin	Kuantan River Basin	Johor River Basin
HPS	2JR01,2JR02,2JR03, 2JR04,2JR05,2JR06, 2JR07	4KN03,4KN04	3JH10,3JH46
MPS	2JR10,2JR11,2JR12	4KN01,4KN02,4KN075, 4KN07	3JH05,3JH06,3JH09,3JH18,3JH32, 3JH35
LPS	2JR08,2JR09	4KN06,4KN08,4KN09, 4KN10,4KN11	3JH03,3JH07,3JH08,3JH1,3JH12, 3JH13,3JH15,3JH16,3JH19,3JH20,3JH22,3JH25,3JH27,3JH28,3JH30,3JH33,3JH36,3JH37,3JH40,3JH42,3JH43,3JH44,3JH45,3JH47

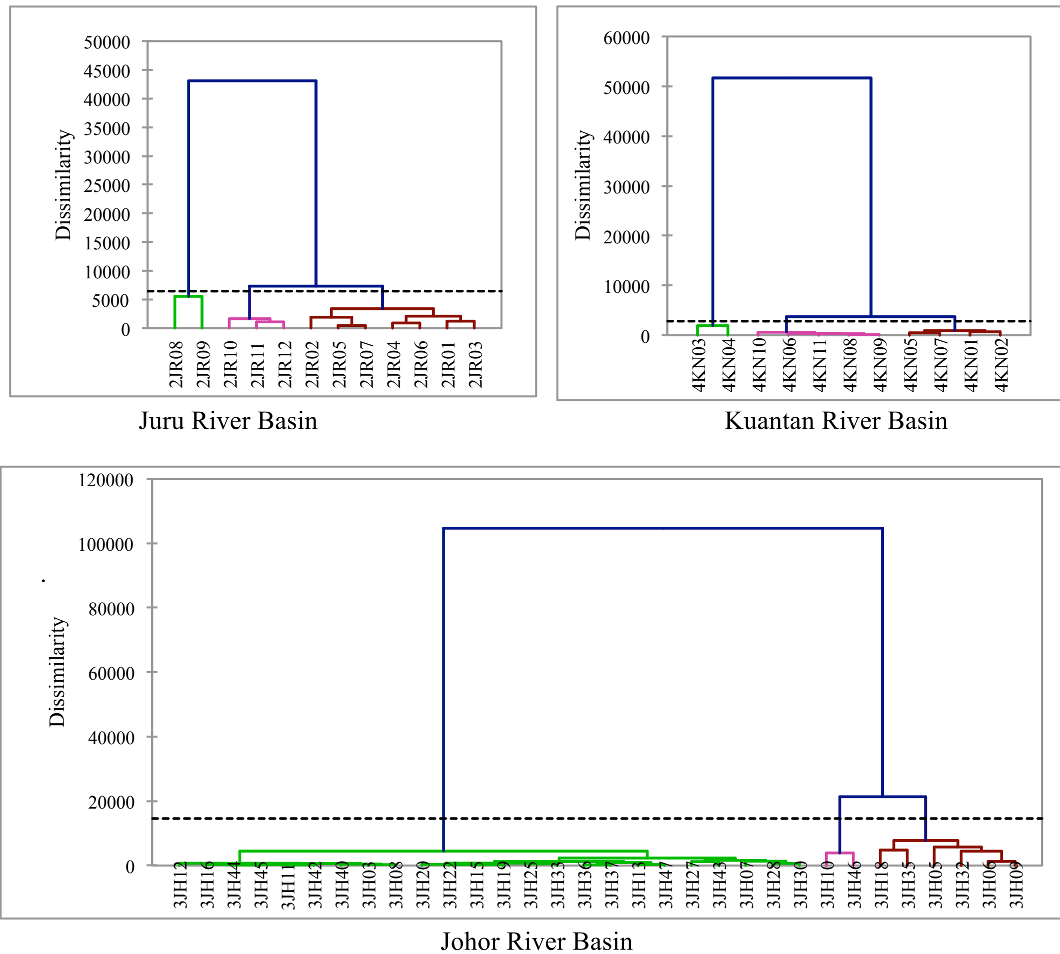

Figure 1. Dendogram shows sampling stations that had been classified in each three river basins

Among the three river basins, Juru River Basin is proved to be the most polluted river basin as 7 of its monitoring stations are clustered under high pollution sources (HPS) group. This is due to the fact that this river basin receives heavy pollution loadings from nearby urbanized area that densely populated by humans and multiple types of industrial located along the basin. In Kuantan River Basin, two monitoring stations are clustered under high HPS group which are mainly being polluted by sediment deposition and siltation that resulted from anthropogenic activities. For Johor River, two stations are clustered under HPS group as there are many oil palm plantation and land development located in the surrounding area which may influence to the river water quality of the two rivers.

3.2 Spatial variation of river water quality
3.2.1 Discriminant Analysis

The accuracy of spatial classification all the three regions of the studied river basins were 79.33% (13 discriminant variables) for standard mode, 79.33.74% (9 discriminant variables) for forward stepwise mode and 76.73% (10 discriminant variables) for backward stepwise mode. In forward stepwise mode, DO, NH_3-N, CI, pH, NO_3^-, BOD, COD and PO_4^{3-} were determined to be the significant variables which indicate that all these nine parameters have high variation upon their spatial distribution in the region of all the three studied river basins. For backward stepwise, *E.coli* exist as the tenth parameter that have high distribution upon the spatial variation. The result of DA which had been illustrated in a classification matrix for each clustered region is shown in Table 2. Box and whiskers plot of some water quality parameters in five years periods (2003-2007) is shown in Fig.2.

Table 2. Classification matrix for DA of spatial variations in the three studied river basins

Sampling regions of 3 studied river basins	% Correct	Regions assigned by DA		
		HPS	LPS	MPS
Standard DA mode (13 variables)				
HPS	75.34%	32	2	20
LPS	95.91%	0	635	13
MPS	39.23%	6	59	97
Total	79.55%	38	696	130
Forward stepwise mode (9 variables)				
HPS	77.58%	32	2	20
LPS	95.91%	0	634	14
MPS	39.23%	5	59	98
Total	79.70%	37	695	132
Backward stepwise mode (10 variables)				
HPS	75.34%	32	2	20
LPS	95.91%	0	634	14
MPS	39.23%	6	60	96
Total	79.33%	38	696	130

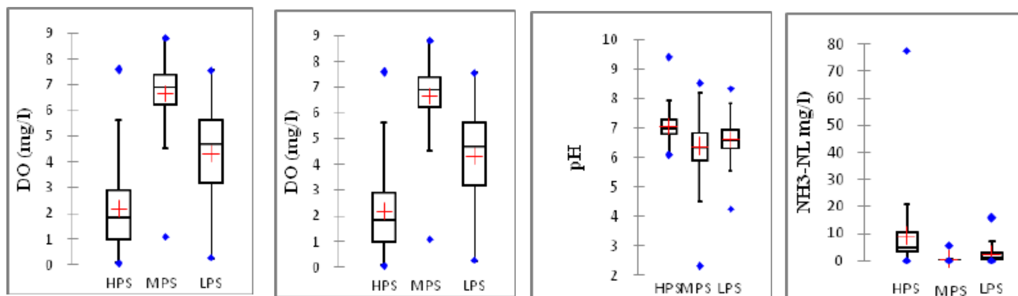

Figure 2. Box and whisker plots of some parameters separated by spatial DA associated with the three river basins. The crosses are mean values, top and bottom of whiskers indicate maximum and minimum values, respectively while horizontal lines of the boxes from top to bottom indicate the third quartile, median, and first quartile, respectively.

3.2.2 Principal Component Analysis

Five PCs were obtained for each HPS, MPS and LPS regions, with the concerned amount of eigenvalues (larger than 1) sum up the total variance of data set almost 81.9%, 78.8% and 74.1% respectively. Varimax rotation that had been performed through this PCA technique managed to obtain five varimax function (VF) in each HPS, MPS and LPS region.

3.2.2.1. High Pollution Source (HPS)

For HPS region, among five VFs, VF1 accounts for 25.6% of the total variance, which have strong positive loadings on BOD, COD and SS. In this region, loading of BOD and COD are assumed to be contributed by the direct discharges from nearby pig farm which are not equipped with proper sanitary treatment system (UPUM, 2002). It is reported that Juru River flow through largely urbanized areas where it had been polluted by domestic waste and discharges from pig farms (Lim and Kiu, 1995). The present of COD in the river basins was assumed to come from the anthropogenic activities that arise from the nearby industrial areas that discharge their industrial waste into these three rivers. The strong loading on SS are possibly originated from the high load of soil runoff and also from wood industry (Zali *et al.*, 2011) nearby these three river basins. VF2, account 20.4% total variance with the positive loading of three variables which are DS, TS AND Cl. The two variables DS and TS can be assumed to be originated from point sources (PS) and non-point sources (NPS) (USGS, 1999; Ha and Bae, 2001) as these river basins receive a lot of changes in the land development that also depends on the seasonal variation in studied area. Cl^- on the other hand, is identified to be originated from the mineral salt content in the river. VF3 explaining 15.4% of the total variance, has strong loading on *E.coli* and coliform which indicate the microorganism parameters in the HPS region of these three rivers. In Juru River for example, source of *E.coli* and coliform are possibly originated from Juru sewage pond located near the river and also from nearby residential areas as human settlements including squatters along the river banks at Juru River are not equipped with proper sanitary systems.VF4, explaining 10.8% of the total variance and has high loading of NH_3-N and PO_4^{3-}. The NH_3-N indicates that the HPS region of these three river basins experienced from pollution that caused by livestock waste and as well as the agricultural and domestic sewage waste. For example, PO_4^{3-} loads were mainly originated from agricultural runoff such as fertilizers at Juru River flow nearby the Prai Industrial Estate. VF5, explaining 9.5% of the total variance and has strong loading on NO_3^-. The loading of NO_3^- is possibly due to the runoff from agriculture land along the HPS region of these three river basins.

This NO_3^- is mainly originated from commonly used nitrogen and potassium fertilizers at the crop planted area of this HPS region.

3.2.2.2. Medium Pollution Source (MPS)

For MPS, among five VFs, VF1 accounts for 25% of the total variance which include BOD, COD, NH_3-N and PO_4^{3-}. BOD and COD are among organic factors that assumed to be attributed from anthropogenic activities such as farming and timber logging activities that take place along the river basin. The presence of NH_3-N in the river is probably due to the excessive runoff from the agricultural area nearby the basin regions. The PO_4^{3-} loading is assumed to be originated from phosphate fertilizer that contain in soils from the agricultural farm area located nearby the MPS region of these three river basins. VF2, explaining 23% of the total variance, has strong loadings on DS, TS AND NO_3^-. Farming and construction were more frequent near these river basins area and had resulted into sediment deposited. Thus, the loading of DS and TS in this MPS region are possibly due to extreme river bank erosion that usually occur during the storm flow which eventually cause the bedload sediment enter the river region (Bolstad and Swank 1997). This assumption is reasonable especially to the river water in Kuantan River Basin which is mainly polluted due to land development through agricultural, timber logging and forest clearing activities. Strong positive loading on NO_3^- is expected to originate from the cultivation area (Vega *et al*., 1998), where crops are planted and the use of inorganic fertilizers such as ammonium nitrate is rather frequent (Juahir *et al*., 2010). NO_3^- may also arise from decomposition and degradation of organic matters containing nitrogen (USGS, 2007). The organic matters contained in the municipal waste include urea and protein from the wastewater discharges which enters this MPS region of the three river basins. VF3, explaining 14% of the total variance, has strong loading on *E.coli* and coliform which are related to domestic waste and treatment plant from paper manufacturing industry, rubber and palm oil refineries that located near the river. Normally, faecal contamination from human occurred when structural and technical flaws in the sewerage system that causing the sewage to be flowed into the river which then leads to the present of *E.coli* and coliform.VF4, accounts the total variance 8.6%, showing loading on SS that can be attributed from high loads of soil and waste disposal runoff. The last one is VF5 which accounts 7.9% of the total variance and include NO_3^- as the positive strong loading variable. NO_3^- is expected to arise from vegetables farm, oil palm and rubber plantation that are located along the MPS region of these three river basins. The nitrate content in river water is caused by agricultural activity that is commonly associated with the use of chemical fertilizer to facilitate the growth of trees. Thus, when surface runoff occur during rainy season, waste chemical fertilizer will flow into these basins and caused increasing of NO_3^- content in the river.

3.2.2.3. Low Pollution Source (LPS)

For LPS region, among five VFs, VF1 represent 26.7% of the total variance, explaining strong loadings on DO, DS, TS and Cl^-. The strong negative loading on DO is cause by the presence of *E.coli* in this LPS region of the three river basins which consumed large amount of oxygen in order to undergo anaerobic fermentation. The negative loading of DO explained that the LPS region in these three river basins had been polluted by municipal waste, oxidation ponds and animal husbandry. DS and TS can be assumed as the sediment accumulation result that happened due to anthropogenic activities at these three river basins such as sand mining operation that is operated at Johor River area. The loading of Cl^- is probably comes from the mineral constituent in the water of this LPS region. VF2 represent the total variance of 18.1% and show the strong positive loading of BOD and COD. The presence of these BOD and COD in this LPS region of the three river basins is believed to be attributed from the influence of point source organic pollutants from sewerage network of the cities located nearby the river. VF3, explain 12.5% of the total variance and has strong loadings on *E.coli* and coliform that signify the contribution of domestic waste to this LPS region. VF4, explaining 9% of the total variance and has strong loading on pH and NO_3^-. The strong loading of pH is expected to arise from several causes such as industrial effluent discharges and other environmental factors. The decrease of pH range into acidic condition are mainly caused by the industrial effluent that release acidic discharges into the river while the significant increase in pH level into alkaline condition are possibly resulted from environmental factors such as the rapid algae growth which remove carbon dioxide from the water during the process of photosynthesis. The NO_3^- loading may additionally derived from agricultural area where inorganic nitrogen fertilizer are in common use such as at vegetable farm near the river. VF5, accounts for 7.7% of total variance, showing strong loadings on NH3-NL and PO_4^{3-}. NH_3-N indicates that the LPS region of the three river basins experienced from pollution that caused by livestock waste and as well as the agricultural and domestic sewage waste while a large amount of PO_4^{3-} loading is possibly originated from the contamination of fertilizer and pesticide discharges from vegetables farm located nearby the river basin.

4. CONCLUSION

Environmetric analysis techniques managed to determine spatial variation among the three studied river. Cluster analysis has successfully classified the cluster that enables the designation of sampling strategy which can reduce the number of sampling stations and also the monitoring cost as on one station in every cluster is enough to represent the accurate rapid assessment of

spatial water quality for the whole region among the group. Discriminant analysis on the other hand also gives encouraging results upon discriminating the data of every monitoring stations with discriminant variables while principal component analysis that applied on the data set for each classified regions had managed to identify the pollutant loading variation due to land use and anthropogenic activities in the three studied river basins. Generally, this study had showed the ability of environmetric techniques for conducting the analysis and interpretation of a large complex data set for water quality assessment and as well as the identification of pollution sources. This analysis is also useful upon investigating spatial variations of water quality as an effort toward a more effective river basin management.

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Environment & Resource Management **ERM-04**

Cadmium and Copper Effects on Reproductive Success of Selected Marine Invertebrates

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ABSTRACT

Aquatic ecosystems are persistently exposed to numerous xenobiotics from anthropogenic sources. Elevated metal concentrations are one form of chemical pollution prevalent in most coastal seas. This research examines the effect of copper and cadmium on sperm motility and fertilisation success of three species of marine invertebrate; the polychaete worm, *Arenicola marina*, and the echinoderms *Psammechinus miliaris* and *Asterias rubens*. Exposure of sperm to copper concentrations ≥10µg/l significantly reduced the average swimming speed and percentage of sperm motility for all test species. For cadmium, a significant reduction was only observed when exposed to 1000µg/l. Incubation of gametes with ≥10µg/l of copper significantly reduced the fertilisation success of all test species. Cadmium proved less toxic with fertilisation inhibited at 1000µg/l for *A. rubens*and *A. marina* and 100µg/l for *P. miliaris*. Pre-incubation of sperm in copper inhibited fertilisation at ≥10µg/l, while pre-incubation in cadmium inhibited fertilisation at concentrations of 100µg/l for *P. miliaris* and 1000µg/l for *A. rubens*and *A. marina* respectively. The effects of pre-incubation of oocytes in copper and cadmium were concentration and time dependent. A significant reduction of fertilisation success was only observed when oocytes were pre-incubated in 1000µg/l of copper for *A. rubens* and ≥100 µg/l for *P. miliaris* and *A. marina.* Pre-incubation with cadmium inhibited fertilisation at 1000µg/l for all species. In conclusion, the potential exists for impairment of echinoderms and polychaete reproductive success in metal contaminated sites, possibly threatening the species population in those environments.

***Keywords**:* metals, reproductive success, polychaete, echinoderms

1. INTRODUCTION

Metals have long been recognised as major marine pollutants. They are slowly released into water bodies and are generally found in very low concentrations (Ansari *et al*., 2004). Some metals are essential for life and some are merely beneficial, whereas many are highly toxic (Uthus, 2003, Zeng *et al*., 2005). Metals have a tendency to accumulate in the tissues of organisms and can be amplified along food chains (Watling, 1983). It is therefore important to continually monitor the levels of metals within the environment. Metal toxicity varies depending on the response of different organisms to metal exposure. At low levels some metals such as copper or cobalt are essential for enzyme function but will readily become toxic at higher concentrations.

Bryan (1976) reported that several aquatic species are sensitive to copper in the concentration range 1-10μg/l, while Nelson *et al*. (1988) observed that 2μg/l of copper dichloride (CuCl2) had significant toxic effects on young bay scallops and surf clams. Even though cadmium is not an essential element for any organism, cadmium reportedly increases phytoplankton photosynthesis and growth at a concentration of 100μg/l (Miao and Wang, 2006). However, cadmium has been listed by the US EPA and the EC's priority list as one of the most toxic metals in industrial discharges and has been reported to affect reproductive success of marine invertebrates (Filosto *et al*., 2008). Metal contaminants may affect the reproductive success of organisms by reducing the quality and/or quantity of gametes and consequently affect fertilisation success, embryo development, larval viability, and species fitness and survival (Filosto *et al*., 2008, Arizza *et al*., 2009).

This research examines the effects of cadmium and copper on sperm motility and fertilisation success of a polychaete worm, *A. marina* and two echinoderms, *A. rubens* and *P. miliaris*. The objectives are: 1) to determine the sperm motility which look at swimming speed (curvilinear velocity, VCL); and 2) to study fertilisation success when exposed to certain concentrations of copper and cadmium. The results will increase knowledge of the effects of metal exposure on reproductive processes of ecologically important marine invertebrates.

2. MATERIALS AND METHODS

A. rubens collected using fishing creels from the Amble coast, Northumberland, UK (55.32˚N, 1.55˚W) from the end of March to early May 2010-2012, were transported in seawater to the laboratory and held in a flow-through seawater aquarium at 5˚C with constant darkness until required. *P. miliaris* were collected in July from two locations on the west coast of Scotland: the Isle of Cumbrae, UK (55.76˚N, 4.94˚W) in 2010 and Oban, UK (56.41˚N, 5.47˚W) during 2011 and 2012. Urchins were transported to the laboratory in

tanks filled with ambient seawater and aerated by a portable electric pump. In the laboratory they were held in flow-through tanks at 10˚C and 12L: 12D photoperiod until required. *A. marina* were collected by digging during low tide, using a flat pronged fork from beaches at Alnwick, Northumberland, UK (55.38˚N, 1.60˚W) during late October to late December 2010-2012. Once removed from the sand, they were placed into buckets containing small amounts of seawater and sand, returned to the laboratory where they were sexed by observation of the gametes present in the coelomic cavity under bright illumination (Pacey and Bentley, 1992). The spawning protocols were followed as published elsewhere; *A. rubens* (Caldwell *et al*., 2002; Williams and Bentley, 2002); *A. marina* (Pacey and Bentley, 1992); and *P. miliaris* (Caldwell *et al*.,2004). Gametes were collected in Eppendorf tubes and stored on ice until required.

For chemicals preparation; all experiments were performed with analytical grade metal salts, CdCl2 and CuSO4 (purchased from Sigma Aldrich UK), dissolved in 0.22μm filtered fresh seawater (FSW) and then serially diluted to obtain a concentration range (0.1, 1, 10, 100, 1000μg/l). All experiments included a control of filtered seawater only. Glassware and equipment were acid washed before use. Exposure durations of up to one hour were used for *A. rubens* and *P. miliaris* and up to two hours for *A. marina*.

Sperm motility was measured using computer assisted sperm analysis (Caldwell *et al*., 2011). Between six to nine replicate sperm suspensions (1000 ml) were prepared for each treatment according to the times and concentrations required and mounted on clean, concave glass slides. Curvilinear velocity (VCL; mm/s) was assessed, which represents the time-averaged velocity of the sperm head along the actual trajectories of individual spermatozoa.

To test for the effects of oocyte pre-incubation, two hundred and fifty oocytes were incubated in 1 ml of test medium; either solvent control, CdCl2 and CuSO4 at set concentrations and times in Eppendorf tubes at 15˚C. After incubation, the oocytes were washed three times with FSW and transferred into 24-well microplates to which unexposed sperm, pooled from three males, was added to give a final concentration of $2.5x10^6$ sperm/ml. The effects of sperm pre-incubation was tested by incubating sperm pooled from three males at a concentration of $5x10^6$ sperm/ml in set concentrations of the solvent control, CdCl2 and CuSO4 at 15˚C. The exposed sperm were then added to unfertilised oocytes that were not previously exposed to the test chemicals. After each time point, 250 ml of sperm was added to the unfertilised oocytes to give a final concentration of 2.5x106 sperm/ml.

A final experiment whereby both oocytes and sperm were separately pre-incubated was conducted using the same conditions as per individual gamete-type pre-incubations. In all fertilisation trials formalin (10% v/v) was added to stop development and preserve the embryos. Fertilisation success was scored by the presence of embryonic cleavage at 60 min post fertilisation using a Zeiss inverted microscope.

All statistical analyses were performed using SPSS (v17). Percentage data were arcsine transformed, while log transformation was used for VCL analysis. All data were back transformed for presentation. No-observed-effect (NOEC) and lowest-observed effect concentrations (LOEC) were determined by analysis of variance (ANOVA) when assumptions for normality and homoscedasticity were met (Shapiro-Wilk and Levene tests, respectively). The significance level was set at 0.05%. Significant ANOVAs were followed by a Dunnett's post hoc test to compare treatment means with control means. Steel's many-one rank test was applied to determine the NOEC or LOEC endpoints if tests for normality and homoscedasticity failed. Later, the Tukey honest significant difference post hoc test was used to identify differences among groups and identify any interaction effect between times and chemical concentrations. Data that did not fulfil normality and homoscedasticity assumptions were subjected to non-parametric Kruskal-Wallis tests followed by a Wilcoxon-Mann-Whitney post hoc test. All figures and tables present the mean ± standard error. Probit analysis was used to calculate the EC50 (half minimal effective concentration) value with 95% confidence limits and fitting the regression equation arithmetically by taking the log of the concentrations used verses the probit value of percentage of immotile sperm and unfertilised oocytes. If the immotile or unfertilised percentage in the control was more than 10% the results with treatment samples were corrected using Abbot's formula (APHA, 1981):

Corrected % ¼ (Pe-Pc)/(100-Pc) x 100 (Abbott, 1925)
Pe = Experimental percentage
Pc= Control percentage

3. RESULTS

Table 1. Lowest-effect concentration (LOEC) and effective concentration 50 (EC_{50}) value of copper and cadmium toxicity.

Metal	Test	*P.miliaris*		*A.rubens*		*A.marina*	
		LOEC (µg/l)	60 minutes EC_{50} (µg/l)	LOEC (µg/l)	60 minutes EC_{50} (µg/l)	LOEC (µg/l)	60 minutes EC_{50} (µg/l)
Copper	Sperm Motility	10.00	58.16	10.00	304.88	10.00	274.83
	Pre-Incubation of sperm	10.00	118.33	10.00	248.13	10.00	345.30
	Pre-Incubation of oocytes	10.00	277.61	1000.00	1176.82	10.00	1281.97
	Exposure of sperm and oocytes	10.00	275.73	10.00	278.35	10.00	541.76
Cadmium	Sperm Motility	100.00	144.63	1000.00	1660.54	1000.00	4624.69
	Pre-Incubation of sperm	100.00	787.25	1000.00	3327.87	1000.00	6003.82
	Pre-Incubation of oocytes	1000.00	7366.62	1000.00	3123.17	1000.00	6190.82
	Exposure of sperm and oocytes	100.00	1932.81	1000.00	3178.84	1000.00	4020.33

The LOEC and EC_{50} values are presented in Table 1. The LOEC for the *P. miliaris* sperm motility test exposed to copper sulphate and cadmium chloride were 10.0 and 100.0µg/l, respectively; whereas the 60 minutes EC_{50}s were 58.16 and 144.63µg/l, respectively. In fertilisation tests, *P. miliaris* sperm pre-incubated in copper and cadmium had LOECs of 10.0 and 100.0µg/l, respectively, while the 60 minutes EC50 values were 118.33 and 787.35µg/l, respectively. The LOECs for pre-incubated oocytes were 10.0 and 1000.0µg/l, respectively for copper and cadmium and the 60 minutes EC_{50} values were 277.61 and 7366.62µg/l, respectively. The comparative values for when both sperm and oocytes were pre-exposed to the metals were 10.0 and 100.0µg/l (LOEC) and 275.73 and 1932.81µg/l for the 60 minutes EC_{50}, for copper and cadmium respectively. The sperm motility LOECs for *A. rubens* exposed to copper and cadmium were 10.0 and 1000µg/l, while for sperm pre-incubation they were 10.0 and 1000.0µg/l respectively. The 60 minutes sperm motility EC_{50} values were 304.88 and 1660.54µg/l and 248.13 and 3327.87µg/l for the sperm pre-incubation test. The LOECs for pre-incubated were both 1000.0µg/l whereas the 60 minutes EC50 values were 1176.82 and 3123.17µg/l. The figures for when both sperm and oocytes were pre-exposure were 10.0 µg/l and 1000 µg/l (LOEC) and 378.35 and 3178.84 µg/l (EC_{50}). The LOEC sperm motility values for *A. marina* were 10.0 and 1000µg/l (copper and cadmium respectively); sperm pre-incubation generated the same values, whereas the EC50 values for the sperm motility and sperm pre-incubation tests were 274.83 and 345.3 µg/l for copper and 4624.69 and 6003.83 µg/l for cadmium. Similar values were determined for oocytes pre-incubation - LOECs were 10.0 and 1000.0µg/l while EC_{50} values were 881.97 and 6190.82µg/l, respectively. Pre-incubation of both sperm and oocytes in copper and cadmium produced EC_{50} values of 541.76 and 4020.33µg/l respectively.

4. DISCUSSION

4.1 Toxicity of metals to gametes of marine invertebrates

In this present study, a comparison of the toxicity of copper and cadmium against sperm motility and fertilisation success clearly showed that copper was the more toxic metal. This conclusion supports prior work on the sea urchin, *Diadema setosum* by Thongra-Ar (1997) who reported a 20 minutes EC50 based on sperm toxicity for fertilisation success of 17µg/l for copper and 628µg/l for cadmium. In terms of EC50 values for cadmium toxicity to marine invertebrate sperm, adverse effects on fertilisation capacity were shown to arise due to sperm toxicity, for example in the sea urchin, *Anthocidaris crassispina* (30 minutes EC50 of 1700µg/l) (Vaschenko *et al*., 1999). Many other studies report sperm toxicity EC50 values for a variety of sea urchin species ranging between 380-1700µg/l (Kobayashi, 1994). A study on the serpulid polychaete

Hydroides elegans, found the EC50 for embyrogenic inhibition to be 47µg/l when oocytes were exposed to copper prior to fertilisation (Xie *et al*., 2005). An EC50 for gametes of the polychaete *Alitta* (*Nereis*) *virens* exposed to copper was reported at 139.8µg/l (Caldwell *et al*., 2011b); however, Watson *et al*. (2008) reported that exposure to copper, at up to 500µg/l, for the same species, resulted in no reduction in fertilisation success. As copper and cadmium concentrations in seawater range between 0.05-0.25µg/l and 0.08-0.25µg/l (OSPAR, 2002), sperm and oocytes of these three species are not likely under threat from these metals as the EC50 values obtained in this study were higher than those measured in the environment.

4.2 Gamete sensitivity to metals between species

In terms of gamete sensitivity to metals across the three test species, *P. miliaris* sperm were more sensitive to copper than sperm from *A. rubens*; *A. marina* sperm were less sensitive to copper. For oocytes, copper was more toxic to *P. milaris* than *A. rubens* but less toxic for *A. marina*. *A. marina* sperm were the most resistant to copper, followed by *A. rubens* with *P. milaris* sperm the most affected. Conversely, *P. miliaris* oocytes were less sensitive to cadmium followed by *A. marina* then *A. rubens*. The differences in the sensitivity of gametes from each species might be explained by the different trophic levels they occupy. Moreover, as the polychaete worm is generally found living in more contaminated sites, and particularly as it is a sediment dweller (sediments have a higher metal burden that overlying water), it is perhaps unsurprising that *A. marina* gametes were less sensitive to copper and cadmium (except the oocytes with cadmium exposure) compared to the echinoderms. In addition, this can be explained by the metal detoxification system and tolerance of this worm to these metals. (Mouneyrac *et al*., 2003) indicated that the polychaete *Hediste diversicolor,* can increase its tolerance to cadmium, copper, and zinc by secreting mucus that prevents or reduces the metal availability.

5. CONCLUSIONS

This research was examined the effects of copper and cadmium on reproductive success, concluding that both copper and cadmium reduced sperm motility and fertilisation success in a dose and time dependent manner. Copper proved more toxic than cadmium; this agrees with the scientific literature based on other bioassays. The finding was similar to Campbell *et al*. (2014), where it has been proven that copper negatively affected fertilisation success of *A. marina*. In terms of an environmental context, the species used in this study provides an effective and sensitive tool for detecting metal toxicity by detecting the reduction of sperm motility and fertilisation success. The present study also adds to the significant weight of evidence for use in regulatory frameworks and environmental quality standards by setting individual concentration limit in

aquatic environments. However, in the natural environment metals are not present alone but in mixture. Therefore, experimental data based on single compound exposure should be interpreted with an element of caution.

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Environment & Resource Management **ERM-06**

Association of Ambient Light Intensity and the Depth Distribution of Giant Sea Anemone, *Heteractis magnifica*

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ABSTRACT

A study was conducted on the distribution of giant sea anemone, *Heteractis magnifica*, according to depth at four sampling locations: two locations in the northern Straits of Malacca (Pulau Intan Besar and Pulau Lembu) and two locations in the Andaman Sea (Pulau Weh and Pulau Rubiah). The anemone colonies were found at a depth range of 1-3 m at Pulau Intan Besar 1-4 m at Pulau Lembu, 3-6 m at Pulau Weh and 6-12 m at Pulau Rubiah. The distribution of *H. magnifica* in these locations is restricted to the depths where they can receive sufficient sunlight for the endosymbiotic zooxanthellae to photosynthesize. This resulted in the presence of *H. magnifica* colonies in deeper reef areas in a clear water environment (low turbidity). However, the number of colonies found in the deeper reef area were less than in the shallower reef area. The relationship of depth and the size of colonies will also be discussed in this study.

Keywords: anemone; depth distribution; light intensity; Straits of Malacca; Andaman Sea

1. INTRODUCTION

The giant sea anemone, *Heteractis magnifica* or also known as magnificent anemone, is native to the tropical and sub-tropical waters of the Indo-Pacific. It belongs to the family of Stichodactylidae, alongside with the genus *Stichodactyla* (carpet anemones). Subtidal coral reef regions serve as habitats for these giant sea anemones, while hosting anemonefish (usually from the genera *Amphiprion* and *Premnus*, Pomacentridae) and contain intracellular symbiotic algae (zooxanthellae) in their tissue cell (Dunn, 1981; Fautin, 1991). As giant sea anemones are more or less dependent on the photosynthate produced by the endosymbionts (Dunn 1981; Steen 1988), shallow reefs can be better habitats for these species than deep reefs. However, several species of giant sea anemones are found at depths of 40 m or more (Fricke 1974; Dunn 1981; Brolund et al. 2004). Furthermore, small individuals are frequently found in shallow reefs (Dunn 1981; Fautin and Allen 1992; Richardson et al. 1997; Srinivasan et al. 1999). The present study was

performed to examine the association of enviromental parameters such as the ambient light intensity, which is crucial for their photosynthetic activity, to the distribution patterns of the giant sea anemone, *Heteractis magnifica.*

2. MATERIALS AND METHODS

This study was carried out at four sampling stations in the Northern Straits of Malacca: Pulau Intan Besar and Pulau Lembu (Malaysia), Pulau Weh and Pulau Rubiah (Indonesia) by SCUBA diving surveys. Identification of *Heteractis magnifica* was done after Dunn (1981). For each anemone, the approximate depth was measured using a dive computer, and the long and short axial lengths of the tentacle crown were recorded. Sizes of anemone (the area covered by the tentacles) were regarded as an oval (following Hirose, 1985) where,

$$\frac{(\text{long axial length}) \times (\text{short axial length}) \times \pi}{4}$$

Measurements of light intensity using HOBO™ logger and turbidity were taken at the same depth and time (1200h, sunny day) for all sampling locations for comparison purposes. Due to the high variation in spectral irradiance at 0 to 2 m depth, the depth chosen for the measurement of light intensity is fixed at 4 m depth following the maximum depth of the shallowest reef in this study.

3. RESULT

3.1 Distribution pattern of *Heteractis magnifica*

The *H. magnifica* were distributed over the coral reef from the reef edges to the inshore reef flat but mostly were distributed at the reef edge zones. Most anemones (68.4%) inhabited reefs at depths of less than 4 m (Figure 1). No small anemones less than 500 cm2 inhabited reefs at depths of more than 4 m, and large anemones measuring more than 1000 cm2 inhabited the deepest parts of the reef edges up to a depth of 12 m. There were no large anemones in shallow reefs at depths of less than 4 m (Figure 2). The sizes of the anemones were significantly correlated with their depth distribution (Spearman's correlation analysis, $R2 = 0.62354$, $P < 0.05$, $n = 38$) (Figure 2).

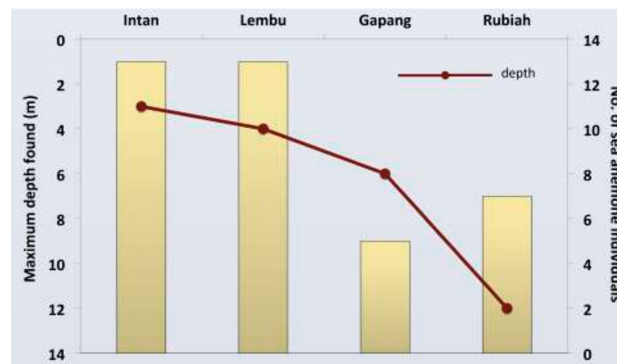

Figure 1. The number of sea anemone individuals and the maximum depths they were found at different sampling locations.

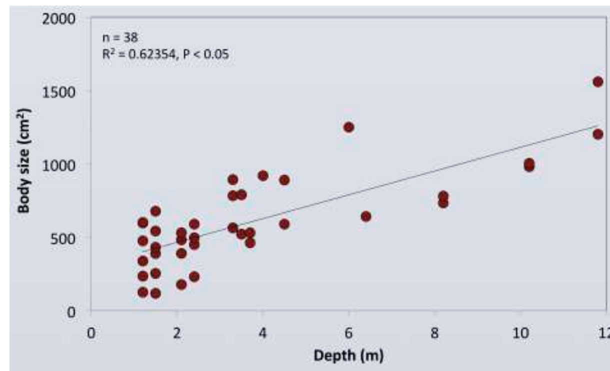

Figure 2. The association between the body size of *H. magnifica* and the depth distribution.

3.2 Association of depth distribution of *H. magnifica* with turbidity and light intensity

The depth range of *H. magnifica* increases as the turbidity decreases (Figure 3). *H. magnifica* colonies can be found in deeper reef areas when the turbidity is low. The light intensity increases when the turbidity decreases, which allow more light to penetrate the water column and reach the colonies in deeper waters such as in Pulau Rubiah. However, the number of colonies found in deeper areas are less than in the shallower area.

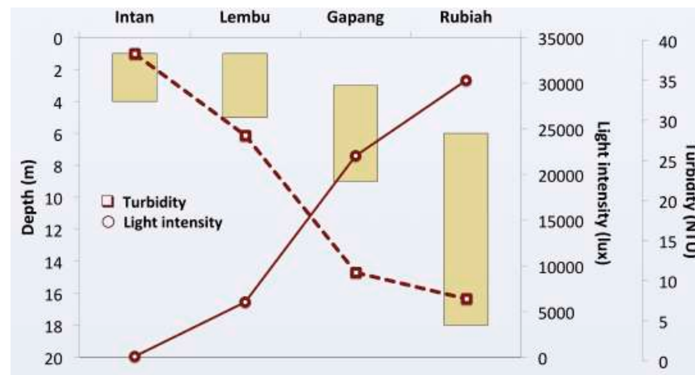

Figure 3. The association between depth distribution of *H. magnifica* with turbidity and light intensity in this study.

4. DISCUSSION

The sizes of anemones were significantly correlated with their depth distribution. These data suggests that deep reef edges are suitable habitats for *H. magnifica* in this study. In a deeper habitat of clearer water column, large individuals of *H. magnifica* can be found at the depth of 40 m in the Red Sea (Brolund et al., 2004). Smaller individuals are often found at the shallow reef areas but they cannot grow large because of the high levels of physiological stress (extreme water temperature, wave action) in this habitat (Sebens, 2002). Giant sea anemones, which are found in

deeper waters, are dependant on the photosynthate produced by the endosymbionts (Dunn, 1981; Steen, 1988). Anemone fish residents also indirectly benefit the anemone by enhancing photosynthesis by the zooxanthellae by supplying nutrients and protecting the anemone. Thus, the anemone could maintain the oral disk and tentacles in an expanded position for maximum interception of light (Fautin, 1991).

5. CONCLUSION

From this study, large individuals of *H. magnifica* were found in the deeper and clearer water of Pulau Weh and Pulau Rubiah. This may indicate that there is enough sunlight for the zooxanthellae to photosynthesize and the anemone may maintain the expansion of the oral disk and tentacles with protection from the resident false clownfish, *Amphiprion ocellaris*.

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Environment & Resource Management **ERM-07**

Responses of Coral Holobiont to Elevated Temperature and Light

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ABSTRACT

Coral reefs in Thai Waters have been seriously affected by coral bleaching events. However, mortality and their ability to recover vary. This is based on their thermal history. Some corals were able to adapt and subsequently resist higher seawater temperature. The aim of this study was to investigate response and resistant ability of the corals *Pocillopora damicornis* to elevated temperature and lightCoral nubbins from Panwa Cape and Maiton Island, Phuket, Thailand were exposed to four treatments: 1) ambient temperature, ambient light 2) ambient temperature, high light intensity, 3) high temperature, ambient light, and 4) high temperature, high light intensity. The results of photosynthesis performance measured by PAM flurometry showed that F_v/F_m of corals from high temperature and light slightly was reduced. Remarkably, corals from the extreme reef at Cape Panwa seemed to be more resistant compared to corals from Maiton Island. During the recovery phase, the F_v/F_m of corals from Panwa Cape partially recovered while F_v/F_m from Maiton Island had low ability to recover. This study suggested that *P. damicornis*from Cape Panwa might be more resistant to heat stress, which enhanced its survival after coral bleaching event.

Keywords: coral bleaching, light stress, heat stress, PAM flurometry, Thailand

1.0 INTRODUCTION

Temperature is important factor influencing coral growth and photosynthesis (Osinga et al., 2012) and effects of the high temperature of seawater causes corals response to temperature stress. Normally, the coral can live in a narrow temperature range and when temperature changes, corals become stressed. This has leaded to

bleaching and imbalance of mutualistic relationship which is a loss of zooxanthellae in their tissue (Miththapala, 2008). Reduction in photosynthetic performance might occur and may lead to coral mortality. The effect of temperature on corals might be influent by irradiance as well (Lesser & Farrell, 2004). It has been found that corals in higher irradiance are more susceptible to elevated temperature comparing to lower irradiance. However, some corals might be able to adapt and resist to elevated temperature (Fautin & Buddemeier, 200). The ability of coral recovery from temperature stress might depend on their thermal history (Putchim, 2017).

Response of coral holobiont to elevated temperature and light were observed e.g., reduction in zooxanthellae density (Caroselli & Levy, 2015), change in photosynthetic performance (Lesser & Farrell, 2004; Middlebrook & Dove, 2012), and changing in reaction oxygen species (ROS) (Baird & Takahashi, 2008; Ros et al., 2016). The photosynthetic performance of photosystem II can be measured by chlorophyll *a* fluorescence using pulse amplitude modulated (PAM) fluorometer (Maxwell & Johnson, 2000; Ralph & Gademann, 2005). PAM fluorometry can measure the fluorescence parameters such as: 1) effective quantum yield of PSII ($\Delta F/F_m'$) which indicates the efficiency of photochemical process in PSII when under the experimental light conditions, and 2) maximum quantum yield of PSII (F_v/F_m) which represents the maximum efficiency that light absorbed by photosystem II and indicates stress or damage in PSII (Maxwell & Johnson 2000; Ralph & Gademann, 2005). It is a non-destructive, non-invasive and rapid method. Light energy which is absorbed by light harvesting complexes (LHCs) of PSII is separated into three parts: 1) transfer to PSII reaction center and drive photosynthesis, 2) dissipate in the form of non-photochemical quenching (NPQ) as heat, and 3) re-emitted at a longer wavelength as chlorophyll fluorescence (Maxwell & Johnson, 2000). The aim of this study was to investigate the effect of temperature and light on photosynthetic performance of corals, *Pocillopora damicornis* from Maiton Island and Panwa Cape. Responses of corals during stress and recovery phases were elucidated.

2.0 MATERIALS AND METHODS

2.1 Experimental design

Colonies of *P. damicornis* from Maiton Island (7°45'43.94"N; 98°28'35.37"E) and Panwa Cape (7°48'6.26"N; 98°24'23.75"E), Phuket, Thailand were collected and maintained in an indoor aquarium system (artificial seawater, temperature 27°C, light intensity 150 µmol photons m^{-2} s^{-1}) for acclimation. Light was set at 12:12 h light:dark cycle on and off at 6 a.m. and 6 p.m., respectively. After that, coral colonies were cut into nubbins of 3 - 5 cm. using bone cutter and allocated (*n* = 4) to 4 treatments: 1) ambient temperature, ambient light intensity (ATAL; 27°C, 150 µmol photons m^{-2} s^{-1}), 2) ambient temperature, high light intensity (ATHL; 27°C, 300 µmol photons m^{-2} s^{-1}), 3) high temperature, ambient light intensity (HTAL; 33°C, 150 µmol photons m^{-2} s^{-1}), and 4) high temperature, high light intensity (HTHL; 33°C, 300 µmol photons m^{-2} s^{-1}) for 13 days. In high temperature

treatments (HTAL and HTHL), temperature was increased 1°C each day from 27°C to 33°C (stress phase) and then temperature was decreased 1°C per day from 33°C to 27°C (recovery phase) as shown in **Table 1**.

Table 1. Experimental design shows temperature in each treatment at each day of experiment in stress phase and recovery phase.

Treatments	Day / Temperature (°C)													
	Stress phase								Recovery phase					
	0	1	2	3	4	5	6	7	8	9	10	11	12	13
ATAL (T27, L150)	27	27	27	27	27	27	27	27	27	27	27	27	27	27
ATHL (T27, L300)	27	27	27	27	27	27	27	27	27	27	27	27	27	27
HTAL (T33, L150)	27	28	29	30	31	32	33	33	32	31	30	29	28	27
HTHL (T33, L300)	27	28	29	30	31	32	33	33	32	31	30	29	28	27

Note: T = temperature in unit of °C, L = light in unit of µmol photons $m^{-2} s^{-1}$

2.3 Detection of photosynthesis performance

Photosynthetic performance was determined through a measurement of maximum quantum yield of PSII (F_v/F_m) and effective quantum yield of PSII ($\Delta F/F_m$') at day 0, 3, 5, 7, 9, 11 and 13. Days 3 - 7 were stress period and days 9 - 13 were recovery period. Maximum quantum yield of PSII (F_v/F_m) and effective quantum yield of PSII ($\Delta F/F_m$') were measured at 5 a.m. and 10.30 a.m., respectively using Pulse Amplitude Modulated (PAM) Fluorometer and WinControl software version 3.26 (Junior PAM, Walz, Germany) (PAM settings: measuring intensity <0.15 µmol photons $m^{-2} s^{-1}$, saturating intensity >4500 µmol photons $m^{-2} s^{-1}$, saturating width = 0.8 s, gain = 2, damping = 2).

2.4 Statistics analysis

Data of maximum quantum yield were calculated in term of changing percentage in order to compare change of initial value and each day using a formula as:

$$\text{Change of maximum quantum yield} = \frac{\text{Day}_i - \text{Day}_0}{\text{Day}_0} \times 100$$

where Day_i = value of maximum quantum yield of each day,
Day_0 = average initial data of all treatments

All data were met with assumptions of normality (Kolmogorov-Smirnov test) and equal variance (Levene's test), except maximum quantum yield, which was transformed using X^ (1/4). To determine any significant differences among

treatments, one-way ANOVA tests were performed with a significant level of 95% for maximum quantum yield and effective quantum yield. The change of maximum quantum yield was tested two-way ANOVA for influences of treatments and study sites. The Tukey's HSD post hoc tests was used to determine the statistically distinct groups.

3.0 RESULTS AND DISCUSSION

Maximum quantum yield (F_v:F_m) and effective quantum yield ($\Delta F/F_m$') of corals both Maiton Island and Panwa Cape in the control treatment were constant throughout the experiment. During stress period, a significant decrease in F_v:F_m and $\Delta F/F_m$' were found in corals from both study sites when exposed to elevated temperature, ambient light (HTAL) and elevated temperature and high light (HTHL) on day 7 ($p < 0.05$, 0.01; **Figure 1** and **Figure 2**). F_v:F_m and $\Delta F/F_m$' of corals in HTAL and HTHL treatments were still low on day 9, although temperature was reduced from 33 to 31°C. Besides, F_v:F_m of corals from ambient temperature and high light (ATHL) continually decrease on day 9 even though the temperature was reduced from 33 to 31°C. These results suggested there was cumulative stress on corals, which required longer recovery time. Similar results during recovery phase were also presented in other corals, e.g., *Montipora* and *Acropora* (Higuchi & Yuyama, 2015; Saxby & Hoegh-Guldberg, 2003). At the end of the experiment (day 13), there were no significant difference in F_v:F_m and $\Delta F/F_m$' among treatments suggesting F_v:F_m and $\Delta F/F_m$' can recover after the temperature is reduced to ambient. However, some Maiton Island's corals visually bleached suggesting that this was a critical period that lead to coral mortality or survival.

Significantly decreased in maximum quantum yield in ATHL, HTAL and HTHL treatments since day 3 as shown in **Figure 3** suggested that corals from Maiton Island more Maiton Island was more sensitive to high temperature and high light. On the other hand, maximum quantum yield in ATHL, HTAL and HTHL from Panwa Cape corals was significantly lower on day 7 ($p < 0.01$) indicating that corals from Panwa Cape seemed to be more resistant to elevated temperature and light than corals from Maiton Island. The differences in response, resistance and recovery ability of corals from two locations might be from their thermal history (Putchim, 2017) or their experiences with extreme environmental conditions such as run-off and sedimentation as Panwa Cape is closer to the shoreline and has higher rate of run-off and sedimentation. This study also confirms the ability of PAM fluorometry in timely detection of physiological stress in corals.

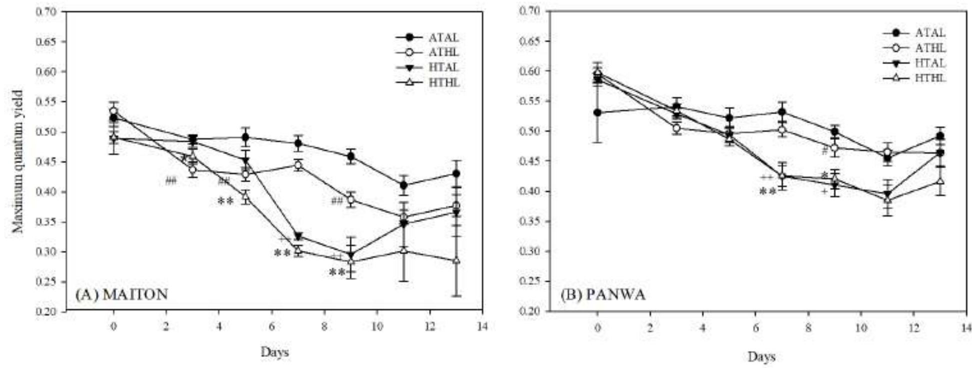

Figure 1. Maximum quantum yield (mean ± SE) of each treatment among study sites: (A) Maiton Island and (B) Panwa Cape. Difference among treatments were determined by one-way ANOVA and post hoc Tukey's HSD test. *, # and + indicate significant differences with the control (ATAL) and others: ATAL VS ATHL ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$) ATAL VS HTHL ($^{*}p < 0.05$, $^{**}p < 0.01$) and ATAL VS HTAL ($^{+}p < 0.05$, $^{++}p < 0.01$), respectively.

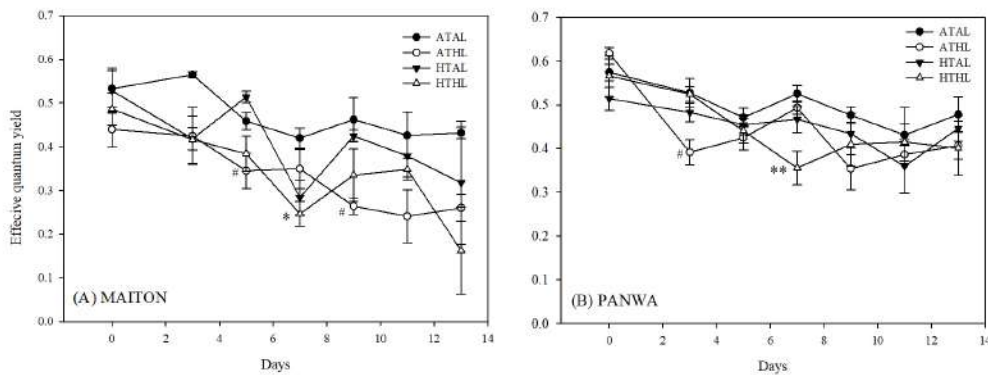

Figure 2. Effective quantum yield (mean ± SE) of each treatment among study sites: (A) Maiton Island and (B) Panwa Cape. Difference among treatments were determined by one-way ANOVA and post hoc Tukey's HSD test. *, # and + indicate significant differences with the control (ATAL) and others: ATAL VS ATHL ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$) ATAL VS HTHL ($^{*}p < 0.05$, $^{**}p < 0.01$) and ATAL VS HTAL ($^{+}p < 0.05$, $^{++}p < 0.01$), respectively.

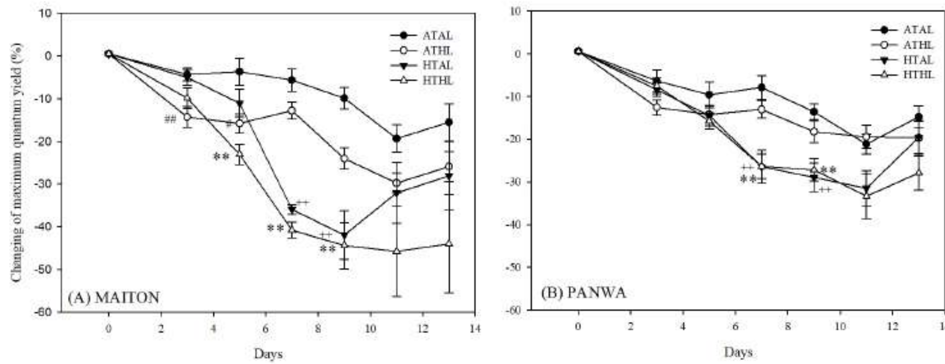

Figure 3. Change of maximum quantum yield (mean ± SE) of each treatment among study sites(A) Maiton Island and (B) Panwa Cape. Difference among treatments were determined by two-way ANOVA and post hoc Tukey's HSD test. *, # and + indicate significant differences with the control (ATAL) and others: ATAL VS ATHL ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$) ATAL VS HTHL ($^{*}p < 0.05$, $^{**}p < 0.01$) and ATAL VS HTAL ($^{+}p < 0.05$, $^{++}p < 0.01$), respectively.

4. CONCLUSION

Our finding demonstrates the combination of elevated temperature and light leaded to a reduction in photosynthetic performance of *P. damicornis* from Phuket and these corals had an ability to recover when temperature was returned to ambient. However, the degree of stress response and recovery ability were different between corals from Maiton Island and Panwa Cape.

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Pests & Diseases of Men **PDM-01**

Laboratory Evaluations of Different Insecticide Formulations against the Field-Collected Tropical Bed Bugs, *Cimex hemipterus* (F.)

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ABSTRACT

At present, insecticide resistance in bed bugs is the major issue contributing towards control failure on this obnoxious urban pest. In this study, the resistance status of several field-collected tropical bed bugs (Kuala Lumpur strain and Queensland strain), *Cimex hemipterus* (Fabricius) were evaluated at several life stages, using several novel and conventional insecticide formulations. Comparison was made with a susceptible strain (Monheim strain) of *Cimex lectularius*, due to the lack of susceptible strain of *C. hemipterus.* The insecticide formulations chosen were pyrethroid-neonicotinoids (A [Thiametoxam (11.6%) + Lambda-cyhalothrin (3.5%)] and B [Imidacloprid (10.5%), Beta-cyfluthrin (10.5%)]), organophosphate formulation (C [Fenitrothion (20%)]), pyrethroid mixture (D [d-tetramethrin (4.4%) + Cyphenothrin (13.2%)]), and pyrethroid only (E [d-phenothrin (10%)]). Among all the formulations tested, the % mortality of eggs from the resistant strains tested was lower than 20%. The results could possibly be explained by the egg shells acting as the protective shield and also the presence of metabolic enzymes at the embryo stage. From the Kaplan-Meier Survival Analysis, adult insects of the resistant strains survived significantly longer than that of first instars ($P < 0.05$) when exposed to the label rate-treated surface. From the results, resistance level of bed bugs towards insecticides was shown to be stage-specific. Hence it is proposed that evaluation on insecticide resistance status on bed bug should be determined by careful assessment of its different developmental stages.

Keywords: bed bugs, insecticide resistance

1.0 INTRODUCTION

Insecticide resistance has been proposed as one of major contributing factors for the resurgence of bed bugs in the 1990s. Bed bug, a hematophagous and nocturnal ectoparasite has been adapted in human dwellings, with human as host for no less than 3000 years (Paragiotakopulu & Buckland, 1999). Both species of bed bugs, which are the common bed bug (*Cimex lectularius*) and tropical bed bug (*Cimex hemipterus*), were under control with the widespread use of DDT and malathion during World War II (Usinger, 1966). Little did people know that overuse of insecticides on bed bugs acted as a selection pressure, escalating the prevalence of insecticide resistance in bed bugs. Bed bug bites triggered allergic reactions in hypersensitive individuals, the reaction ranging from no visible reaction to visible redness, swellings and itchiness. Association with bed bugs infestations over an extended period could lead to medical complications, for instance, anemia and iron deficiency were reported in infants and elders (Pritchard & Hwang, 2009; Koganemaru & Milller, 2013). People often engaged with pest control services to get rid of these nuisance pests. Nonetheless, most of the pest control operators do not have up-to-date knowledge on management of bed bugs. Despite insecticidal control failures are frequently heard, the main adopted approach bed bugs control remains as insecticidal control. In order to be able to control bed bug better, more effort needs to be executed on the study of insecticide resistance and the underlying resistance mechanisms in bed bugs. To date, most laboratory evaluations were done on the adult bed bugs (Romero et al., 2010; Kilpinen et al., 2011; Lilly et al., 2016; Dang et al., 2017). The resistance status of other developmental stages was hardly reported. Better understanding into insecticide resistance at different developmental stages and against different insecticide classes are urgently warranted. A monitoring bioassay to attain the same level of understanding on the insecticide resistance of bed bugs is also required. In this study, different developmental stages of bed bugs were used for investigation. Several formulations with one or more active ingredients from different insecticide classes were chosen for the evaluations to detect the differences in the insecticide resistance status among various developmental stages.

2.0 MATERIALS AND METHODS

Insects and experimental condition

Bed bugs were reared under laboratory conditions of 27 ± 2°C, 70 ± 5% RH and a 12-hour photoperiod. The bed bugs were kept in glass jars with folded brown paper that acted as harborage. Two field-collected *C. hemipterus* strains chosen for this study were the Queensland and Kuala Lumpur strain. The Monheim strain (*C. lectularius*) was used as the susceptible strain due to absence of a susceptible strain of *C. hemipterus*.

Insecticide formulations

Five commercial insecticide formulations were chosen in the study. All formulations were diluted with distilled water according to the recommendations from the manufacturers. The details of the formulations are shown in Table 1.

Table 1 Formulations Used in the Study

Formulations	Insecticide classes
A	Mixture of Neonicotinoid and Pyrethroid
B	Mixture of Neonicotinoid and Pyrethroid
C	Organophosphate
D	Mixture of Pyrethroids
E	Pyrethroid

Bioassay

The insecticide resistance status of the selected strains at the first instar and adult stage were examined using petri dish assay (Dang et al., 2017). The responses of the bed bugs were recorded at regular time intervals and defined as dead when the bed bugs showed no response within a minute upon a gentle probe. Treatments for the bed bug eggs were delivered using immersion method. Percentage mortality of the eggs treated with insecticides following manufacturer's recommendations as well as a series of different concentrations were assessed. Control were treated with distilled water. All data were pooled and subjected to probit analysis.

3.0 RESULTS AND DISCUSSION

The field-collected strains demonstrated high resistance ratio for both formulations D and E when compared with other formulations (Table 2). The results suggested potential presence of *kdr* mutations and metabolic resistance in the bed bugs tested. Liberal use of DDT and pyrethroids in the past, has led to the development of widespread resistance of modern bed bugs towards organochlorines and pyrethroids. Formulation C showed slower reaction time compared to other formulations tested. This is because longer time is required for the conversion of organophosphate to organophosphate-oxon, a metabolite that responsible for the intoxication (Fukuto, 1990). Nevertheless, all the bed bugs tested still showed a relatively high mortality after treatment with formulation C (Table 3). When comparison was made on first instars, adult bed bugs of field-collected strains exhibited higher resistance ratio towards all the formulations tested (Table 2).

Of all the formulations tested at label rates, eggs of both field-collected strain showed low mortality of less than 20% (Table 3). The lethal concentrations of the five formulations tested on both field-collected strains cannot be obtained as the mortality of the eggs was <40% even after treatment with high concentrations. The low efficacy was likely due to the protective layer of the egg shells, slowing down the penetration of insecticides. In addition, embryo of insects also possessed the

ability to detoxify insecticides due to the presence of metabolic enzymes (Bell et al., 1977; De Villar et al., 1980).

Table 2 Resistance Ratio of Several Bed Bug Strains against Formulations Tested.

Formulations	Strain name	Resistance ratio [a]	
		First instar	Adult
A	Monheim	-	-
	Queensland	+	+
	Kuala Lumpur	+	+
B	Monheim	-	-
	Queensland	+	+
	Kuala Lumpur	+	+
C	Monheim	-	-
	Queensland	+	+
	Kuala Lumpur	+	+
D	Monheim	-	-
	Queensland	+	+ + +
	Kuala Lumpur	+ +	+ + +
E	Monheim	-	-
	Queensland	+ +	+ + +
	Kuala Lumpur	+ +	+ + +

[a] Resistance ratio: 0 -30 = '+'; 31 – 100 = '+ +'; 101 and above = '+ + +'.

Table 3 Percentage Mortality of Several Bed Bug Strains against Formulations Tested.

Formulations	Strain name	Percentage Mortality (%) [a]		
		Egg	First instar	Adult
A	Monheim	+ + +	+ + +	+ + +
	Queensland	+	+ + +	+ + +
	Kuala Lumpur	+	+ + +	+ +
B	Monheim	+ + +	+ + +	+ + +
	Queensland	+	+ + +	+ +
	Kuala Lumpur	+	+ +	+
C	Monheim	+ + +	+ + +	+ + +
	Queensland	+	+ + +	+ + +
	Kuala Lumpur	+	+ + +	+ + +
D	Monheim	+ + +	+ + +	+ + +
	Queensland	+	+ + +	+
	Kuala Lumpur	+	+ + +	+
E	Monheim	+ + +	+ + +	+ + +
	Queensland	+	+ + +	+

	Kuala Lumpur	+	+ + +	+

[a] Percentage mortality (%): 0 -30 = '+'; 30 – 80 = '+ +'; 80 - 100 = '+ + +'.

CONCLUSION

The present findings suggested that insecticide resistance status of tropical bed bugs should not be assessed solely at one life stage. Tropical bed bugs exhibited different resistant status at different developmental stages. The results provide an insight in developing a protocol of insecticide bioassay to monitor insecticide resistance.

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Pests & Diseases of Men **PDM-02**

Knockdown Resistance (*KDR*) Genotyping for V1016G and F1534C Mutations in Voltage Gated Sodium Channel (VGSC) in *Aedes aegypti* Populations across Malaysia

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ABSTRACT

Emergence of insecticide resistance in *Aedes aegypti* population worldwide is becoming worrisome especially to the public health authorities. This happens due to the resilience of mosquitoes to withstand certain type of insecticides that has been exposed to them over a period of time, thus leading to the failure in vector control program, despite proactive mitigation efforts done by public health authority. Having knowledge in understanding resistance mechanisms of targeted vector is one of the components in insecticide resistance management, therefore the decision-making in using an appropriate insecticide for control programmes could be made. Not many comprehensive studies has been conducted on Malaysian population of *Aedes aegypti* through molecular characterization, thus this study will provide information on current status of target site mutation in three states in Malaysia. The susceptibility status of *Aedes aegypti* population from Penang Island, Selangor and Kelantan were determined through WHO adult bioassay. These populations were exposed to 0.25% permethrin and 0.21% pirimipos-methyl. Permethrin-resistant and susceptible populations were then genotyped for V1016G and F1534C *kdr* mutations and we found both point mutations in the Malaysian population of *Aedes aegypti* from Penang, Selangor and Kelantan. The findings are useful in providing information on current *kdr* point mutations that occur at VGSC gene in different strains of *Ae. aegypti* in Malaysia.

Keywords: insecticide resistance; *Aedes aegypti*; *kdr* mutation

1. INTRODUCTION

Aedes aegypti is the primary vector for human arboviral diseases such as dengue fever, dengue haemorrhagic fever, yellow fever, chikungunya and also Zika. In Malaysia, dengue is still holding our attention as one of the major public health problems (Rose, 2015). Application of the insecticides to the target mosquitoes is one of the way to combat this public health problem since it shows the fast-acting action. But, the insecticide resistance problem in the targeted vectors arises due to the high dependent on using this method in the vector control program.

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mutation in the voltage gated sodium channel (VGSC) gene is one of the resistance mechanisms that is usually associated with pyrethroid-resistant *Ae. aegypti* population. This resistance mechanism occurs when there is an alteration of amino acid in the VGSC gene, causing less binding of pyrethroid insecticides thus, leading to the survival of the mosquitoes after the application of insecticides (Kasai *et al*., 2014).

In *Aedes aegypti*, the *kdr* mutations that are usually associated in the pyrethroid resistance occur in domains I (Segment 6), II (Segment 5 & 6), III (Segment 6) and IV (Segment 5) of the VGSC gene. Several non-synonymous mutations have been identified in the VGSC gene of *Ae. aegypti*, and the *kdr* mutations of S989P, V1016G, I1011M and F1534C are the most frequent mutations that have been reported to be associated with the pyrethroid-resistant mosquitoes. Recently, a point mutation of V410L has been reported to occur in a Brazilian *Ae. aegypti* population (Haddi *at al*., 2018). All of these point mutations are proven to confer resistance towards pyrethroid insecticides (Haddi *et al*., 2018; Saavedra-Rodriguez *et al*., 2007; Yanola *et al*., 2011). In Malaysia, the occurrence of V1016G and F1534C mutations in this species have been reported by Ishak *et al*. (2015) and Rasli *et al*. (2018).
By understanding the underlying insecticide resistance mechanisms involved in *Ae. aegypti*, it will help in instigating better insecticide resistance management in the future. Hence, this study was conducted to detect the presence of several point mutations reported to occur at the voltage gated sodium channel in Malaysian *Ae. aegypti* populations.

2. MATERIALS AND METHODS

2.1 Sampling of Mosquitoes.

Field strains of *Aedes aegypti* mosquitoes were sampled from three different states in Malaysia; Penang, Selangor and Kelantan by placing the ovitraps in

residential houses. After five days, these ovitraps were then collected and brought back to the insectary where the eggs obtained were hatched for culturing purposes. *Aedes aegypti* were morphologically identified based on the band patterns on the thorax. The populations were cultured continuously until the supply of the eggs were enough for the next assay.

2.2 WHO Adult Bioassay

Female adult *Ae. aegypti* mosquitoes of F_1 generation from each strain, aged three to five days old were exposed to type I pyrethroid, permethrin 0.25% following WHO 2016 guidelines.

2.3 Genomic DNA Extraction

The surviving mosquitoes form the WHO adult bioassay were then extracted to obtain the genomic DNA (gDNA) for genotyping purposes (Livak, 1984).

2.4 Genotyping of V1016G and F1534C mutations

In this study, the gDNA of the surviving mosquitoes were genotyped individually for mutations at the position 1016 at domain IIS6, and 1534, at domain IIIS6 to detect V1016G and F1534C mutation using multiplex PCR assay (Saingamsook *et al*., 2017).

3. RESULTS AND DISCUSSION

Multiplex PCR was performed on 30 female *Ae. aegypti* permethrin-resistant mosquitoes from three different states in Malaysia. Figure 1 shows the results of the genotyping of V1016G and F1534C mutations in the VGSC gene from Selangor, Penang and Kelantan strains. The genotyping of this species from these states shows the occurrence of V1016G and F1534C mutations with frequency ranging from 45% to 70% for V1016G and 35% to 45% for F1534C respectively. The analysis of genotypic allele frequencies from Malaysian *Ae. aegypti* populations shows that Selangor strains has the highest frequency of the mutant allele (1016Gly) for mutation V1016G which is 0.7, followed by strains from Kelantan (0.45) and Penang (0.25). However, for F1534C mutation, the 1534Cys allele frequencies from Penang strains displayed is 0.45 which is the highest among the three states. Occurrence of mutant allele in V1016G is more dominant compared to the mutant allele of F1635C mutation within these states.

The present study provides new updates on target site mechanisms from selected samples of *Ae. aegypti* populations in Malaysia. From the findings, it shows the occurrence mutations of V1016G and F1534C from the chosen states in Malaysia. The presence of both mutations in Malaysian population of *Ae. aegypti* might be one of the contributing factors that leads to the resistance

towards pyrethroid. In Malaysia, insecticides from the class of pyrethroid has been used widely in vector control program and due to high reliance on this insecticide, the arise of point mutations V1016G and F1534C has been detected in Malaysian *Ae. aegypti* populations (Ishak *et al*., 2015 & Rasli *et al*., 2018). In other studies, the distribution of V1016G and F1534C has been detected in *Ae. aegypti* populations from Singapore, Thailand, Indonesia and Vietnam (Pang *et al*., 2015; Stenhouse *et al*., 2013; Wuliandari *et* al., 2015; Kawada *et al*., 2009). The co-occurrence of two or three *kdr* mutations such as S989P, V1016G and F1534C in *Ae*. *aegypti* species from the Asian countries shows the effects of the long-term usage of the pyrethroid insecticides (Kawada *et al*., 2009) during the vector control program and it might result to the high level of resistance.

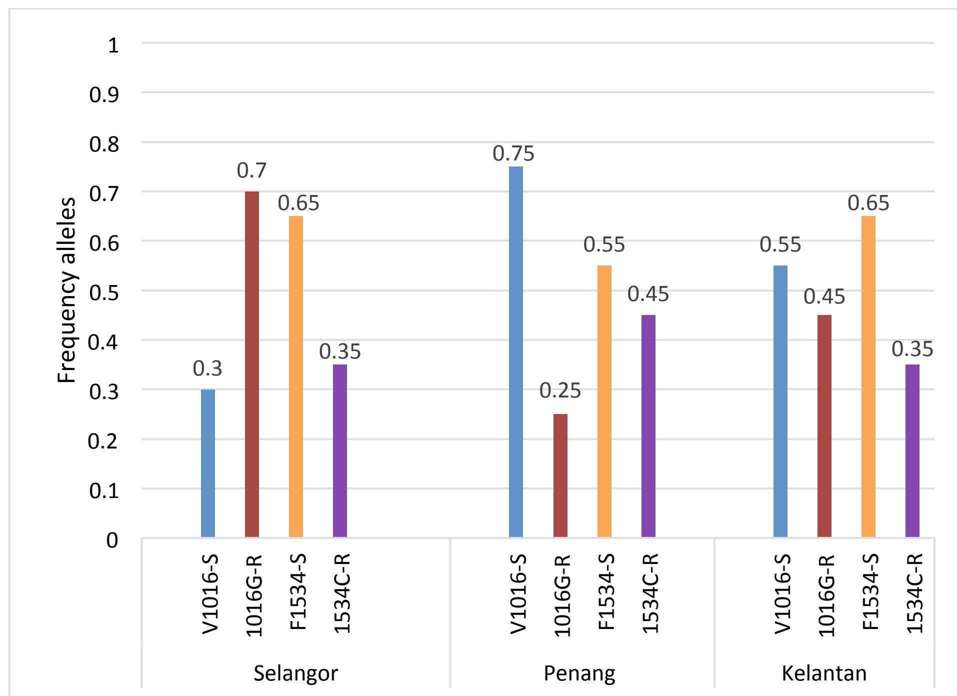

Figure 1. Distribution of genotypic allele frequencies of V1016G and F1534C for permethrin-resistant *Aedes aegypti.* S: susceptible and R: resistant

4. CONCLUSION

This study provides the latest information on mutations occurring in *Ae. aegypti* populations from Malaysia that might be the major reason contributing to target site insensitivity in the VGSC gene. Both *kdr* mutations have been detected in the resistant samples from the three states in Malaysia. Therefore, detecting and monitoring the spread of *kdr* mutations is a must to ensure insecticide resistance management would be successful.

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Pests & Diseases of Men **PDM-03**

Time Dependent Metabolic Enzyme Activity Responses of *Aedes Albopictus* Mosquitoes upon Induction with Malathion and Permethrin

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ABSTRACT

Differential expression levels of metabolic enzymes in *Aedes albopictus* larvae upon acute treatment of malathion as well as permethrin were determined. LC dosages of malathion and permethrin towards the laboratory strain of *Ae. albopictus* larvae were established according to WHO diagnostic determination protocol to be used as standard dosage for this research. Induction of early fourth larvae with 100ppm of malathion as well as 40ppm of permethrin were done for a range of different durations. The induced larvae were harvested and subjected to biochemical analysis to observe the level of metabolic enzyme activities (Gluthathione S-transferases, Acetylcholinesterases and Non-specific Esterases). Differing levels of expressions as well as activities of metabolic enzymes were analysed and compared between treated larvae and susceptible larvae to observe the effect of acute treatment of malathion as well as permethrin on the metabolic enzyme mechanisms in the larvae. From the analysis, the level of specific activity of the enzymes have no significant difference ($p>0.05$) between the induced hours but differed significantly ($p<0.05$) when compared to susceptible strain. Therefore, it can be concluded that the enzyme activities will be elevated upon induction with the test insecticides. The results obtained may provide more knowledge about the effect of metabolic enzymes in *Ae. albopictus* mosquitoes which can be useful for public sector so that proper control measures can be taken to overcome insecticide resistance issue.

Keywords: Metabolic enzymes; *Aedes albopictus*; induction; metabolic resistance

1. INTRODUCTION

The Asian Tiger Mosquito *Ae. albopictus* is a major vector for a variety of viral diseases, such as dengue fever and chikungunya, which threaten over 2.5 billion people worldwide (Griroraki et al. 2015). The prevalence *Ae. albopictus* correlates to dengue fever incidence across Malaysia (Ahmad et al., 1997; Chen et al. 2005). The control of *Ae. albopictus* usually relies on the combination of a few measures such as clean-up campaigns by reducing the larval breeding sites, repellents as well as application of insecticides which includes larvicides and adulticides (Griroraki et al. 2015). Considering the absence of a vaccine and due to unavailability of specific treatment for dengue, the main vector control strategy which have been practised worldwide is the periodic application of insecticide such as ultra-low-volume application, thermal fogging, and indoor residual spraying (Koou et al. 2014). Dengue control routine in Malaysia is predominantly by fogging activities with permethrin and malathion (Wan-Norafikah et al. 2013). However, prolonged exposure to insecticides leads to insecticides resistance which poses serious challenge to the dengue vector control strategies (Ranson et al. 2009). There are four major mechanism of resistance which have been evolved by insect vectors which are metabolic resistance, insecticide target site modification, behavioural resistance as well as cuticular resistance (Hemingway, et al. 2004). The mode of action of metabolic resistance relies on the enzyme systems that allow insects to ensure the natural detoxification of not only insecticides but also all foreign elements (Sokhna et al. 2013). These mechanisms work in two ways; it predominantly functions either by metabolically detoxifying the insecticide before it reaches its target site, or the sensitivity of the target site will change so that it is no longer susceptible to insecticide inhibition (Hemingway 2000). Three main groups of enzymes involved in metabolic based resistance are glutathione s-transferases, esterases, cytochrome P450 monooxygenases and acetylcholinesterases (Sokhna et al. 2013). Insensitive AChE was revealed in *Ae. albopictus* field populations which shows the emergence of organophosphates and carbamates resistance (Dhang et al. 2013). Permethrin tolerance in the *Ae. albopictus* populations in Malaysia have been revealed to be caused by oxidases activity while another study revealed that permethrin resistance of *Ae. albopictus* from Penang is conferred primarily by cytochrome P450 monooxygenase. (Nazni et al. 2000; Chan & Zairi 2013). The focus of this research will be the role of metabolic enzymes responsible in conferring insecticide resistance of *Ae. albopictus* and mechanisms of toxicology challenge towards *Ae. albopictus* larvae. The study of insecticide resistance mechanism will be greatly assisted by the availability of these information. It will also encourage research to determine points for development of novel control tools.

2. MATERIALS AND METHODS

2.1 Diagnostic testing and determination of toxicity parameters

VCRU strain of susceptible *Ae. albopictus* which had been maintained without insecticide pressure were used in this study. After dosage determination, early 4th instar larvae were treated 100ppm malathion and 40ppm permethrin for a range of durations; 1 hour, 2 hours, 4 hours as well as 6 hours. The treated larvae were used for biochemical assays

2.2 Biochemical assays

Varying levels of enzymes in VCRU strain larvae as well as field strain were determined according to WHO standard procedure (Hemingway, 1998). Induced early 4th instar larvae were individually homogenized in 200 µl of season water on ice. 25µl of homogenate were used for the acetylcholinesterase assay. The rest of the homogenate were centrifuged at 14K, 4°C for 30 s, and the supernatant were used as a source of enzyme for the rest of enzyme assays. The assays were performed in a 96-well microplate on ice and the absorbance OD values were measured on the microtitre plate reader. Specific enzyme activities were determined according to the procedure below.
All biochemical assays were performed according to Hemingway J, & Brogdon W 1998 protocol. AChE specific activity was expressed as percentage insensitive AChE activity after propoxur inhibition. Non-specific esterases enzyme activities were expressed as nmole of α-naphthol or β-naphthol/min/mg protein. GST activity were calculated following Beer's Law ($A = \epsilon cl$) and reported as mmole of CDNB/min/mg protein.

2.3 Protein Assay

Protein concentration were used as a standard correction factor for the analysis of all enzymes activities, to account for size variances among individuals. The protein concentration was calculated and transformed from the bovine serum albumin standard curve.

2.4 Statistical analysis

The data obtained were analysed statistically using ANOVA as well as independent t test through SPSS program.

3. RESULTS AND DISCUSSION

Table 1: Mean ± (SE) levels of gluthathione S-transferase (GST) activities (mMole) of *Ae. albopictus* mosquitoes upon induction with malathion and permethrin for different durations.

Strain	Susceptible	Malathion induced	Permethrin induced
1 hour	0.0850±0.0078	0.1070±0.0034*	0.1005±0.0007*
2 hours	0.0893±0.0050	**0.1195±0.0173***	**0.1074±0.0028***

4 hours	0.0825±0.0044	0.1142±0.0145*	0.0991±0.0010*
6 hours	0.0784±0.0026	0.0926±0.0048*	0.0909±0.0016*

Enzyme activities expressed as reaction rate of mMole of CDNB/min/mg protein. Mean followed by asterisk symbol are significantly higher when compared to its respective susceptible strain of *Ae. albopictus* ($p<0.05$). Bolded numbers indicate the highest specific enzyme activities.

Based on table 1, there is a significant difference ($p<0.05$) in the expression levels of GST between different hours and its respective susceptible strains. Enzyme activity levels are at the peak upon induction for 2 hours and subsequently decreases as the induction duration are increasing. However, GST activities are not significantly different ($p>0.05$) when comparison are done between different induction periods. An increase in the production of GST has been reported to be one of the mechanism responsible in conferring resistance in mosquitoes (Hamzah & Alias 2016).

Table 2: Mean ± (SE) levels of Nonspecific Esterases (α-EST) activities of *Ae. albopictus* mosquitoes upon induction with malathion and permethrin for different durations.

Strain	Susceptible	Malathion induced	Permethrin induced
1 hour	0.0828±0.0062	0.0873±0.0072	0.0893±0.0114
2 hours	0.0893±0.0053	**0.1069±0.0146***	**0.1071±0.0136***
4 hours	0.0856±0.0064	0.1066±0.0020*	0.1044±0.0160*
6 hours	0.0833±0.0070	0.0968±0.0114*	0.1026±0.0153*

NSE activity with substrate alpha-naphthyl acetate (µmole of 1-naphthol produced/min/mg protein). Mean followed by asterisk symbol are significantly higher when compared to its respective susceptible strain of *Ae. albopictus* ($p<0.05$). Bolded numbers indicate the highest specific enzyme activities.

From table 2, the α-EST enzyme activities differ significantly ($p<0.05$) between treated and susceptible strains for its specific time duration but did not show significant difference ($p>0.05$) between different hours. *Ae. albopictus* larvae that survived induction with malathion as well as permethrin for 2 hours during the larval bioassay expressed the highest levels of enzyme activity compared to others.

Table 3: Mean ± (SE) levels of Nonspecific Esterases (β-EST) activities of *Ae. albopictus* mosquitoes upon induction with malathion and permethrin for different durations.

Strain	Susceptible	Malathion induced	Permethrin induced
1 hour	0.0778±0.0088	0.0809±0.0053	0.0808±0.0098
2 hours	0.0781±0.0061	**0.1263±0.0102***	**0.1057±0.0099***
4 hours	0.0748±0.0115	0.1135±0.0046*	0.1023±0.0129*
6 hours	0.0793±0.0048	0.0984±0.0066*	0.0894±0.0016*

NSE activity with substrate beta-naphthyl acetate (µmole of 2-naphthol produced/min/mg protein). Mean followed by asterisk symbol are significantly higher when compared to its respective susceptible strain of *Ae. albopictus* ($p<0.05$). Bolded numbers indicate the highest specific enzyme activities.

Similar pattern of results was obtained for β-EST enzyme activities by which the enzyme activities are most elevated for larvae which have been induced with test insecticides for 2 hours. Enzyme activities for all induction

duration shows significant difference ($p<0.05$) compared to its susceptible strain except for larvae induced for 1 hour for both insecticides. There are reports confirming the association of elevated oxidase enzyme activity with the permethrin resistance development in field population of *Ae. albopictus* (Wan-Norafikah et al., 2013).

Table 4: Mean ± (SE) levels of Acetylcholinesterase (AChE) activities of *Ae. albopictus* mosquitoes upon induction with malathion and permethrin for different durations.

Strain	Susceptible	Malathion induced	Permethrin induced
1 hour	28.71%±1.4914	45.81%±7.9365*	**54.44%±2.1611***
2 hours	27.64%±1.7686	37.14%±2.0008*	44.43%±5.1133*
4 hours	29.76%±1.6280	38.25%±5.5000*	40.67%±0.6970*
6 hours	29.01%±2.1057	**51.02%±2.7983***	38.91%±0.8014*

Enzyme activities expressed as percentage insensitive acetylcholinesterase activity after inhibition by propoxur. Mean followed by asterisk symbol are significantly higher when compared to its respective susceptible strain of *Ae. albopictus* ($p<0.05$). Bolded numbers indicate the highest specific enzyme activities.

From table 4, the percentage of insensitive AChE enzyme activities are markedly higher ($p<0.05$) between susceptible and induced larvae from its respective induction duration as well as between different treatment hours. Percentage values more than 30% are considered to be highly elevated. Induction period of larvae with malathion for 6 hours results in the highest elevated percentage of insensitive acetylcholinesterase activities. Different reaction is obtained for larvae treated with permethrin which shows the highest activity after been induced for 1-hour. Enhanced levels of monooxygenases, glutathione S-transferase, monooxygenases and carboxylesterases indicate the emergence of tolerance and resistance to all four main classes of insecticide that have been used to combat vectors (Brogdon and McAllister 1998).

4. CONCLUSION

In conclusion, acute induction of *Ae. albopictus* larvae with malathion as well as permethrin for different time duration enhanced the specific enzyme activities of the analysed metabolic enzyme and this may subsequently contribute in enhancing the tolerance of *Ae. albopictus* to insecticides in the field.

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Pests & Diseases of Men **PDM-04**

Occurrence of *Blastocystis* ST1 from Different Host in Peninsular Malaysia

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ABSTRACT

Blastocystis sp. is ubiquitous, globally distributed intestinal protozoan parasite in avian, mammalian, reptile, insect as well as human hosts. Faecal-oral transmission is the most common pathway with the cystic form as the infective form transmitted through food and waterborne. The mode of transmission is believed to occur by animal-to-animal, human-to-human, animal-to-human and possibly, human-to-animal routes. To date, the genus *Blastocystis* can be classified into 17 small subunits ribosomal RNA (SSU-rDNA) lineages known as subtypes (STs). In Malaysia, ST1 was shown to be the second most common subtype among colorectal cancer (CRC) patients and schoolchildren. In view of the close association between humans and animals, this study was conducted to determine the prevalence and association of *Blastocystis* ST1 in 175 individual faecal samples (including 20 humans, 11 canine, 50 feline, 47 chickens, and 47 wild rats). Based on DNA barcoding methods, a zoonotic subtype *Blastocystis* ST1 was detected in all the human samples (18/20; 90%), eleven feline samples (11/50; 22.0%), one chicken samples (1/47; 2.1%), two wild rat samples (2/47; 4.3%). However, none of the canine faecal samples in this study were positive for this protozoan parasite (0/11; 0%). These data obtained in the present study improve our understanding of the host range of *Blastocystis* ST1 in the investigated areas.

Keywords: *Blastocystis*; canine; chickens; feline; wild rats

1. INTRODUCTION

Blastocystis sp. is a protozoan parasite which commonly colonizes the gastrointestinal tract of humans and variety of animals. This organism has been extensively studied in Malaysia particularly infections in humans (Suresh et al. 1997; Rajah et al. 1999; Tan & Suresh 2006a, b; Chandramathi et al. 2010; Kumarasamy et al. 2014). However, there are also studies on this organism in wildlife (Chandrasekaran et al. 2014), domestic animals (Tan et al. 2013; Farah et al. 2014; Samseh et al. 2017) and water sources (Ithoi et al. 2011; Samseh et al. 2016).

By the application of DNA barcoding method, subtyping *Blastocystis* isolates has revealed an extensive genetic diversity within this genus. Currently, 17 distinct subtypes (ST1-ST17) have been identified in mammals, birds and reptiles with eight subtypes (ST1-ST8) co-occurring in humans and animals. It was found that humans and animals have been infected with the same subtypes, such as ST1 in zoo keeper and one wombat in Australia (Parkar et al. 2010) and ST5 in pig farmer and pigs in Australia (Wang et al. 2014).

In Malaysia, ST1 was shown to be the second most common subtype among colorectal cancer (CRC) patients (Kumarasamy et al. 2014) and schoolchildren (Nithyamathi et al. 2016). Relatively few data are available on *Blastocystis* ST1 distribution in animal hosts. Interestingly, this subtype was mainly associated with zoonotic transmission. Therefore, it is hoped that this present study will add essential information on the host range of *Blastocystis* ST1 in Peninsular Malaysia.

2. MATERIALS AND METHODS

2.1 Ethics statement

All animals were handled according to protocols approved by the Institutional Animal Care and Use Committee (IACUC), University Malaya (Case No.: ISB/31/01/2013/SNMZ (R)). Meanwhile, human ethical approval was obtained in accordance with University Malaya Medical Centre research policy (Reference No.: 2054-12181).

2.2 Collection of faecal samples

Fresh faecal samples of 11 canine and 50 felines were collected from animal shelters, pet shops as well as pet owners around Penang Island whereas 20 human faecal samples were obtained from patients in the University Malaya Medical Centre (PPUM), Kuala Lumpur. The faecal samples were stored in stool containers and processed within 12 hours after collection. Meanwhile, 47

positive *Blastocystis* isolates were isolated from chicken and wild rats, respectively during the prevalence study in Perak and Selangor by Farah Haziqah et al. (2018a, b).

2.3 Molecular techniques

Genomic DNA was extracted using QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) following to the manufacturer's instructions. All samples had been barcoded according to the method of Mohd Zain et al. (2017). All barcode sequences were then identified to allele level using the sequence query facility at www.pubmlst.org/blastocystis.

3.0 RESULTS AND DISCUSSION

In this preliminary study, 18.3% (32/175) of faecal samples were positive for *Blastocystis* ST1 by DNA barcoding method in which human (18/20; 90%) had higher infection rates than animals. Our findings concur with the previous findings that this subtype was the most common subtype in humans in many countries including Malaysia (Wang et al. 2014). Generally, person infected with ST1 had symptoms such as diarrhoea, abdominal pain, abdominal bloating and constipation (Nithiyamathi et al. 2016).

The presence of this subtype was also observed in eleven feline samples (11/50; 22.0%), one chicken samples (1/47; 2.1%) and two wild rat samples (2/47; 4.3%). ST1 has low specificity and is found in a wide range of animals worldwide, including dogs, chickens, cattle, pigs and non-human primates (Alfellani et al. 2013; Ramirez et al. 2014). Others suggested that this subtype appears to be linked to zoonotic transmission from farm animals (Tan, 2008). In Malaysia, Tan et al. (2013) reported that this subtype is the most predominant *Blastcystis* sp. subtype harboured by locally reared goats from different farms in Selangor. Remarkably, this study revealed new sight on *Blastocystis* ST1 reservoirs in Malaysia namely in domestic cats, the companion animal. However, none were detected in the canine samples in this study. This could be due to limited number of samples.

Meanwhile, allele calling using the *Blastocystis* 18S data allowed the identification of allele 4 and 2 within ST1. The most commonly identified was allele 4, occurring in all isolates accept for one isolate from human (H4) which was allele 2.

4. CONCLUSION

This study provides the preliminary data on the occurrence of *Blastocystis* ST1 from different hosts in Peninsular Malaysia. ST1 was the most common subtypes that can be found in humans and most of the animal hosts sampled except canine. Further investigations are essential to better understand this protozoan parasite as well as their host range in Malaysia. In addition, proper health education and animal handling safety are recommended to reduce the prevalence of *Blastocystis* sp.

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Plastics & Waste Management **PWM-01**

Abundance and Distribution of Plastic Debris at Pulau Songsong, Kedah in the Northern Straits of Malacca

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ABSTRACT

Plastic debris were collected during the low tide at Pulau Songsong, Kedah along a 20 m × 1 m transect line on each of the four zones. The zones are backshore, strandline, before strandline and water edge of the exposed beach. The plastic debris were categorized according to 1) sizes, 2) forms and 3) seven major plastic market segment in Malaysia, such as packaging, electrical and electronic, household, automotive, construction, agriculture, and others. Total number of macroplastics (> 2.5 cm) and mesoplastics (2.5 cm – 5 mm) found were 109 items. The highest number of macroplastics and mesoplastics were found at strandline zone, with 2.15 item/m^2 and 1.35 item/m^2, respectively. Most macro- and mesoplastics found were in the form of fragment followed by foam, which constitutes 55.9% and 22.0%, respectively, of the total items. Percentages of 36.6% of the plastic debris found on the beach were from the "packaging" market segment followed by 35.7% of unknown segment. Three items of plastiglomerate (> 2.5 cm, stone that contains mixtures of natural debris that is held together by hardened molten plastic) were also recorded at the strandline. The presence of this material shows the anthropogenic influence which has great potential to form a marker horizon of human pollution.

Keywords: Straits of Malacca, Malaysia, island, marine debris, plastics, microplastics

1.0 INTRODUCTION

Eight out of the top ten countries listed in the top 20 countries of mismanaged plastic waste were Asian countries including Malaysia as the top 8 (Jambeck *et al.* 2015). In Malaysia, studies had been done in mangrove areas of Selangor and Penang, beaches of Penang, Sarawak, Negeri Sembilan, Terengganu, and Sabah to determine the abundance and distribution of microplastics (<5 millimeter (mm)) (Barasarathi *et al.* 2014; Nur Izzati Izyan 2018, Noik & Tuah 2015; Fauziah *et al* 2015). Less studies have been done specifically on macro- (>2.5 centimeter (cm)) and mesoplastics (2.5 cm – 5 mm) especially on island, with only studies done on three beaches of Penang coastline and two at Sarawak (Nur Izzati Izyan 2018; Noik & Tuah 2015). Studies done on the floating marine debris in the Straits of Malacca and Bay of Bengal showed that 97 percent (%) of the debris were found at the Straits of Malacca, indicating the high occurrence potential of plastic debris pollution at the beaches in the vicinity of the straits (Ryan 2013). Besides beaches, remote island can be affected by the pollution too. For example, plastics debris were also found at the remote island of Canary, Spain due to the ocean wave and wind (Herrera *et al.* 2018). Looking at the negative impacts such as entanglement of organisms (NOAA Marine Debris Program, 2014) that will causes them death, we need to access the current status to understand the risk of the plastic pollution for a particular habitat and find a suitable mitigation strategy. For this study, the study site was Pulau Songong and the objectives are to determine the abundance and distribution of macro- and mesoplastics at the exposed beach of Pulau Songsong and to categorise the plastic debris found according to forms and major plastic market segment in Malaysia.

2.0 MATERIALS AND METHODS

Field Sampling

The sampling was conducted during the Highland to Ocean (H_2O) Songsong-Jerai Expedition on 29 September 2017 at the low tide of the day at the eastern side of Pulau Songsong where an exposed sandy beach can be found (5°48'39.28"N, 100°17'50.49"E) (Figure 1). The water edge zone of the beach was identified and a 100 meter transect tape was laid along the zone. An area of 20 meter (m) × 1 m along the transect was randomly identified and possible plastic debris found within the area was using a sample bag (Lippiatt *et al*. 2013 with modification). The procedures were repeated for other zones at the beach, namely before strandline, strandline, and backshore zones. All samples collected were brought back to Marine Science Laboratory and Aquatic Laboratory of School of Biological Sciences for further analysis.

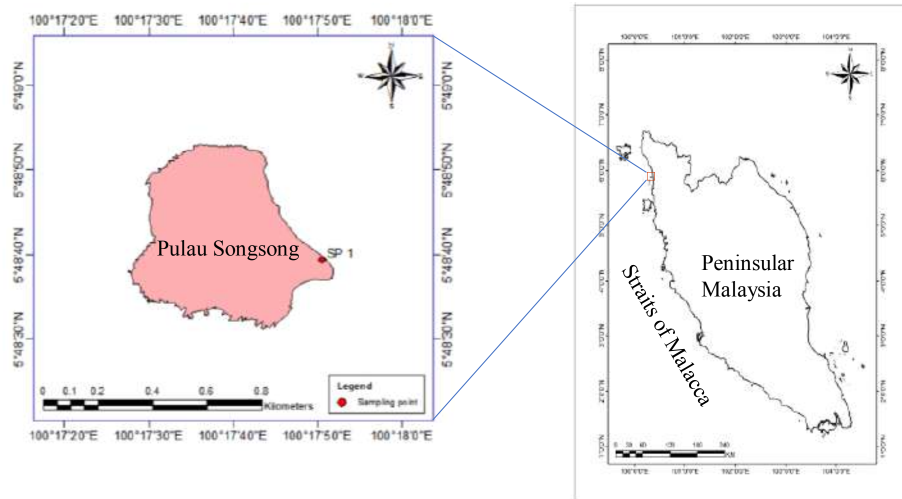

Figure 1. Sampling Location of Pulau Songsong, Kedah.

2.2. Laboratory Analysis

The collected samples were washed with freshwater to remove any sediment and natural debris not associated with the plastics. The samples were then air dried and identity was given, such as plastic bottle, plastic cup, plastic bag, rubber, toys, cable tie, plastic straw, rope and other debris that found and can be identified. Debris that was unsure of whether it is a plastic was tested with hot needle test based on De Witte *et al.* (2014), which was discussed in section 2.2.1. The debris were then photographed and the longest dimension of the debris were measured with ruler and sorted to either mesoplastics or macroplastics. The form of the debris were identified to either fragment, fibre, sphere, film, pellet, and foam (Crawford & Quinn 2017). The debris were also categorised according to major plastic market segment in Malaysia, which are packaging, electrical and electronic, household, automotive, construction, agriculture and others (National Solid Waste Management Department (NSWMD) 2011).

2.2.1. Hot Needle Test

A small fragment of the object which was unsure of whether it is made of plastic or not, was isolated by cutting. The object of interest was put under a stereomicroscope with appropriate magnification that ease the observation of the changes on the object's surface. A metal needle was contacted with flame until the needle became orange or red in colour. Then the hot needle was quickly used to contact the surface of the object. The object was then observed for melting or curling event which would indicate the possibility of plastic component.

3.0 RESULTS AND DISCUSSION

The total number of macroplastics and mesoplastics recorded were 77 items and 32 items respectively, yielding total of 109 items found at the beach. The highest abundance of macroplastics and mesoplastics were found at strandline, which were 2.15 items/m^2 and 1.35 items/m^2 respectively, while backshore had the second highest abundance of plastic debris (Figure 2). There was no plastic debris found at the water edge zone. This trend can be explained by the direction of pounding of ocean wave to the debris along with the natural debris such as leaf and wood debris from the water edge to the strandline and backshore zones. The ocean may transport those thrown or accidentally discarded debris in the ocean to the beach. Sungai Muda and Sungai Merbok rivers that flow to the ocean where the island situated could bring debris too as the rivers support aquaculture and agricultural activities. Visitors and fisherman of the island may brought along plastic made materials and discarded here too.

The form of plastic debris found was mostly fragment, constituting 56% of the total items found while foam recorded the second highest with 22% (Figure 3(a)). Fragments have hard and sharp edges which could injure living fauna if ingested (Kovač Viršek *et al*. 2016) (Figure 4). Foam debris may originated from styrofoam food packaging. It constitutes 45% of the plastics found on the island categorized into packaging market segment. Packaging market segment of plastics polluted the beach the most while secondly unknown segment of plastics (Figure 3(b)). Packaging plastics such as single use plastic bags, cups and plastic bottles were found (Figure 4) and dominated 42% of the plastic market segment share which stands as the largest plastic market segment in Malaysia (NSWMD, 2011). Those debris which their origins and possible functions cannot be guessed were categorised into unknown segment. Those are most probably fragmented from larger plastic pieces, through embrittlement by ultraviolet light and various form of mechanical stress (Cooper & Corcoran 2010). The remaining debris at the beach might one day be embrittled and fragmented from its larger counterpart, generating microplastics especially at the strandline. This increases the bioavailability to organisms living around the area.

Two international packaging products, which are Tetra pak beverage and bottle wrap were also found, which indicates the transboundary capability of plastic pollution. It alarms the importance of international cooperation on implementing suitable policy and strategy to solve this emerging issue. Total of three items of plastiglomerate were also found at the strandline zone (Figure 5). Burning or camp fire activities maybe the possible local reason of the formation of the material since no volcanic activity was detected so far. The presence of plastiglomerate indicates the anthropogenic influence towards the habitat. This material has potential to mark the horizon of human pollution (Corcoran *et al.* 2014).

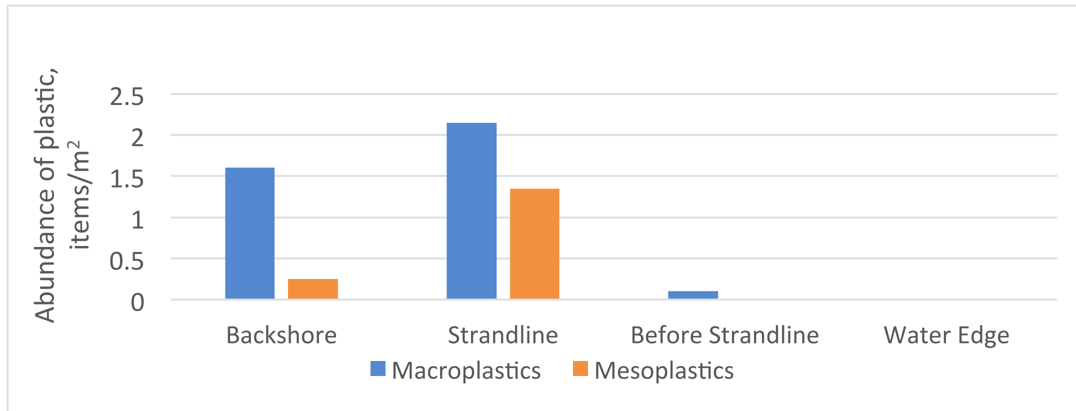

Figure 2. Abundance and distribution of Macroplastics & Mesoplastics at each transect zone at Pulau Songsong.

4. CONCLUSION

Strandline zone was the most polluted zone at the beach at the eastern side of Pulau Songsong. Fragment form of plastic was the form that most commonly found at the beach, Packaging market segment was the plastic market that most polluted the beach. Island can be reached by plastics too. The determination of abundance and distribution of microplastics at the beach and selected organisms of Pulau Songsong is progressing and will help broaden the current status and understanding of the risk of the pollution.

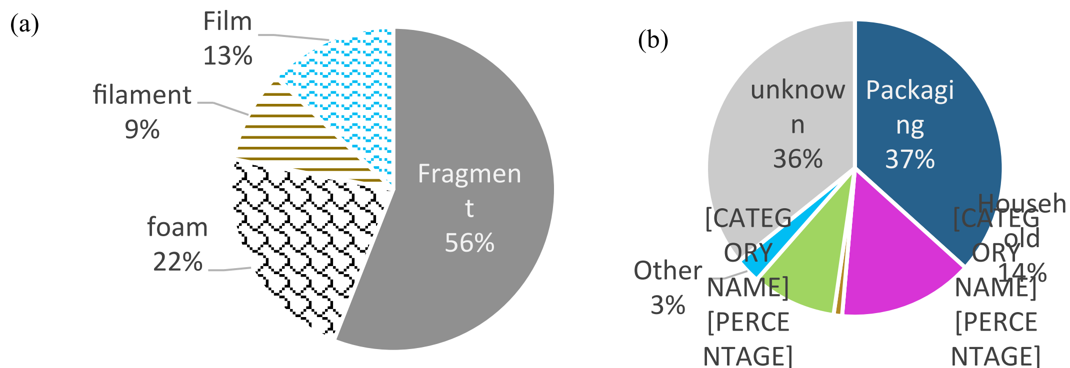

Figure 3. (a) Percentage distribution of forms of plastic debris found at Pulau Songsong; (b) Percentage distribution of the plastic major market segment identified at Pulau Songsong.

(c)

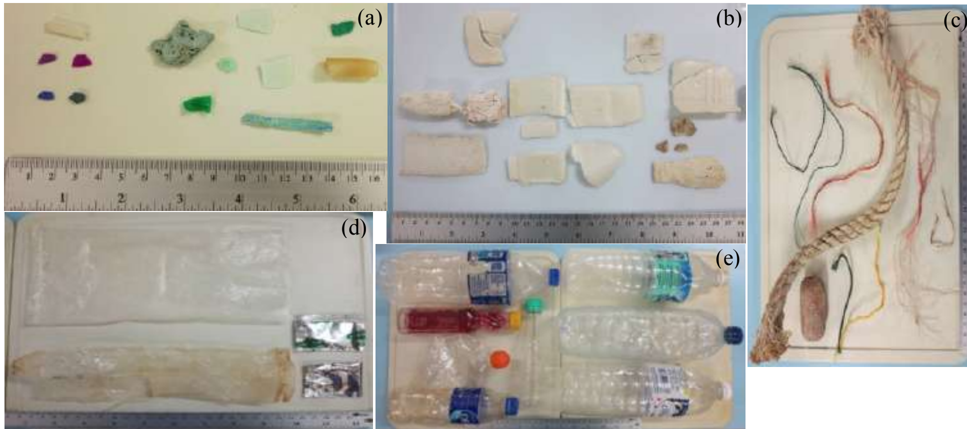

Figure 4. Various forms of plastic debris: (a) fragment; (b) foam (c) filament (d) film. Debris in photo (b), (d), and (e) are examples of debris categorised into packaging market segment.

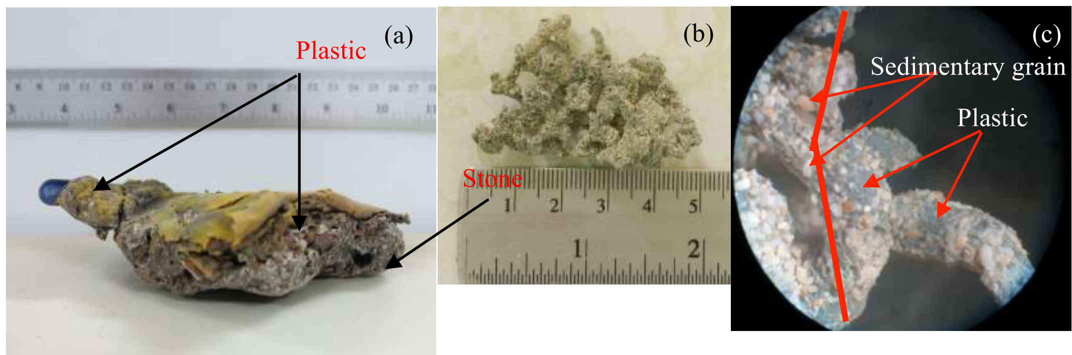

Figure 5. Examples of plastiglomerate that are found at strandline. Plastiglomerate in (a) comprises of yellow and blue colour plastic. Plastiglomerate in (b) has plastic in green colour and (c) is one part of it viewed under stereomicroscope.

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Bioinformatics & Genetics **BG-01**

Polymorphism of 12-BP Insertion/Deletion (InDel) of IPS-Related OCT4 Gene in Southern Thai Indigenous Pigs

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ABSTRACT

InDel markers have been successfully utilized in genetic diversity, population structure analysis, phylogenic relationships, classification of a breed, and fine mapping of target traits in mammals. The genetic variation of 12-bp insertion/deletion (indel) of ips-related oct4 gene in 230 pigs including the indigenous pig populations were randomly collected from seven provinces in southern Thailand: Suratthani (n = 30), Nakhonsi Thammarat (n = 30), Phang Nga (n = 15), Trang (n = 15), Phatthalung (n = 15), Songkhla (n = 20) and Pattani (n = 15), one province in northeast of Thailand (Sakon Nakhon, n = 30), and two commercial pig breeds: Large White (n= 28) and Landrace (n= 32). The genotypes were detected using PCR methods and were classified into 3 genotypes, ins/ins (II) = 344 bp, del/del (DD) = 332 bp and ins/del (ID) = 344/332 bp. Hardy-Weinberg equilibrium were tested by chi-square test. Genotype frequency of II (0.02), ID (0.23) and DD (0.75) and allele frequency of D (0.87) were highest in indigenous pig. The allele frequencies of I in Large White and Landrace pigs were 0.54 and 0.62, respectively. The southern Thai indigenous pigs were at Hardy-weinberg equilibrium.

Keywords: indel, oct4 gene, indigenous pig, Southern Thailand

1.0 INTRODUCTION

Thai native pigs are lard type breeds that have poor growth rates, lower loin eye area and carcass length traits, higher back fat thickness and produce small litters. The main features of these are well adapted to a tropical climate, maintain high utilization of low-quality feed, are perhaps more resistant to disease and internal parasites, and thrive under semi-intensive and intensive management systems. At

present, most of the southern Thai native pigs are integrated pig rearing in oil palm or rubber plantations by smallholder and used for meat production and agro-tourism. However, no information is available on the population structure and genetic diversity of southern Thai native pig. The in formations can be used for Thai native pig preservation and unforeseen breeding requirements in the future.

Various types of molecular markers used in detecting the genetic diversity of Thai native pig, such as mitochondrial DNA, microsatellite or simple tandem repeated (Chaiwatanasin *et al*. 2002; Charoensook *et al*. 2009; Yang *et al*. 2012) and SNPs markers (Klomtong *et al*. 2015) have been reported. Recently, insertion deletion polymorphisms (InDels) and single nucleotide polymorphism (SNP) are becoming important genetic markers, easy and cheap to genotype for genetic analysis of natural populations (Xiao *et al*. 2017). Insertion and/or deletion (indels) polymorphisms are diallelic markers with the potential for use in the study of genetic diversity and phylogenic relationships. Several studies have shown that many genes, such as *Oct4* (Ren *et al*. 2017), *FSHβ*, *MUC13* and *POU1F1* genes (Ren *et al*. 2017), possessed indels in pigs. *Oct4* (octamer-binding transcription factor-4) is essential for the self-renewal of embryonic cells and is also expressed in induced pluripotent stem cells (iPSCs) (Kobayashi *et al*. 2016). In this study, we present a test of the usefulness of indel markers (12-bp indel in the *Oct4* gene) for genetic diversity in the southern Thai indigenous pig populations and two commercial pig breeds.

2.0 MATERIALS AND METHODS

2.1. Sample collections and DNA extraction

A total 230 pigs including the indigenous pig populations were randomly collected from seven provinces in southern part of Thailand (ST): Surat Thani (n = 30), Nakhonsi Thammarat (n = 30), Phang Nga (n = 15), Trang (n = 15), Phatthalung (n = 15), Songkhla (n = 20) and Pattani (n = 15), one province in northeast of Thailand (Sakonnakhon, n = 30), and two commercial pig breeds: Large White, LW (n= 28) and Landrace, LR (n= 32) (From *Surat Thani livestock research* and breeding center). Genomic DNA of the above samples was isolated from the white blood cell samples using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific Baltics UAB, Lithuania).

2.2 Primer design and PCR amplification

A PCR primer for Oct4 was designed according to Ren *et al*. (2017). PCR reactions were performed in 1 μL of 10x PCR buffer, 20 ng genomic DNA, 1 μL of 10 pM of each primer, 0.8 μL of 25 mM $MgCl_2$, 1 μL of 10 nM dNTPs, 0.5 U Taq DNA polymerase (MBI Fermentas) and added ddH2O to 10 μL. The PCR reaction was performed at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, then the reaction was

extended at 72°C for 7 min. The PCR products were separated by electrophoresis in 3.5% agarose gel and stained with Gel star.

2.3 Statistical analysis

For each population, *Oct4* allele frequencies, the effective allele number (N_e), observed heterozygosity (H_o), and expected heterozygosity (H_e) were calculated by GenAlEx 6.5 program (Peakall & Smouse 2012). Polymorphism information content was calculated.

3.0 RESULTS AND DISCUSSION

Two alleles, allele I (344 bp inserted 12-bp fragment) and allele D (332bp) and 3 genotypes, II (344bp), ID (344 bp and 332 bp) and DD (332bp) were observed (Figure 1). The genotypes and allele frequencies, as well as the population parameters in the Oct4 gene indel, were calculated based on agarose gel electrophoresis results and analyzed by the HWE test to determine the distribution of genotypes among pig breeds (Table 1).

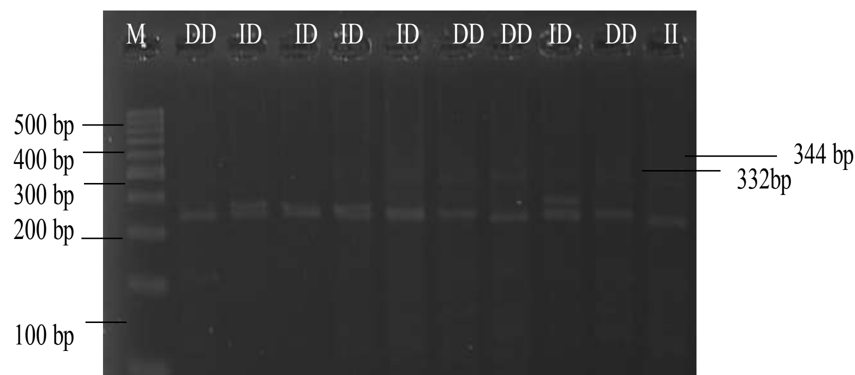

Figure 1. Polymorphism caused by a 12 bp indel in the *Oct4* gene detected by 3.5% agarose gel electrophoresis. Three genotypes of II, ID and DD were resolved.

Our results indicated that the II genotype of *Oct4* gene had a high frequency in LW and LR breeds, the DD genotype had a high frequency in southern Thai native pigs. Furthermore, the I allelic frequencies in LR and LW breed was 0.62 and 0.55, respectively. The frequency of allele D is much higher than allele I in southern Thai native pig. Values for mean heterozygosity and expected heterozygosity (gene diversity) in ST, LW and LR were as follows: 0.22, 0.63, and 0.75 and 0.27, 0.49 and 0.46 respectively. These values are higher than those reported by Ren *et al.* (2017) in LW and LR pig breeds. The PIC value showed that the ST pigs possesses low genetic diversity ($0 < PIC < 0.25$), whereas the LW and LR breed has moderate genetic diversity ($0.25 < PIC < 0.5$) in the indel locus. However, the *effective*

number of *alleles* (N_e) ranged from 1.36 to 1.88. The $\chi 2$ test in ST and LR showed that the genotypic frequencies of *Oct4* gene are in agreement with the HWE (P <0.05), except LW pigs. LW and LR pigs showed three genotypes designated as II, ID, and DD, the frequencies of allele “I” in LW and LD pigs were moderate according to Ren *et al*. (2017). The frequencies of allele “I” in LW and LD pigs were 0.587 and 0.648, respectively. The 12-bp insertion/deletion (indel) polymorphism (NC 010449: g.2759-2760 ins GGTTTTTGTCTA) within the *Oct4* gene was identified in Large White (LW) and Landrace (LD) breeds, showed three genotypes designated as II, ID, and DD. In the previous paper, the *Oct4* gene indel in LW and LR pigs found a non-target fragment when detected 3.5% agarose gel electrophoresis (Ren *et al*. 2017). However, in this study not found. In domestic chicken, Maw *et al*. (2012) reported that found that genetic variability was higher among native chicken populations than in Red jungle fowl (*Gallus gallus bankiva*) and Green jungle fowl (*Gallus varius*) using indels polymorphisms.

Table 1. Genotype and allele frequencies and population indexes for pig *Oct4* gene.

Breed	Size	Genotype frequencies			Allele frequencies		HWE	Population parameters			
	N	II	ID	DD	I	D	P values	H_o	H_e	N_e	PIC
NT	170	0.026	0.27	0.704	0.16	0.84	P<0.05	0.22	0.27	1.36	0.23
LW	28	0.529	0.169	0.303	0.55	0.45	ns	0.63	0.49	1.98	0.37
LR	32	0.391	0.141	0.469	0.62	0.37	P<0.05	0.75	0.46	1.88	0.35

Note: HWE, Hardy-Weinberg equilibrium; H_o, homozygosity; H_e, heterozygosity; N_e, effective allele number, PIC, Polymorphism information content, NT, Thai native pig, LW, Large white pig and LR, Landrace pig.

4. CONCLUSION

The 12-bp insertion/deletion (indel) of ips-related *Oct4* gene was investigated by genetic polymorphism in southern Thai native pigs and two commercial pig breeds. The low genetic diversity was found in southern Thai native pigs and moderate in Large white and Landrace pigs (PIC, H_o and H_e). However, only one indel marker might be poor indicators of genetic diversity in southern Thai native pigs. Thus, future research should be *increasing indels marker* availability.

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