

“AGRICULTURE: TOWARDS A BETTER FUTURE”



.....

PROCEEDINGS

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20 & 21 SEPTEMBER 2018

UNIVERSITI MALAYSIA SABAH (SANDAKAN CAMPUS)



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Soil organic and inorganic nitrogen fractions availability following application of urea and rice straw compost

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Abstract

Most of N in soils are in the unavailable organic form. Soil organic N fractions are proteins, cell wall constituents (amino sugars and their polymers, chitin, and peptidoglycan), and nucleic acids. Only a small fraction of the total soil organic N mineralizes to contribute to the pool of mineral soil N. Soil N fractions mineralized from composts are scarcely described, thus, compost recommendations have been made on the basis of cropping or economic factors rather than soil testing. A soil incubation study was carried out to determine the effects of using different rates of urea and rice straw compost on soil N fractions availability over a period of 90 days. At 30, 60, and 90 DAI, soil was characterized for soil total N, hydrolyzable $\text{NH}_4\text{-N}$, (NH_4^+ + amino sugar)-N, amino sugar-N, amino acid-N, exchangeable NH_4^+ , and available NO_3^- . Combined use of urea with rice straw compost improved soil total N, exchangeable NH_4^+ , and available NO_3^- compared with urea alone. Soil total hydrolyzable $\text{NH}_4\text{-N}$, (NH_4^+ + amino sugar)-N, amino sugar-N, and amino acid-N soils increased in soils with urea and rice straw compost suggesting that decomposition of soil fraction N into available forms of N (NH_4^+ and NO_3^-) was affected by the addition of rice straw compost. Urea can be amended with rice straw compost to regulate availability N for optimum crops use.

Keywords: Amino-acid; amino-sugar; ammonium; hydrolyzable nitrogen; nitrogen fractions.

Introduction

The availability of N from organic source such as composts is low because majority of total composts N is bound to the organic N-pool (Amlinger et al., 2003). The beneficial effects of composts warrant that it is used in conjunction with urea to improve urea-N use efficiency. Studies on determining organic-N fractions following composts application are limited. Mulvaney et al. (2001) demonstrated a laboratory approach for determining N fractions in soils that has been considered by some soil testing laboratories (Sawyer et al., 2003) as a means of determining N fertilizer application rates. It has been recognized that in the organic fractions, significant amounts of the soil N can be identified including such simpler monomer compounds of N forms such as amino acid and amino sugar (Stevenson, 1994). It has been shown that added organic residues decompose rapidly in soils, and it is assumed that much of the organic N which remains is of microbial origin (Kelley and Stevenson, 1985). A soil incubation study

was carried out to determine the effects of using different rates of urea and rice straw compost on soil organic and inorganic N fractions availability over a period of 90 days.

Materials and Methods

A soil incubation study was carried out for 90 days at the Research Complex Soil Laboratory of Universiti Putra Malaysia Bintulu Sarawak Campus, Malaysia. A 250 g of soil was weighed using a digital balance into 500 mL beaker. There were ten treatments with three replications tested in this study. Different rates of urea and rice straw compost were applied without applying P and K fertilizers because the fractions of soil N fractions are the main focus of this study. The rates of the urea (MARDI, 1993) and rice straw compost (John et al., 2013) used in this study were scaled down from the standard fertilizer recommendation for *Zea mays* L. cultivation.

The details of ten treatments evaluated in the soil incubation study are summarized as follows:

1. T0 : soil only (no urea)

Different rates of urea

2. U3 : soil + 130 kg ha⁻¹ urea

3. U2 : soil + 97.5 kg ha⁻¹ urea

4. U1 : soil + 65 kg ha⁻¹ urea

Different rates of rice straw compost

5. RC1 : soil + 0.192 kg ha⁻¹ compost

6. RC2 : soil + 0.385 kg ha⁻¹ compost

7. RC3 : soil + 0.577 kg ha⁻¹ compost

Different rates of urea + rice straw compost

8. RU3C1 : soil + 130 kg ha⁻¹ urea + 0.192 kg ha⁻¹ compost

9. RU2C2 : soil + 97.5 kg ha⁻¹ urea + 0.385 kg ha⁻¹ compost

10. RU1C3 : soil + 65 kg ha⁻¹ urea + 0.577 kg ha⁻¹ compost

The treatments were mixed thoroughly in 500 mL beakers. The treatments were afterwards covered with parafilm to regulate moisture loss. The treatments were incubated for 30, 60, and 90 days, respectively at 26 °C. However, the parafilm was perforated to enable good aeration. Each treatment had 3 replications (that is, 30 samples for 30 days of incubation, 30 samples for 60 days of incubation, and 30 samples for 90 days of incubation). At 30, 60, and 90 days of incubation (DAI), soil samples were air-dried and analyzed for total N using Leco CHNS Analyzer. Soil organic N fractionation was carried out using the method described by Mulvaney et al. (2001) and Stevenson (1996). The method of Keeney and Nelson (1982) was used to extract soil exchangeable NH₄⁺ and available NO₃⁻ after which their contents were determined using steam distillation. The soil incubation study was arranged in completely randomized design (CRD) in triplicates. Analysis of variance was used to detect treatment effects whereas Tukey's test was used to compare treatment means at $P \leq 0.05$. The Statistical Analysis System version 9.2 was used for the statistical tests.

Results and Discussion

Higher total N in soils with combined use of urea and rice straw compost (RC1, RC2, RC3, RU3C1, RU2C2, and RU1C3) at 60 DAI and 90 DAI (Figure 1) was partly because of the organic matter of rice straw compost. Organic matter provides substrate for microbes to decompose organic N as according to Mulvaney et al. (2001), changes in soil total N are related to the substrate supply (from organic amendment) for microbial growth and activity resulting increased in soil total N at 60 DAI and 90 DAI (Figure 1). Organic matter of rice straw compost also serves as sources of microorganisms for decomposition of organic N in soils. The higher organic matter of the rice straw compost ensured retention of NH_4^+ on the negative charges of organic matter of this organic amendment (Figure 2). This also explains the higher of hydrolyzable $\text{NH}_4\text{-N}$ in the soils with urea and rice straw compost (RC1, RC2, RC3, RU3C1, RU2C2, and RU1C3) compared with urea alone (U3, U2, and U1) at 60 DAI and 90 DAI (Figure 2). According to Stevenson (1994), NH_4^+ in acid hydrolysates is derived partly from the decomposition of organic N-compounds and partly from the release of fixed NH_4^+ from organic matter complexes during acid hydrolysis. Furthermore, high CEC of rice straw compost ($86 \text{ cmol}_c \text{ kg}^{-1}$) (Latifah et al., 2017) improved the capacity of the soils with urea and rice straw compost (RC1, RC2, RC3, RU3C1, RU2C2, and RU1C3) to adsorb NH_4^+ compared with unamended urea (U3, U2, and U1).

There was no significant difference of soil NH_4^+ + amino sugar-N in U3 (100% urea) and RU3C3 (50% urea + 15 t ha^{-1} compost) at 30 DAI (Figure 3). The lower soil (NH_4^+ + amino sugar)-N in RC1, RC2, RC3, RU3C1, and RU3C2 compared with U3 at 30 DAI was because of the lower hydrolyzable $\text{NH}_4\text{-N}$ in these soils at 30 DAI (Figure 2). This is possible because (NH_4^+ + amino sugar)-N is derived from hydrolyzable $\text{NH}_4\text{-N}$ which is detected together with amino sugar-N. Mulvaney et al. (2001) reported amino sugar-N is determine as the difference between hydrolyzable $\text{NH}_4\text{-N}$ and (NH_4^+ + amino sugar)-N. However, at 60 DAI and 90 DAI, soil (NH_4^+ + amino sugar)-N was higher in the soils with urea and rice straw compost (RC1, RC2, RC3, RU3C1, RU2C2, and RU1C3) than in urea without rice straw compost (U3, U2, and U1) (Figure 3). The higher (NH_4^+ + amino sugar)-N in the soils with rice straw compost (RC1, RC2, and RC3) and combined use of urea and rice straw compost (RU3C1, RU2C2, and RU1C3) is related to higher hydrolyzable $\text{NH}_4\text{-N}$ content. Thus, suggesting that mineralization rate is influenced $\text{NH}_4\text{-N}$ availability. According to Reddy and DeLaune (2008), at low soil $\text{NH}_4\text{-N}$ level, mineralization rate is slower due to assimilation of inorganic N into microbial biomass. As the level of soil $\text{NH}_4\text{-N}$ increases, rate of mineralization also increases and this is indicated by the higher (NH_4^+ + amino sugar)-N in the soils with rice straw compost (Figure 3).

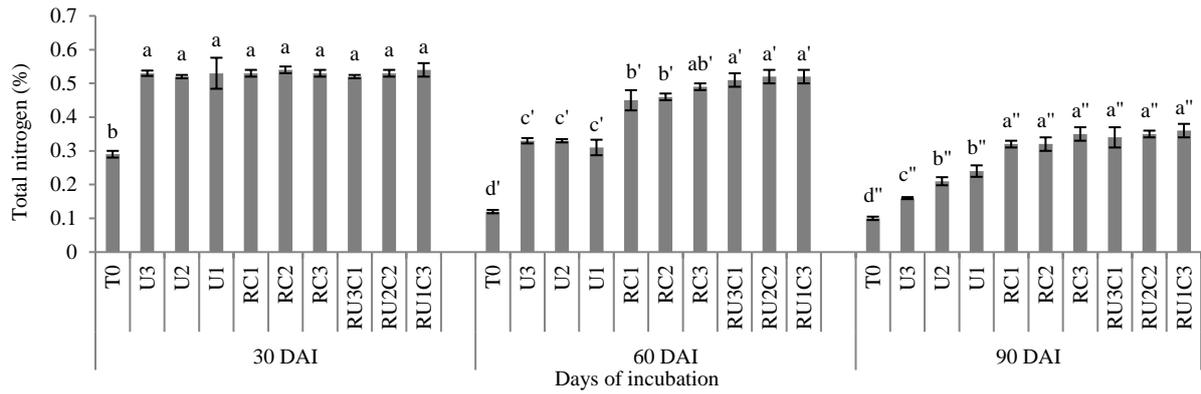


Figure 1: Effects of rice straw compost on soil total nitrogen at 30, 60, and 90 days of incubations. Means with the same letter are not significantly different by Tukey's test at $P \leq 0.05$. Letters without prime represent thirty days of incubation, single prime superscript represents sixty days of incubation, and double prime superscript represents ninety days of incubation.

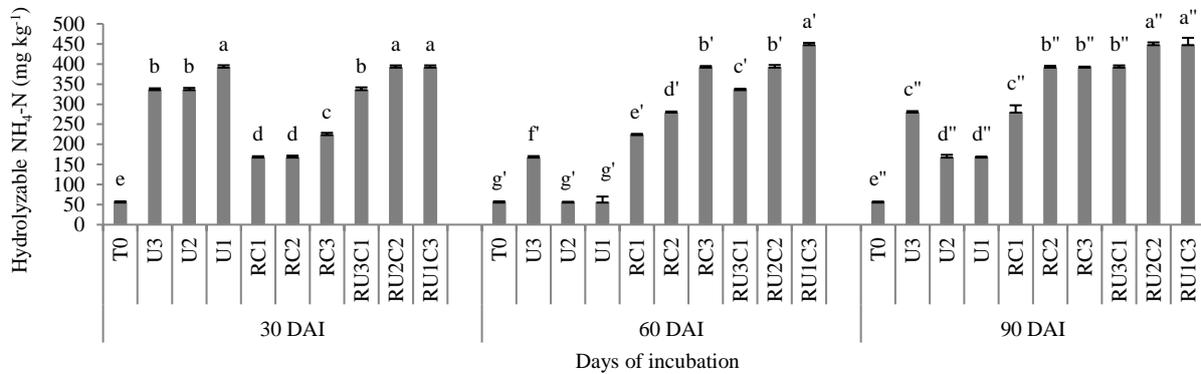


Figure 2: Effects of rice straw compost on soil total hydrolysable NH₄-N at 30, 60, and 90 days of incubations. Means with the same letter are not significantly different by Tukey's test at $P \leq 0.05$. Letters without prime represent thirty days of incubation, single prime superscript represents sixty days of incubation, and double prime superscript represents ninety days of incubation.

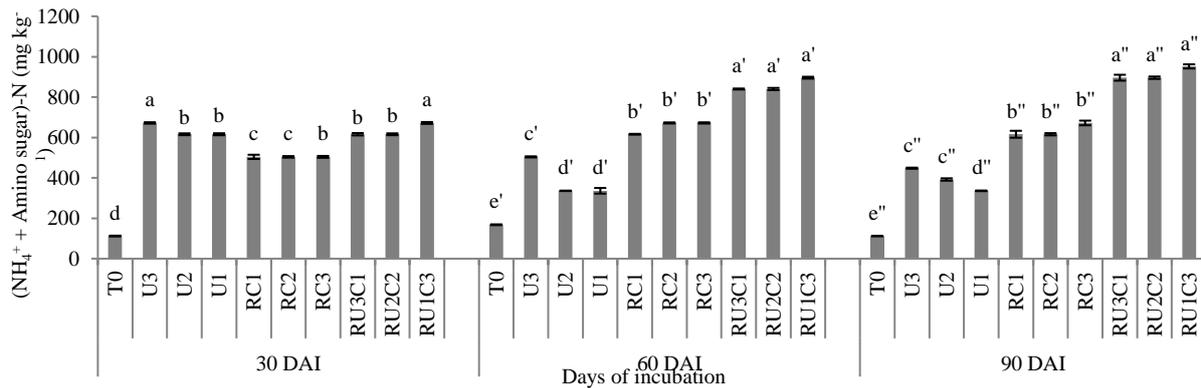


Figure 3: Effects of rice straw compost on soil total (NH₄⁺ + amino sugar)-N at 30, 60, and 90 days of incubations. Means with the same letter are not significantly different by Tukey's test at $P \leq 0.05$. Letters without prime represent thirty days of incubation, single prime superscript represents sixty days of incubation, and double prime superscript represents ninety days of incubation.

At 30 DAI, higher soil amino sugar-N was observed in RC3 compared with U3, U2, and U1 (Figure 4). At 60 DAI, RC2, RU3C1, RU2C2, and RU1C3 significantly increased amino sugar-N contents compared with T0 and U3, U2, and U1. Whereas, at 90 DAI, all of soils with urea and rice straw compost (RC1, RC2, RC3, RU3C1, RU2C2, and RU1C3) were higher in soil amino sugar-N compared with urea alone (U3, U2, and U1) (Figure 4). The functional groups of humic acids in rice straw compost might have affected the higher soil amino sugar-N (Figure 4) and amino-acid-N (Figure 5) because humic substances are mixture of a large number of chemical individuals of a polymeric feature which are formed microbial degradation of lignin (Reddy and DeLaune, 2008). The increase in amino sugar-N is also related to a buildup of cell wall material associated with microbial biomass production, as amino sugar contents have been employed to estimate soil microbial biomass (Zelles et al., 1990) or the quality of soil organic matter (Guggenberger et al., 1999). A relatively high amount of amino sugars and amino acids N in soils are incorporated into humic acids (Guggenberger et al., 1999).

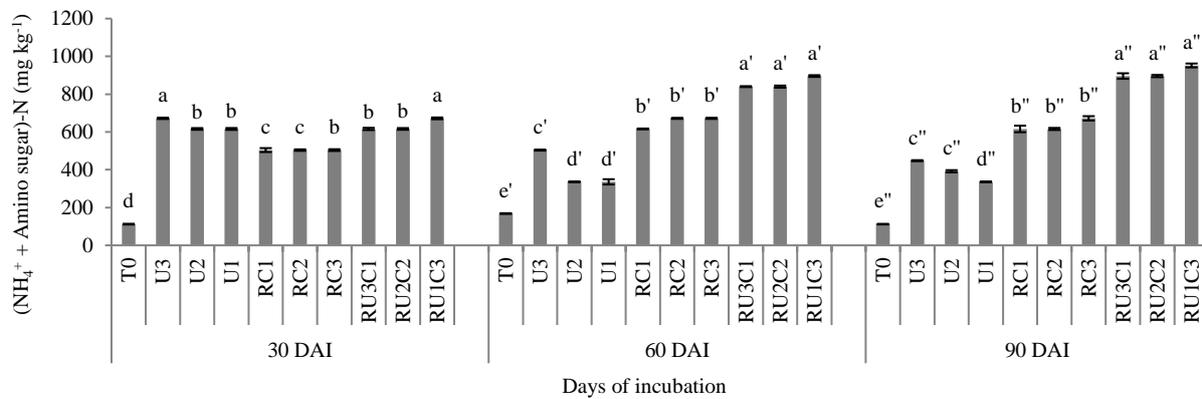


Figure 4. Effects of rice straw compost on soil amino sugar-N at 30, 60, and 90 days of incubations. Means with the same letter are not significantly different by Tukey's test at $P \leq 0.05$. Letters without prime represent thirty days of incubation, single prime superscript represents sixty days of incubation, and double prime superscript represents ninety days of incubation.

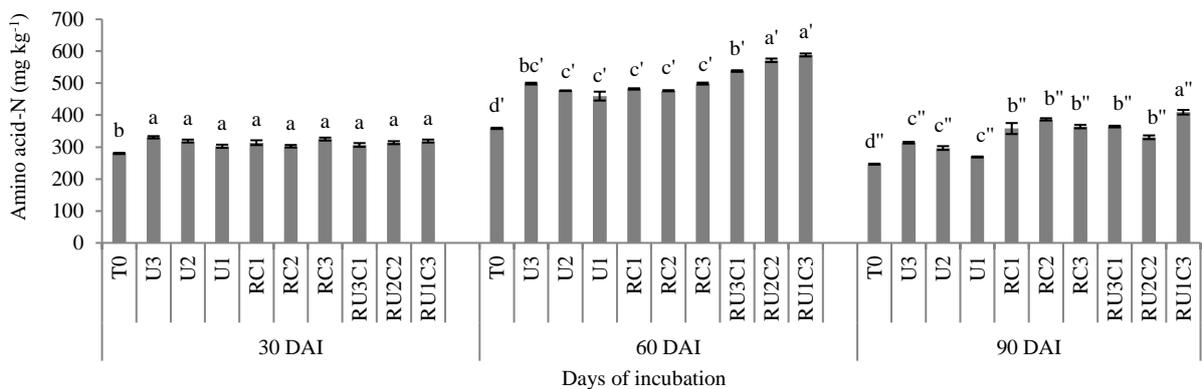


Figure 5. Effects of rice straw compost on soil amino acid-N at 30, 60, and 90 days of incubations. Means with the same letter are not significantly different by Tukey's test at $P \leq 0.05$. Letters without prime represent thirty days of incubation, single prime superscript represents sixty days of incubation, and double prime superscript represents ninety days of incubation.

Urea with rice straw compost (RC1, RC2, RC3, RU3C1, RU2C2, and RU1C3) increased soil exchangeable NH_4^+ and available NO_3^- compared with urea alone (U3, U2, and U1) irrespective of incubation period (Figures 6 and 7). The higher contents of soil exchangeable NH_4^+ and available NO_3^- in soils with rice straw compost were partly due to the higher total hydrolyzable $\text{NH}_4\text{-N}$, (NH_4^+ + amino sugar)-N, amino sugar-N, and amino acid-N at 60 DAI and 90 DAI in these soils. This suggest that the soil organic N fractions released by acid hydrolysis (total hydrolyzable $\text{NH}_4\text{-N}$, (NH_4^+ + amino sugar)-N, amino sugar-N, and amino acid-N) are related to the availability of mineralizable soil inorganic N (exchangeable NH_4^+ and available NO_3^-). According to Mulvaney et al. (2001), amino sugar-N serves as the major source of readily mineralizable soil N, therefore prolonged incubation might have caused the decrease in amino sugar-N as observed at 90 DAI in this study. The decrease also occurred in total hydrolyzable $\text{NH}_4\text{-N}$ at 90 DAI (Figure 2). Gonzalez-Prieto et al. (1997) observed that urea-N was transformed mostly into hydrolyzable organic N fractions as the major source of crop available N.

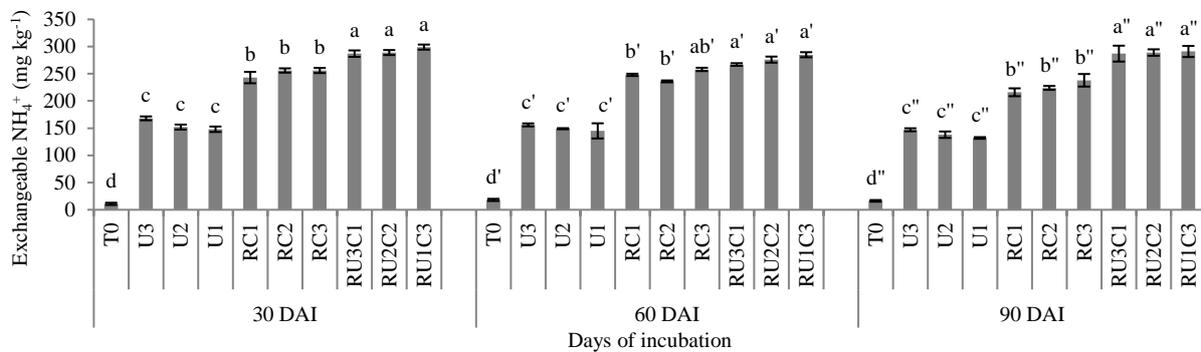


Figure 6. Effects of rice straw compost on soil exchangeable NH_4^+ at 30, 60, and 90 days of incubations. Means with the same letter are not significantly different by Tukey's test at $P \leq 0.05$. Letters without prime represent thirty days of incubation, single prime superscript represents sixty days of incubation, and double prime superscript represents ninety days of incubation.

The humic acids of rice straw compost (15.2%) (Latifah et al., 2017) also affected the release of NH_4^+ and NO_3^- (Figures 6 and 7). This is partly because of the humified organic matter and the colloidal properties of the humic substances which are related to fractions of amino acids and amino sugars N, precursors of humic substances formation (Amlinger et al., 2003). Sorption properties of the humic acids are determined by the presence of functional groups such as carboxyl and hydroxyl groups, constant, and the degree of their dissociation (Cassman et al., 2002).

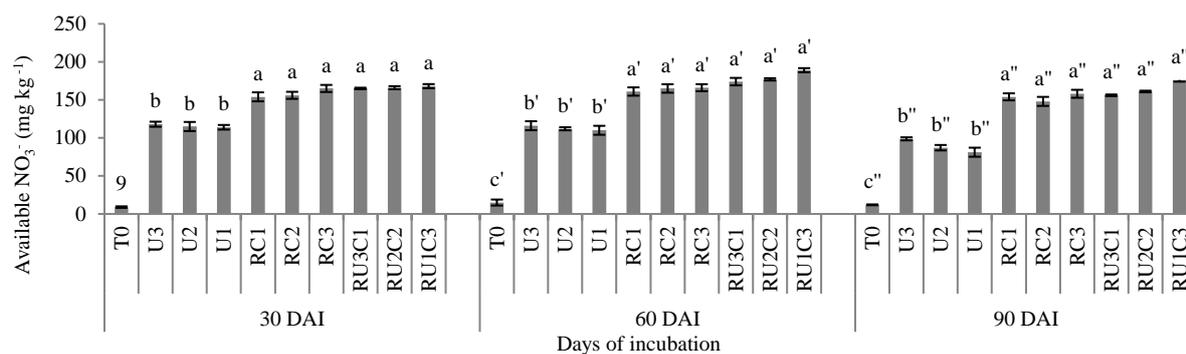


Figure 7. Effects of rice straw compost on soil available NO₃⁻ at 30, 60, and 90 days of incubations. Means with the same letter are not significantly different by Tukey's test at $P \leq 0.05$. Letters without prime represent thirty days of incubation, single prime superscript represents sixty days of incubation, and double prime superscript represents ninety days of incubation.

Conclusions

Combined use of urea with rice straw compost improves soil total N, exchangeable NH₄⁺, and available NO₃⁻ compared with urea alone. Soil total hydrolyzable NH₄-N, (NH₄⁺ + amino sugar)-N, amino sugar-N, and amino acid-N soils were increased in soils with urea and rice straw compost suggesting that decomposition of soil fraction N into available forms of N (NH₄⁺ and NO₃⁻) was affected by the addition of rice straw compost. Urea can be amended with rice straw compost or paddy husk compost to regulate availability N for optimum crops use.

Acknowledgement

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Microbial Carotenoid derived from *Phaffia rhodozyma* and its Antioxidant Activity of Enzymatically Synthesized Carotenoid Glycoside

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Abstract

Phaffia rhodozyma is known as a good source of carotenoids-producing yeast, which is useful as natural antioxidant and antimutagen substitute for synthetic antioxidants that have shown to cause toxic and carcinogenic effects. Biotransformation is one of synthesis methods in producing high yield microbial carotenoid while improving its physicochemical and functional properties by means of biosynthesis of carotenoid glycoside. Present study was conducted to evaluate ability of *P. rhodozyma* to catalyze biosynthesis of carotenoid glycosides through biotransformation using culture of *P. rhodozyma* in the presence of carotenoid extracted from *P. rhodozyma*. We have successfully identified the profile of the microbial carotenoids derived from *P. rhodozyma*. Cells were cultivated in a glucose based media separately under aerobic batch culture system and the carotenoid profiles were analyzed by high performance liquid chromatography. As the results, medium composition strongly affects the profile of *P. rhodozyma* carotenoids represented by ratio of astaxanthin and β -carotene. A carotenoid glycoside as the product of biotransformation was analyzed by thin layer chromatography and extracted using organic solvent and furthermore tested for antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity method. Carotenoid glycoside was found to have EC₅₀ of 109.59 \pm 4.15 μ g/mL which was higher antioxidant activity than that of carotenoid extract (137.18 \pm 5.05 μ g/mL), and the percentage of inhibition against mutagen of synthesized carotenoid glycoside (97.1%) was higher as well than that of carotenoid extract (71.6%). The result obtained from separate studies using commercial astaxanthin and β -carotene found that carotenoid extract at approximately 300ppm to 600ppm showed its antimutagenic activity (42.4% and 45.4%) higher than that of commercial astaxanthin (41.1% and 28.4%) and β -carotene (58.0% and 37.2%). However, inhibition activity of carotenoid extract at approximately 300ppm to 600ppm against antimutagen (57.6% and 54.6%), slightly lower than that of commercial astaxanthin (58.9% and 71.6%) and β -carotene (42.0% and 62.8%).

Keywords: *Phaffia rhodozyma*, carotenoid, biotransformation, antioxidant, antimutagen.

Introduction

Carotenoids are pigments synthesized by photosynthetic and non-photosynthetic organisms. These substances are of commercial interest as coloring agents for food, pharmaceuticals, cosmetics, and animal feed. Because of their antioxidant and antimicrobial properties (Jaswir

et al. 2011, Young and Lowe, 2018), some of them have been proposed to act in the prevention of chronic diseases. Several carotenoids are currently produced for commercial purposes by chemical synthesis. However, there is tendency to avoid the usage of synthetic materials for establishing safety and healthy food or feed supplements. For example, synthetic astaxanthin (3,3'- dihydroxy- β , β -carotene- 4,4'-dione) have been rejected on approval by the Food and Drug Administration (FDA), United of States for the addition those materials for improving fish quality (Dwyer *et al.*, 2018). There is a challenge to promote carotenoids dealing with food safety, quality and healthy for human consumption by applying microbial resources for mass production of microbial carotenoids, since microbes are easier to be handled and modified under controlled condition than plants (Fiedor and Burda, 2014; Zielinska *et al.*, 2017).

Phaffia rhodozyma also known as *Xanthophyllomyces dendrorhous* is the most promising yeast stands out as a natural source of microbial carotenoids. It has a pattern of relatively rapid growth and nutritional quality as well as being approved as a Generally Recognized as Safe (GRAS) microorganism by the Department of Health and Human Services of the Food and Drug Administration (FDA, 2000). *P. rhodozyma* produces different carotenoids depending on the growth conditions. This yeast produces astaxanthin and so far it is the only known natural source of this stereoisomer. It is also capable of producing β -carotene, which is a dicyclic carotene produced from neurosporene through lycopene (Rodriguez-Amaya *et al.*, 2008; Grewe *et al.*, 2007; Schmidt *et al.*, 2010; Chang *et al.*, 2015; Xiao *et al.*, 2015).

The yeast *P. rhodozyma* has some advantageous properties such as yielding carotenoid as a principal microbial carotenoid, utilizing many kinds of saccharides under both aerobic and anaerobic conditions, and growing at high rate. Considering the physiological aspects aforementioned, *P. rhodozyma* is attractive for commercial carotenoid production in large scale. Previous studies reported the promising application of *P. rhodozyma* cultivation for producing microbial carotenoid (Johnson 2003; Moriel *et al.*, 2004; Hui *et al.* 2007).

Overall, this manuscript aims at providing information in terms of the free radical scavenging, antioxidant, and antimutagen properties of microbial carotenoid. To the best of our knowledge, biotransformation of carotenoid glycoside by application of yeast culture induced with microbial carotenoid has not been reported so far. In this study, we evaluated the biotransformation carried out by fungal culture of *P. rhodozyma* containing microbial carotenoids derived from *P. rhodozyma* as its acceptor for the synthesis. The synthesized biotransformation products were then applied for antioxidant and antimutagenic properties through *in vitro* analyses.

Materials and Methods

Strain and maintenance

Phaffia rhodozyma Golubev (MUCL 31142) was maintained on YMA slants containing 10.0 g/L glucose, 5.0 g/L bacto-peptone, 3.0 g/L malt extract, 3.0 g/L yeast extract and 20.0 g/L agar and the strain was stored at 4°C until use.

Production of carotenoids

Productions of microbial carotenoids of *P. rhodozyma* were carried out in three cultivating media; glucose medium containing 3g yeast extract, 3g malt extract, 5g bactopectone, and 10 g glucose in 1L water and two media, i.e. molasses medium containing 20 g molasses, 40 ml bean sprout extract in 1 L coconut water and coconut water medium containing 40 g sucrose in 1 L coconut water. *P. rhodozyma* cells were grown at 22°C in conical flasks with 250 ml of broth media on a rotary shaker at 150 rpm for 168 h. Samplings were conducted at every 24 h.

Biomass measurement.

Cell growth was monitored by determining the dry cell weight according to the method of Sutton (2011). To collect the cell pellet, 5.0 ml of culture from the flask was transferred to a 15 ml corning tube and washed twice with distilled water by centrifugation at 12.000 x g for 5 min. The biomass was weighed after drying cells at 80°C for 36 h. Growth kinetics of *P. rhodozyma* cells growing in media were calculated as follows: $\mu = \ln (N_1 - N_0) / \ln (T_1 - T_0)$, where μ is the specific growth rate (h^{-1}), N_0 is the biomass at initial growth (g), N_1 is the biomass at final growth (g), T_0 is time at initial growth (h), and T_1 is time at final growth (h)

Extraction of microbial carotenoids

One milliliter of homogenous cell suspension was centrifuged at 10.000 x g, 4°C for 10 min and washed twice with distilled water. Two milliliters of dimethyl sulfoxide (DMSO) (Merck, Schuchardt, Germany) was added to the dried pellet incorporation with one milliliter of 1 M phosphate buffer (pH 7.0) and in 15 ml corning tube with glass bead (5 mm in diameter). After centrifugation, cell pellet was added with 5 ml petroleum ether (Mallinckrodt Baker Inc., Phillipsburg, USA) in reaction tube and homogenized again for 10 min. After centrifugation at 10,000 x g, 4°C for 10 min, carotenoid in petroleum ether phase was transferred to clean reaction tubes and dried. Finally, aliquot of 2 ml methanol (Merck KGaA, Darmstadt, Germany) was added for analysis of carotenoid concentration and profiles.

Analysis of total carotenoids

To calculate the total extracted microbial carotenoids were analyzed by high-performance liquid chromatography (HPLC, LC-20AB) (Shimadzu Co. Inc., Tokyo, Japan) on a Spherisorb ODS2 4.0- by 250 x 4.6 mm C18 column (Luna 5u Silica (2) 100 A) by using a 15-min gradient of ethyl acetate (0 to 100%) in Hexan-Aceton (86 : 14, v/v) at a flow rate of 2 ml min^{-1} in 20°C. Absorption spectra for individual peaks were obtained with a photodiode array detector. Carotenoid peaks were identified by their absorption spectra and by their typical retention times at λ 474 nm. Standards of 10 ppm astaxanthin (Sigma, Germany) and β -carotene (Sigma, Germany) were applied to identify the peak of those substances in sample.

Synthesis and determination of carotenoid glycoside

Reaction mixture was prepared by adding 50 ml of Na-phosphate buffer pH 7.0 containing 5.0g soluble starch as substrate, 2.5g microbial carotenoid extract as acceptor and 5-days incubated fungal culture. Incubation of reaction mixture was performed for 24 h at 40°C in a water bath. The biotransformation culture was then extracted with diethyl ether to remove the excessive carotenoid acceptor which were synthesized into glycosides during biotransformation and remaining solvent was then removed by using rotary evaporator (Nazir *et al*, 2018). Obtaining biotransformation products were determined by using thin layer chromatography (TLC) which was developed using ethyl acetate, acetic acid and distilled water (3: 1: 1, v/v). The TLC plate was heated at 80-90°C for 1 h prior to spray with a reagent of 20% H₂SO₄ in methanol, and then heated at 150°C for 5-10 min (Nazir *et al*, 2018).

DPPH radical scavenging assay

Free radical scavenging activity of biotransformation synthesized carotenoid glycoside (CG) was determined against 1, 1- diphenyl-2-picryl hydrazyl (DPPH) as defined by Shekhar and Anju (2014) and Molyneux (2004). Briefly, DPPH solution of 0.1Mm in methanol was prepared. One ml of this solution was added into 3ml of CG dissolved in (10-100 µg/ml) methanol. The mixture was shaken strongly and permitted to leave at ambient temperature for 30 min. Absorbance was determined at 517 nm using spectrophotometer. The IC₅₀ value of the respective compounds needed to inhibit 50% of the DPPH was determined.

Determination of antimutagenic activities.

The antimutagenic effect of CG toward Trp-P-1 in *Salmonella typhimurium* TA98 was assayed by the preincubation method with some minor modifications and using an S9 mix as previously described (Trakoontivakorn *et al*, 2001). The activity was calculated using the formula defined by Kanazawa *et al* (1995). The antimutagenic activity was examined according to the method of Kanazawa *et al* (1995).

Results and Discussion

Extraction and determination of microbial carotenoid

We observed that cells grow well in all tested media used in this study. Generally, the highest biomass was obtained at 96h of cultivation. Scanning electron microscopy of cells had been treated with DMSO method for disruption of cell was shown in Figure 1. Compared with intact cells before disruption, most of the cells treated by solvent were broken, but some cells kept intact after disruption, thus indicating that the loss of carotenoid during treatment process came from two sources whether from carotenoid degradation either underutilization of encysted cells. Mendes-Pinto *et al* (2001) studied different cell disruption processes on encysted cells of

Haematococcus pluvialis and autoclave treatment (30 min, 121°C) was verified to be the most effective cell disruption process in terms of carotenoid extraction and availability.

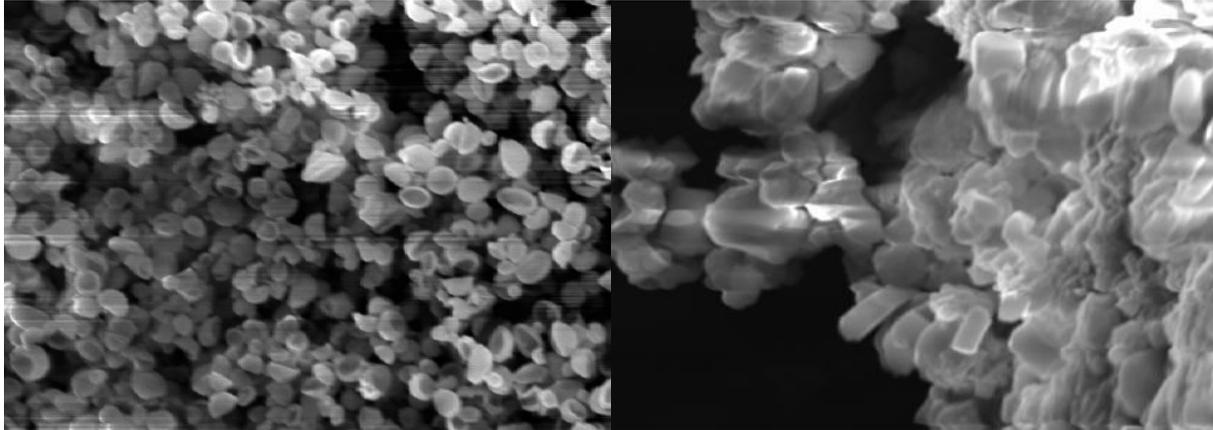


Figure. 1. SEM of *P. rhodozyma* cells before disrupting (*left*) and after treatment with dimethyl sulfoxide DMSO (*right*).

In our work, autoclave treatment was also examined, yet the result was against expectation. Carotenoid was hardly extracted from processed cells of *P. rhodozyma* (data not shown). Compared with control cells before disruption, the shape of yeast cells after autoclave treatment changed a little but still remained intact as illustrated in Figure 1, meaning that no carotenoid could be extracted from encysted cells because of hindrance by cell wall and cell membrane. With more and more problems arising from raw material of doubtful origin in the usage of chemical additives, there is growing concern about food security. A natural food pigment, carotenoid, is safe and nutritive when used as food additive. However, being one of the most important natural pigment sources of microbial carotenoid, *P. rhodozyma*'s thick cell wall hinders digestion of by the animal (Mendes-Pinto *et al*, 2001). Therefore, an effective method of cell disruption is essential for commercial applications.

The growth rate of *P. rhodozyma* cells in coconut water medium was higher than those of in molasses medium and glucose medium, respectively (*data not shown*). Furthermore, the measurement of carotenoid was conducted at 96h based on the highest biomass. Two major microbial carotenoid in *P. rhodozyma* culture, β -carotenoid (MC-1) and astaxanthin MC-2, were determined in this study by HPLC. Based on HPLC analyses, the two main types MC were detected clearly from extracted cell of *P. rhodozyma* which was cultivated in glucose medium. However, there were few peaks beside MC on the chromatogram which are indicated the assortments of carotenoid cultivated in other two other media (Figure 2).

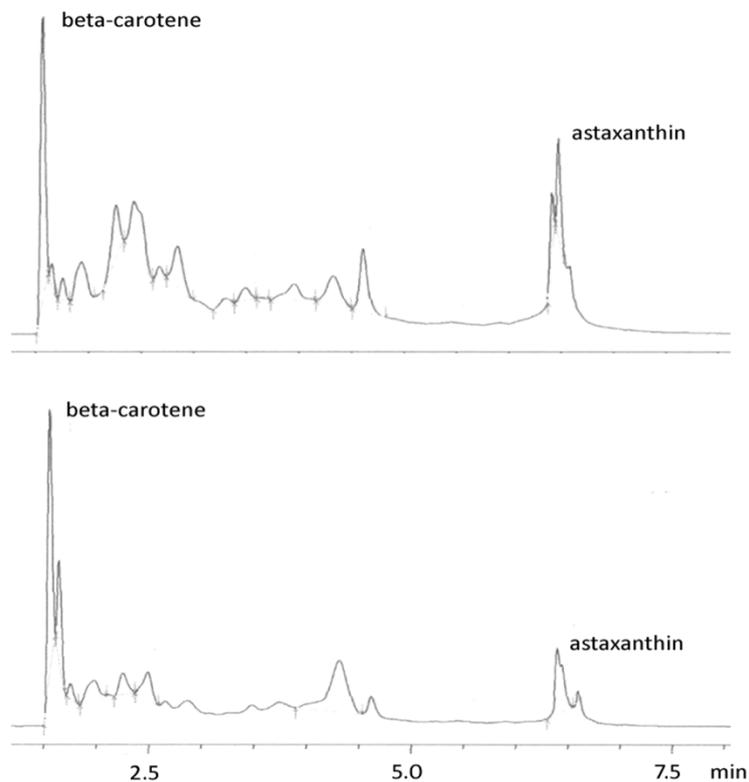


Figure. 2. Chromatogram of microbial carotenoids extract derived from culture of *P. rhodozyma* using cell disruption with DMSO. LC-20AB photodiode array detection system 474 nm. Chromatography Spherisorb ODS2 4.0 (250 x 4.6 mm) C18 column.

The high level of specific growth of *P. rhodozyma* cell in medium indicated the effective ability of substrate usage and/or other component of the medium to yield about 12 g l^{-1} cell biomass. Carbon source influenced the yield and carotenoid compositions. Similar phenomenon of different condition on accelerating biomass and microbial carotenoid production was reported previously in term of C/N ratio. Braunwald *et al* (2013) and Yamane *et al.* (1997) reported that carotenoid production was enhanced by an initial high carbon/nitrogen ratio (C/N ratio) present in the medium, but cell growth was inhibited by a high glucose concentration. Total astaxanthin produced in this study using glucose medium is similar to previously reported which ranged between $300 - 600 \mu\text{g g}^{-1}$ dry cell (Ramirez *et al.* 2001).

Biotransformation of carotenoid glycoside

Microbial culture of *P. rhodozyma* containing soluble starch as substrates, microbial carotenoid extract as acceptor were reincubated to synthesize carotenoid glycosides as biotransformation products. They were synthesized via intermolecular glycosylation reaction, where is a process to transfer glucose residues to any acceptor having OH- group. After conducting the biotransformation using culture of *P. rhodozyma* containing carotenoid extract was applied as its acceptor, we found two biotransformation products could be determined from two different media were used for biotransformation through TLC analysis (Figure 3).

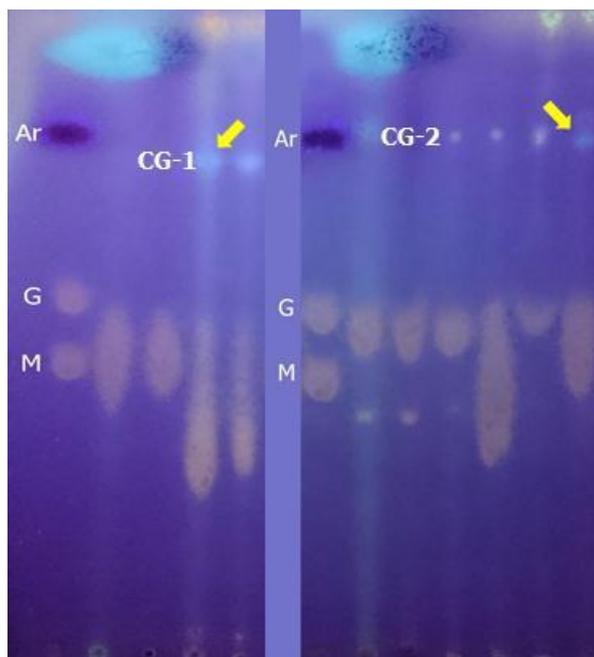


Figure 3. Chromatogram of biotransformation products in the presence of microbial carotenoid extract derived from *P. rhodozyma* culture-1 (A) and culture-1 (B) observing under UV light irradiation. Arbutin (Ar), glucose (G), maltose (M) were used as reference for authentic standard solution and CG-1 and CG-2 indicated to biotransformation products.

Arbutin (4-hydroxyphenyl-D-glucanopyranoside) was applied as an authentic standard component for determination of existing biotransformation product on the TLC chromatogram. *P. rhodozyma* culture showed capability to synthesize two carotenoid glycosides through biotransformation in the presence of carotenoid extract which could play a role as acceptors for the biotransformation. The spots of biotransformation products those were parallel corresponding to RF value of the spot of arbutin were considered as the synthesized carotenoid glycosides. The results exhibited that *P. rhodozyma* culture had shown high activity in transferring glucose units to the carotenoids due to effect of strong interaction between OH-groups of donor-substrate and the OH-group of carotenoids-acceptor by synthesizing carotenoid glycosides.

Carotenoids exhibit instability due to oxidation, light and biochemical changes, which was indicated by occurrence of browning reaction. For that reason, while it is once oxidized then its properties and the use as biological compound will modify, decreased or even vanished. As compare to carotenoid glycosides that were enzymatically synthesized, they were able to demonstrated quite high stability toward the chemical changes (Aramsangtienchai *et al*, 2011). The glycosylation of carotenoids into its glycosides has a number of advantages in contrast to chemical synthesis, which include low cost production, easiness on handling microbial strains and its culture containing enzyme for the purpose of synthesis of bioactive compounds. The chemical synthesis of glycosides is well developed, but remains a laborious task, as many protection and selective deprotection steps are required to solely address single functional groups in order to avoid side product formation (Nicolaou and Mitchell, 2001; Douglas *et al*,

1998; Cheung *et al*, 1997). Complete anomeric control of the newly formed bond is also a prerequisite to avoid lengthy separation methods.

In contrast to conventional chemical synthesis that usually requires tedious protection/deprotection manipulations in order to achieve regio- and stereo-selectivity, biotransformation through enzymatic glycosylation usually provides perfect control of the anomeric configuration and high regio-selectivity without the need of any protecting groups (Crout and Vic, 1998). Biotransformation permits insoluble and less stable bioactive compounds to be changed into the resultant soluble and more stable compounds during suitable single-step biological glucosylation (Shimoda *et al*, 2006). Additionally, glucosides of physiologically bioactive substances, i.e. vitamin glucosides, have been suggested to be functional anti-allergic agents (Sato *et al*, 2001).

Throughout nature the synthesis of glycosidic structures is accomplished by the enzyme group of glycosyltransferases. These enzymes transfer activated sugar nucleotide donor molecules selectively onto an acceptor molecule. Their use in biocatalysis has been extensively researched, but is still limited for a large scale by the high cost of the nucleotide-phosphate donors and difficulty in expression and handling of these mostly membrane bound enzymes (McArthur and Chen, 2016). Alternative carbohydrate active enzymes are glycohydrolases or glycosidases, of which a high variety are readily available due to their occurrence in the metabolic pathways of all organisms (Talens-Perales *et al*, 2016). This group of enzymes naturally degrade glycosidic structures and are defined into two groups depending on their catalytic mechanism. Glycosidases can be utilized in synthetic reactions by reverse hydrolysis or transglycosylation methods (Danby and Withers, 2016; Bissaro *et al*, 2015).

Nevertheless, the ability of the glycosidase in hydrolyzing the produced product leads to strongly diminished yields. To overcome the disadvantage of product hydrolysis Mackenzie *et al* (1998) and also Malet and Planas (1998) that reported the production of genetically engineered glycosidases, namely glycosynthases, which were lacking a nucleophilic residue and therefore void of hydrolytic activity. However, the intact structure of the enzyme allowed the formation of glycosidic bonds in high yields in the presence of activated glycosyl donors such as glycosyl fluorides. The biotransformation and enzymatic synthesis has proven itself to be a promising alternative to the laborious chemical synthesis of glycosides by avoiding the necessity of numerous protecting group strategies. Among the biocatalytic strategies, glycosynthases, genetically engineered glycosidases void of hydrolytic activity, have gained much interest in recent years, enabling not only the selective synthesis of small glycosides and glycoconjugates, but also the production of highly functionalized polysaccharides.

Radical scavenging activity against DPPH

Moreover, we have determined carotenoid glycoside (CG) due to similarity on its RF value with that of arbutin to conduct further study regarding with its antioxidant capacity assessment. Herein, carotenoid extract was utilized as acceptor in the biotransformation to catalyze the synthesis of carotenoid glycoside was furthermore evaluated for its *in vitro* antioxidant activities. Free radical scavenging activity of CG against DPPH was determined as compared

to the capacity of carotenoid extract and ascorbic acid that were used as commercial products supposed to have antioxidant activity. As shown in Figure 4, it showed that CG exhibited considerably strong antioxidant capacity as compared to ascorbic acid. The CG exhibited strong free radical scavenging activity with a value of IC₅₀ was 109.59 µg/ml as compared to the ascorbic acid (38.98 µg/ml), however lower than carotenoid extract (137.18 µg/ml) (Table 1).

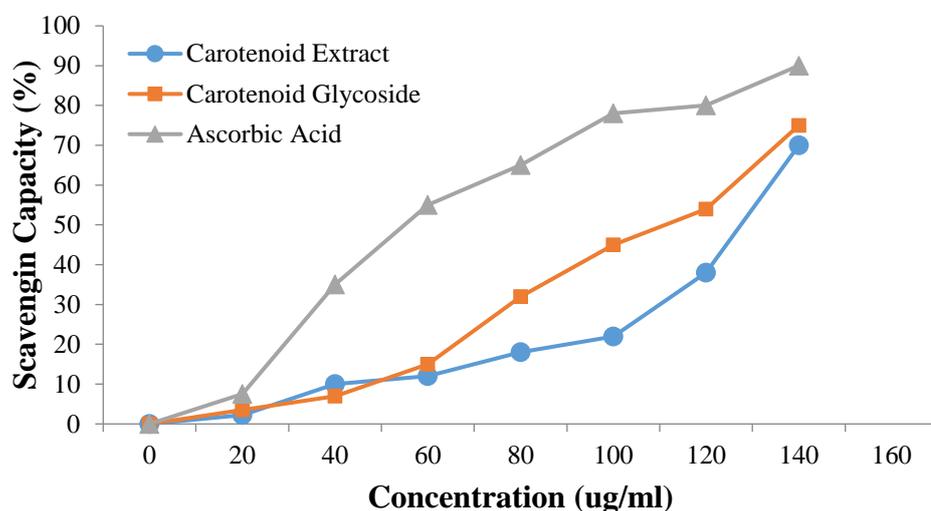


Figure 4. Radical scavenging capacity (%) of carotenoid and carotenoid glycoside extracts against radical DPPH.

Table 1. The IC₅₀ value of ascorbic acid, carotenoid extract and carotenoid glycoside.

Samples	IC ₅₀ Value (µg/mL)
Carotenoid extract	137.18 ± 5.05 ^a
Carotenoid glycoside	109.59 ± 4.15 ^b
Ascorbic acid	38.98 ± 6.43 ^c

The recorded IC₅₀ value of CG was lower as compare to the carotenoid extract but still indicating strong antioxidant potential considerably higher to the ascorbic acid used as standard. The IC₅₀ value was concluded from the schemed graph of DPPH scavenging effect against concentration. IC₅₀ value is described as amount of the effectiveness of a compound in inhibiting a definite biochemical or biological function. This computable measure designates how much of a specific inhibitor or drug is required to scavenge a given biochemical process by half. Rendering to the FDA, IC₅₀ signifies the concentration of a drug that is needed for 50% inhibition in vitro; the lesser value of IC₅₀ specifies greater radical scavenging activity. DPPH is a stable free radical, due to the delocalization of the spare electron on the whole molecule. Thus, DPPH does not dimerize, as happens with most free radicals. The delocalisation on the DPPH molecule determines the occurrence of a purple colour, with an absorbtion band around 520nm. When DPPH reacts with a hydrogen donor, the DPPH reduced form is generated, accompanied by the disappearance of the violet colour. Therefore, the absorbance diminution depends linearly on the antioxidant concentration (Thaipong et al, 2006; Pisoschi, 2009).

Recently, Edge and Truscott (2018) review the most recent work on the interaction between singlet oxygen, free radicals, and carotenoids and retinoids. Recent research by the authors demonstrates that carotenoids can switch from antioxidant to pro-oxidant behavior as a function of oxygen concentration. Employing a cell-based model system, they observed total protection from exposure to high energy-radiation by lycopene at 0% oxygen, but zero protection at 100% oxygen. This may have implications for the behaviour of carotenoids in tissues where different partial pressures of oxygen are present. The physical organization of the carotenoid is an important consideration that affects its antioxidant abilities, through its interactions with reactive oxygen species themselves as well as with other antioxidants such as α -tocopherol and vitamin C. The antioxidant properties of the carotenoid astaxanthin are studied by Focsan *et al.* (2017). This pigment is bound to the white muscle of salmonids and is found in the pigment-protein complexes of the carapace of a number of crustacea. Astaxanthin also accumulates in the freshwater microalga *Haematococcus pluvialis* under stress conditions. Focsan and colleagues (2017) indicate that a range of factors influence the antioxidant activity of astaxanthin. These include: the formation of chelate complexes with metals; esterification and its inability to aggregate in the ester form; a high oxidation potential; and the formation of neutral radicals under high irradiation in the presence of metal ions.

Antimutagenic properties of carotenoid glycoside

The antimutagenic activity of the carotenoid glycoside was evaluated in experiments involving application of mutagen sodium azide to *S. typhimurium* TA98, which resulted in the formation of mutant colonies. In control plates, no incidence of mutant colonies was observed. Likewise, application of carotenoid extracts and carotenoid glycoside did not cause formation of mutant colonies of *S. typhimurium* TA98. On the other hand, application of sodium azide led to marked incidence of mutations. Sodium azide at a certain concentration induced irreversible mutant cells. The viability of the *S. typhimurium* TA98 cells was estimated by counting the total colony cell number affected by mutagen compared to that of the control plate.

In accordance with the *Salmonella typhimurium* TA98 strain genotype, the presence of colony in biotin-histidine medium and absence one in control biotin medium show that these strains are dependent to histidine. The results showed that carotenoid extract and carotenoid glycoside could inhibit mutagenic agents of sodium azide (Table 2, 3). Carotenoid extract and carotenoid glycoside with the inhibition of 71.6% and 97.1% sodium azide, respectively showed high potential in decreasing mutagenic agents. Negative controls and positive controls have been implemented in this test. The number of revertant colonies that grow on the negative control plate was 2.67 ± 1.15 , however, the mutant strain *S. typhimurium* TA98 was unable to synthesize histidine as amino acid needed for growth since the strain may undergo spontaneous mutations naturally without mutagen addition. Otherwise, onto positive control plate, the mutagen was added to induce the revertant colony, thus the number of revertant colonies on positive control plate (124.33 ± 7.77) was significantly increased (Table 2, 3). The presence of mutagen promotes irreversible mutation to bacterial cells and causes the formation of revertant colonies growing on the plates. This is because of the recovery of the *S. typhimurium* TA98

bacterial gene function and can produce histidine. A newly mutated bacterial cells can grow in the absence of histidine to form a revertant colony (Tejs, 2008; Mortelmans and Zeiger, 2000).

Table 2. Antimutagenesis activity of carotenoid extracts and carotenoid glycoside.

Samples	Revertant colony (CFU)
Positive control	124.33 ± 7.77 ^c
Negative control	2.67 ± 1.15 ^a
Carotenoid extract	35.33 ± 9.07 ^b
Carotenoid glycoside	3.67 ± 1.15 ^a
Comercial glycoside	1.33 ± 0.58 ^a

Table 3. Percentage of carotenoid extract and carotenoid glycoside inhibition against mutagen.

Samples	Inhibition (%)
Carotenoid extract	71.6
Carotenoid glycoside	97.1
Comercial glycoside	98.9

The percentage of inhibition of carotenoid glycoside (97.1%) was higher than carotenoid extract (71.6%). This is because carotenoid glycosides are more likely to exhibit the biological and pharmacological functions of carotenoid aglycone (Lu *et al.*, 2015). Therefore, carotenoid glycoside has shown higher antimutagenic effect rather than its carotenoid aglycone extracted from *P. rhodozyma*. The percentage of inhibitions for arbutin and carotenoid glycoside were not significantly different due to their slightly difference (1.8%). This means that both tested samples could exhibit the same potential antimutagenic activity. Arbutin was used as a standard since it is one of the types of commercial glycoside compound.

According to the results of assay on antimutagenic activity as shown in Figure 5, indicated that a certain concentration of carotenoids showed different antimutagenic capability. It shown that different source of carotenoid exhibited different inhibition level against antimutagen. Providing commercial asthaxantin, β-carotene and carotenoid extract at approximately 300ppm could induce antimutagenic activity of 41.1%, 58.0% and 42.4%, respectively. Increased in concentrations to 600ppm resulted in decreasing of antimutagenic activity of commercial astaxanthin and β-carotene to 28.4% and 37.2%, respectively, however could increase the antimutagenic activity of carotenoid extract to 45.4%. This might be occurred due to it instability of commercial carotenoid against oxidation and irradiation when they were exposed or influenced by light, oxygen, or chemical reaction, thus led to reducing in functional properties and less effective in inhibiting cell mutations.

In contrast, providing commercial asthaxantin, β-carotene and carotenoid extract at approximately 300ppm showed inhibition activity against mutagen were 58.9%, 42.0% and 57.6%, respectively. Increasing in concentrations to 600ppm resulted in increasing of inhibition activity of commercial astaxanthin and β-carotene against mutagen up to 71.6% and 62.8%,

respectively, however the inhibition activity of carotenoid extract against mutagen was slightly decreased to 54.6% (Figure 6).

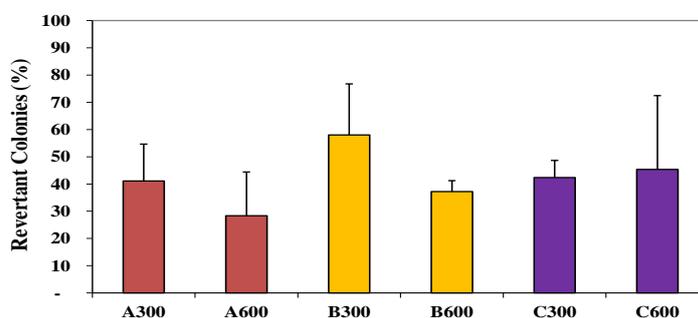


Figure 5. Antimutagenesis activity of different source of carotenoid.

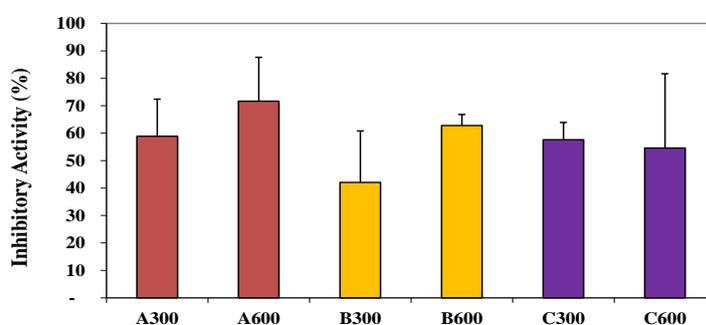


Figure 6. Percentage of different carotenoid source inhibition against mutagen.

Bhagavathy *et al.* (2011) investigated Antimutagenic assay of carotenoids from green algae *Chlorococcum humicola* using *Salmonella typhimurium* by Ames mutagenicity assay in the most sensitive *Salmonella* test strains. This test was performed in both the absence and the presence of a rat liver metabolizing system (S9 mix), providing a very sensitive study of potentially mutagenic pathways for the metabolism of these materials. It has been suggested that the use of antimutagen in daily life will be the most effective procedure for preventing human cancer and genetic disease (Pounikar and Dawande, 2010). These compounds interfere with mutagen metabolism or they may act as mutagen scavengers. They may also inhibit either the initiation or promotion phase of the carcinogenic process. It was reported that the potential activity of carotenoid extracts for its antimutagenicity using *S. typhimurium* assay was mainly based on the reversion of mutant cells by mutagenic agents. From the results it was found that the carotenoid extracts of green algae *C. humicola* could inhibit the revertant formation produced by direct acting mutagens such as sodium azide, daunomycin, phenylhydrazine and B(a)P.

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Physicochemical properties and oxidative stability of fish emulsion sausage incorporated with surimi and fish protein hydrolysate

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Abstract

This study aims to determine the physicochemical properties, and oxidative stability of shortfin scad (*Decapterus macrosoma*) emulsion sausage incorporated with surimi and silver catfish (*Pengasius*) protein hydrolysate. Fish emulsion sausage was prepared by different fish mince and surimi ratio; (A) 100 fish mince: 0 surimi; (B) 60 fish mince: 40 surimi; (C) 40 fish mince: 60 surimi; (D) 0 fish mince: 100 surimi with the addition of protein hydrolysate. The results obtained showed that the proximate composition of fish emulsion sausage were in the range of 67.85-71.82%, 14.09-21.61%, 2.25-3.89%, 1.26-2.53%, and 4.12-10.58% for moisture, protein, fat, ash and carbohydrate, respectively. The increase of surimi ratio was increased the lightness (L*) and decreased the redness (a*) of sausage significantly (p<0.05). The higher fish mince ratio in sausage caused an increased in texture profile and more emulsified microstructure with less void compared to the control. The use of fish protein hydrolysate could maintain the oxidative stability as it has antioxidant properties.

Keywords: Fish emulsion sausage; surimi; fish protein hydrolysate; physicochemical properties; oxidative stability.

Introduction

Emulsion sausage such as frankfurters widely consumed in both Western and Asian countries and it made from beef, pork or chicken with fat contents 25-30% (Panpipat and Yongsawatdigul, 2008). Recently, manufactures has begun to use fish mince incorporation with surimi as raw material in emulsion fish sausage, particularly in Asian countries (Panpipat and Yongsawatdigul, 2008). The purpose of adding surimi in sausage manufacturing because these products have desirable texture and cost effective.

Physicochemical properties measurement of sausage was important in determining consumer acceptance. According to Food Act 1985 (2015), sausage composition should contain <1.7% nitrogen and not >30% fat. Texture properties were changed by biochemical degradation because of storage time (Dincer and Cakli, 2015). Many studies have been conducted in incorporating of non-meat ingredients to enhance physicochemical and sensory properties then promote a healthier meat sausage such as vegetable (Syuhairah *et al.*, 2016) and fish protein hydrolysate (Zakaria and Sarbon, 2018). Shelf life of sausage product was determined by microbiological growth, changes in colour and texture, development of undesirable rancid

flavours caused by lipid oxidation, lipolysis and other reactions (Valencia *et al.*, 2007). The oxidative stability and shelf life of sausage products could be improved by incorporation of natural antioxidant and material containing low sources of fat content.

Fish protein hydrolysate (FPH) act as antioxidant, which function to against free radical and able to improve properties of product in term of physicochemical and functional properties such as fat absorption, water holding capacity and emulsifying properties without losing its nutritional value (Halim *et al.*, 2016). The addition of FPH was reported to improve the structure properties of fish sausage and act as preservative to increase shelf life (Ren *et al.*, 2011). Studies on incorporating fish protein hydrolysate in sausage such as Snakehead (*Channa striata*) (Zakaria and Sarbon, 2018) and Channel catfish (*Ictalurus punctatus*) (Ren *et al.*, 2011) were successfully conducted.

Shortfin scad was known as ‘Selayang’, ‘Curut’ and ‘Sardin’ in Malaysia. Shortfin scads was a type of fish that has good source of protein, vitamin and minerals which make it suitable to be used as a source of food product such as keropok lekor and fish emulsion sausage (Zakaria and Sarbon, 2018). Ishak and Sarbon (2017) reported that FPH from shortfin scad waste has high potential of bioactivity. Therefore, the aim of this study was to determine the physicochemical properties and oxidative stability of shortfin scad (*Decapterus macrosoma*) emulsion sausage incorporated with surimi and silver catfish (*Pengasius*) protein hydrolysate.

Materials and methods

Materials

Fresh Silver catfish (*Pengasius sp*) was obtained from local market in Kuala Terengganu while Shortfin scad (*Decapterus macrosama*) and surimi was bought from MAPEROW Sdn. Bhd., Kuala Terengganu. All samples were stored in iced condition during transportation to the laboratory. Other ingredients for making sausages were purchased from local hypermarket in Kuala Terengganu. All chemicals used were of analytical grade.

Methods

Sample preparation

Silver catfish (*Pengasius sp*) was decapitated, eviscerated and washed with ice water to avoid the fish become deteriorate. The bones and skin was removed then the flesh were stored at -40°C until further use. The flesh was grounded by using food processor.

Preparation of Silver Catfish protein hydrolysate

Silver catfish protein hydrolysate was prepared following Zakaria and Sarbon (2018). Fish flesh (55g) was suspended in distilled water and pre-incubated in water bath shaker at 85°C (20 min) for inactivation of endogenous enzyme in fish muscle prior to enzymatic hydrolysis.

The hydrolysis reaction was initiated by the addition of 20g Alcalase and the hydrolysis was taken at 50°C at pH 7.89 for 84 min. The mixture was then placed into water bath at 85°C (15 min) to terminate the enzymatic reaction before centrifuged at 6000 rpm (20 min) at 4°C. The supernatant of hydrolysate were then filtered and freeze dried.

Development of fish emulsion sausage

The frozen flesh of shortfin scad and surimi was thawed overnight in a chiller (4°C). Table 1 showed the different formulation of fish sausage from shortfin scad with surimi incorporation at different ratio which was formulation A (100 fish mince: 0 surimi), B (60 fish mince: 40 surimi), C (40 fish mince: 60 surimi) and D (0 fish mince: 100 surimi). The ingredients included vegetable oil, icy cold water, isolated soy protein, and corn starch were mixed and homogenized for 30 sec. Silver catfish protein hydrolysate was added with chilled water and further ground for 30. The mixture was placed into the sausage stuffer and pumped into cellulose casing. The casing was tied at 10-12cm of each sausages and were boiled for 25 min. The sausage samples were vacuum packed in polyethylene bag and were kept chilled at -4°C for further oxidative stability analysis while prepare fresh for proximate and microstructure analysis (Zakaria and Sarbon, 2018).

Table 1. Formulation of shortfin scad (*Decapterus macrosoma*) sausage incorporated with surimi and silver catfish (*Pengasius*) protein hydrolysate

Formulations	A	B	C	D
Ratio	100:0	60:40	40:60	0:100
SC mince: Surimi (%)	63:0	37:26	26:37	0:63
SCPH (%)	3	3	3	3
Vegetable oil (corn oil) (%)	7	7	7	7
Egg white powder (%)	3	3	3	3
Isolated soy protein (%)	2	2	2	2
Sugar (%)	0.5	0.5	0.5	0.5
Salt (%)	1.0	0.8	0.8	0.8
White pepper (%)	1.3	0.5	0.5	0.5
Garlic (%)	1.0	1.0	1.0	1.0
Corn flour (%)	5.0	5.0	5.0	5.0
Sodium tripolyphosphate (%)	0.5	0.5	0.5	0.5
Water + ice (%)	12.70	12.70	12.70	12.70
Total	100	100	100	100

Physicochemical properties

Proximate analysis

Determination of chemical composition (moisture, protein, fat and ash) of fish emulsion sausage was conducted following AOAC (2002) methods. Carbohydrate were determined by calculation from the remaining percentage of moisture, fats, protein, and ash as follows:

$$\text{Carbohydrate (\%)} = [100 - \text{moisture (\%)} - \text{crude protein (\%)} - \text{crude fat (\%)} - \text{ash (\%)}]$$

Physical Analysis

Texture profiles

Texture profile of emulsion fish sausage produced was determined using a texture analyser (Stable Micro Systems, Godalming, Surrey, UK) with load cells 50kg. Cylindrical aluminium probe with diameter 75mm was used. The sample were cut into cylindrical-shape (25 mm height x 20 mm diameter) and run into two compression cycles. The parameter such as hardness, cohesiveness, springiness, chewiness, gumminess and resilience were measured as the force that was required to compress the sample by a pre-set distance. The texture profile of sample at 4°C were analysed at 0, 6, 12 days (Intarasirisawat et al., 2014).

Colour Analysis

Colour analysis of fish emulsion sausage produced was determined following Ismail et al. (2014) using a colorimeter (Hunter Lab, Model colour Flex, Reston, VIRG, USA). The sample preparation was conducted by slicing the sausage into 5mm thick. The values were reported in the CIE colour profile system as lightness (L*), redness (a*) and yellowness (b*).

Scanning electron microscopy (SEM)

The microstructure of prepared fish sausage were analyzed by Scanning Electron Microscope (SEM) (JEOL JSM -6360LA, Tokyo, Japan) following Intarasirisawat et al. (2014). Fish sausage with a thickness of 2-3mm were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer in 2 to 4 hours. The dried sample were mounted on a bronze and sputter-coated with gold (JEOL JFC-1600, Tokyo, Japan). The specimen were observed with scanning electron microscope at an acceleration voltage of 5kv.

Determination of oxidative stability

Peroxide value

Determination of peroxide value was conducted following AOAC (2002). The peroxide value was calculated based on the following formula:

$$PV \text{ (mEq peroxide/kg fat)} = \frac{(S-B) \times M \times 1000}{\text{Sample weigh (g)}} \quad \text{thiosulphate}$$

Thiobarbituric acid reactive substances (TBARS)

Thiorbabituric acid reactive substance were conducted following Burge and Aust (1978) with slightly modification. TBARS were calculated from a standard curve of malondialdeyde (MDA) that was freshly prepared by the acidification of TEP (tetramethoxypropane) in the range of 2 to 10ppm and expressed as mg of MDA per kg of sample.

Statistical Analysis

All the measurement were conducted in triplicate for each sample. Statistical analysis was performed using one-way analysis of variance (ANOVA) to determine difference between the

mean of samples. The significant differences among the means were identified by using Fischer's Least Significant (LSD). Data analysis was performed using Minitab Statistical Software, version 18.0 (Minitab Inc).

Results and discussion

Proximate composition of shortfins scad emulsion sausage

The proximate composition of shortfin scad (*Decapterus macrosoma*) sausage incorporated with surimi and silver catfish (*Pengasius*) protein hydrolysate (SCPH) were shown in the Table 2. The moisture and carbohydrate of shortfin scad sausage were significantly increased ($p < 0.05$) with the increased ratio of surimi while protein, fat and ash were significantly decreased with the increased ratio of surimi in shortfin scad sausage. There was a significantly difference between all formulations in term of moisture, protein, fat, ash and carbohydrate ($p < 0.05$).

Table 2. Proximate Composition of shortfin scad (*Decapterus macrosoma*) emulsion sausage incorporated with surimi and silver catfish (*Pengasius*) protein hydrolysate

Formulation	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Carbohydrate (%)
A	67.85±0.04 ^d	21.61±0.41 ^a	3.89±0.03 ^a	2.53±0.12 ^a	4.12±0.41 ^a
B	68.40±0.05 ^c	18.06±0.24 ^b	3.24±0.52 ^{ab}	1.79±0.21 ^b	8.51±0.61 ^b
C	70.30±0.25 ^b	16.85±0.10 ^c	2.59±0.53 ^{bc}	1.33±0.11 ^b	8.93±0.60 ^b
D	71.82±0.06 ^a	14.09±0.8 ^d	2.25 ±0.58 ^c	1.26±0.58 ^b	10.58±0.84 ^c

*Mean ± SD (n=3).

**Different superscripts (a-d) in the same column indicate the significant differences ($p < 0.05$)

*** (A = 100 fish mince: 0 surimi) (B = 60 fish mince: 40 surimi) (C = 40 fish mince: 60 surimi) (D = 0 fish mince: 100 surimi).

Texture properties of fish sausage

The texture profile analysis of shortfin scad sausage incorporated with surimi and SCPH were shown in Table 3. The data showed that, formulation A has the highest hardness, gumminess and chewiness value at day 0, 6 and 12 compared to other formulations ($p < 0.05$). The decrease of hardness in fish emulsion sausage was due to higher moisture content of surimi incorporated. The increased of hardness in formulation A was likely due to water loss and increased of fat and protein content in fish emulsion sausage which contributed to increase of gumminess and chewiness the sausage (Andres, 2006). In addition, the functional properties of FPH such as emulsifying, binding, gelling properties and water holding capacity might stabilised the matrix of fish sausage and increased the hardness of sausage. SCPH has an emulsifying activity that could stabilised the matrix of fish emulsion sausage. In general, the incorporation of surimi in fish sausage significantly affect the hardness, gumminess and chewiness since the rest parameter was not significantly difference during storage ($p > 0.05$).

Table 3. Texture profile analysis of shortfin scad sausage incorporated with surimi and silver catfish protein hydrolysate.

Storage day	Formulations	Texture parameter					
		Hardiness (kg)	Springiness (cm)	Cohesiveness (ratio)	Gumminess (kg)	Chewiness (kg cm)	Resilience (ratio)
0	A	9.26±0.26 ^a	0.918±0.01 ^b	0.697±0.00 ^a	6.68±0.21 ^a	6.41±0.36 ^a	0.33±0.04 ^c
	B	7.60±0.34 ^b	0.923±0.00 ^{ab}	0.720±0.01 ^a	5.47±0.27 ^b	5.05±0.24 ^b	0.37±0.01 ^{ab}
	C	7.27±0.27 ^b	0.926±0.00 ^{ab}	0.729±0.00 ^a	5.65±0.75 ^b	4.74±0.22 ^b	0.38±0.01 ^a
	D	4.86±0.06 ^c	0.936±0.01 ^a	0.731±0.05 ^a	3.78±0.31 ^c	3.54±0.33 ^c	0.35±0.03 ^{bc}
6	A	8.11±0.38 ^a	0.927±0.00 ^b	0.735±0.00 ^a	5.97±0.25 ^a	5.53±0.23 ^a	0.36±0.01 ^b
	B	7.07±0.10 ^b	0.927±0.05 ^b	0.736±0.00 ^a	5.20±0.06 ^b	4.82±0.04 ^b	0.37±0.01 ^{ab}
	C	6.93±0.08 ^b	0.939±0.02 ^a	0.745±0.01 ^a	5.16±0.03 ^b	4.85±0.04 ^b	0.37±0.03 ^{ab}
	D	4.44±0.39 ^c	0.939±0.14 ^{ab}	0.724±0.01 ^a	3.22±0.31 ^c	3.01±0.25 ^c	0.40±0.01 ^a
12	A	7.78±0.44 ^a	0.934±0.00 ^a	0.810±0.03 ^a	6.03±0.42 ^a	5.63±0.43 ^a	0.44±0.02 ^a
	B	7.06±0.27 ^{ab}	0.946±0.00 ^a	0.733±0.00 ^b	5.49±0.24 ^a	5.31±0.32 ^a	0.44±0.01 ^a
	C	6.12±0.74 ^b	0.936±0.00 ^a	0.748±0.00 ^b	3.14±0.44 ^b	2.89±0.43 ^b	0.44±0.01 ^a
	D	3.91±0.51 ^c	0.836±0.19 ^a	0.809±0.05 ^a	3.397±0.20 ^b	2.04±0.21 ^c	0.46±0.06 ^a

Values are mean±SD (n=3). Different superscripts (^{a-c}) within the same storage time in the same column denote the significant differences (p<0.05). *(A = 100 fish mince: 0 surimi) (B = 60 fish mince: 40 surimi) (C = 40 fish mince: 60 surimi) (D = 0 fish mince: 100 surimi).

Colour properties of fish sausage

The lightness (L*) value of fish sausage incorporated with surimi and SCPH were significantly increased as storage time increased (p>0.05). Meanwhile, the redness (a*) value of fish sausage incorporated with surimi and SCPH were significantly decreased as storage time increased (Table 4). The highest lightness value (L*) of shortfin scad emulsion sausage incorporated with surimi and SCPH in Formulation D may due to the color properties of surimi and the elimination of heme pigments by washing process which increased the lightness of surimi (Ismail et al., 2014). In addition, the decreased in redness (a*) of shortfin scad sausage with the increased ratio of fish mince to surimi was due to the myoglobin content in fish mince since it contribute to 80% of meat color (Huda et al., 2012). Meanwhile during storage, the processes of lipid oxidation that occur contribute to the additional factor that changes the colour, aroma, taste and nutritional value.

Table 4. Color Analysis of shortfin scad (*Decapterus macrosoma*) sausage incorporated with surimi and silver catfish (*Pengasius*) protein hydrolysate

Storage time (days)	Formulations	Color Analysis		
		L*	a*	b*
0	A	60.95±0.38 ^d	3.763±0.17 ^a	13.86±0.29 ^a
	B	67.15±0.45 ^c	2.413±0.11 ^b	13.55±0.35 ^a
	C	70.85±0.58 ^b	1.790±0.11 ^c	13.59±0.10 ^a
	D	79.67±0.18 ^a	0.25±0.036 ^d	13.80±0.63 ^a
6	A	61.64±0.78 ^d	2.74±0.12 ^a	13.66±0.65 ^a
	B	66.11±0.79 ^c	2.07±0.13 ^b	14.52±0.24 ^{ab}
	C	70.41±0.08 ^b	1.60±0.04 ^c	14.36±0.46 ^b
	D	79.21±1.66 ^a	0.59±0.07 ^d	12.86±0.08 ^c
12	A	59.76±0.87 ^d	2.02±0.12 ^a	14.23±0.48 ^a
	B	66.44±1.29 ^c	1.62±0.074 ^b	14.45±0.25 ^a
	C	71.28±0.25 ^b	1.42±0.090 ^c	14.71±0.22 ^a
	D	78.91±0.23 ^a	0.46±0.037 ^d	14.67±0.24 ^a

*Values are mean±SD (n=3). Different superscript (^{a-c}) within the same column in the same storage indicate the significant differences (p<0.05).

** (A = 100 fish mince: 0 surimi) (B = 60 fish mince: 40 surimi) (C = 40 fish mince : 60 surimi) (D = 0 fish mince : 100 surimi).

*** (L*) lightness, (a*) redness, (b*) yellowness.

Microstructure of fish sausage

Microstructure of fish emulsion sausage developed were shown in Figure 1. The microstructure of Formulation A contained more voids compare to other formulation. As the surimi content in fish emulsion sausage increased, it enhance the structure of fish emulsion sausage with the evidence of less voids presence on the surface of fish emulsion sausage. The voids disappears as the surimi ratio increase in fish emulsion sausage. Formulation B, C and D showed less dense matrix compared to control that explained why this formulation was softer and easier to eat than control. The microstructure also influenced by the composition of moisture content in all formulations. Furthermore the incorporation of protein hydrolysate in fish emulsion sausage contribute to emulsify, binding and gelling properties which could formed interfacial film around fat globules (Intarasirisawat *et al.*, 2014).

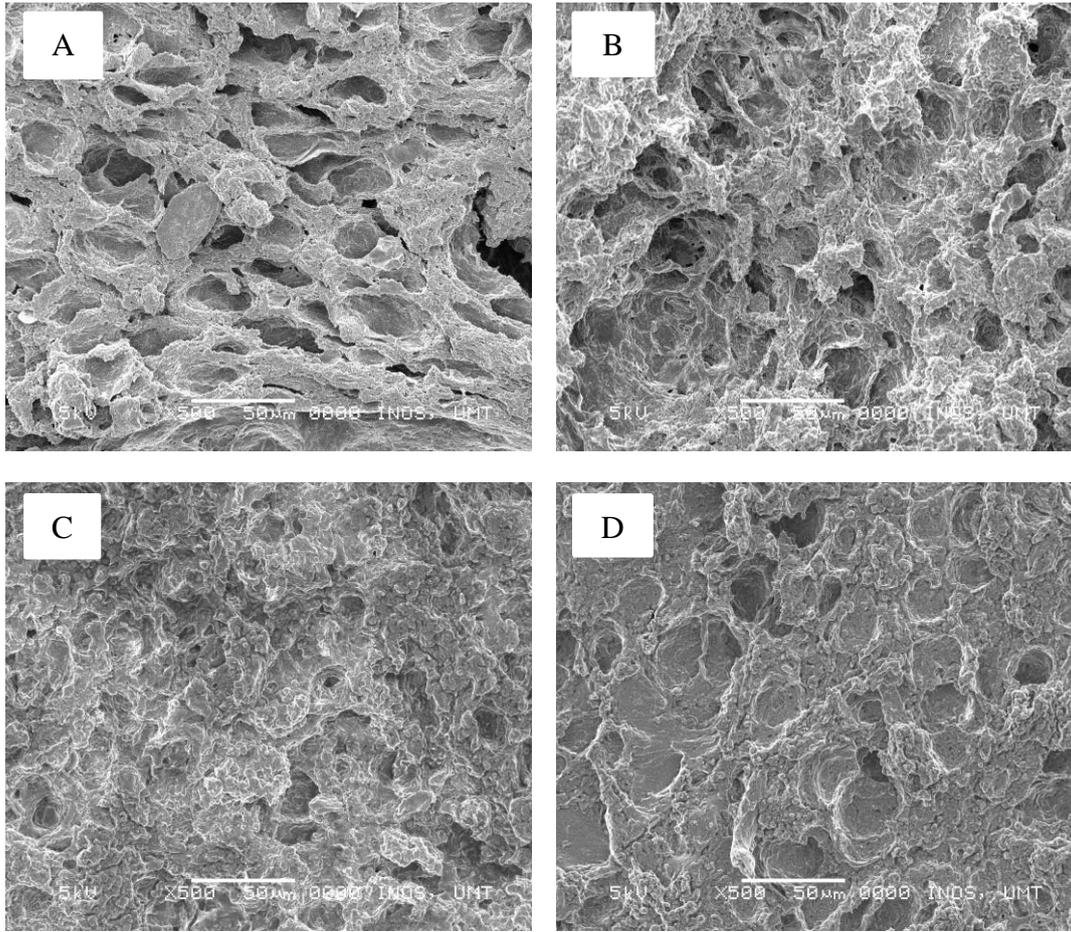


Figure 1. Scanning Electron Micrograph of shortfin scad sausage incorporated with surimi and silver catfish protein hydrolysate:

* (A) control (without surimi) 100 fish mince: 0 surimi (B) 60 fish mince: 40 surimi (C) 40 fish mince: 60 surimi (D) 0 fish mince: 100 surimi.

Lipid oxidation of shortfin scad sausage incorporated with surimi and silver catfish protein hydrolysate

Peroxide value of fish sausage

Table 5 showed the peroxide value for shortfin scad sausage incorporated with surimi and SCPH during 12 days of refrigerated storage (4°C). The initial value of PV at 0 days was significantly decreased when the ratio of surimi increased in shortfin scad emulsion sausage. The sharp increase was noticeable in all samples up to 6 days during the refrigerated storage for all formulations except formulation D that significantly increased until day 9 then decreased until 12 days of storage. Peroxide value (PV) value were significantly difference for all formulations during 12 days of storage ($p < 0.05$).

Table 5. The peroxide value of for shortfin scad sausage incorporated with surimi and silver catfish protein hydrolysate

Samples	Day 0	Day 3	Day 6	Day 9	Day 12
mEq lipid peroxide / kg of sample					
A	1.86±0.64 ^a	3.08±0.57 ^a	5.44±0.79 ^a	5.37±1.0 ^a	4.51±0.30 ^a
B	1.66±0.41 ^a	3.19±0.35 ^a	4.30±0.30 ^{ab}	4.04±0.40 ^b	4.05±0.48 ^a
C	0.47±0.12 ^b	2.38±0.21 ^b	3.83±0.31 ^b	3.66±0.43 ^b	2.52±0.69 ^b
D	0.28±0.10 ^b	1.05±0.12 ^c	2.59±0.92 ^c	3.52±0.30 ^b	2.49±0.31 ^b

*Values are mean±SD (n=3). Different superscript (^{a-c}) within the same column in the same storage indicate the significant differences (p<0.05).

** (A = 100 fish mince: 0 surimi) (B = 60 fish mince: 40 surimi) (C = 40 fish mince: 60 surimi) (D = 0 fish mince: 100 surimi).

The peroxide value obtained in this study was lowered than the range of noticeable rancidity which was 20-40 mEq/kg (Pearson, 1976). During the 12 days storage, formulations A showed the highest peroxide value because of higher fat content in formulation A compared to other formulations. Formulations D showed the lowest PV value due to the higher surimi ratio which has the lowest fat content because of washing process. This also due to the SCPH that incorporated in the shortfin scad emulsion sausage. Then, the decreased in PV values during 12 days of storage was due to the decomposition of hydroperoxides, which produce variety of decomposition products including aldehydes and ketones (Chaijan *et al.*, 2006).

TBARS value of fish sausage

Table 6 showed the result of thiorbabituric acid of shortfin scad sausage incorporated with surimi and SCPH. There were significantly increased in TBA value at day 0 until day 9 for sample A and D then it was significantly decrease until day 12. The result showed that TBA value during storage were significantly difference during 0, 6 and 12 days of storage (p<0.05). The TBARS value obtained in this study was lower than the range of rancidity (0.5 to 2.0 mg MDA/kg) (Gray and Pearson, 1987). The increased in TBA value for formulation A compared to other formulation was due to high fat level in fish mince compared to surimi. The higher amount of fat and unsaturated fatty acids cause the higher TBA value (Tang *et al.*, 2001). TBARS values of most of the samples increased up to day 9 of storage followed by a decreased until the end of storage period. The small increased in TBA value during 12 days of storage was due to the storage condition.

Table 6. The 2-thiobarbituric acid (TBA) of for shortfin scad sausage incorporated with surimi and silver catfish protein hydrolysate

Formulations	Day 0	Day 3	Day 6	Day 9	Day 12
(mg malonaldehyde/kg sample) x 10⁻³					
A	8.2±0.92 ^a	10.6±0.14 ^a	10.5±0.16 ^a	11.7±0.29 ^a	8.75±0.08 ^a
B	6.98±0.17 ^b	6.27±0.30 ^c	6.23±0.30 ^c	8.47±0.74 ^b	8.68±0.05 ^a
C	6.02±0.05 ^b	7.56±0.47 ^b	7.56±0.47 ^b	5.51±1.32 ^c	3.87±0.27 ^b
D	1.52±0.56 ^c	1.25±0.05 ^d	1.25±0.05 ^d	2.10±1.43 ^d	1.84±1.64 ^c

*Values are mean±SD (n=3). Different superscript (^{a-d}) within the same column in the same storage indicate the significant differences (p<0.05).

** (A = 100 fish mince: 0 surimi) (B = 60 fish mince: 40 surimi) (C = 40 fish mince : 60 surimi) (D = 0 fish mince : 100 surimi).

Conclusions

In conclusion, shortfin scad emulsion sausage incorporated with surimi and SCPH was successfully produced and investigated. The physicochemical properties was affected with the incorporation of surimi in shortfin scad emulsion sausage with fish protein hydrolysate. The higher ratio of shortfin scad mince in fish sausage contribute to greater hardness, gumminess and hardness. Meanwhile, high shortfin scad ratio compared to surimi reduce the lightness value (L^*), while increased in redness (a^*). The greater hardness of fish emulsion sausage contributed to compact structure and greater voids formation which in agreement with composition of fat content. Meanwhile, the increase addition of surimi in shortfin scad emulsion sausage lowering the lipid oxidation with evidence of reduction of PV and TBARS value as it below the range of rancidity during 12 days of storage. Formulation B with 60 fish mince and 40 surimi ratio was the most acceptable compared to control and other formulations.

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Biomass and Flavonoid Production in Cell Suspension System of *Ficus deltoidea* var. *kunstleri*

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Abstract

Ficus deltoidea, which is widely known as Mas Cotek among the Malay community is being used traditionally to treat diabetes and cardiovascular diseases. Nevertheless, this medicinal plant has gained attention lately due to its ability to cure chronic diseases without causing side effects. Therefore, in order to propagate the plant in a short time and in large quantity, plant tissue culture was introduced. The callus culture was induced using MSB5 basal media supplemented with 30 g/L sucrose, 2.75 g/L gelrite and pH adjusted to 5.75. In addition, different types of auxins were also used for the callus growth. Based on the results produced on callus initiation, picloram was the fastest to produce callus in 27 days with the fresh and dry weight of 1.066 and 0.056 g/25 mL of media, respectively. Different concentration of picloram was tested and found that 2 mg/L of picloram produced the highest fresh and dry weight with 1.721 and 0.076 g/25 mL of media after four weeks incubation. In addition, picloram was also integrated with different types of cytokinins to produce more callus. Kinetin at the concentration of 1 mg/L was found to be the best compared to the others cytokinin by producing the highest fresh and dry weight of callus. Then, the callus was subcultured and transferred to the cell suspension culture for flavonoid production. The results showed a bell-shaped curve with the highest production of flavonoid on day 12 with 2.1 mg RE/g DW. In conclusion, it is proven that bioactive compounds can be produced from plant tissue cultures. Hence, enabling medicinal plants from the nature to be conserved for the next generation.

Keywords: *Ficus deltoidea*; callus culture; cell suspension; biomass; flavonoid

Introduction

Ficus deltoidea which is commonly called as Mas Cotek among the Malay community in Malaysia is an evergreen shrub used traditionally to treat diabetes and cardiovascular diseases (Hakiman and Maziah, 2009). This plant belongs to the Moraceae family which can grow up to seven meters with broad spoon-shaped to ovate-shape leaves (Corner, 1969). The term Mas Cotek was coined as Mas means gold, while Cotek means dot, as there are gold dots on the upper surface of the plants' leaves (Hakiman et al., 2012). Moreover, the extract of *F. deltoidea* has been discovered to possess the potential to lower blood sugar level, blood pressure, cholesterol, and also reduce the risk of cancer (Aris et al., 2009).

Production of bioactive compounds from medicinal plants has now become acclaimed in pharmaceutical industries. However, the downside to its fame is the excessive harvesting of the plant to support the consumer demands which causes its presence in the nature to become limited (Bartarya et al., 2009; Aoyagi, 2011). In order to overcome this problem, biotechnological approaches can be applied to produce bioactive compounds in large scales through plant tissue culture technique (Fulzele, 2005). Recently, callus culture gained popularity due to its novelty by culturing cells as independent selection units rather than the whole plant (Mahmood et al., 2012). The cultured callus was then used for bioactive compounds production in cell suspension culture. Plant cell suspension culture is able to produce many types of bioactive compounds in short time and large quantity (Shinde et al., 2009). This study was carried out to determine the best plant growth regulators and its concentration on biomass and flavonoid production of *F. deltoidea* var. *kunstleri*.

Materials and Methods

2.1 Preparation of basal media and plant material

Induction media for callus initiation was prepared using macro- and micronutrients formulation by Murashige and Skoog (1962) and vitamins by Gamborg et al. (1968) with the basal media labeled as MSB5. The basal media was supplemented with 30 g/L sucrose, 2.75 g/L gelrite and the pH was adjusted to 5.75 prior to autoclave. The 6-month-old leaf explants of *F. deltoidea* underwent surface sterilization using 30% Clorox with two drops of Tween 20 and were shaken for 20 min. Aseptic leaf explants were then excised into 0.5 cm x 0.5 cm cubes and were cultured onto the media. All cultures were incubated at a temperature of 25 ± 2 °C under a photoperiod of 16 hrs light and 8 hrs dark with 2000 lux irradiation.

2.2 Initiation of callus from leaf explants

Different auxins consisting of picloram, dicamba, 2,4-Dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) were used to facilitate the process of callus initiation. Each auxin at a concentration of 1 mg/L was supplemented in MSB5 basal media to study the callus initiation rate of each auxin. The data on days to form callus, percentage of explants forming callus, callus morphology, fresh and dry weight were recorded after four weeks of incubation.

2.3 Optimum concentration of picloram

Based on experiment at subsection 2.2, picloram was chosen because it produced callus at a faster rate compared to the other auxins. Thus, the optimum concentration of picloram needed was studied. The concentrations of picloram in this study ranged from 0 to 3.0 mg/L with 0.5 mg/L interval in MSB5 basal media. Data on fresh and dry weight of callus were recorded after four weeks of incubation for cultures in picloram.

2.4 Integration of picloram and cytokinins

Based on experiment at subsection 2.3, 2 mg/L picloram was found to be the best concentration for callus initiation. Hence, 2 mg/L of picloram was selected to proliferate the callus with the

integration of cytokinins: kinetin, 6-benzylaminopurine (BAP), thidiazuron (TDZ) and zeatin. One milligram per liter of each cytokinin was supplemented in the basal media and callus was incubated for four weeks and the data on fresh and dry weight were recorded.

2.5 Integration of picloram and different concentration of kinetin

MSB5 media supplemented with 2 mg/L picloram and 1 mg/L kinetin were found to be the best for callus production based on the findings from a previous study in subsection 2.4. Hence, kinetin was chosen to be integrated with 2 mg/L picloram. Different concentrations of kinetin ranging from 0 to 3.0 mg/L with 0.5 mg/L interval were used. Data was collected after four weeks of incubation culture.

2.6 Callus growth curve

Callus growth curve was plotted out to determine the growth pattern of the callus. The callus was subcultured in MSB5 basal media supplemented with 2 mg/L picloram, 1 mg/L kinetin, 30 g/L of sucrose and 2.75 g/L of gelrite at pH 5.75. The cultures were harvested in three vessels every week until week six or until the growth curve displayed a sigmoid pattern. During every harvest, the callus were carefully taken out and placed in an oven at a temperature of 50 °C and were left to dry for a minimum of two days or until the weight was constant. The dry weight of the callus was then measured to plot the graph.

2.7 Establishment of cell suspension culture

For the establishment of cell suspension culture, the 3-week-old callus with 2 ± 0.2 g weight was used to initiate the cell suspension culture. The cell suspension culture used a similar formulation of MSB5 basal media supplemented with 2 mg/L of picloram, 1 mg/L of kinetin and 30 g/L of sucrose with pH 5.75 without the addition of gelrite. The cultures were incubated at 25 ± 2 °C with continuous agitation at 120 rpm in an orbital shaker for two weeks.

2.7.1 Cell suspension culture growth curve

The growth curve of cell suspension was obtained by randomly harvesting three culture vessels once every four days. The cell suspension was filtered using filter paper where the harvested cells were placed in the oven at 50 °C for two days or until the weight measured was constant and the growth curve was plotted.

2.7.2 Production of flavonoid in cell suspension culture

The flavonoid production was determined using the dried cell suspension obtained in the experiment at subsection 2.7.1.

2.7.2.1 Preparation of extract

The extraction of total flavonoid was carried out according to Marinova et al. (2005) with minor modifications. A total of 0.5 g of cell suspension was homogenized using pestle and mortar with distilled water at room temperature. Then, the crushed cells were transferred into covered flasks and centrifuged at 14000 rpm for 5 min. The supernatant was used for flavonoid quantification.

2.7.2.2 Total flavonoid content

The flavonoid content was determined based on the method explained by Marinova et al. (2005) using Aluminium Chloride Colorimetric Method. Briefly, 1 mL of extract with 4 mL of distilled water and 0.3 mL of 5% sodium nitrite were added into a test tube. Followed by the addition of 0.3 mL of 10% aluminium chloride at the fifth minute and 2 mL of 1 M sodium hydroxide at the sixth minute. Finally, the volume was made up to 10 mL with the addition of 2.4 mL of distilled water. The mixture was then mixed thoroughly and the absorbance was measured at 510 nm. The flavonoid was expressed as mg rutin equivalent (RE)/g samples.

2.8 Statistical analysis

All the data were analyzed by using Analysis of Variance (ANOVA) to compare the significant difference between the treatments by using Statistical Analysis Software version 1.4 (SAS). The analysis of mean was performed by using Least Significant Difference (LSD) with $P < 0.05$.

Results and Discussion

3.1 Initiation of callus from leaf explants

Callus initiation of *F. deltoidea* var. *kunstleri* using 1 mg/L of different auxins are tabulated in Table 1. Based on Table 1, 1 mg/L of picloram exhibited the shortest time to form callus in 27 days. Meanwhile, no callus induction was observed from the MSB5 basal media supplemented with 1 mg/L of IBA, IAA and control treatment. This was expected because auxins such as IBA and IAA are primarily used for root formation instead of callus induction. In addition, picloram produced the highest callus score with callus forming on three or more edges of the leaf section. The percentage of explants forming callus was also the highest for picloram with 80% of the produced callus were yellowish and friable. Therefore, picloram was determined as the best auxin to induce faster response rate and callus morphology. In another study that was conducted by Kiong et al. (2007), the MS media that was supplemented with 3 mg/L picloram produced the highest degree of callus formation in 10 days compared to IBA and 2,4-D treatments. That finding was in agreement with this study as picloram is the best auxin in callus induction of *F. deltoidea* var. *kunstleri*.

Table 1. Effects of Different Auxins at a Concentration of 1 mg/mL on Callus Initiation of *F. deltoidea* var. *kunstleri*.

Auxins (1 mg/L)	Days to form callus	Callus score	Percentage explants forming callus (%)	Callus morphology
MSO	na	na	na	na
2,4-D	41 ± 5	++	70	Whitish, friable
Picloram	27 ± 3	+++	80	Yellowish, friable
Dicamba	29 ± 3	++	70	Whitish, friable
IBA	na	na	na	na
IAA	na	na	na	na
NAA	33 ± 4	+	50	Whitish, friable

Legend; na: Data not available, +: One edge of leaf section forming callus, ++: Two edges of leaf section forming callus and +++: Three or more edges of leaf section forming callus.

3.2 Callus growth

In order to find the most suitable auxin for *F. deltoidea* callus initiation, six different auxins were used at concentration of 1 mg/L. Based on the result in Table 1, leaf derived callus which was subcultured three times in 1 mg/L picloram to produce more callus. The callus was then separated from the leaf and transferred again into a media with 1 mg/L of different auxin to obtain fresh and dry weight of the cell. Referring to Table 2, the highest fresh and dry weight were significantly produced by the treatment of 1 mg/L 2,4-D with 1.922 g/25 mL of media and dicamba with 0.092 g/25 mL of media. Although 1 mg/L picloram exhibited highest percentage of callus formation (80%) in just 27 days, it yielded lower fresh and dry weight compared to 2,4-D and dicamba. However, picloram was chosen because it formed better morphological characteristics of callus for *F. deltoidea* var. *kunstleri* compared to the other treatments. A previous study on leaf-derived callus of *F. deltoidea* using 2,4-D, NAA, IBA and picloram at concentrations of 1, 3 and 5 mg/L demonstrated that 3 mg/L of picloram produced the highest callus induction rate with 45.5% (Kiong et al., 2007).

Table 2. Effects of Different Auxins at a Concentration of 1 mg/L on Callus Biomass of *F. deltoidea* var. *kunstleri*.

Auxins (1 mg/L)	Callus fresh weight of 1 culture vessel (g/25 mL of media)	Callus dry weight of 1 culture vessel (g/25 mL of media)
MSO (Control)	0.671 ± 0.013 ^c	0.048 ± 0.005 ^c
2,4-D	1.922 ± 0.051 ^a	0.066 ± 0.006 ^b
Picloram	1.066 ± 0.032 ^c	0.056 ± 0.005 ^{bc}
Dicamba	1.879 ± 0.046 ^a	0.092 ± 0.007 ^a
IBA	1.191 ± 0.036 ^b	0.068 ± 0.006 ^b
IAA	0.827 ± 0.027 ^d	0.048 ± 0.004 ^c
NAA	0.746 ± 0.021 ^e	0.051 ± 0.004 ^c

^{abc} Means value (n=3) ± standard deviation of the mean. Means on a same column followed by the same letter were not significantly different at P<0.05. Differences between means were separated using the LSD test.

3.3 Optimum concentration of picloram

Table 3 demonstrates the effects of picloram at different concentration in order to find the optimum concentration of picloram for callus production. The fresh and dry weight of callus increased gradually from control treatment to 2.0 mg/L of picloram before dropping in weight. The results shown there were significantly differences on the fresh and dry weight of the callus as affected by different rates of picloram. The highest fresh and dry weight was recorded at the 2 mg/L picloram with 1.721 and 0.076 g/25 mL of media, respectively. Hence, 2 mg/L of picloram was chosen for the subsequent experiment as it produced the highest fresh and dry weight of callus.

Table 3. Effects of Picloram at Different Concentrations on Callus Biomass of *F. deltoidea* var. *kunstleri*.

Concentration of picloram (mg/L)	Callus fresh weight of 1 culture vessel (g/25 mL of media)	Callus dry weight of 1 culture vessel (g/25 mL of media)
MSO (Control)	0.671 ± 0.013 ^e	0.048 ± 0.005 ^c
0.5	1.076 ± 0.033 ^d	0.055 ± 0.005 ^{bc}
1.0	1.066 ± 0.032 ^d	0.056 ± 0.005 ^{bc}
1.5	1.207 ± 0.041 ^c	0.057 ± 0.005 ^{bc}
2.0	1.721 ± 0.060 ^a	0.076 ± 0.006 ^a
2.5	1.337 ± 0.047 ^b	0.064 ± 0.005 ^b
3.0	1.384 ± 0.049 ^b	0.062 ± 0.006 ^b

^{abc} Means value (n=3) ± standard deviation of the mean. Means on a same column followed by the same letter were not significantly different at P<0.05. Differences between means were separated using the LSD test.

3.4 Integration of picloram and cytokinins

An integration of auxins and cytokinins is required to maintain the callus in optimum condition. Hence, in this study the callus was cultured onto the MSB5 basal media supplemented with 2.0 mg/L of picloram and 1 mg/L of different cytokinins. Based on Table 4, the fresh and dry weight of the callus was significantly affected by different types of cytokinin. MSB5 basal media supplemented with 2 mg/L of picloram and 1 mg/L of kinetin significantly produced the highest fresh and dry weight of callus, 2.456 and 0.110 g/25 mL of media. Meanwhile, control treatment (MSO) yielded the lowest callus weight mass. In an integration of auxins and cytokinin, the auxins will stimulate elongation and division of callus cell while cytokinins induce cell division for shoot regeneration.

Table 4. Effects of Integration between 2 mg/L Picloram and 1 mg/L Cytokinins on Callus Biomass of *F. deltoidea* var. *kunstleri*.

2 mg/L picloram + 1 mg/L cytokinin	Callus fresh weight of 1 culture vessel (g/25 mL of media)	Callus dry weight of 1 culture vessel (g/25 mL of media)
MSO (Control)	0.671 ± 0.013 ^d	0.048 ± 0.005 ^d
BAP	2.062 ± 0.078 ^b	0.096 ± 0.008 ^{ab}
Kinetin	2.456 ± 0.083 ^a	0.110 ± 0.009 ^a
TDZ	2.012 ± 0.096 ^{bc}	0.088 ± 0.007 ^{bc}
Zeatin	1.865 ± 0.070 ^c	0.081 ± 0.007 ^c

^{abc} Means value (n=3) ± standard deviation of the mean. Means on a same column followed by the same letter were not significantly different at P<0.05. Differences between means were separated using the LSD test.

3.5 Integration of picloram and different concentration of kinetin

Different concentrations of kinetin were also integrated with 2 mg/L picloram to study its effectivity against callus growth of *F. deltoidea* in this study. Following the findings from Table 4, 2 mg/L of picloram and 1 mg/L of kinetin produced the highest biomass. Thus, additional experiments were conducted to determine the optimum concentration of kinetin to produce higher callus biomass. From the results (Table 5), different concentrations of kinetin were significantly affected the fresh and weight of the callus produced. The highest biomass was produced by 2 mg/L of picloram with 1 mg/L of kinetin with 2.456 and 0.110 g/25 mL of media for fresh and dry weight, respectively. Meanwhile, the lowest biomass was produced in control treatment with 0.671 and 0.048 g/25 mL of media for fresh and dry weight. Concentrations of kinetin other than 1.0 mg/L did not produce high biomass. A previous study on callus culture of *Paspalum scrobiculatum* found that 1 mg/L of picloram and 1 mg/L of kinetin produced the highest fresh weight of callus (585 mg/culture vessel) compared to their other treatments (Kaur and Kothari, 2004)

Table 5. Effects of Integration between 2 mg/L Picloram with Kinetin at Different Concentrations on Callus Biomass of *F. deltoidea* var. *kunstleri*.

2 mg/L picloram + different concentration of kinetin (mg/L)	Callus fresh weight of 1 culture vessel (g/25 mL of media)	Callus dry weight of 1 culture vessel (g/25 mL of media)
MSO (Control)	0.671 ± 0.013 ^g	0.048 ± 0.005 ^e
0	1.721 ± 0.060 ^e	0.076 ± 0.006 ^{cd}
0.5	1.965 ± 0.075 ^{cd}	0.088 ± 0.007 ^{bc}
1.0	2.456 ± 0.083 ^a	0.110 ± 0.009 ^a
1.5	2.280 ± 0.076 ^b	0.093 ± 0.007 ^b
2.0	2.043 ± 0.065 ^c	0.090 ± 0.007 ^{bc}
2.5	1.850 ± 0.063 ^d	0.078 ± 0.006 ^{cd}
3.0	1.568 ± 0.055 ^f	0.073 ± 0.005 ^d

^{abc} Means value (n=3) ± standard deviation of the mean. Means on a same column followed by the same letter were not significantly different at P<0.05. Differences between means were separated using the LSD test.

3.6 Callus growth curve

According to Table 5, MSB5 basal media supplemented with 2 mg/L picloram and 1 mg/L kinetin produced the highest biomass of callus. Thus, it was chosen to maintain and subculture the callus in order to obtain a large amount of biomass. The callus dry weight was measured every week to plot the growth curves and to find the best period to harvest the callus. Based on Figure 1, a lag phase for callus growth was observed from week 0 to week 1, followed by an exponential phase from week 1 to week 3. The dry weight of callus produced in week 1 was 0.085 g/25 mL of media. At week 3, the dry weight increased by two-fold to 0.250 g/25 mL of media, indicating that the callus underwent differentiation exponentially during that incubation period. At week 4, the callus was in stationary phase with 0.290 g/25 mL of media, while, from week 5 onwards, the callus reduced in biomass indicating a death phase. Therefore, week 3 was chosen as the suitable incubation period to produce large amounts of callus because the callus undergoes active differentiation during this period and is observed as an optimal condition to subculture.

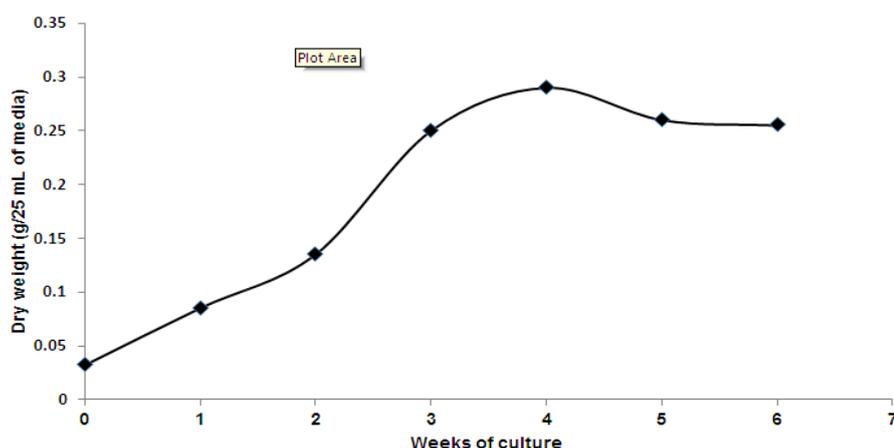


Figure 1. Callus Growth Curve of *F. deltoidea* var. *kunstleri*.

3.7 Cell suspension culture growth curve

The establishment of cell suspension culture of *F. deltoidea* var. *kunstleri* was initiated using subculture 0.5 g of 3-week-old callus into MSB5 basal media supplemented with 2 mg/L picloram and 1 mg/L kinetin without the addition of gelrite as a gelling agent with the aid of constant agitation on an orbital shaker. Theoretically, it helps to break the callus clumps to produce single cells. According to Figure 2, the cell suspension culture was in lag phase from day 0 to day 4. At day 4, the dry weight of the cell suspension was estimated at 0.056 g/25 mL of media. Following that, the cell suspension was in an exponential phase from day 4 to day 12. During this stage, the cell suspension actively divided and differentiated to produce more cells. The dry weight of cell suspension increased from 0.071 to 0.120 g/25 mL of media for day 8 and day 12, respectively. The cell suspension was at its highest and reached the stationary phase with 0.160 g/25 mL of media. Then, the dry mass of cell suspension gradually decreased from 0.140 to 0.09 g/25 mL of media from day 20 onwards. This phenomenon indicated that the cell suspension had undergone death phase. In previous study by Abdullah et al. (2013) on

F. deltoidea var. *trennganuensis* revealed that cell suspension produced the highest dry cell weight at day 15 (10.27 mg/mL) prior to the drastic drop in the dry cell weight which indicated cell death phase. The growth curve produced by Abdullah et al. (2013) was almost the similar to the findings in this study which recorded the maximum dry cell weight on day 16.

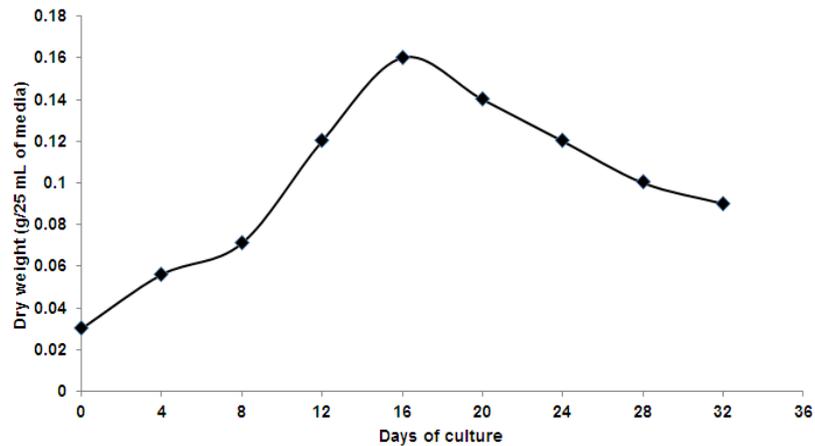


Figure 2. Cell Suspension Growth Curve of *F. deltoidea* var. *kunstleri*.

3.8 Flavonoid production of Cell suspension culture

Upon measuring the dry weight of the cell suspension, the dried cell suspension was used to profile the flavonoid production (Figure 3). The flavonoid production exhibited a bell-shaped curve with the highest production observed on day 12 with 2.1 mg RE/g DW. The flavonoid production of cell suspension was not proportional with the biomass produced. This is because the highest biomass was produced on day 16 while the highest flavonoid was produced on day 12. The increment in flavonoid production can be observed from day 4, 8 and the peaked on day 12 with 1.48, 1.64 and 2.1 mg RE/g DW, respectively. However, the production of flavonoid decreased from day 12 to day 16 by 0.1 mg RE/g DW. This was indicative of cell death phase mainly due to nutrient depletion. The death phase became more prominent from day 20 until day 32. Besides, the flavonoid production was recorded at 1.8, 1.4, 1.3 and 1.1 mg RE/g DW on day 20, 24, 28 and 32, respectively. Furthermore, Ong et al. (2011) discovered 3 flavonoid compounds in *F. deltoidea* extract which were rutin, quercetin and naringenin. The compounds were quantified using HPLC analysis.

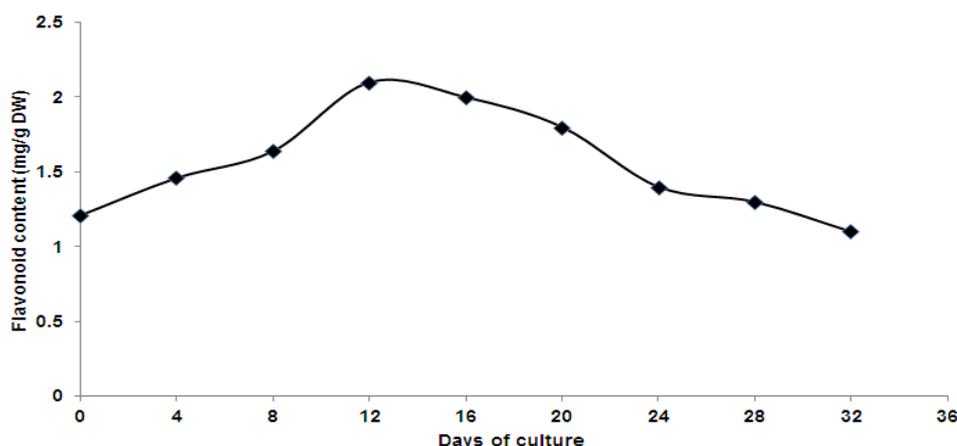


Figure 3. Flavonoid Production of Cell Suspension of *F. deltoidea* var. *kunstleri*.

Conclusion

Callus and cell suspension cultures of *F. deltoidea* var. *kunstleri* were successfully established. Different plant growth regulators and concentrations were experimentally undertaken to obtain the suitable plant growth regulators for the mass propagation of *F. deltoidea* var. *kunstleri*. Based on the findings in this study, MSB5 basal media supplemented with 2 mg/L picloram and 1 mg/L kinetin was found to be the most suitable media for the propagation and flavonoid production of *F. deltoidea* var. *kunstleri*. This finding may fulfill the demand for this plant in the pharmaceutical industries. Hence, the wild *F. deltoidea* plants can be conserved.

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An Economic Analysis of Anthropogenic Climate Change on Rice Production in Malaysia

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Abstract

Rice is an important staple food in Malaysia and represents a substantial household expenditure. Malaysia, which imports about 35 percent of its rice, is the 13th largest importer of rice in the world. This makes Malaysia susceptible to global rice crisis, similar to the one in 2008. Climate change is crucial in affecting rice production in tropical countries especially Malaysia as climate projections have shown that climate change will affect countries in the tropics most negatively with increased temperature and flooding due to anthropogenic carbon dioxide emissions. This study analysed the effect of anthropogenic carbon dioxide emissions on rice production in Malaysia during the period 1970-2013. The analysis incorporated the following variables: total local rice production, carbon dioxide emissions, precipitation, land used for paddy farming, total rice imports, and global average crude oil prices. The results indicated that in the estimated model the level of carbon dioxide does not affect rice production in the short-run. However, increased carbon dioxide emissions can influence rice production indirectly by affecting the level of precipitation. Precipitation and area of irrigated land are significant variables in determining level of rice production. Policies for reducing carbon emissions is however crucial for ensuring long run sustainability in rice production.

Keywords: rice production, climate change, temperature rise, precipitation, carbon dioxide emission.

Introduction

The agriculture sector is an important component in any country as it provides food and economic opportunities for the people. Due to its importance, various policies and budget allocation have been implemented to ensure its sustainability. Although this has been the case, it has been projected that in the coming two decades, developing countries would be affected by crop production problems following changes in global temperatures and weather (Lobell & Field, 2007). These climate changes stemming from anthropogenic activities such as mass deforestation, urbanization and vehicle pollution would contribute to food and economic problems in the country. Anthropogenic climate change is defined as “a change of climate which is attributed directly or indirectly to human activity that alters the composition of the global atmosphere and natural climate variability observed over comparable time periods.” (United Nations Framework Convention on Climate Change, 2006).

Malaysia imported RM45 billion worth of food in year 2015 (Carvalho et al., 2016), thus food security is an important problem to be given attention. Anthropogenic activities such as burning of forests for cultivation, land reclamation, carbon emission from industrial factories and housing development can cause the agriculture activities to decline. The decline in agricultural outputs may cause Malaysia to import even more food in the future especially rice from other nations such as Vietnam and Thailand. Rice is crucial to Malaysia as rice is a staple food in Malaysia accounting for some 25 percent of the food expenditure budget share (Sheng et al., 2008). This study examines the impact of anthropogenic climate change on rice production in Malaysia and suggests some policy implications to manage the anthropogenic activities in Malaysia.

Rice in Malaysia

Rice is the major staple food in Malaysia, making up the largest portion in food expenditure in an average household (Ishida et al., 2003). Due to its importance, the Malaysian government allocates incentives and subsidies to local farmers to increase production. With these efforts, production has increased from 1,318,000 tonnes in the year 1980 to 1,820,000 tonnes in the year 2016 (Department of Statistics Malaysia, 2016). Although Malaysia has seen increase in rice production, demand has so far outweighed local output. In the year 2017, Malaysians demanded 2.75 million tonnes of rice whereas the domestic production was only 1.8 million tonnes (USDA, 2017). This puts local rice supply well below sufficiency to meet the populations' demand with a supply deficit of close to a million tonnes. To overcome this deficit in rice supply, the Malaysian government imported rice from neighbouring countries such as Thailand, Vietnam, Cambodia, Pakistan and India. Among these countries, Thailand and Vietnam are the top exporters of rice to Malaysia (USDA, 2017). Comparatively, Malaysia is also ranked thirteen in total import of rice globally with an import of 950,000 metric tonnes (USDA, 2017). Thus, rice is an important commodity for the Malaysian people, and the government relies heavily on imports to meet the high national demand.

The high dependency on rice imports has in the past, negatively affected Malaysia, such as in the case with the 2008 global rice and cereal crisis. The rice crisis, which was due to number of factors, namely constraints in trade by major exporters, the panic purchase of stock by importers, high crude oil prices, and a weakening dollar, caused rice prices to increase substantially (USDA, 2017). Together with a weak Malaysian currency, meeting the country's demand for rice proved costly for the Malaysian government, as rice was a controlled commodity in the country (Arandez-Tanchuling, 2011). Realizing that Malaysia is over dependent on rice imports, the Malaysian Government then prompted to increase rice sufficiency level to 90 percent by the year 2010 but was later reduced to 70 percent in the Tenth Malaysian Plan (2011 to 2015). Thus, being overly dependent on food imports can be detrimental, as any regional food crisis would affect the country's food security.

Methodology

The framework of impacts of anthropogenic climate change on rice production is presented below:

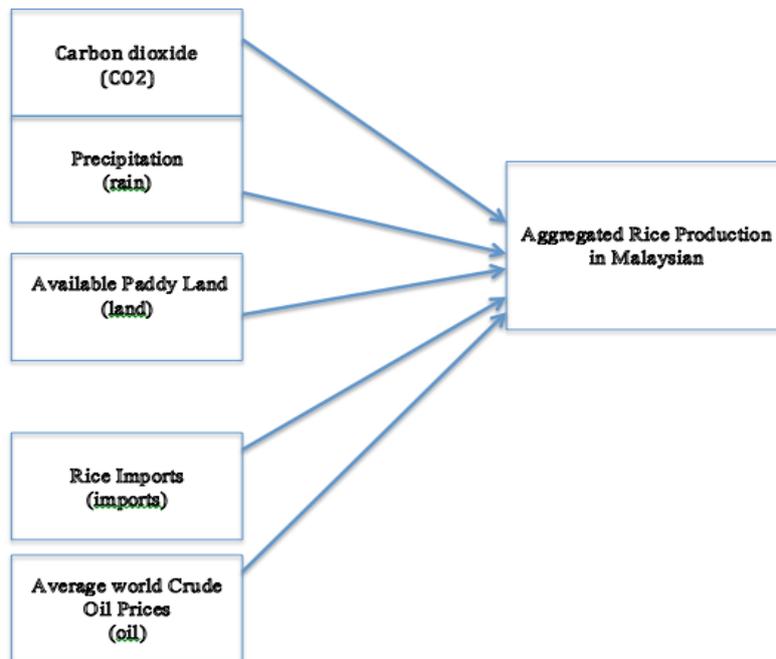


Figure 1. Determinants of Rice Production in Malaysia

The dependent variable is aggregated rice production in Malaysia. Independent variables on the other hand will include carbon dioxide emissions, precipitation levels, amount of available irrigated paddy land, amount of rice imports into the country, and average crude oil prices.

The model of rice production is as follow:

Rice = f (CO2, rain, land, imports, oil)

$$\Delta \ln rice = \beta_0 + \beta_1 \Delta \ln CO2_{t-1} + \beta_2 \Delta \ln rain_{t-1} + \beta_3 \Delta \ln land_{t-1} + \beta_4 \Delta \ln imports_{t-1} + \beta_5 \Delta \ln oil_{t-1} + \beta_6 ECT_{t-1} + \varepsilon_t \quad (1)$$

Where:

Rice = total local rice production (tonnes)

CO2 = concentration of carbon dioxide the atmosphere (parts per million, ppm)

Rain = precipitation (millimetres)

Land = available paddy land (% of total land)

Imports = total rice imports in Malaysia (tonnes)

Oil = average world crude oil prices (USD per barrel)

ECT= Error Correction Term

ε_t = error term

t = time period

β_0 = intercept

$\beta_1, \beta_2, \beta_3, \beta_4, \beta_5, \beta_6$ = coefficient for the explanatory variables

The data collected covers a period from 1970 to year 2013. The data of rice production and area of land were obtained from Department of Statistics Malaysia. The data of precipitation was obtained from the World Bank, data of carbon dioxide from United Nations Statistics Division's World Energy Data Set and data of petroleum price from world Brent crude oil price. The Vector Error Correction Model was used to examine the effect of anthropogenic climate change on rice production in Malaysia.

Results

The Table 1 below shows the results of Vector Error Correction Model of Rice Production

Table 1. Model of Rice Production

Variables	Coefficient (p-values)
1. Carbon dioxide (lagged one year)	0.045 (0.398)
2. Carbon dioxide (lagged two years)	-0.035 (0.421)
3. Number of rainfall (lagged one year)	0.545 (0.002)***
4. Number of rainfall (lagged two years)	0.548 (0.0001)***
5. Area of irrigated paddy land (lagged one year)	-0.645 (0.296)
6. Area of irrigated paddy land (lagged two years)	3.00 (0.015)**
7. Imported rice (lagged one year)	-0.137 (0.014)**
8. Imported rice (lagged two years)	0.01 (0.410)
9. Average world crude oil prices (lagged one year)	-0.050 (0.162)
10. Average world crude oil prices (lagged two years)	-0.02 (0.299)
11. Error correction term	-0.106 (0.004)***

Note: The values in the brackets indicate the p-values for the regression results. * denote 10% significance level, ** denote 5 % significance level and *** denote 1% significance level.

From Table 1, it can be observed that only rain, irrigated paddy land, imports of rice, and the error correction term are deemed significant at the least 5% level of significance. Rain, area of irrigated paddy land and imports of rice all show significant impacts on rice production in Malaysia. From the regression results, every 1% increase in the change of rainfall in country during the previous year will result in a 0.55% increase in rice output within the country. This

is considered to be an inelastic effect as the lagged one year rain variable's coefficient is less than one unit. An increase and well distributed rain-fall throughout the year is expected to increase rice output as farmers are not required to fully rely on the countries' irrigation system (Saseendran et al., 2000). Not being dependent on irrigation results in less strain for the rice crop. Thus, allowing the aforementioned system to water a larger area. Saseendran's results similarly found positive long-term effects of rain on rice production in the order of below unity.

From Table 1, besides the effect of rain, the p-value for the area of irrigated paddy land is proven to be significant at 5% level with a p-value of 0.014. Furthermore, analyzing the coefficient, available irrigated paddy land can be observed to have an elasticity value of 3.00. In other words, a 1% increase in area of available irrigated paddy land will result in a 3 percent increase in total rice output in the short-run. This can be interpreted as the demand for food increases, stress on the country's food producing sector will prompt the opening of additional agricultural land for paddy production (Lambin & Mayfroidt, 2011). Increase in the opening of irrigated paddy land may result in the increase in rice production.

Increase droughts in the country will result in low yielding crop seasons. Lower yields will then prompt the need to increase food import to meet increasing demand. Increase in food imports will hinder and reduce the rice production in Malaysia as the country will rely on food import and put less focus on developing the country's rice production to ensure self-sufficiency. This is seen from the significant negative coefficient of 0.13 between the imported rice and Malaysian rice production.

Lastly, the utilization of the vector error correction model necessitates the analysing of the error correction term. This term allows the model's system to correct any disequilibrium that may potentially occur in the system and adds adjustments to bring the model back to equilibrium. In the model above, the estimated coefficient for the error correction term is significant at 5% level and thus points to the validity of the existence of a long-run relationship between the tested variables. With a coefficient of 0.106, the speed of adjustment during events of shock can be said to be slow. In other words, the system has a speed of adjustment of about 10.6% during shocks. The model is also expected to be stable as any corrections made to reach the long-run equilibrium during short-run disequilibrium will not cause the model to explode and increase exponentially as the coefficient possesses a value smaller than one ($a < 1$). Besides that, the value for the coefficient of the error term is also negative, falling into the expected range of values that the error term should be, to keep the model stable. What this entails is that whenever the model encounters shocks, after a period of time, the model will shift back to equilibrium and not grow exponentially and break down. For example, crude oil spikes, droughts, and floods are a few commonly encountered shocks.

Comparing the effects of increase carbon dioxide with the other variables, it can be observed that the other variables are more significant. Carbon dioxide is found to be insignificant in affecting the rice production in Malaysia. On the other hand, rain or precipitation has a significant impact on rice output within the country. The increase in carbon dioxide does not seem to have an impact on rice production. This could be due to the fact that in the case of

Malaysia the increase in carbon dioxide emissions is not large enough to cause a drop in rice production. That is, the emission of carbon dioxide is still well below the threshold level to cause a reduction in production of rice. Due to this, it can be inferred that precipitation is still the major factor in affecting rice production and if emissions from carbon dioxide were to reduce precipitation than one can expect a reduction in rice production. Thus, the impact of greenhouse gasses on precipitation would be more important in determining rice production. Besides that, land allocation for farming has also been found to be more significant than increase in carbon dioxide emissions. Thus it can be said that increase in agriculture intensification may be able to counteract the effects of increased greenhouse gasses in the atmosphere.

Conclusion

Various researchers have cautioned the existence of global warming from recent increases of greenhouse gases in the atmosphere (Garcia, & Randel, 2008; Van Groenigen, Osenberg & Hungate). The rising temperature has been found to have detrimental negative effects on plants and various food crops around the world. Malaysia is expected to experience food production reductions in the near future. The results from this study however show that the impact of greenhouse gases is not significant in the case of rice production. However if the continued increase in greenhouse gases were to effect temperature and rainfall than there will be a reduction in rice production as rainfall is a significant variable in determining production of rice. Thus policies for monitoring the level of greenhouse gas emissions and the control of its emissions should be put in place to reduce the impacts of anthropogenic climate change on rice production in Malaysia.

Although it has been shown that the effects of carbon dioxide are not as profound as compared to other factors, it is still important that efforts are taken to manage the country's carbon emissions. This is because without any intervention, carbon dioxide emission may reach levels that would cause severe temperature rise over the coming decades. Among policies that can be applied are carbon taxes, increased budget on biofuel development, improved mass public transport, better urbanization management, and reduce deforestation.

The results also show that the opening of new agricultural land on the other hand, would also be able to increase rice output within the country by means of enlarging the agricultural capacity of the country. Although it is a quick way to achieve the target of self-sufficiency, the cutting down of forest may exacerbate the effects of global warming with the release of more carbon dioxide and the loss of carbon sinks. Instead of the opening of new agriculture land, a bigger budget should be allocated to agriculture research and development, allowing of a greater yield in a smaller hectare of paddy land. Greater yield of rice production in Malaysia would reduce the food imports from other nations. Reducing food imports from other nations are crucial for the development of local rice producers and ensure food security of the nation is sustained in the future.

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The Relationship between SPAD Chlorophyll and Disease Severity in *Ganoderma*-infected Oil Palm Seedlings

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Abstract

Establishment of disease in oil palm seedlings through artificial inoculation of *Ganoderma* are widely used for studies of various aspects of plant pathology, including epidemiology, etiology, disease resistance, host-parasite interaction and disease control. The estimation of chlorophyll content in the infected seedlings possibly could provide a good indicator for degree of disease or infection, and changes during pathogenesis. Thus, the objective of this study was to evaluate the relationship between disease severity index (DSI) and chlorophyll content in *Ganoderma* infected oil palm seedlings. Three-month-old oil palm seedlings were infected with *Ganoderma* inoculum on rubber wood block (RWB), where 44 isolates of *Ganoderma* were tested. Disease severity index (DSI) and chlorophyll content using a single-photon avalanche diode (SPAD) meter were recorded at 4 weeks interval for a period of 24 weeks after inoculation (WAI). Pearson's correlation analysis and regression analysis were performed to evaluate the relationship between the variables. It was found that the relationship between DSI and SPAD chlorophyll value was inversely proportional ($R = -0.92$) in a linear trend ($R^2 = 0.85$). Furthermore, the increasing trend of the DSI across the weeks were fitted in a quadratic model ($R^2 = 0.99$). In contrast, the SPAD chlorophyll value declined in a linear trend ($R^2 = 0.98$). The SPAD chlorophyll value could be considered as a better alternative over the DSI as the SPAD chlorophyll value was strongly related to DSI, as well as able to detect physiological changes in the infected oil palm seedlings at the early stages of pathogenesis.

Keywords: Disease-plant physiology relationship; Disease severity; *Ganoderma*; Oil palm; SPAD chlorophyll

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is an important plantation crop in the Southeast Asia, especially in Indonesia, Malaysia, and Thailand (USDA, 2018). However, the sustainability of oil palm in that region is threaten by *Ganoderma* species, a basidiomycete fungus causing stem rot disease in oil palm (Rees *et al.*, 2012; Rakib *et al.*, 2014). Establishment of disease by artificial inoculation is essential for studies of various aspects of plant pathology, including epidemiology, etiology, disease resistance, host-parasite interaction and disease control. Internal and external disease signs and symptoms are the common indicator used to evaluate the establishment of disease in plants. However, there are other alternative physiological

characteristics of plant could be used to evaluate the disease establishment, such as the use of SPAD chlorophyll value to estimate the chlorophyll content in the leaf of a plant using a SPAD chlorophyll meter (Uddling *et al.*, 2007; Chang *et al.*, 2015; Goh *et al.*, 2016).

Evaluation of disease severity based on the external disease signs and symptoms which correspond to disease scales require tedious work and careful observation for data recording, as well as time consuming. The use of the SPAD chlorophyll meter device could provide better alternative to evaluate disease severity in a plant. Thus, the objective of this study was to evaluate the relationship between the values of disease severity index and SPAD chlorophyll in *Ganoderma* infected oil palm seedlings.

Materials and Methods

Ganoderma isolates and planting materials

A total of 44 isolates of *Ganoderma* from stem rot infected oil palm were obtained (Rakib *et al.*, 2014). Planting materials used in this study were 3-month-old oil palm (*Dura x Pisifera*) seedlings planted in mixed top soil and sand medium in polyethylene bags.

Inoculation of Ganoderma on oil palm seedlings

Rubber wood block (RWB) sitting technique was used for inoculation of *Ganoderma* on the oil palm seedling as described in Rakib *et al.* (2015). Each *Ganoderma* isolate was tested with three seedlings per replicate in four blocks for a total of 12 seedlings per isolate. The total of 528 experimental units were arranged in a randomized complete block design (RCBD) under a rain-shelter facility with temperature and relative humidity ranging from 25-35 °C and 60-80%, respectively.

Collection and analysis of data

Data were recorded at 4 weeks interval for a period of 24 weeks after inoculation (WAI). The disease severity index (DSI) was assessed according to Ilias (2000), where it was calculated based on the numerical values of disease scale correspond to external disease signs and symptoms of infected seedlings (Table 1). Disease severity index (%) = $[\sum (A \times B) / \sum n \times 5] \times 100$, where 'A' is the disease scale (0 to 5), 'B' is the number of seedling showing the disease scale per treatment, 'n' is the total number of replication, and 5 is the constant representing the highest scale of assessment. SPAD chlorophyll values were measured using a SPAD chlorophyll meter (SPAD-502 meter, Konica Minolta, Japan) (Chang *et al.*, 2015; Goh *et al.*, 2016) at the third lanceolate or bifurcate leaf from the top leaf of the oil palm seedling. The data were subjected to Pearson's correlation analysis and regression analysis to evaluate the relationship between the variables using the Statistical Analysis System (SAS v 9.2) program.

Table 1. Numerical disease scales and their corresponding disease signs and symptoms

Scale	Disease signs and symptoms on oil palm seedling
0	Healthy plant with green leaves and no fungal mass development on any part of the plant
1	Appearance of 1-3 chlorotic leaves with no fungal mass development on any part of the plant
2	Appearance of fungal mass with or without chlorotic leaves
3	Appearance of >3 chlorotic leaves, necrotic leaves (dead leaves) with or without fungal mass development on any part of the plant
4	At least 50% of total leaf number showing severe chlorosis or necrosis with or without fungal mass
5	Dead plants with or without fungal mass

Results and Discussion

All 44 *Ganoderma* isolates tested showed positive signs and symptoms of disease infection on the oil palm seedlings. Leaf symptoms were observed as the infection progressed over time and eventually the seedlings died. Generally, the external disease signs and symptoms appeared at 12 weeks after inoculation (WAI) and in several seedlings, the symptoms appeared as early as 8 WAI. The external infection symptoms observed include leaf chlorosis (yellowing leaf) which eventually became necrotic (brown or dead leaf). Mycelia mass of *Ganoderma* appeared at the base of the seedlings which progressively develop into small white button that eventually formed bracket of *Ganoderma* (Figure 1). The progression of infection as observed in this study was similar to those reported by Sariah *et al.* (2007) and Kok *et al.* (2013).



Figure 1. Infection progression of *Ganoderma*-inoculated oil palm seedlings, where (a) to (f) arranged according increasing order of infection severity. Source: Rakib *et al.*, (2015)

The DSI and SPAD chlorophyll values of the oil palm seedlings inoculated with *Ganoderma* at the 24 WAI ranged between 10.77 - 68.33% and 12.10 - 30.80, respectively. Pearson's correlation analysis of the data (N = 44) recorded on the 24 WAI indicated that the DSI and SPAD chlorophyll values were highly correlated ($p < 0.0001$, $R = -0.92$). Further regression analysis of the data showed strong relationship ($R^2 = 0.85$) between DSI and SPAD chlorophyll values, where increase in the DSI caused linear declining trend in the chlorophyll value. The trend of the relationship can be written as SPAD chlorophyll value = $-0.2893 \text{ DSI} + 34.177$ (Figure 2).

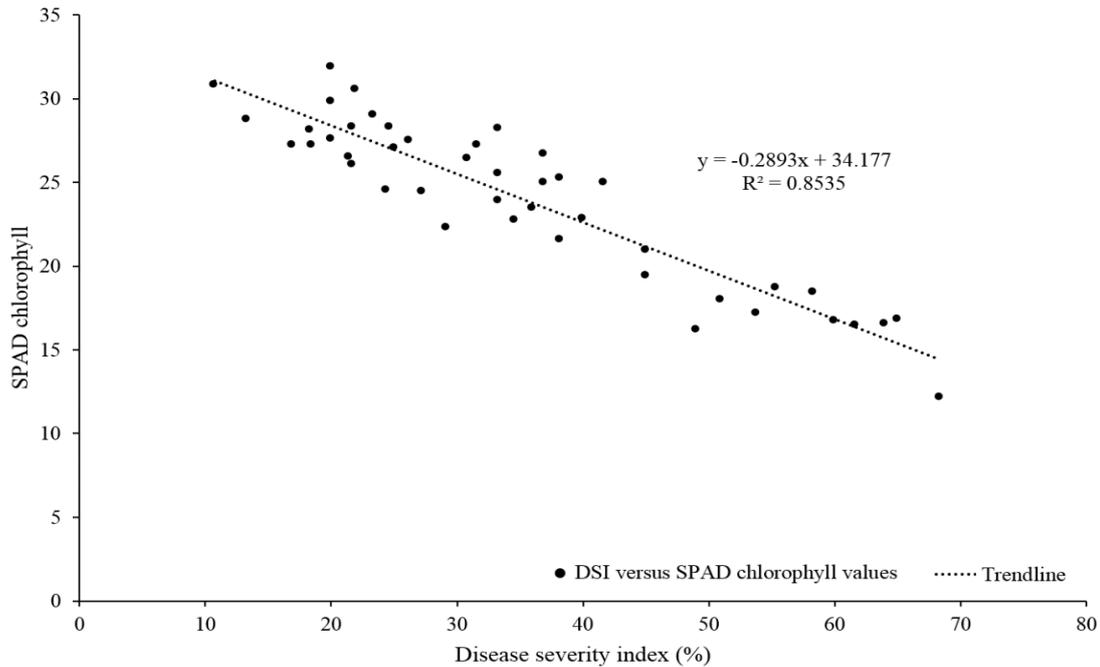


Figure 2. Regression between disease severity index (DSI) and SPAD chlorophyll values on the 24 week after inoculation with *Ganoderma* on the oil palm seedlings (N = 44)

Figure 3 shows the trend of the average DSI and SPAD chlorophyll values of the *Ganoderma* infected oil palm seedlings (a total of 44 isolates of *Ganoderma* tested, or N = 44) at 4-week interval for a period of 24 WAI. The DSI was low for the first 8 WAI (0 - 0.76%), and the DSI values increased rapidly at the 12 WAI until the 24 WAI (4.31 - 35.14%). Meanwhile, the SPAD chlorophyll values decline constantly from 56.20 on the 0 WAI until 24.01 on the 24 WAI. This indicate that the SPAD chlorophyll value was more sensitive for detection of disease especially in the early stage when the external disease signs and symptoms are not obvious to the naked eyes. Pearson's correlation analysis showed that the DSI was positively correlated across the weeks ($p = 0.01$, $R = 0.90$), while the SPAD chlorophyll value was negatively correlated across the weeks ($p < 0.0001$, $R = -0.99$). The increasing trend of the DSI across the weeks were fitted in a quadratic model (Average DSI = $0.0942 \text{ Week}^2 - 0.8785 \text{ Week} + 0.9804$), with a R^2 value of 0.99. In contrast, the SPAD chlorophyll value declined in a linear trend (Average SPAD chlorophyll = $-1.2955 \text{ Week} + 53.537$), with a R^2 value of 0.98.

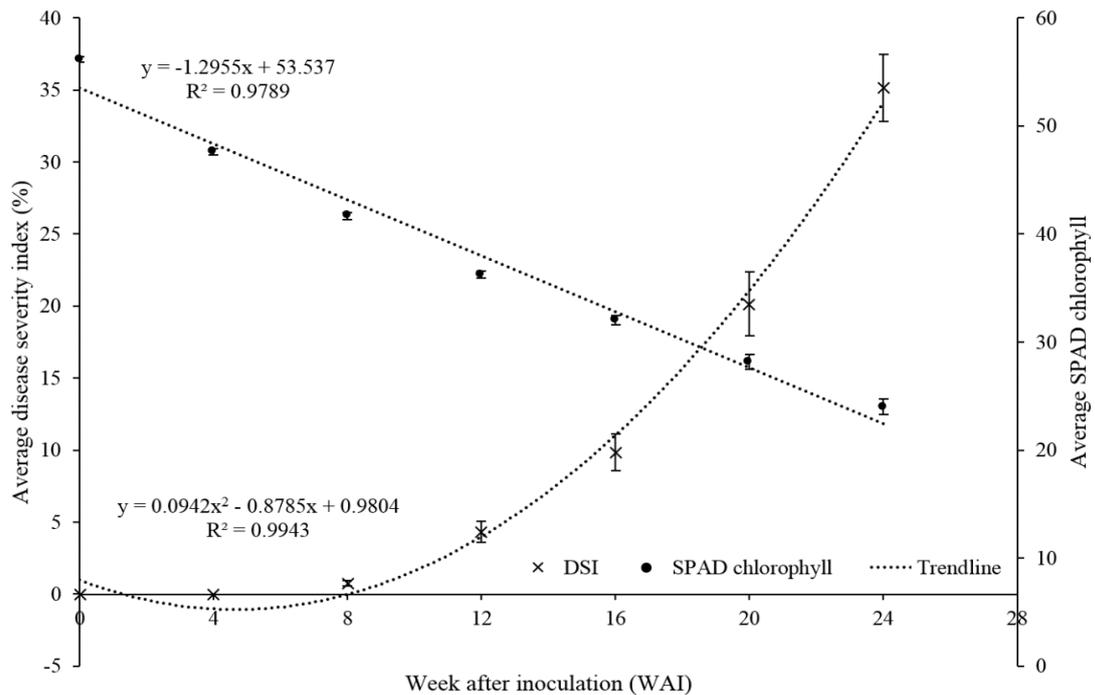


Figure 3. Trends of average disease severity index (DSI) and SPAD chlorophyll values (\pm standard error) across 24 weeks after inoculation with *Ganoderma* on the oil palm seedlings

Goh *et al.* (2016) reported that the SPAD chlorophyll values decline as the disease progress or during pathogenesis of *Ganoderma* in the oil palm seedlings. Similarly, Chang *et al.* (2015) also reported that increasing disease in different stages of plant growth caused reduction in the SPAD chlorophyll value. Significant reduction in the chlorophyll content could be related to the injury in the oil palm seedling's root and vascular system caused by the fungus infection (Shafri *et al.*, 2011; Goh *et al.*, 2016), which possibly caused reduction in nutrient adsorption, especially nitrogen (N) and magnesium (Mg). Nitrogen and magnesium are related to the biosynthesis of chlorophyll (Rissler *et al.*, 2002), and both nutrients have been positively correlated with the chlorophyll content in plants (Bojovic and Markovic, 2009; Alberto *et al.*, 2014). Furthermore, the shortage of N and Mg caused chlorosis, where the leaves appeared yellowish as observed in this study (Figure 1).

Conclusion

The relationship between DSI and SPAD chlorophyll value was inversely proportional in a linear trend with confidence level of 85%. The decline in the SPAD chlorophyll values were detected on the early stages of pathogenesis (0 to 8 WAI) although no obvious changes was recorded in the DSI values during that early stages. This conclude that SPAD chlorophyll value could be used as an alternative as well as better alternative indicator to evaluate the disease establishment in the oil palm seedlings due *Ganoderma* species.

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Propagation of Babagon Pineapple (*Ananas comosus* cv Babagon) By Stem Cuttings

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Abstract

This study was conducted at the Faculty of Sustainable Agriculture, Universiti Malaysia Sabah to determine the types of pineapple stem cuttings that could potentially produce a large number of pineapple planting materials. The experiment highlighted on the germination and growth performances of pineapple sucker, where both were used Completely Randomized Design with a single factor. Germination of sucker had five replications and growth performances had 20 replications. The pineapple stem was cut 3 cm lengthwise and were subjected to 3 treatments; unsplit stem (T1), halved stem (T2), and quartered stem (T3). Data were collected on germination of suckers, number of suckers, and leaves, leaf fresh and dry weights, stem fresh and dry weights, root fresh and dry weights, plant fresh and dry weights, shoot-root ratio and analysis of regression on weight and length of Babagon pineapple suckers for 180 days after planting. The results were analyzed using SAS 9.4 and the treatment means were compared by using Least Significance Difference at 5% significant level. Sucker germination, number of suckers and number of leaves have significant differences on T1, which were 32.8, 54.8 and 31.65 respectively. The plant biomass also showed there were significant differences of the T1, where the plant fresh weight and plant dry weight recorded 172.42 g and 21.91 g respectively. Root-shoot ratio did not show any significant difference. The relationship between fresh weight and height of pineapple by regression analysis showed most of the suckers produced were between small to medium grades. Hence, it could be concluded that unsplit stem is the best choice for mass production of sucker from pineapple stem.

Keywords: Pineapple sucker, stem cutting, sucker germination, sucker development, Babagon pineapple

Introduction

Pineapple (*Ananas comosus* L. Merr.) is a tropical fruit native to Latin America around Brazil, Paraguay, and Uruguay (Malip and Sapii, 2010). These monocotyledonous plants belong to the Bromeliaceae family. There are five groups of pineapples grown around the world, namely Spanish, Queen, Cayenne, Pernambuco, and Perolera. Three groups of pineapple that is grown in Malaysia are Spanish, Queen, and Cayenne (Mohammed Selamat, 1996). Based on the Agrofood Statistics Report, the Ministry of Agriculture and Agro-based Industry of Malaysia (2014), the total area of pineapple plantation in Sabah 2012 was 1,317 hectares with production of 24,119 tones. The size and production of pineapple in Sabah have decreased in 2014, with a total area of 1,366 hectares and production of 23,512 tones (Ministry of Agriculture and Agro-based Industry Malaysia, 2014). Propagation through stumps and crowns for commercial use was slow where one pineapple tree can only supply one to three stumps and crowns for a

harvest cycle of 18 months. In view of the need for commercial pineapple planting, it requires about 42,000 planting materials per hectare (Fitchet and Van de Venter, 1988). Pineapple tissue culture can produce many plantlets and there are free of insects and diseases. However, tissue culture method requires high financial cost and technology (Agogbua and Osuji, 2011). The most common problem in pineapple industry is the difficulty of obtaining uniform planting materials in large quantities. This is due to financial constraints, and questionable quality of planting materials needed for large-scale pineapple planting (Mohd Johaary, 2010). An alternative method of production of pineapple planting materials according to the desired uniformity, abundance, and high-quality planting materials can be done through cutting and sectioning of pineapple stems (Marie, 2001). Therefore, the study was carried out to assess effect of stem cuttings on the number and quality of sucker produced from Babagon pineapple as planting materials.

Methods and Materials

The experiment was conducted in a rain shelter and insect proof netted structure located at the Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, Sandakan Campus, between February 2017 until December 2017.

The stem cut were obtained from freshly harvested Babagon pineapple cultivars with thickness of three cm. A total of 600 stems were used in this experiment. All the stems were subjected to three types of treatments which included:

- 1). Control (T1): Unsplit stem
- 2). Treatment 2 (T2): Halved stem
- 3). Treatment 3 (T3): Quartered stem

All the stem cuttings were treated with fungicide TOPSIN M (Thiophanate-methyl 70%) and weighed using electronic balance and separated according to the type of stem cut for planting.

Germination phase

Forty stem cuttings were used for each treatment and established in a completely randomized design with five replications. Forty stem cuttings were planted in a nursery tray. Top soil was used as medium for planting. Seedlings were watered twice daily.

Growth performance phase

At sixty days after planting (DAP), the sucker that emerged from the stem was detached and sorted according to treatment. It was then individually transplanted to a polybag. Twenty replications were used for each treatment arranged in a completely randomized design.

Regression Analysis

At 115 DAP and 213 DAP the plant fresh weight and height were taken to be analyzed as regression relationship. Resultant data was evaluated based on the classification of the pineapple planting material grades as below:

Size	Length, cm	Weight, g
Small	30-44	110 -125
Medium	45-59	180 – 200
Large	60	280-370

Source: Mohammed Selamat and Abdul Rahman, 1996

Data collection and analysis

Data was collected on the days to emergence, number of suckers, number of leaves, fresh weight, dry weight, root-shoot ratio on dry weight basis and regression analysis for fresh weight and height. The data was subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) to test for the significance of effects of treatment on the parameters measured. Differences between treatment means were compared using the least significant difference test.

Results and Discussion

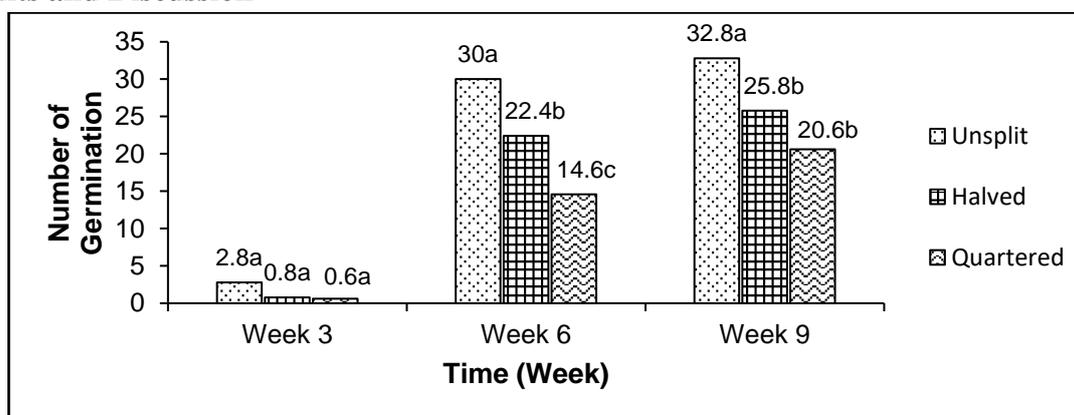


Figure 1. Mean number of weeks to sucker emergence.

Table 1. Mean of effect of stem cutting on number of suckers, number of leaves, fresh weight, dry weight, and root-shoot ratio on dry weight basis.

Treatment	Number of suckers	Number of leaves	Fresh weight, (g)	Dry weight, (g)	Root-shoot ratio
Unsplit stem	54.80 ^a	31.65 ^a	172.42 ^a	21.91 ^a	8.94 ^a
Halved stem	33.00 ^b	26.00 ^b	115.22 ^b	14.93 ^b	8.03 ^a
Quartered stem	22.40 ^c	26.20 ^b	94.90 ^b	11.52 ^b	10.70 ^a

Means followed by the same letters within the column are not significant different at 5% level after Least Significant Difference test.

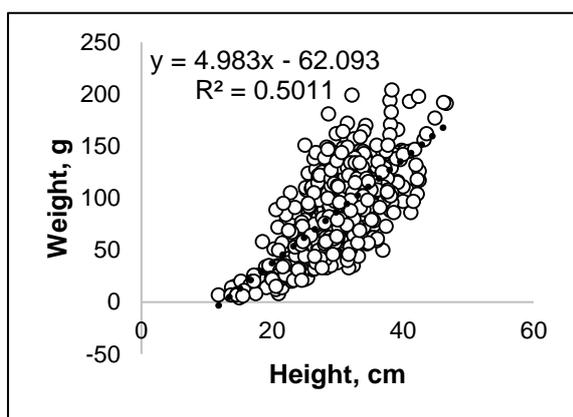


Figure 2. 50.11% regression line of weight and height, at 215 DAP

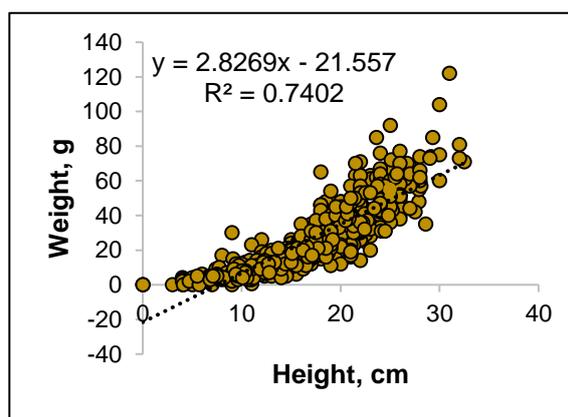


Figure 3. 74.02% regression line of weight and height, at 115 DAP.

Germination occurred as early as second to third week. Studies by Weerasinghe and Siriwaranda (2006); Heekenda and Samarathunge (1993) found that germination occurred only in week four to five. According to Soler and Dole (2006), shoots began to grow between the second and third week after planting. This perhaps due to shade factor which also affected shoot emergence. Shoot emergence is proportional with the degree of shade the pineapple crops are subjected unto. On the contrary, high light intensity promoted rapid growth of the shoots.

Larger-sized or heavier plant materials showed rapid growth in comparison with small and medium-sized plant materials. Similar result was reported in previous studies (Agogbua and Osuji, 2011; Reinhardt *et al.*, 2003; Norman, 1976). According to Milhomem *et al.* (2011), large pineapple stem has high nutrient and food storage, this may affect the initial stages of growth. In addition, small-sized pineapple stems were very delicate than the large-sized pineapple stems. This was reflected in fewer number of suckers in quartered Babagon pineapple compared to the unsplit and halved stems.

At 180 DAP, the finding were similar obtained by Milhomem *et al.* (2011), where the number of leaves increases proportionally with the weight and length of pineapple stems. This study suggests that in the early germination phase of Babagon pineapple stems, there was a difference in the growth rate and size of the suckers (Reinhardt *et al.*, 2003). The pineapple stem size greatly influenced the growth of the suckers. This is due to the complete dependence of food

storage in the pineapple stem. When suckers reached a stage where the process of photosynthesis depended entirely on the leaves, this affected the growth of the suckers and number of leaves. Small leaf area has a lower rate of photosynthetic capacity, causing the growth and development of the suckers are different between treatments.

These results were also similar with the study on effect of plant material size on the production of dry weight and fresh weight of pineapple stem at 60 DAP conducted by Reinhardt *et al.* (2003). The study suggested that the different volumes of food storage and low growth rates affected the plant biomass. In addition, plants that grow on soils with high humidity levels had high dry matter compared to less damp soil. This was due to good soil moisture maintenance (Martinez *et al.*, 2005).

The highest root-shoot ratio on dry weight basis of pineapple material was 10.70, from the quartered pineapple stem, while the halved pineapple stem shows the lowest which is 8.03. Roots, stems and leaves were interdependent. These three plant organs maintained a dynamic balance of plant biomass, which reflected the relationship between light source and carbon dioxide gases obtained through shoots with water sources and nutrients derived from root zones (Poorter *et al.*, 2012).

There were positive relationship between fresh weight and height of pineapple plant. The 74.15% increase in weight of plants was directly proportional to the increase in the height of the plants. Likewise, positive relationship was seen at 213 DAP but at this stage the R^2 regression coefficient is only 50.11%. This showed that the quality of planting material has decreased in comparison with the quality of plant at 115 DAP. The decrease in quality of plant material might be the result of increased competition between plant to obtain light due to increasing number of suckers. Based on the regression analysis at 213 DAP and 115 DAP, it was determined that the resulting suckers were mostly in range of small to medium planting material grade.

Conclusions

From this study, it can be concluded that the unsplit stem cuttings was the best source for sucker production. On the average, the unsplit stem can produce 1.4 number of suckers. A hectare of pineapple cultivation with 30,000 trees after harvesting could produce 420,000 plant materials from its stem. In terms of quality, it can be seen through the germination from the unsplit stem where it began to germinate as early as the third week after planting. Therefore, the unsplit pineapple stems is suitable for mass production of plant materials. The other treatments were not suitable because low sucker production. Germination and growth performance of pineapple sucker are largely influenced by some of the previously mentioned factors, but the most significant factors were the size and food storage volume of Babagon pineapple stems.

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Endophytic Bacteria to Control Bacterial Wilt Disease Caused by *Ralstonia solanacearum*

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Abstract

Ralstonia solanacearum has been identified as the second most important bacterial phytopathogen due to its aggressiveness and vast host range. Biological control approach seems to be an effective solution to reduce the bacterial wilt incidence primarily the endophytes. In this study, *R. solanacearum* strain was isolated from an infected eggplant in Selangor, Malaysia. *In-vivo* pathogenicity test was carried out on eggplants to confirm its pathogenicity. Potential bacterial endophytes were isolated from healthy stem of tomato, eggplant and chilli in cultivation field and subjected to *in-vitro* antagonism bioassay. The most dominant endophytes were subjected to cultural, morphological and molecular identification. In the pathogenicity test, the infected eggplants demonstrated 80% wilting symptoms on the third week. A total of 116 bacterial endophytes were isolated and 55 exhibited an ability to restrain *R. solanacearum* growth in the dual culture plate. Statistical analysis showed that isolate UPMBC1 and UPMBE2 were the most superior bacterial endophytes with 14.6 mm and 12.6 mm inhibition radius respectively. UPMBC1 was identified as *Enterobacter tabaci* and UPMBE2 as *Bacillus cereus*. This study reports for the first time on a novel bacterium, *E. tabaci* isolated in Malaysia and as the first case as antagonist globally.

Keywords: *Ralstonia solanacearum*; bacterial wilt disease; bacterial antagonist; *Enterobacter tabaci*; *Bacillus cereus*.

Introduction

Achaari and Ramesh (2014) reported that *R. solanacearum* (Smith) is able to infect more than 200 different crops species worldwide that covers 50 families. Profound disease severity was reported in the tropical climate (Mondal *et al.*, 2014). This phytopathogen vigorousness destroys the infected plants and led to more than 90% yield losses (Artal *et al.*, 2012). The prominence effects of bacterial wilt (BW) disease can be seen in tomato, banana, eggplant and potato where the severity ranging from 80% to 100%. Mondal *et al.* (2014) reported that BW disease was a major constraint on tomato and brinjal major-growing areas in India. Meanwhile in south-western Nigeria, 80% of the tomato yields were greatly reduced due to BW infection (Popoola *et al.*, 2014).

Once was a biological control agent against Kahili ginger (Anderson and Gardner, 1999), and now *R. solanacearum* has been noted as the second most important bacterial pathogen after *Pseudomonas syringae* (Yuliar *et al.*, 2015). The first infection of BW disease took place in Guyana in 1840 (Thurston and Galindo, 1989). However, Burrill was the first to discover the causal agent in 1890 when BW disease infected potato in Japan (Monther and Kamaruzaman, 2010). Studies on finding the effective control with respect to the environment and human safety are still ongoing. This non-sporulate bacterium is capable to live for years in drought soil or in the absence of the host (Stander *et al.*, 2013; Hammes 2013). Infection may also occur even if the population of the pathogen is low (Nair, 2013; Hammes, 2013). It is able to live for years in the host even after the host has died that eventually provides inoculum for the next infection cycle (Scherf *et al.*, 2010). The variability of the strain and the widely distributed host makes it difficult to resist BW infection (Blomme *et al.*, 2017).

Since there is no effective control to wilt disease in general, most farmers are still relying on the use of resistant cultivars (Artal *et al.*, 2012). Unfortunately, resistant cultivars are often unstable and have low endurance (Liu *et al.*, 2009). To date, biological control method was proven to efficiently control soil-borne diseases (Lwin *et al.*, 2006). Thus, the study was carried out with the following objectives: 1) to isolate *R. solanacearum* from infected crops, 2) to test the virulence of *R. solanacearum* via pathogenicity test, 3) to isolate and evaluate endophytic bacteria with antagonistic activity against the *R. solanacearum*, and 4) to identify the most potential antagonistic agent via cultural, morphological and molecular methods.

Materials and Methods

Isolation of the BW causal pathogen

R. solanacearum was isolated from a wilted eggplant in Kuala Selangor, Malaysia. The wilted stem was surface sterilized by dipping it in 10% of sodium hypochlorite (NaOCl) for 30 s and washed using sterile distilled water for 1 min. Briefly, 1 cm of each end of the wilted stem was discarded. The remaining length was dipped and slightly shook in sterile distilled water for the ooze. One loop of the infected distilled water was streaked on the Kelman tetrazolium chloride (TZC) agar to confirm the pathogen (French *et al.*, 1995). Normally, the colony of virulence *R. solanacearum* exhibits pink or light red with whitish margin and fluidal on the TZC agar.

Pathogenicity test

R. solanacearum was inoculated in casein-protease-glucose (CPG) broth and shaken at 140 rpm for 48 h at 25 °C. The concentration of bacterial cells were calculated using the plate count method and altered to 10¹⁰ cfu/ml. Approximately, 30 ml of the broth was soil-drenched in each pot containing a 30-day-old eggplant seedlings, Antaboga. Pots drenched with 30 ml of sterile distilled water were served as the control. Seedlings were monitored up to three weeks for disease severity following Winstead and Kelman (1952) wilting index.

Isolation of endophytic bacteria

Healthy stems of eggplant, chili and tomato were randomly collected from eight states in Peninsular Malaysia and surface sterilized following El-Deeb et al. (2013) with modifications. Approximately, 11 cm of main stem's length for each plant was excised and the bark was discarded. The excised stem was dipped in 20% NaOCl for 1 min and rinsed using a sterile distilled water for five times. The rinsed water was streaked onto the nutrient agar (NA) as a control. One cm of each end of the stem was discarded. The remaining stem was cut shorter to 3 cm and cut open to expose the internal parts. The pieces were transferred into 200 ml 1xPBS (phosphate buffered saline) buffer with 7.4 pH and shook. After 48 h, the buffer was streaked on NA and incubated for 4 days at 25 °C. At the end of incubation period, different colonies of endophytes were selected based on their shape, color, colony and texture, and subsequently purified onto NA until single colony was obtained.

Cultural and morphological characterization

The promising bacterial endophytes were characterized by evaluating the colony diameter, margin, pigmentation, opacity, texture and colony's form. Gram staining was also performed to record the Gram reaction and cell shape.

In-vitro antagonistic assessment

Isolated endophytic bacteria were subjected to antagonism bioassay against *R. solanacearum* via Ramesh and Phadke (2012) protocol with modifications. Endophytic bacteria and *R. solanacearum* were cultured in 5 ml of nutrient broth (NB) and CPG broth respectively and shook. Approximately, 250 ml of NA was prepared and 150 ul of *R. solanacearum* was added in every 100 ml of NA, swirled and poured into Petri plate. Three wells were made for each plate using a sterile one cm cork borer. Briefly, 25 ul of endophyte suspension was pipetted into each well and plates were sealed. Sterilized distilled water was used in control plates. All plates were incubated at 25 °C for 4 days and the radius growth of the endophyte was measured from the edge of the well (in mm).

Statistical analysis

Bacterial endophytes with consistent growth inhibition activity were selected for statistical analysis. Tukey's mean separation of ANOVA was performed using the Statistical Analysis System (SAS) software 9.4 (SAS Institute, United States) with ($P>0.05$).

Molecular identification

The most promising antagonists were subjected to molecular identification using colony-PCR (Polymerase Chain Reaction) method (Walch *et al.*, 2016) with slight modifications. Approximately, 50 ul of Master mix consists of 1.25 ul of *Taq* polymerase, 1.0 ul of dNTPs, 2.5 ul of reverse primer (5' GGTTACCTTGTTACGACTT 3'), 2.5 ul of forward primer (5'

AGAGTTTGATCCTGGCTCAG 3'), 5 ul of PCR buffer and 37.73 ul of distilled water was prepared in 0.5 ml PCR tube. The endophyte colony was lightly touched using a sterilized 10 ul pipette tips and transferred to the Master mix. The Master mix without the presence of endophyte was served as a control. All PCR tubes were amplified in a PCR machine (c1000 Bio-Rad Thermal Cycler, United States). For amplification, reaction started with initialization (94 °C for 5 min), denaturation (94 °C for 30 s), annealing (55 °C for 40 s) and elongation (72 °C for 1 min). The reactions were repeated 32 times.

One ul of 100 bp ladder (Thermo Fisher Scientific, United State) was mixed with one ul of loading dye and loaded in the first well of the agarose gel. One ul of each PCR product was mixed with one ul of loading dye before pipetted into the individual wells. The 1 % of agarose gel was used and electrophoresis was run 40 min at 85 V till the dye was seen approaching the end of the electrophoresis gel. The gel was viewed under the ultraviolet (UV) light using Bio-rad Gel Doc XR Imaging System for bands. Approximately, 30 ul of the PCR product was outsourced and sent to First BASE company for sequencing in order to identify the endophyte based on the 16S rRNA sequence.

Results

Pathogen isolation and pathogenicity test

Only 1 strain of *R. solanacearum* was successfully isolated from the infected eggplant. The colony of *R. solanacearum* also exhibited light red in color at the center with whitish color on the margin on the TZC agar and presented in Figure 1 (A). Besides, it also exhibited a fluidal appearance indicates a virulent strain. In the pathogenicity test, BW disease severity was recorded. On the first and second week, the eggplant showed low wilting severity with only 20% of wilting index. Within this duration, no wilting symptoms were exhibited by the seedlings. However, the BW disease progressed aggressively where on the third week, the treated seedlings showed a sudden wilting and disease severity hiked up to 80% of the wilting index. As for the untreated seedlings (control), no wilting symptoms were shown. The comparison of untreated and treated eggplant seedlings were presented in Figure 1 (B).

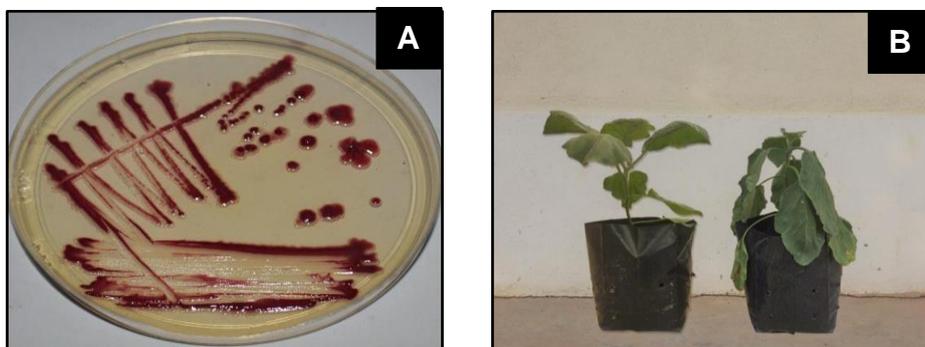


Figure 1. (A) Culture plate of *Ralstonia solanacearum* strain streaked on tetrazolium chloride agar. (B) The untreated (left) and treated (right) eggplant seedling in the pathogenicity test.

Cultural and morphological characters of isolates UPMBC1 and UPMBE2

E. tabaci isolate UPMBC1 stained pink color with cocci cell shape, while *B. cereus* isolate UPMBE2 stained violet purple color with rod shape in Figure 2 (A and B). The colony appearances for isolates UPMBC1 and UPMBE2 were shown in Figure 3 (A and B) and the cultural characterizations for both colonies were displayed in Table 1.

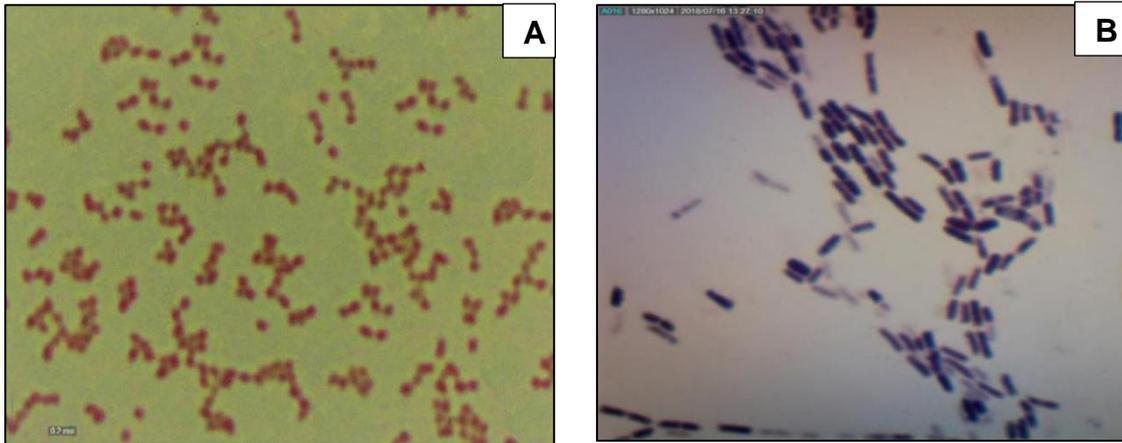


Figure 2. The cell shape close-up viewed via Dino-Lite 2.0 software. (A) *Enterobacter tabaci* isolate UPMBC1 demonstrating cocci shape. (B) *Bacillus cereus* isolate UPMBE2 in rod shape.

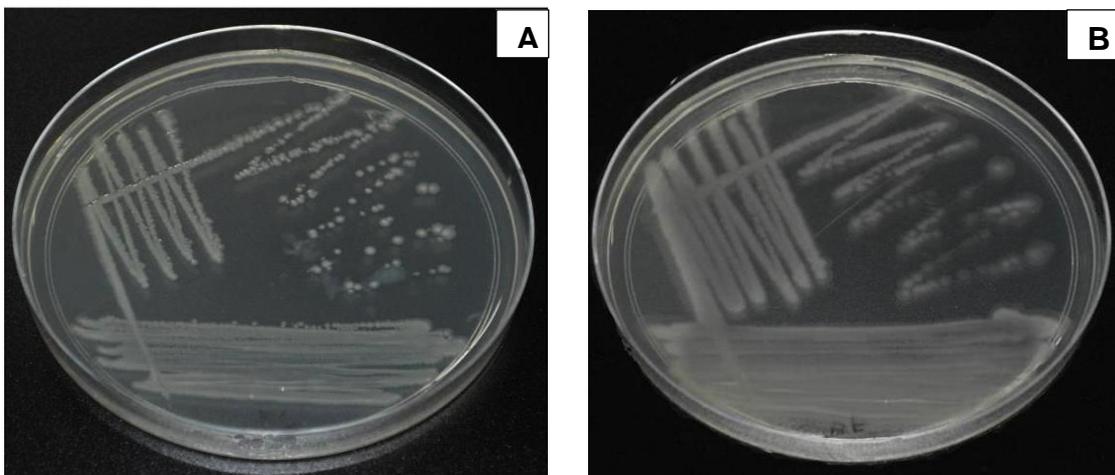


Figure 3. The colony of (A) *Enterobacter tabaci* isolate UPMBC1 and (B) *Bacillus cereus* isolate UPMBE2 streaked on nutrient agar.

Table 1. The characteristics of the *Enterobacter tabaci* isolate UPMBC1 and *Bacillus cereus* isolate UPMBE2 colonies

Characteristics	<i>E. tabaci</i> isolate UPMBC1	<i>B. cereus</i> Isolate UPMBE2
Diameter (mm)	2	4
Margin	Round	Round
Pigmentation	Yellowish	White
Form	Smooth and translucent	Rough and opaque
Texture	Butyrous	Dry

Antagonism bioassay

A total of 116 endophytes were isolated, however, only 55 demonstrated inhibition against the growth of *R. solanacearum*. Among the 55 isolates, 27 isolates showed consistent inhibition activity towards *R. solanacearum* growth in all replicates (Figure 4).

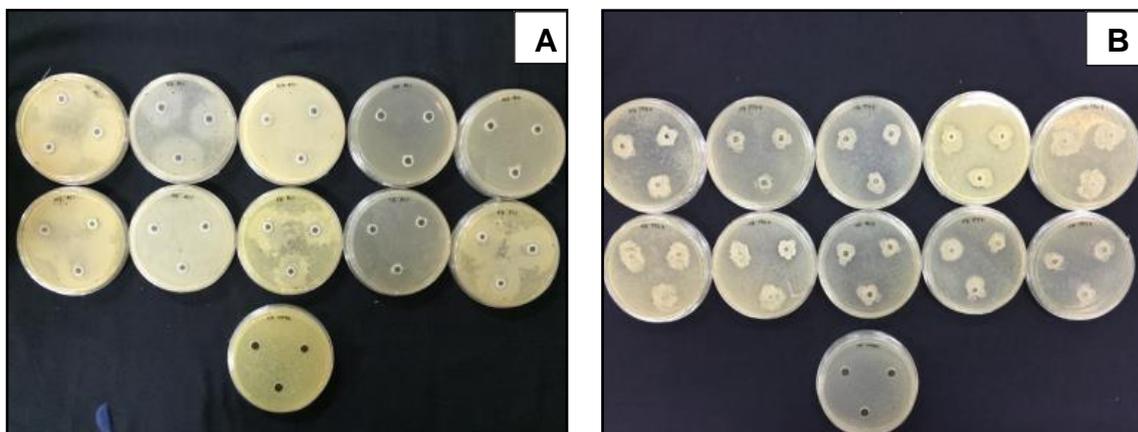


Figure 4. *In-vitro* antagonism bioassay for bacterial endophytes (A) *Enterobacter tabaci* isolate UPMBC1 and (B) *Bacillus cereus* isolate UPMBE2. Control was at the bottom for A and B. Both endophytes of isolate UPMBC1 and isolate UPMBE2 outgrowth the *Ralstonia solanacearum* in all replicates.

Statistical analysis

Out of 27 bacterial endophytes, only two endophytes exhibited the highest inhibition radius against *R. solanacearum* (>10mm) with significant differences (Table 2) which coded as UPMBC1 and UPMBE2. UPMBC1 was extracted from chili in Selangor, while UPMBE2 was from eggplant in Selangor.

Table 2. Mean radius of 27 potential bacterial endophytes

Bacterial endophyte isolates	Radius (mm)
UPMBC1	14.64 ^{±a}
UPMBE2	12.55 ^{±ab}
UPMJTCe2	9.72 ^{±abc}
UPMBER4	9.19 ^{±bc}
UPMBER5	8.47 ^{±bcd}
UPMATTO2	7.97 ^{±bcde}
UPMAC3	6.84 ^{±cdef}
UPMAC1	6.61 ^{±cdef}
UPMJTCe4	6.51 ^{±cdef}
UPMBC2	6.49 ^{±cdef}
UPMATTO3	5.72 ^{±cdef}
UPMAC2	5.50 ^{±cdef}
UPMNTPi3	5.26 ^{±cdef}
UPMJTCe3	4.98 ^{±cdef}
UPMPE2	4.54 ^{±cdef}
UPMPE4	4.39 ^{±cdef}
UPMCE7	3.57 ^{±def}
UPMBER6	3.53 ^{±def}
UPMPC3	3.45 ^{±def}
UPMCEI5	3.40 ^{±def}
UPMCE6	3.27 ^{±def}
UPMCC1	2.86 ^{±ef}
UPMPE1	2.72 ^{±ef}
UPMNTPi2	2.49 ^{±f}
UPMCE5	2.44 ^{±f}
UPMBE6	2.09 ^{±f}
UPMJC4	1.56 ^{±f}

* The same alphabet denotes no significant difference ($P>0.05$)

Molecular identification

Both isolates displayed intact single bands on gel electrophoresis. The size of 16S rRNA gene amplified for both bacterial endophytes is 1500 bp (Figure 5). Isolate UPMBC1 was 99% identified as *E. tabaci* and was designated as *E. tabaci* isolate UPMBC1. The sequence from isolate UPMBE2 was 98% identical to *B. cereus* and was designated as *B. cereus* isolate UPMBE2. The 16s rRNA sequence of *E. tabaci* (Accession No: MH 794127) obtained in this study was aligned to the sequence of *E. tabaci* (Accession No: NR 146667.2) isolated from tobacco stem in China by Duan et al. (2015), yielded 99% sequence homologous.

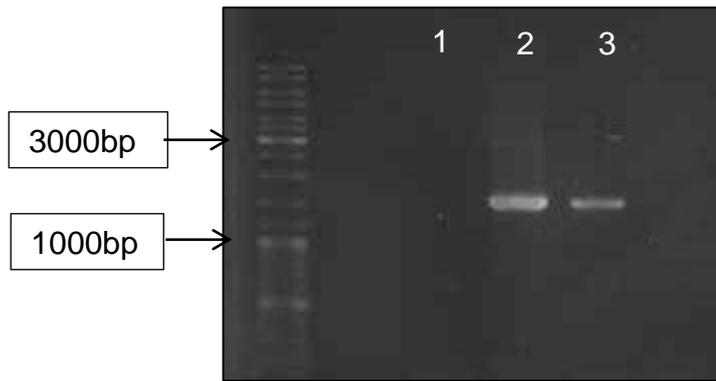


Figure 5. The agarose gel shows intact band for *Enterobacter tabaci* isolate UPMBC1 (Lane 2) and *Bacillus cereus* isolate UPMBE2 (Lane 3). Control (Lane 1).

Discussions

A sum of 116 of endophytic bacteria were isolated from the stem of the tomato, eggplant and chili. *R. solanacearum* was also freshly isolated from infected eggplant since it loses virulence and viability easily when continuously sub-cultured onto the laboratory media and stored at 4 °C (Champoiseau *et al.*, 2009). Only 2 bacterial endophytes were subjected to identification since both were the most efficient in outgrowth the *R. solanacearum* in dual culture assay. The 16S rDNA was performed to identify the endophytes as the colony phenotype cannot be trusted 100% (Sousa *et al.*, 2013). Besides, Young (2007) also proposed that environment may influence the bacterial characteristics.

The molecular identification was carried out to support the cultural and morphological identification. The diameter of *E. tabaci* single colony is two mm. The colony appearances of the *E. tabaci* matched to the study by Duan *et al.* (2015) which has smooth looking, yellowish in color and round shape margin. *E. tabaci* is a Gram-negative bacterium with a thin peptidoglycan layer in its cell wall. Even with a thin cell wall layer, the bacterium is capable to endure the ultimate pH and temperature (Beveridge, 1999) as well as to withstand a three atmospheric pressure (atm) turgor pressure (Koch, 1998). Nevertheless, the cocci shape of *E. tabaci* in this study is in contrast to the rod shape of the Enterobacteriaceae family. This can be explained by the abiotic and biotic stresses from the environment imposed upon the bacterium (Philippe *et al.*, 2009). *Escherichia coli* is often used as the model in shape changing study (Kerksiek, 2009; Philippe *et al.*, 2009; Pilizota and Shaevitz, 2014). The shape changes from rod to cocci when there is a difference in surrounding osmotic pressure. Besides, to endure a harsh environment, it triggers the *E. coli* to reduce its size from rod to cocci. It is because when under stress condition, the binding protein that involved in synthesizing the peptidoglycan is distracted. The distraction disturbs the formation of cell wall and leads to a reduced size bacterium. Therefore, it can be deduced that the odd shape of *E. tabaci* in this study is due to evolution and adaptation from the changing environment the bacterium faces.

The size of *B. cereus* single colony is 4 mm. The characteristics of *B. cereus* in this study match to the Bacillaceae. The colony has a dry texture and opaque. The cell is rod-shaped and stained

purple in the Gram test indicating a Gram-positive bacterium. Gram positive bacterium has thick peptidoglycan layer, thus more rugged compares to the Gram negative bacterium.

Based on the previous literature, *B. cereus* was reported to secrete several antibiotics such as zwittermicin A and kanosamine to suppress the *Phytophthora medicagini* growth and inhibit oomycete of certain fungi and bacteria (Stabb *et al.*, 1994; Milner *et al.*, 1996). *B. cereus* also was reported to induce resistance in *Arabidopsis thaliana* by triggering expression of several pathogenesis-related (PR) protein and suppress the growth of *Pseudomonas syringae* pv. *tomato* (Niu *et al.*, 2011). Since not much discoveries on the inhibition mechanisms by *E. tabaci*, we assumed it exerted much less antagonistics mechanism as the other *Enterobacter* sp. to defy the phytopathogen. *Enterobacter agglomerans* was recorded to excrete pyrrolnitrin, an antibiotic to inhibit bacterial phytopathogen such as *Clavibacterium michiganense* (Chernin *et al.*, 1996). Other than pyrrolnitrin, the performance of antibiotic named 2,4-diacetylphloroglucinol (DAPG) by *Enterobacter cloacae* was recorded to suppress the *R. solanacearum* growth successfully (Ramesh *et al.*, 2009). Meanwhile, *Enterobacter asburiae* colonized the chaff and seed of *A. thaliana* and out competed and reduced the bacterial cells of *Escherichia coli* and *Salmonella enterica* (Cooley *et al.*, 2003).

Conclusion

This study yielded 2 promising biocontrol agent against BW disease caused by *R. solanacearum* which were identified as *E. tabaci* and *B. cereus*. The study also reported for the first time on the novel bacterium, *E. tabaci* isolated from chili in Malaysia as well as potential biocontrol agent globally. For the future works, the mechanisms of inhibition exerted by the *E. tabaci* and *B. cereus* obtained in this study will be carried out.

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Effect of organic materials application on production of cocoa pods and bean quality

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Abstract

A study has been carried out to determine the effects of different fertilizer treatments on cocoa matured tree as listed; T1 – mix of inorganic fertilizer, T2 - chicken manure, T3 - cow manure, T4 – empty fruit bunch and T5 – cocoa pod husk on the pod and bean quality toward its production. The result obtained showed that the organic materials application had given a similar effect as inorganic fertilizer on the pod and bean quality, hence consistently produced a positive production similar to the inorganic fertilizer. Therefore, this study has indicated the potential effect of organic materials application, particularly on the pod and bean qualities toward its production of cocoa matured trees.

Keywords: Cocoa, Fertilizer, Organic materials, Production, Waste by-product

Introduction

Fertilizers are organic or inorganic materials which are added to the soil to supply certain essential elements to plants for growth (Panda, 2010). They have played an important role in increasing crop production, including cocoa. Global consumption of fertilizers is steadily increasing every year as the demand from growers is continuously rising particularly for inorganic fertilizers. However, with the recent increase in the price of inorganic fertilizers and environmental issues, the best alternative is using organic fertilizers optimally.

Organic fertilizers are naturally occurring materials of biological or mineral origin (Allen, 2010). Although organic fertilizers are low in nutrient concentration or solubility or both, but the slow release of nutrients makes them available for a longer period. In plantation and livestock farming, there is abundance of waste by-products (biomass and manure) available that can be used as a source of nutrient. The application of these organic materials is fundamentally important to supply various kinds of plant nutrients, including micronutrients, improve soil physical and chemical properties, nutrient holding and buffering capacity, and enhance microbial activities (Suzuki, 1997).

Earlier study by Thong and Ng (1978) found that the nutrient uptake by cocoa is quite high (400 kg N, 40 kg P and 500 kg K with 5-years old matured cocoa). However, some of these

requirements have been met through the appropriate use of cocoa pod husk, poultry manure and mineral fertilizer in the release of nutrient for crop production and yield in Southwestern Nigeria (Ayeni et al., 2008). Another study also has shown the effectiveness of nutrient-rich oil palm residues, coconut husks and other agricultural wastes in a cocoa plantation (Sharifuddin and Zaharah, 1987). With these studies, it showed that the application of organic materials such as crop residues and livestock manure has been found to bring about a steady improvement in soil productivity and crop performance especially in cocoa. Therefore, the objective of this study was to determine the effects of different composted materials, including chicken manure, cow manure, empty fruit bunch and cocoa pod husk in comparing with a mix of inorganic fertilizer of Urea, TSP and MOP, on the pod and bean qualities, and towards its production. The result from this study was expected positively contributed to the use of organic fertilizer (materials) in cocoa cultivation and its significant effects on the qualities of the pod and bean, and eventually the production.

Materials and Methods

The trial was conducted at Malaysian Cocoa Board Research and Development Centre in Madai, Kunak. The design of the trial was Randomized Complete Block Design (RCBD) with three replicates. Planting material was clone BR 25 (Class II Clone; Pod Weight (PW): 395 g, No. Bean per Pod (BNP): 40, average of dry bean weight (ADBW): 1.00 g, and pod value (PV): 25) and approximately 7 years old. The treatments in the study based on cocoa nutrient uptake – 400 kg N, 40 kg P and 500 kg K on the cocoa matured tree. The treatments were; T1 – Mix of inorganic fertilizer (0.86 ton/ha/year Urea (46% N) + 0.086 ton/ha/year TSP (46% P₂O₅) + 1.0 ton/ha/year MOP (50% K₂O)), T2 – Chicken manure (3% N, 1% P, 2% K = 13.3 ton/ha/year), T3 – Cow manure (2% N, 1% P, 2% K = 20.0 ton/ha/year), T4 – Empty fruit bunch (1.5% N, 0.02% P, 1.28% K = 26.6 ton/ha/year) and T5 – Cocoa pod husk (2.18% N, 2.15% P, 3.54% K = 18.3 ton/ha/year). Each treatment was applied every two months onto 35 trees and total of the experimental size was about 0.5 ha. All harvested pods were cut, and beans fermented in a wooden box for five (5) days with one turning on day 3. Then the beans were sun-dried for seven days attaining 6 to 7% moisture content. Samples of the dried bean from each treatment were taken to the laboratory for bean analyses. All experimental plots were given similar agronomic practices according to the field program; including pruning activity, application of weedicide and pesticide. Below were the parameters taken for the study:

i. Pod and bean analysis

Pod and bean analyses were conducted according to MCB (n.d.). Five (5) pods were sampled out from each plot during peak harvest and measured for their length, diameter and girth of pods (cm), weight of harvested pods (kg), average number of beans/pods, single dry bean weight (g) and pod value (number of pods to produce 1 kg dry beans).

ii. Production

Production were evaluated according to Osman et. al. (1994). The number of pods per tree was taken every month to determine the production of pods. Successful pods having a perimeter of 20 – 25 cm were counted and marked with blue paint. The number of pods were recorded

starting from 2 months after all treatments applied. Mature pods were harvested twice a month or 10-14 days interval and total number of harvested mature pods were recorded to determine the actual yield of harvested mature pods.

iii. Soil sampling

Soil and leaf sampling were conducted according to Denamany & Rosinah (1994). Soil samples from depths of 0 – 20 cm were taken at the end of project. They were sent to the laboratory for analysis of soil pH, total nitrogen (%), available P (ppm) and exchangeable K (cmol (+) kg⁻¹).

Statistical analysis

Statistical analysis was carried out for one-way ANOVA and Tukey's multiple comparison tests for all data obtained using Statistical Product and Service Solutions (SPSS 21.0) software.

Results and Discussion

Effect of Different Types of Fertilizer Application on Pod and Bean Quality

The results of pod qualities such as length, diameter, width and weight of pod suggest that there were no significant differences ($p > 0.05$) between the treatments (Table 1). This indicated that all treatments did not give major effect on the qualities of the pod parameters. Despite that, all treatments except T2 had slightly affected the pod by increasing its weight, whereas the mean value was ranging from 408.0 g to 451.5 g, which was beyond the expected average pod weight of 395.0 g. It was thought once that such increased weight could give a positive effect on the quality of the bean. However, according to Wood and Lass (1985), pod characteristics were probably the last factor that could affect the cocoa production, but it is relatively important, particularly in the breeding area for determining the pod quality and its identification.

Table 1. Total mean on the pod and bean qualities for five (5) different types of fertilizer

Variables	T1	T2	T3	T4	T5
Pod Qualities					
Pod length (cm)	15.74 ± 0.25 ^a	16.00 ± 0.80 ^a	16.27 ± 0.19 ^a	16.07 ± 0.61 ^a	16.35 ± 0.41 ^a
Pod diameter (cm)	25.33 ± 0.05 ^a	24.83 ± 0.39 ^a	25.11 ± 0.15 ^a	24.75 ± 0.33 ^a	24.55 ± 0.17 ^a
Width (cm)	7.85 ± 0.04 ^a	7.67 ± 0.17 ^a	7.86 ± 0.05 ^a	7.74 ± 0.13 ^a	7.64 ± 0.08 ^a
Weight of pod (g)	451.5 ± 15.50 ^a	380.5 ± 6.50 ^a	439.5 ± 15.12 ^a	408.0 ± 20.65 ^a	418.0 ± 3.79 ^a
Bean Qualities					
Number of beans/pod	29.58 ± 2.86 ^a	28.72 ± 2.94 ^a	26.56 ± 0.41 ^a	29.46 ± 1.28 ^a	26.91 ± 0.55 ^a
Average dry bean weight (g)	0.91 ± 0.05 ^a	0.94 ± 0.07 ^a	0.84 ± 0.04 ^a	0.83 ± 0.02 ^a	0.87 ± 0.04 ^a
Pod value	44.76 ± 7.47 ^a	43.10 ± 2.22 ^a	48.27 ± 3.32 ^a	46.38 ± 2.24 ^a	44.45 ± 3.47 ^a

Means with same letter (superscript) within rows are not statistically different using Tukey's at $P > 0.05$ probability level. (mean ± S.E.). Treatments - T1– Inorganic fertilizer, T2 – Chicken manure, T3 – Cow manure, T4 –Empty fruit bunch and T5 – Cocoa pod husk.

As for parameters of bean qualities such as number of beans per pod (BNP), average of dry bean weight (ADBW) and pod value (PV), the results also indicated that there are no significant differences ($p>0.05$) between the treatments. This suggests that all treatments had a similar effect on the bean qualities. For the BNP, all treatments were ranging between 26.91 to 29.58 beans per pod which below the average of 40. Average of dry bean weight (ADBW) for all treatments also between 0.79 g to 0.92 g, less than the expected average bean weight of 1.00 g. Consequently, these two parameters have given a relatively higher pod value ranging from 43.10 to 48.27, beyond the range of an acceptable pod value of 25 and below, thus might affect the yield of cocoa production. However, according to Mainstone and Thong (1978), potassium that often associated with an increase in the fresh pod weight, could also be the factor in increasing the number of pods and at the same time decrease the weight of the pod contents by reducing either the mucilage weight, or the number of beans, or the bean weight, which correlate with pod value. Therefore, the reason for high pod value for all treatments can be related to the availability and enough uptake of potassium in the soil.

Effect of Different Types of Fertilizer Application on Crop Pattern and Production

Generally, pods can be harvested throughout the year. Nevertheless, there are two main fruiting seasons for cocoa depending on the climate condition. As the rainfall distribution pattern at experimental site uniform throughout the year with a mean total annual rainfall almost 2000 mm (Table 2), it clearly illustrated that the peak months of cropping for all treatments in Figure 1 were less pronounced and appeared to be the same (July – September and December – March). Besides that, this similar crop pattern when it is expressed in average of dried beans per month showed no significant difference ($p>0.05$) between the treatments as stated in Figure 2.

The production of cocoa for twenty-one (21) months after its first application of all treatments had shown that the effect of treatments on the yield production of total cumulative dried bean appeared to be consistent throughout the study. Surprisingly, the highest production was in T2 (1743.7 kg/ha) followed by T3 (1734.4 kg/ha), T4 (1707.6 kg/ha), T1 (1691.5 kg/ha), and T5 (1447.7 kg/ha). The difference in production among the treatments was influenced by yield components, as has been shown in T2 where it produced the highest of ADBW and made the lowest pod value among the treatments that eventually affect the production of cocoa. However, these varying results gave no significantly different ($p>0.05$) between the treatments. Therefore, it is reasonable to assume that all treatments have produced the same result of cocoa production.

Table 2. Rainfall distribution per month from year 2015 – 2016

Month	2015	2016
	Rainfall Distribution (mm)	
January	593.8	47
February	45	63.1
March	113.1	102.2
April	63.9	102
May	130.4	127.8
June	165.6	120.5
July	70.9	127.7
August	130.5	224.5
September	63.9	244.6
October	107.7	272.6
November	188.4	215.8
December	223.7	217.6
Total	1896.9	1865.4

Source: Cocoa Research & Development Centre of Madai, Kunak, Sabah

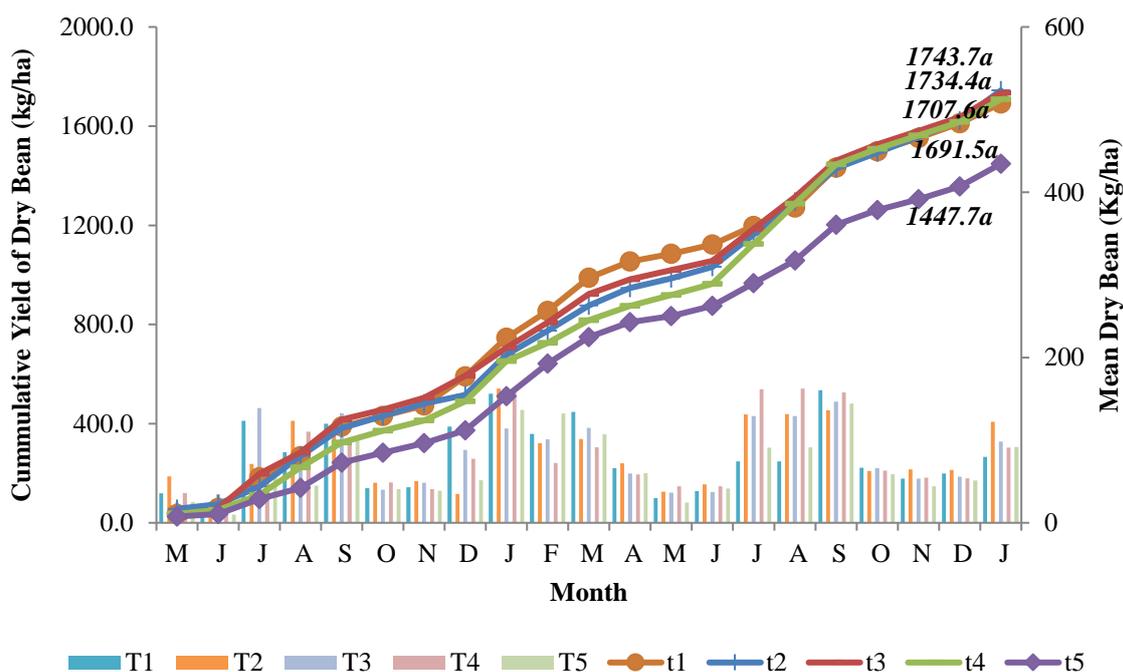


Figure 1. Crop pattern and its cumulative production for (5) different types of fertilizer. Means with same letter are not statistically different using Tukey’s at $p>0.05$ probability level. Crop pattern: - T1 – Inorganic fertilizer, T2 – Chicken manure, T3 – Cow manure, T4 –Empty fruit bunch and T5 – Cocoa pod husk; Cumulative yield dry bean: - t1– Inorganic fertilizer, t2 – Chicken manure, t3 – Cow manure, t4 –Empty fruit bunch and t5 – Cocoa pod husk.

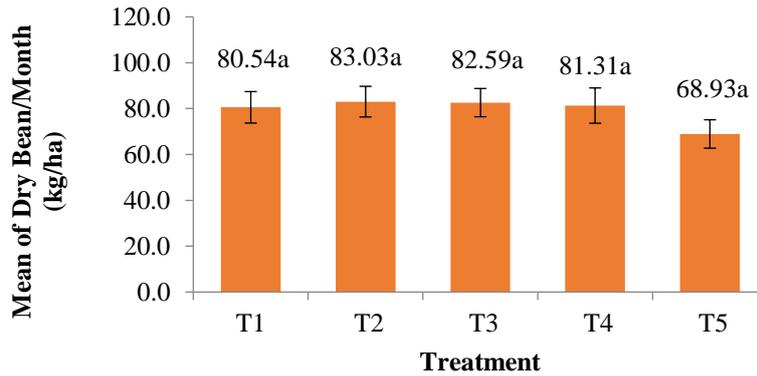


Figure 2. Mean of dried bean per month for twenty-one (21) months period. Means with same letter are not statistically different using Tukey's at $p > 0.05$ probability level. Treatments - T1– Inorganic fertilizer, T2 – Chicken manure, T3 – Cow manure, T4 –Empty fruit bunch and T5 – Cocoa pod husk.

Effect of Different Types of Fertiliser Application on Soil

Results from the soil analysis (Table 3) has shown that there were no significant differences ($p > 0.05$) for all nutrient levels between the treatments. The result also indicated that soil pH was low and has probably affected the availability of plant nutrients, though lime was applied onto the soil twice annually in correcting the soil pH. This can be seen in the concentration of nitrogen in soil as it was lower than adequate range where the mean value ranging from 0.09 % to 0.15 % for 0.16 %. For phosphorus, the concentration nutrient showed that all treatments having less than adequate range (> 15.0 ppm) with a mean value between as low 2.80 ppm to 13.25 ppm. However, for potassium, all treatments have shown higher concentration nutrient than adequate range (0.24 cmol/kg) which ranging between 0.44 cmol/kg to 0.59 cmol/kg. By taking enough potassium from the soil, the development of fruit and seeds should be improved, as has been shown in bean qualities of this study on number bean per pod (BNP), average dry bean weight (ADBW) and its pod value (PV) towards the production of cocoa.

Table 3. Adequate range and total mean of soil analysis for five (5) different types of fertilizer applied

Treatment	pH (H ₂ O)	Total N (%)	Available P	Exch. K (cmol)
Adequate	5.5 -6.5	> 0.16	> 15	> 0.24
T1	4.80 ± 0.25^a	0.10 ± 0.01^a	18.50 ± 9.65^a	0.52 ± 0.08^a
T2	4.73 ± 0.28^a	0.11 ± 0.01^a	22.80 ± 10.46^a	0.42 ± 0.09^a
T3	4.50 ± 0.00^a	0.11 ± 0.008^a	7.20 ± 0.50^a	0.43 ± 0.05^a
T4	4.53 ± 0.06^a	0.11 ± 0.003^a	16.70 ± 13.70^a	0.36 ± 0.09^a
T5	4.53 ± 0.20^a	0.09 ± 0.008^a	26.53 ± 13.70^a	0.35 ± 0.04^a

Means with same letter (superscript) within columns are not statistically different using Tukey's at $P > 0.05$ probability level. (mean \pm S.E.) Treatments - T1 – Inorganic fertilizer, T2 – Chicken manure, T3 – Cow manure, T4 –Empty fruit bunch and T5 – Cocoa pod husk. *Source: Wong, I. F. T. (1974) (revised) – Soil-crop suitability classification for Peninsular Malaysia, Soils and Analytical Services Bulletin Nr.1, Ministry of Agriculture, Kuala Lumpur)

Conclusion

Overall, this present study has clearly demonstrated the potential effect of organic materials application, particularly on the pod and bean qualities toward its production of cocoa matured trees. Soil nutrients, particularly potassium, also essentially affect the efficacy of the organic materials and its intake by cocoa matured trees.

Acknowledgements

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Effect of silicon and nitrogen fertilizer application on growth, lodging and yield of rice (var. Serendah Merah)

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Abstract

Lodging of rice plants in paddy fields can reduce yield and cause economic loss to the farmers. A field experiment was conducted at the Faculty of Sustainable Agriculture (FSA) University Malaysia Sabah, Sandakan campus to determine whether the application of a high rate of nitrogen and silicon can improve the yield of traditional rice variety by reducing the effect of lodging. The experimental design used was factorial completely randomized design (CRD) by using 60 kg/ha, 120 kg/ha and 180 kg/ha of Nitrogen fertilizer and 0 kg/ha, 200 kg/ha and 400 kg/ha of the silicon fertilizer with three replications in each treatment. Data were analyzed with two-way analysis of variance (ANOVA) by using Minitab 16 Statistical Analysis Software. The means were compared at $P < 0.05$ by using Tukey's test. The results revealed that application of nitrogen at the rate of 120 kg/ha produced the highest 1,000 grains weight (27.09 g) and highest extrapolated yield (5.42 t/ha) but showed no significant effect between nitrogen rates. In the case of silicon, the largest culm diameter (10.29 mm) was from 400 kg/ha Si. Although there were no significant differences for the interaction between N and Si on all yield parameters, the highest Si rates of 400 kg/ha showed a significant effect on culm diameter. Hence in view of the needed resilience from the impact of climate change, the application of silicon fertilizer is recommended in order to strengthen the culm of the rice plant to withstand lodging.

Keywords: Sabah traditional rice, fertilizer rates, lodging, grain yield, culm size

Introduction

Rice is the most commonly produced and consumed food grain in Malaysia. But rice production in Sabah is considered very low, which is only 2.8 t/ha compared to the national average production of 5 t/ha (The Star Online, 2016). In Sabah, a huge diversity of local photoperiod-sensitive rice is still grown (Mackill, *et al.*, 1996). Taller characteristic helps the rice to tolerate the flood-prone areas. The height of traditional rice can exceed 140 cm, hence the taller plant is more susceptible to lodging when there is waterlogging. Rice production can be increased by addition of high nitrogen level, but it can also cause weak and skinny stems that are too feeble to hold themselves up. This is known as the stem lodging effect on plants (Grant, 2016).

Lodging is the phenomenon that rice plants topple over to the ground surface due to high wind speed, heavy rainfall, storm, weaker stem, longer internode, lignin concentration, amount of

internode overlap by leaf sheath, culm diameter, culm wall thickness, soil condition and also heavy spikelet (Wang and Hoshikawa, 1991, Ookawa and Ishihara, 1993 as cited in Mackill, *et al.*, 1996). Hence there is a need to study the resilience of the rice crop in view of the impending impact of climate change on rice production.

Rice is the crop that will uptake large amount of silica for their growth and development (Srivastava and Mahapatra, 2012). Takahashi (1995) reported that silicon application on rice would increase the rice production efficiency by one-third and the increase in un-hulled grain by 450% (cited in Fageria, 2014; Deivaseeno, *et al.*, 2017). Meanwhile, the same authors also mentioned that when Si is applied in the correct amount, plant lodging can be minimized even when a high rate of nitrogen is applied. Silicon enhances the photosynthesis process by reducing the drooping of the leaf blade and improves rice growth by maintaining more erect leaves and thus increase exposure to the solar radiation (Panda, 2010; Fageria, 2014).

Materials and methods

Study Site and duration of study

This experiment was conducted in the net house structure of the Faculty of Sustainable Agriculture (FSA), University Malaysia Sabah (UMS) located at latitude 5° 55'N and longitude 118° 02'E. The period of this study was from February 2017 until October 2017, during which data for the vegetative growth, yield components and soil analysis were collected.

Experimental design and cultural practice

The Serendah Merah variety was used as a local traditional rice variety in this experiment. The rate of Nitrogen, N fertilizer were 60 kg/ha, 120 kg/ha and 180 kg/ha while the rate of Silicon, Si fertilizer were 0 kg/ha, 200 kg/ha and 400 kg/ha.

Parameters

The parameters for vegetative growth were taken at the interval of seven days throughout the various growth stages. The vegetative growth parameters include plant height (cm), number of tillers per hill, the percentage of productive tillers (%), culm diameter (mm), internode length (cm) and panicle length (cm). The yield components were taken once the rice plant reached maturity. The parameters taken were number of grains per panicle, the number of filled grains per panicle, number of empty grains per panicle, the percentage of filled grains per panicle (%), 1,000 grains weight (g), extrapolated yield (t/ha) and dry matter of leaf, stem and root.

Statistical analysis

The collected data was analyzed with two-way analysis of variance (ANOVA) by using Minitab 16 Statistical Software. The means were compared at $P < 0.05$ by using Tukey's test.

Results and Discussion

Nitrogen fertilizer affects vegetative growth and plant height of the rice plants. From the results of this study, 120 kg/ha N contributed to the tallest plant height when compared to 60 kg/ha N, thus in agreement with many earlier researchers since Nitrogen is an important structural constituent of plant cells such as proteins, nucleic acids and amino acids (Srivastava and Mahapatra, 2012; Bhiah *et al.*, 2010). In addition, Chaturvedi (2005) reported that higher rate of nitrogen fertilizer results in increased leaf area with increased photo assimilates thereby more dry matter accumulation, which contributes to higher plant height. Plant height increased gradually from 28 DAT until 42 DAT when the silicon fertilizer rate was increased from 0 kg/ha to 400kg/ha. Ma *et al.* (1989), Fallah (2012), Ghanbari *et al.* (2011) and Pati *et al.* (2016) have mentioned positive effect of silicon on plant height as silicon has the function to make the leaf and stem of rice plant more erect. Results of this study agreed with the findings of the earlier researchers in that the highest rate of silicon fertilizer, 400 kg/ha contributed to the maximum plant height during the vegetative growth starting from 28 DAT until 42 DAT. Figure 1a shows the effect of nitrogen fertilizer on plant height at 21 DAT; Figure 1b shows the effect of silicon fertilizer on plant height at 28, 35 and 42 DAT; and Figure 1c shows the effect of N and Si fertilizers on plant height throughout the growing period.

The number of tillers was the highest when nitrogen rate was highest. This scenario can be explained by the fact that high nitrogen gave high transport efficiency in the vascular bundle and most of the nutrients were retained in the straw for late emerging tillers (Wang *et al.*, 2017). Meanwhile, the data from this study showed that the higher the silicon fertilizer applied, the higher the number of tillers produced by the rice plant and agreed with the research done by Pati *et al.* (2016). This study showed similar results to those done by Deivaseeno *et al.* (2017) that rice plant with silicon application showed increased number of tillers per hill.

This study found significant effect ($P < 0.05$) on the number of tillers during tillering stage at 21 DAT when different treatments of nitrogen and silicon fertilizers were applied. However, this result was contrary to the studies done by Mauad *et al.* (2003) and Ghanbari *et al.* (2011) who found no significant effect for the number of tillers per hill when different treatments of nitrogen and silicon fertilizers were applied. This scenario can be explained by genetic and environmental factors as the number of tillers per hill is dependent on these factors as cited by Mauad *et al.* (2003). Figure 2a shows the effect of nitrogen fertilizer on number of tillers per hill at 70 DAT; Figure 2b shows the effect of silicon fertilizer on number of tillers per hill at 70 DAT; and Figure 2c shows the effect of N and Si fertilizers on number of tillers per hill at 21 DAT.

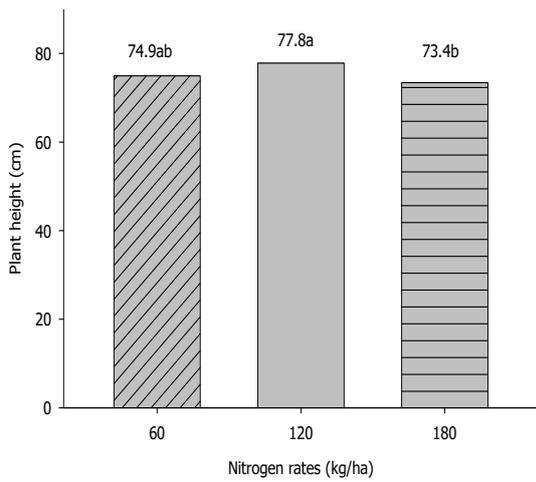


Figure 1a

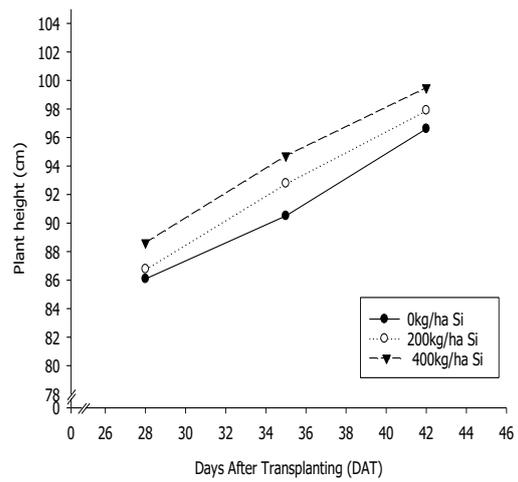


Figure 1b

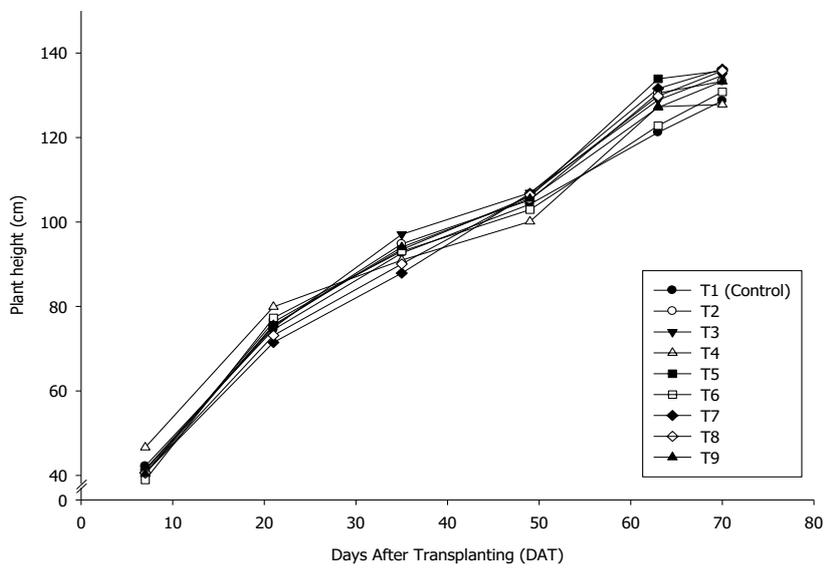


Figure 1c

Figure 1 (a) Effect of nitrogen fertilizer on plant height at 21 days after transplanting (DAT); (b) Effect of silicon fertilizer on plant height at 28, 35 and 42 DAT; (c) Effect of N and Si fertilizers on plant height throughout the planting period.

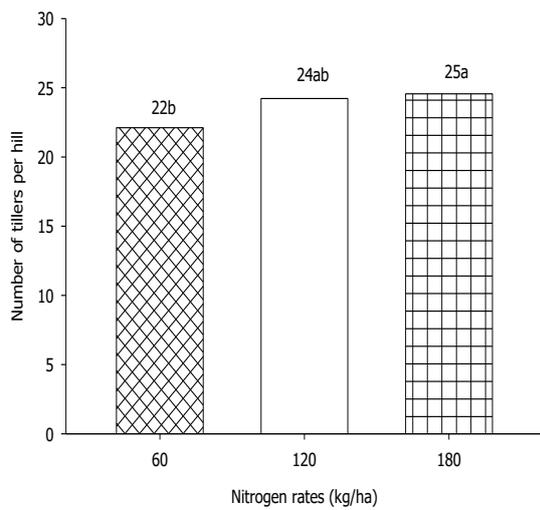


Figure 2a

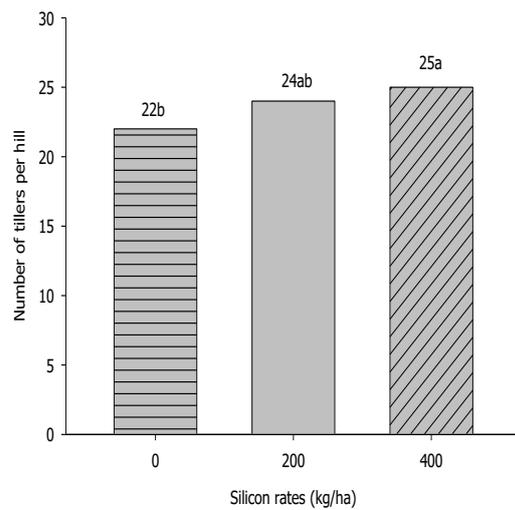


Figure 2b

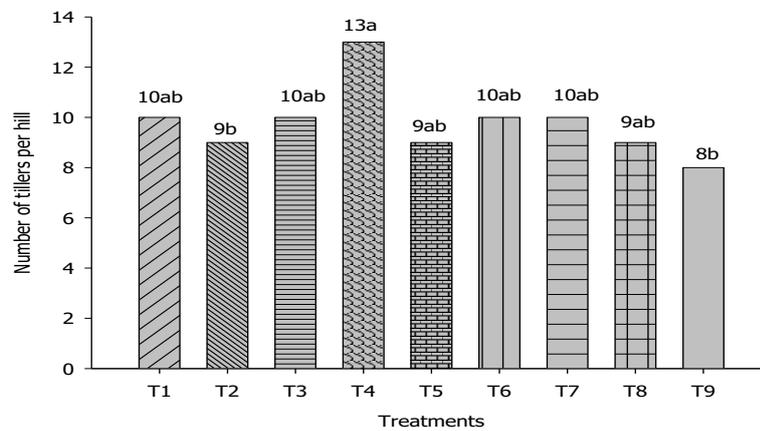


Figure 2c

Figure 2 (a) Effect of nitrogen fertilizer on number of tillers per hill at 70 DAT; (b) Effect of silicon fertilizer on number of tillers per hill at 70 DAT; (c) Effect of N and Si fertilizers on number of tillers per hill at 21 DAT.

There was no significant difference ($P>0.05$) of culm diameter when three different rates of nitrogen fertilizer were applied but the results revealed that the higher the nitrogen rate, the wider the culm diameter. This was consistent with the previous study done by Zhang *et al.* (2016) who described that high nitrogen fertilizer application helps to improve biomass production of the rice plant such as the culm diameter. Table 1 shows the mean values for vegetative growth parameters.

Table 1. Mean values for vegetative growth parameters

Treatment	Culm	Percentage of	Panicle	Internodes
Rates of N (kg/ha)	mm	%	cm	cm
60	9.60 ^a	91.56 ^a	23.0 ^{ab}	39.3 ^a
120	9.73 ^a	87.00 ^a	22.7 ^b	35.3 ^a
180	10.11 ^a	86.95 ^a	24.2 ^a	35.2 ^a
Rates of Si (kg/ha)				
0	9.36 ^b	84.66 ^b	23.2 ^a	34.4 ^a
200	9.80 ^{ab}	92.07 ^a	23.3 ^a	38.4 ^a
400	10.29 ^a	88.79 ^{ab}	23.3 ^a	36.9 ^a
Value of F ²				
Rates of N	0.357 ^{ns}	0.117 ^{ns}	0.018*	0.150 ^{ns}
Rates of Si	0.057*	0.022*	0.979 ^{ns}	0.236 ^{ns}
N x Si	0.947 ^{ns}	0.606*	0.558 ^{ns}	0.464 ^{ns}

Means followed by a common letter do not differ by Tukey test (P<0.05)

There was a positive effect on culm diameter between three different rates of silicon fertilizers ranging from 0 kg/ha until 400 kg/ha of silicon. According to Dorairaj *et al.* (2015) and Deivaseeno *et al.* (2017), silicon is associated with the rigidity of the rice culm and thus lodging could be minimized as silicon increase the mechanical or physical strength of the rice culm by increasing the culm diameter. Moreover, Yoshida *et al.* (1962) mentioned that increased in culm diameter when silicon was applied could be due to the formation of thicker cell walls below the cuticle layer. In this study, rice plant with no silicon fertilizer application gave the minimum culm diameter of 9.36 mm whereas 400 kg/ha silicon gave the maximum culm diameter of 10.29 mm which was consistent with previous studies done by Fallah (2012) and Zhang *et al.* (2016).

Percentage of productive tillers decreased gradually from application of 60 kg/ha N to 180kg/ha N but showed no significant difference (P>0.05) when different nitrogen rates were applied. This situation happened due to the nutrients stored in rice straw for late emerging tillers thus makes the tillers unable to produce grain at a shorter time (Wang *et al.*, 2017). Silicon rate of 200 kg/ha gave the maximum percentage of productive tillers which was 92.07 % whereas rice plant with no silicon fertilizer application contributed the lowest percentage of productive tillers which was 84.66 %.

The results of this study are in agreement with those done by Yogendra (2014) and Deivaseeno *et al.* (2017) who reported that silicon helps to increase percentage of productive tillers; but the results contradicted to the research done by Ahmad *et al.* (2013). Pereira *et al.* (2004) stated that increase in silicon fertilizer helps to improve the percentage of productive tillers of the rice plant. Meanwhile, Yogendra (2014) explained this case occurred because rice plant makes use of the advantage of the nutrients supplied to the plant.

The results also showed that when nitrogen rate was increased from 60 kg/ha to 180 kg/ha panicle length experienced a decreasing trend followed by increasing trend. This finding showed a significant difference ($P < 0.05$) on the panicle length between 120 kg/ha and 180 kg/ha of nitrogen fertilizer application which was consistent with the studies done by Saadati and Fallah (1995) and Salman (2012) who reported that nitrogen had a significant effect on panicle length. According to Asif *et al.* (2000), the increased of panicle length when the nitrogen rates increased is due to sufficient availability of fertilizer at panicle emergence stage. Panicle length is an important indicator to improve the plant architecture and grains yield (Liu *et al.*, 2016). The results showed no significant difference ($P > 0.05$) in the panicle length when different rates of silicon fertilizer were applied. Yao *et al.* (2015) explained that panicle length of rice plant might be affected by the environmental condition and controlled by multiple genes.

According to Mulder (1954), there exist a relationship between basal internode length and the weight of upper parts in relation to the lodging of the rice plant. The result of this study showed no significant difference ($P > 0.05$) on the internode length when rice plants applied with different rates of nitrogen fertilizer. Rice plant applied with 60 kg/ha nitrogen gave the highest internode length of 39.3cm whereas rice plant applied with 120 kg/ha and 180 kg/ha nitrogen contributed 35.3 cm and 35.2 cm of internode length respectively. This result is similar to Li *et al.* (1979) and Ling *et al.* (1994) who stated that basal internode length may be affected by the solar radiation that hit on the basal stem which linked to the factor of leaf canopy and also a maximum number of tillers produced at panicle initiation. This meant that higher nitrogen rates would have bigger leaf canopy and influenced the growth and development of basal stem as the leaf block the sunlight from reaching to the basal stem. In addition, higher nitrogen rates will make the rice plant produce more tillers thereby influence the growth and development of the basal internode. Internode length is an important morphological characteristic to indicate the susceptibility to lodging of the rice plant (Salman, 2012). The result of this study showed no significant difference ($P > 0.05$) on the internode length when different rates of silicon fertilizer were applied to the rice plant. The internode length increased from 34.4cm (0 kg/ha Si) to 38.4cm (at 200 kg/ha Si) but decreased to 36.9cm (at 400kg/ha Si). This result contradicted with the research results obtained by Salman (2012) who reported that silicon application did not contribute to the maximum internode length. According to Windslow *et al.* (1997), this scenario happened can be due to the factor that silicon uptake is different in rice varieties as some rice varieties will absorb more silicon fertilizer but other varieties may not.

Table 2 shows the mean values for the rice yield parameters. The findings showed no significant difference ($P > 0.05$) on number of grains per panicle when different rates of nitrogen fertilizers were applied, and this finding matched with the results of the experiment done by Arf (1993) who reported that an increase in nitrogen rate did not show any increment in number of grains per panicle but contradicted with the result of Barbosa (1987). According to Mauad (2003), the number of grains per panicle decreased from 105 grains to 100 grains when the nitrogen rate was increased from 60 kg/ha to 120 kg/ha may be due to the rice variety as traditional rice variety has a poor response to nitrogen fertilizer. Meanwhile, the results showed no significant difference ($P > 0.05$) on number of grains per panicle when different rates of silicon fertilizers were applied; this result was similar to research done by Carvalho (2000) who

also reported that there was no influence of silicon fertilizer on the number of grains per panicle but contradicted to study done by Deren et al. (1994) and Barbosa (1987) who reported significant difference occurred in the number grains per panicle when different rates of silicon fertilizers were applied. Since there were no significant differences on the number of grains per panicle from nitrogen and also silicon fertilizers, there will be no significant difference also on the number of grains per panicle with the combination of nitrogen and silicon fertilizers.

There was a decrease in the number of filled grains per panicle from 74 grains to 68 grains when nitrogen rate was increased from 60 kg/ha to 120 kg/ha. This case occurred due to mutual shading of the leaf which affected the photosynthesis process and thus reduced the photo-assimilates production (Mauad *et al.*, 2003). However, maximum number of filled grains per panicle (77) came from maximum nitrogen application (180 kg/ha N) as rice crop is positively correlated with the amount of N absorbed by the end of the spikelet initiation stage or by flowering and improved of plant architecture (Fageria, 2014, Deren *et.al.*, 1994). Although the number of filled grains increased then maintained when silicon rates increased from 0 kg/ha to 200 kg/ha and lastly to 400 kg/ha, there was no significant difference ($P>0.05$) on the number of filled grains per panicle. Ma *et al.* (2006) reported that any interruption of silicon supply during the reproductive period would affect the grains fertility. The results of this study agreed with the findings of Jawahar *et al.* (2015) who mentioned that silicon fertilizer facilitates the process of assimilation of carbohydrate in the panicle thereby helps in increasing the number of filled grains.

According to Miles *et al.* (2014), empty grains may be affected by the shorter grains length and grains that matured early as these characteristics tend to reduce the chances of getting empty grains. The findings showed there was no significant different ($P>0.05$) on the number of empty grains per panicle for three different rates of nitrogen fertilizer but the number of empty grains per panicle increased gradually when nitrogen rate was increased from 60 kg/ha to 180 kg/ha of nitrogen. This phenomenon can be related to higher nitrogen rate that increase the vegetative growth thereby causing a delay in heading as the maximum vegetative growth absorbed most of the sugars that otherwise use to fill the grains (Miles *et al.*, 2014; Mauad *et al.*, 2003). Thus, the number of empty grains per panicle increased when nitrogen rate increased even though there was no significant difference. Meanwhile, the number of empty grains per panicle decreased gradually from 0 kg/ha to 400 kg/ha of silicon. Silicon fertilizer helped in making the rice more erect by reducing the leaf angle and thus reduce the chances of the leaf undergoes mutual shading meanwhile improve the exposure to solar radiation (Panda, 2010; Fageria, 2014). This behavior then facilitated the solar energy use efficiency thereby transport those assimilate of carbohydrates for grain filling.

The 1,000 grains weight increased from 26.43 g to 27.09 g and dropped to 26.33 g when the nitrogen rate was increased from 60kg/ha to 120 kg/ha and lastly is 180 kg/ha. According to Mauad (2003), the decreased of the 1,000 grain weight from 27.09 g to 26.33 g when the nitrogen rate was increased probably because the amount of assimilate was not enough to fill the greater number of grains produced. In addition, higher nitrogen rate will increase the vegetative growth thereby maximum vegetative growth absorb most of the sugars that

otherwise used to fill the grains (Miles *et al.*, 2014). The result showed no significant difference ($P>0.05$) on 1,000 grains weight when different rates of silicon fertilizers were applied to rice plant and this finding diverged the results of Mauad (2003) and Pati *et al.* (2016) who stated that silicon significantly increased 1,000 grains weight of rice plant. To explain the result on this finding, Yoshida *et al.* (1962) explained that transportation and deposition of silicon might depend on transpiration rates that occur in different plant organs. This may relate to the silicon use efficiency and deposition of silicon during the vegetative growth like plant height and culm diameter but not in yield.

The leaf dry weight increased gradually when nitrogen rates were increased from 60 kg/ha until 180 kg/ha as nitrogen is the important nutrient for the plant growth and development associated with leafy and vegetative growth. The results of this study are in agreement with those of Srivastava and Mahapatra (2012) that nitrogen helps in lengthening the leaf with a larger width. Meanwhile, the leaf dry weight increased gradually when silicon rates were increased from 0 kg/ha until 400 kg/ha. Results of studies by Ma and Takashahi (2002) showing beneficial effects of silicon exposed through silicon present in the leaf are also similar to the results of this study. Although there was no significant difference in the leaf dry weight, the leaf dry weight increased gradually when silicon rate was increased from 0 kg/ha until 400 kg/ha.

The stem dry weight increased gradually when nitrogen rate was increased from 60 kg/ha to 180 kg/ha. The result of this study agreed with the statement said by Kang (1980), Epstein (1991, 1999) and Datnoff *et al.* (2007) that fresh and dry weight of stem will increase when an appropriate amount of nitrogen is supplied to the rice plant (cited in Fageria, 2014). Meanwhile, stem dry weight decreased then increased when silicon rate was increased from 0 kg/ha to 400 kg/ha. Ma *et al.*, (1989) explained this behavior related to Si-efficient rice genotype as different varieties of rice have different silicon fertilizer use efficiency.

Table 2. Mean values for yield parameters

Treatment	No. of grains per panicle	No. of filled grains per panicle	No. of empty grains per panicle	1,000 grains weight	Leaf dry weight	Stem dry weight	Root dry weight	Percentage of filled grains	Extrapolated yield
Rates of N (kg/ha)	-	-	-	g	g	g	g	%	t/ha
60	105 ^a	74 ^a	31 ^a	26.43 ^a	13.14 ^a	8.62 ^a	32.76 ^a	70.81 ^a	5.29 ^a
120	100 ^a	68 ^a	32 ^a	27.09 ^a	13.57 ^a	9.69 ^a	33.51 ^a	67.91 ^a	5.42 ^a
180	113 ^a	77 ^a	37 ^a	26.33 ^a	14.06 ^a	10.16 ^a	25.03 ^a	67.76 ^a	5.27 ^a
Rates of Si (kg/ha)									
0	106 ^a	72 ^a	34 ^a	26.79 ^a	12.65 ^a	9.75 ^a	28.89 ^a	68.28 ^a	5.36 ^a
200	106 ^a	73 ^a	33 ^a	26.58 ^a	13.81 ^a	9.08 ^a	31.19 ^a	69.01 ^a	5.32 ^a
400	105 ^a	73 ^a	33 ^a	26.48 ^a	14.31 ^a	9.64 ^a	31.23 ^a	69.18 ^a	5.30 ^a
Value of P									
Rates of N	0.088 ^{ns}	0.111 ⁿ _s	0.311 ⁿ _s	0.068 ⁿ _s	0.489 ⁿ _s	0.119 ⁿ _s	0.141 ⁿ _s	0.432 ^{ns}	0.068 ^{ns}
Rates of Si	0.980 ^{ns}	0.962 ⁿ _s	0.913 ⁿ _s	0.645 ⁿ _s	0.106 ⁿ _s	0.611 ⁿ _s	0.838 ⁿ _s	0.935 ^{ns}	0.645 ^{ns}
N x Si	0.568 ^{ns}	0.665 ⁿ _s	0.700 ⁿ _s	0.992 ⁿ _s	0.639 ⁿ _s	0.896 ⁿ _s	0.474 ⁿ _s	0.769 ^{ns}	0.992 ^{ns}

Means followed by a common letter do not differ by Tukey test ($P < 0.05$)

Root dry weight dropped from 33.51 g to 25.03 g when nitrogen rate was increased from 120 kg/ha to 180 kg/ha may be related to climatic factor or loss of nitrogen due to leaching or runoff. The root weight increased gradually when silicon rate was increased from 0 kg/ha until 400 kg/ha. This behavior may be related to nutrient management. Ma *et al.* (1989) mentioned that making use of effective sources of silicon fertilizer will promote plant growth. Next, the nutrient management involved in the method of application like broadcasting, mixed into the soil, band application or foliar spraying. So, proper nutrient management of silicon fertilizer will help in better establishment of the root system.

This study showed that the percentage of unfilled grains increased when nitrogen fertilizer increased. Mauad (2003) reported that nitrogen application for rice plant affect significantly the percentage of filled grains and said that this phenomenon could be related to the fact that rice plant uses a large amount of nitrogen at the vegetative stage to increase the number of tiller and number of panicles. The same author also said that excessive tillering cause overlapping of leaf, reduce the area exposed to solar radiation, thus reduce the production of carbohydrates that otherwise would be directed to grain filling and increase the number of empty grains, consequently reduce the percentage of filled grains. This study also showed that percentage of filled grains increased gradually when silicon rate was increased from 0 kg/ha until 400 kg/ha. According to Ma *et al.* (1989), although silicon played a role in the percentage of filled grains,

there was no known mechanism which silicon would act on this yield component. The result of this study agreed with the research done by Mauad (2003) as the author stated that there was no effect of silicon rates on the percentage of filled grains was observed.

This study found that nitrogen rate of 120 kg/ha gave the maximum extrapolated yield which was 5.42 t/ha compared to only 5.29 t/ha from application of 60 kg/ha nitrogen. Malav and Ramani (2016, 2017) reported that increased rice yield with increased nitrogen rate might be due to the better establishment of the root system and promoted vegetative growth like leaf production thereby effectively converted solar energy into assimilates and led to increase in yield. At the same time, Yoshida (1981) reported that the number of grains per unit area of the rice crop is positively correlated with the amount of N absorbed by the end of the spikelet initiation stage or by flowering (cited in Fageria, 2014). The extrapolated yield showed a decreased trend when the silicon rate was increased from 0 kg/ha to 400 kg/ha. This is contrary to the results of the study by Malav and Ramani (2016) who reported that rice yield was significantly influenced by silicon fertilizer. However, the same authors had mentioned that lower yield of rice plant when treated with silicon may be due to the leaching and fixation loss of silicon in submerged environment which is unable to reach the optimum requirement of silicon by the plant.

Conclusion

This study found that there were no significant differences occurred in all yield parameters even nitrogen, silicon or nitrogen incorporated with silicon fertilizer applied to rice plant. However, due to the significant increase in culm diameter, silicon fertilizer is expected to significantly reduce lodging that can ultimately reduce overall grain yield. The addition of silicon fertilizer is thus highly recommended in view of resilience needed in anticipation of the impact of climate change on rice production.

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Nitrate and nitrite contents, nitrate reductase activity and leaf colour of organically and conventionally grown *Lactuca sativa*

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Abstract

Eating vegetables provides a lot of health benefits and reduced the risk for chronic diseases. However, consumption of vegetables that contained high nitrate and nitrite levels may pose some safety health problems to the human being. Excessive and unregulated application of nitrogen fertilizers could result in luxury uptake of nitrate by the plant leading to an increase of nitrate accumulation in vegetables. Consumers tend to choose dark green leafy vegetables colour to be related to freshness and quality of the vegetables. Due to consumer demand, growers tend to apply a high amount of nitrogen fertilizers and chicken dung to produce such vegetables. Therefore, a study was conducted to determine the effects of organic and conventional cropping systems on nitrate and nitrite contents, nitrate reductase (NR) activity, leaf colour and chlorophyll content of *Lactuca sativa*. Fresh *L. sativa* was harvested from selected conventional and organic farms. The vegetables were sorted, washed, rinsed and dried with paper towels. Nitrate and nitrite contents, NR activity, colour lightness (L*), chromaticity (C*) and hue (h°), and chlorophyll content were recorded. Nitrate content in *L. sativa* produced by ORG1 higher than the other farms. Nitrite content for both organic and conventional cropping systems were inconsistent. The NR activity of ORG1 and ORG2 was the highest compared to NR activity of leaves in the other four farms. Leaf color and chlorophyll content was affected by the cropping system. The value of h°, L* and C* attributed indicated that the colour of organic leaves of *L. sativa* was greyish green while the conventional leaves were pale green. Thus, the consumers' perception regarding dark green leafy vegetables being related to freshness and high quality is questionable. Thus, farmers practicing the organic cropping system should always monitor the nitrogen contents in the soil to be more sustainable.

Keywords: L*, C*, h°, chlorophyll content

Introduction

The demand for vegetables is expected to increase to 2.4 million metric tons in 2020 with a growth of 4.5% per annum (MOA, 2013). In Malaysia, about 90 % of the vegetables are conventionally grown while 10% is produced organically. The conventional vegetable production system usually uses chemical resources and synthetics-based materials (Pussemier et al., 2006) to manage weeds, pests and diseases that can affect consumers'

health. Some consumers would rather choose to pay high prices for the safety attributes in organic produce (Hadi et al., 2010). An organic production system is more environmentally, friendly and sustainable compared to the conventional production system since no synthetic chemicals are used. Most consumers prefer fresh and dark green leafy vegetables. Thus, in both the production systems, high rates (400 to 500 ton/ha) of nitrogen fertilizers are used by growers to produce the dark green vegetables. The colour of leaves has a close liaison with the nitrogen content in the leaves (Tuncay, 2011). The darker the colour of the leafy vegetables, the higher is the nitrate content, as the excessive nitrogen fertilizer contributes to the greenness of the vegetables. The organically grown vegetables are consumed for health benefits. However, the high contents of nitrogen, in the form of nitrate and nitrite, pose a risk of illnesses to consumers, such as gastric cancer and methaemoglobinemia or baby-blue syndrome (ATSDR, 2001; Knobeloch et al., 2000). In vegetables, the nitrate is converted to nitrite and eventually to nitrosamine, a potent carcinogen associated with various forms of cancers (Michaud et al., 2004). Hence, the excessive nitrogen input would lead to nitrate accumulation in vegetables after harvest. Leafy vegetables tend to accumulate the most nitrates compared to other types of vegetables (Chung et al., 2011). Organic vegetables have been reported to contain 30% to 50% lower nitrate than conventional vegetables (Worthington, 2001). However, local organic farmers tend to apply equivalent amount of nitrogen as that of the conventionally grown vegetables. Furthermore, in Malaysia, the quality of most organic fertilizers is not guaranteed (Kala et al., 2011). Thus, excessive and unregulated application of nitrogen fertilizers could result in luxury uptake of nitrate by the vegetables leading to an increase of nitrate accumulation in vegetables. Organic vegetables are consumed to reduce exposure to pesticide residues. However, with the possible increase of nitrate accumulation in such vegetables, the health benefits of organic vegetables become questionable. Therefore, the objective of this experiment was to determine the contents of nitrate, nitrite and chlorophyll, nitrate reductase (NR) activity and leaf colour of *L. sativa* produced on organic and conventional farms.

Materials and methods

Fresh leafy *L. sativa* were obtained at random from the three organic and conventional farms respectively (Table 1). The vegetable was transported to the Faculty of Agriculture, UPM, in a in air-tight, polystyrene icebox within two hours after harvest. After that, all the vegetables were sorted, washed, rinsed and dried with paper towels. Then, the vegetables were selected for uniformity in size and free from damages. Nitrate and nitrite contents, leaf colour, chlorophyll contents and NR activity of the vegetables were measured.

Nitrate and nitrite concentration: Nitrate was quantitatively reduced according to the QuikChem® method (Sechtig, 2003) to nitrite by passage of the sample through a copperized cadmium column (Lachat Part No. 50327) attached to Lachat QuikChem 8000 Flow Injection Analyzer.

NR activity: NR activity were determined according to the method of Zou (1995) was expressed as $\mu\text{mol NO}_2^- \text{h}^{-1} \text{g}^{-1} \text{FW}$.

Leaf colour: was measured by using chromameter to get colour values of hue (h°), lightness (L^*) and chromaticity (C^*)

Chlorophyll content: Chlorophyll was extracted in 80% acetone from fresh leaves. The amount of chlorophyll present in the leaves was calculated, taking the OD of the extract at 645 and 663 nm and using the formula, mg chlorophyll/g of tissue

Table 1. Location, years of planting, cultivation system, type of fertilizers, method of application, fertilizing frequency and rate of fertilization for three organic (ORG1, ORG2, ORG3) and three conventional (CONV1, CONV2 and CONV3) cropping systems.

Cropping System	Location	Years of Planting	Cultivation system	Type of Fertilizer	Method of Application	Fertilizing Frequency	Rate of Fertilization
ORG1	Semenyih	5	Under shelter	Compost	Mixed with soil before planting	1	500 kg/ha
ORG2	Bangi	19	Open field	Bokashi Compost	Broadcast	2	150 kg/ha
ORG3	Ulu Klang	17	Open field	Compost	Broadcast	2	200 kg/ha
				Goat dung	Broadcast	1	500 kg/ha
				NPK Blue 15:15:15	Broadcast	1	200 kg/ha
					Broadcast	3	250 kg/ha
CONV1	Hulu Yam	23	Open field	Chicken dung	Mixed with soil before planting	1	200 kg/ha
				NPK Blue 15:15:15	Broadcast	3	150 kg/ha
CONV2	Serendah	10	Open field	Chicken dung	Mixed with soil before planting	1	200 kg/ha
				NPK Blue 15:15:15	Broadcast	2	200 kg/ha
CONV3	Kundang	18	Open field	Chicken dung	Mixed with soil before planting	1	150 kg/ha

The experiment was conducted in a CRD with five replications (five sub samples/replicate), from three organic and three conventional cropping systems. Data were analysed using ANOVA, and significant treatment means were separated by DMRT at $P \leq 0.05$ (SAS, version 9.4). Correlation analysis between parameters was performed to indicate the relationship between parameters (leaf colour, chlorophyll content, NR activity, nitrate and nitrite content) from the different organic and conventional cropping systems.

Results and discussion

Nitrate and nitrite content: The leaf nitrate content of ORG1 was the highest compared to nitrate contents of leaves in the other five farms (Figure 1a). The nitrate contents of leaves from ORG3, CONV2 and CONV3, were similar and the lowest among the six farms. The nitrite contents of leaves from ORG 1 showed no significant differences with nitrite contents of plants from CONV1, but both ORG1 and CONV1 were significantly lower in nitrite contents than those in ORG2 and CONV3 (Figure 1b). Results from study showed that the

nitrate contents in organically grown crops were higher than conventionally grown crops because of the higher mineralization rates of organic fertilizer and livestock manure, which vary with C/N ratio and lignin content of the leafy vegetables. Therefore, organic fertilizers may lead to higher nitrate content in leafy vegetables, depending on the types and amount of organic fertilizers applied (Muramota, 1999). Excess application of nitrogen fertilizers would cause higher nitrate and nitrite contents in vegetables despite types of vegetables and cropping systems. In this study, the rate of fertilizers per application, frequency during planting and method of fertilizers application of certain farms within the cropping systems were different and could affect the nitrogen content, nitrate uptake to the plant (Guadagnin et al., 2005). The nitrate and nitrite contents in *L. sativa* of organic cropping systems farms were varied since the cultivation practices of each farms were different (Burns et al., 2011).

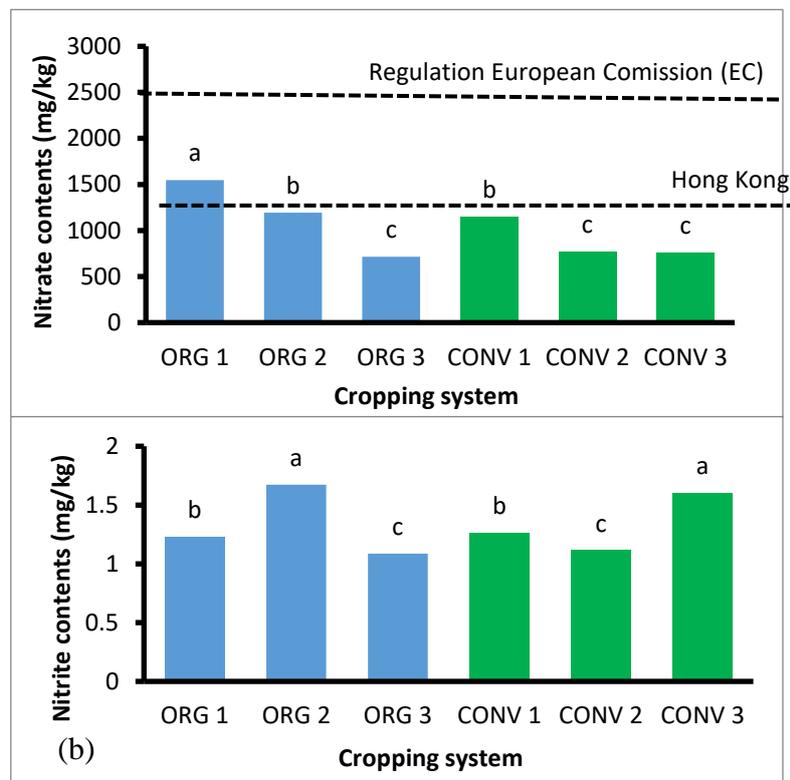


Figure 1. Nitrate (a) and nitrite (b) contents in *L. sativa* produced in three organic (ORG1, ORG2 and ORG3) and three conventional (CONV1, CONV2 and CONV3) farms. Means with the same letter above individual vertical bars are not significantly different by using DMRT, $P = 0.05$ ($n = 25$). The means of nitrate content in *L. sativa* grown in Hong Kong were obtained from Chung et al. (2011) = 1300 mg/kg FW, while the maximum limit of nitrate content from the regulation European Commission (EC) 178/2006 in *L. sativa* shoots = 2500 mg/kg FW (EFSA, 2008).

NR activity: The NR activity of CONV3 showed significantly lower NR activity than those in leaves from the three organic farms studied (Figure 2). However, there were no significant differences between the leaf NR activity of CONV3 when compared to the other two conventional farms, CONV1 and CONV2. The NR activity of leaves from ORG1 and ORG2 was significantly higher than the other conventional cropping system, but there were no significant differences in NR activity of leaves between ORG3 and CONV1 and CONV2.

NR activity was higher in the organic than conventional *L. sativa*. This might be because of higher nitrate content in organic *L. sativa* probably due to the high nitrogen content in organic fertilizer and compost application. Organic fertilizers may cause high nitrate levels in vegetables, depending on the types and amount of organic fertilizer applied. According to Kala et al. (2011) the quality of most organic fertilizers in Malaysia is not ensured as there is no label on the amount or nitrogen content in raw material for organic fertilizers and compost.

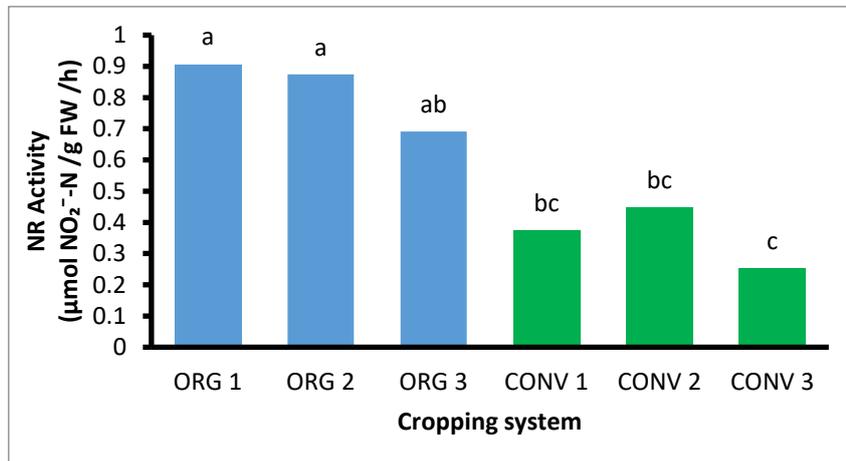


Figure 2. NR activity of *L. sativa* leaves, in three organic (ORG1, ORG2 and ORG3) and three conventional farms (CONV1, CONV2 and CONV3).

Means with the same letter above individual vertical bars are not significantly different by using DMRT, $P = 0.05$ ($n = 25$).

Leaf colour: The h° value of leaves from ORG 2 was significantly higher than the h° value in leaves from ORG1, ORG 3 and CONV3, but no significant differences in h° value of leaves from CONV1 and CONV2 cropping system (Table 2). The L^* value of ORG3 showed significantly higher value than the other five farms. The C^* value of CONV3 was significantly higher than the C^* value of all three farms from organic cropping system, but no significant differences in a C^* value of the leaves from two other conventional farms. In addition, the C^* value of leaves from ORG2 was significantly lower than leaves C^* value from two other organic and all three conventional cropping system farms. Meanwhile, the leaf chlorophyll contents of ORG1 and CONV1 showed no significant differences (Table 3). However, the chlorophyll contents of leaves from ORG2 was significantly lower than leaf chlorophyll contents in ORG1, ORG3 and all three conventional cropping system farms. From the present study, the value of h° , L^* and C^* attributed showed that the colour of organic leaves of *L. sativa* was greyish green while the conventional leaves were pale green. The differences in C^* value could be the contributed to the higher (23.6%) chlorophyll content in organic leaves of *L. sativa* than those of conventional leaves. Chlorophyll content in conventional *L. sativa* was highly significant than organic *L. sativa*. Commonly, leafy vegetables with higher nitrogen content were greener (higher hue angle), darker and duller (higher chromaticity value) than leafy vegetables with lower nitrogen content.

Table 2. Colour values (hue = h°, lightness = L* and chromaticity = C*) and chlorophyll content of *L. sativa* leaves from three organic and three conventional farming systems.

Cropping system	h°	L*	C*	Chlorophyll content (mg/cm ²)
Organic				
ORG1	122.31 d	41.96 c	27.13 bc	26.77 a
ORG2	125.03 a	40.29 c	23.06 d	12.03 e
ORG3	123.49 bcd	54.80 a	25.74 c	15.43 d
Conventional				
CONV1	124.74 ab	47.76 b	29.12 ab	25.88 ab
CONV2	124.32 abc	47.87 b	28.46 ab	22.91 bc
CONV3	123.22 cd	48.42 b	30.13 a	22.21 c

Means, followed by the same small letter among cropping systems and capital letter between the overall mean of organic and conventional, are not significantly different using DMRT, P = 0.05 (n = 25). n = 25 for cropping systems and n = 75 for overall means.

Correlation analysis: There was highly significant positive relationship between nitrate contents and NR activity (Table 3). This result was in agreement with Ivashikina and Sokolov (1997) who reported that there was a positive relationship between nitrate and NR activity. The higher the nitrate contents, the higher NR activity, thus, more nitrate would be reduced. Thus, in this study, NR activity was a good indicator of nitrate contents in *L. sativa* plant. The nitrate contents were also correlated negatively with the L*, C* and h° value of leaves but positively correlated with chlorophyll content. This showed that the brighter and intense colour of *L. sativa* have lower nitrate and chlorophyll contents as well as NR activity. Nitrate reductase response to intense light conditions (Chang et al., 2013) in sweet basil and scallion and thus would affect nitrate concentration in leafy vegetables (Gandreau et al., 1995). This might be because of the close relationship between chlorophyll and nitrogen contents (Tuncay, 2011) would affect the reduction process of nitrate that happen in the vacuole of the leaves in leafy vegetables. The correlation results in Table 3 also showed a significant and positive relationship between L* value and C* value, but a negative relationship between L* value and chlorophyll contents. Hence, it indicated that the brighter and more vivid colour of *L. sativa* would result in lower chlorophyll contents in leaves. This indicated that dark greyish green colour of *L. sativa* would result in higher nitrate contents.

Table 3. Correlation coefficients (r) for each pair of parameters in *L. sativa* obtained from organic and conventional farms

	L*	C*	h°	NR activity	Chlorophyll content	Nitrate
L*	--					
C*	0.500*	--				
h°	-0.154 ^{ns}	-	0.217*	--		
NR activity	-0.44*	-	-	--		
Chlorophyll content	-0.33*	0.125 ^{ns}	0.315*	0.802*	--	
Nitrate	-0.52*	0.001 ^{ns}	0.258*	0.938*	0.801*	--
Nitrite	-0.28*	-	0.349*	0.053 ^{ns}	-0.086 ^{ns}	0.107 ^{ns}

For correlation coefficient, n = 150. * = significant at $P \leq 0.05$.

L* = lightness, C* = chromaticity, h° = hue, NR activity = nitrate reductase activity, and ns = not significant.

Conclusions

The study showed that the nitrate contents of leaves from ORG1 was the highest compared to the rest of the other farms. Meanwhile, the nitrite content for both organic and conventional cropping systems were inconsistent. This is indicated that the accumulation of nitrate in vegetable was depended on the type of cropping system, fertilizers used and cultural practices of the farms. The NR activity in organic *L. sativa* leaves was higher than conventional cropping systems. The NR activity could be used as a nitrate content indicator for an estimation of the nitrate status in *L. sativa* leaves. The value of h°, L* and C* attributed indicated that the colour of organic leaves of *L. sativa* was greyish green while the conventional leaves were pale green. Thus, the consumers' perception regarding dark green leafy vegetables being related to freshness and high quality is questionable. Furthermore, the unknown nitrogen content contained in farm-made organic fertilizers, soil amendments and composts could cause the high nitrogen accumulation in organic soils.

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Ethnic cuisine structure and food handlers' hygiene practices at Rural Minangkabau and Terengganu homestays

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Abstract

One of the main attractions for homestay tourism is utilizing Malay ethnic cuisine and food culture as a marketing tool for international tourists to experience authentic Malay hospitality. Despite this, few studies have examined homestay food provision including traditional dishes and cuisine structure, which tourists perceived as authentic. There's also the problem of unregulated hygiene practice among homestay operators in Malaysia, where its location in rural areas which makes it difficult to be monitored. This study examined the Malay ethnic cuisine structure (local specialities, cooking methods, flavor principle) at rural homestays and food handlers' hygiene practice. A total of 124 respondents from 6 rural homestays in Terengganu and Negeri Sembilan were recruited and responded to the survey questionnaires. Findings suggest keeping cost low impacted menu structure in both homestays; mainly economical and convenient to prepare. Each homestays tend to feature distinct regional ethnic cuisine for breakfast, lunch and dinner. Despite this, Minangkabau did offered Western-style breakfast. Menu were selected based on traditional Malay recipes, although less spicy dishes; *Chicken gulai* (M: 92%, T: 90%) chicken soup (M: 79%, T: 83%) featured heavily in both homestays. Stir fry cooking method is common, besides serving raw local vegetables (75% and 66% respectively). Only age factor was found to affect hygiene practice score, where older respondents have lower hygiene practice score. Attending food safety course significantly affect food safety practices score. No significant differences in hygiene practices were found at Minangkabau and Terengganu homestays. The respective implications of the attributes on homestay cuisine structure and hygiene practices are also discussed.

Keywords: Ethnic cuisine, hygiene practices, homestays, food provision

Introduction

Homestay programs in Negeri Sembilan and Terengganu have been established since 1996 and has been used as a tool in marketing rural destination attractions particularly in rural areas of Malaysia (Kalsom, 2007; Norliza and Salamiah, 2006). Foods appeared to be a central element in homestay programs among Malay hosts. The homestay experience mainly attracts tourists seeking new tourism experience through tasting authentic regional cuisine and learning about the host's food culture (Kalsom, 2007). For instance, the food in Negeri Sembilan is infused with rich Minangkabau culture from Indonesia (Haji Ismail, 1998, 1999) while Terengganu's food is mainly influenced by Thai culture and its coastal area (Haji Hussain, 1992). Therefore, each homestay is unique, independent, and reflective of local culture as well as historical background (Norliza and Salamiah, 2006; Haji Ismail, 1998).

Despite this, studies that examined Malay cuisine structure in homestay food provision remains under reported. Hence, the characteristics of cuisine provided in homestay is relatively unknown, due to the lack of our understanding of the Malay cuisine structure itself. Equally important, the homestay's rural location, raised the issue of unregulated hygiene practice among homestay operators in Malaysia. This study aimed to understand the Malay ethnic cuisine structure within rural homestay establishment and examined food handlers' hygiene practices in rural homestays in Terengganu and Negeri Sembilan, Malaysia.

In this study, the core and complementary food models (Kittler and Sucher, 2004a; Kittler and Sucher, 2004b) were adapted. The model comprised of 3 elements; core foods, secondary foods and flavour principles (Kittler and Sucher, 2004a; Kittler and Sucher, 2004b). Core foods represented the foundation of a diet, which is eaten on a daily basis including rice, wheat, corn, yams and cassava. In Malaysia, rice dishes are often accompanied by assortment of side dishes or *lauk-pauk* (Haji Ismail, 1998, MKNNS, 2005). Secondary foods are those that are consumed widely but less frequently and usually characterize individual's preference. These items include meat, fish, chicken, and local vegetables.

Flavour principles are the way a culture traditionally combines specific ingredients for a unique taste (Kittler and Sucher, 2004b) for instance *palm sugar*, *belacan* (shrimp paste), local herbs and coconut milk. This combination provides a characteristic flavour that foods within the culture shares, and identifies them as originating from that particulate culture.

Food handling malpractices on the part of the food handlers may allow harmful bacteria to come into contact with food and even in some cases to survive and multiply in sufficient numbers to cause illness to consumers (Omemu and Aderoju, 2007; Sammarco *et al.*, 1997). Previous studies asserted mishandling and disregarding hygiene measures during food preparation contributed significantly to food-borne diseases (Redmond and Griffith, 2004, McSwane, 2005 and Trikett, 1997). Thus, this raised concerns among food handlers preparing food in domestic kitchen, since home-cooked food from developing countries were identified as the main contributor to the highest foodborne illness incidences (Jevšnik *et al.*, 2007, Sammarco *et al.*, 1997). A survey of domestic food handling practices in New Zealand estimated that 41% and 28% of respondents would treat knives and surfaces respectively in such a manner to allow the potential for cross contamination (Gilbert *et al.*, 2007). These surveys suggest that domestic food handlers are unaware of the food safety risks presented by food contact surfaces.

Materials and method

The questionnaire was developed to gather food handlers' perception of Malay cuisine structure *vis-a-vis* types of food available in homestays and hygiene practices. The questionnaire was based on Core and Complementary food model (Kittler and Sucher, 2004a) and questions on hygiene practices, personal health and cross-contamination risks were modified from Jevšnik *et al.*, (2007) and Haysom and Sharp (2005). The developed questionnaire was later translated in *Bahasa Melayu*, the official language in Malaysia, since all respondents speaks and understands the language. The translated questionnaires were then piloted and further reviewed by two expert panels in food safety and heritage cuisine respectively.

The study was carried out in Negeri Sembilan (Minangkabau) and Terengganu states, involving six homestays namely Homestay *Kampung Pasir Raja*, Homestay *Kampung Rhu 10* and Homestay *Kampung Teluk Ketapang*, Homestay *Kampung Pelegong*, Homestay *Lonek*, and Homestay *Laman Bangkinang*. These homestays were selected as recommended by previous work (Kalsom, 2007; Norliza and Salamiah, 2006). A convenience sampling technique was used when recruiting respondents, all of which comprised of food handlers from selected homestays.

The data was analysed using the Statistical Package for Social Science (SPSS) version 16.0. For each correct answer on hygiene practice, 1 mark is given and 0 for incorrect or unsure answer. A three-point rating scale (3 = always, 2 = sometimes and 1 = never) is allocated for frequencies including cooking methods, key ingredients and flavours. The score below 61% on hygiene practice is indicative as poor (Jevšnik *et al.*, 2007). Statistical analysis was carried out for descriptive statistics, Chi-square test and Spearman Rho correlation was performed to examine if there are any significant relationship between variables.

Results and discussion

Cooking methods

Results showed more than half (72%) homestay food provisional used stir-fry, boiling in coconut milk (55%) and stewing (67%) cooking methods (Figure 1). Stir-fry cooking method was mainly used for vegetables and poultry. Stewing like curries were mainly for poultry and meat. It reflected traditional Malay approach of cooking, which emphasised on simmering technique for intense flavour, and generous used of local herbs (Haji Hussain, 1992; MKKNS, 2005). Respondents also preferred serving vegetables consisted of local *ulams* raw with condiments such as *sambal belacan* or *budu*.

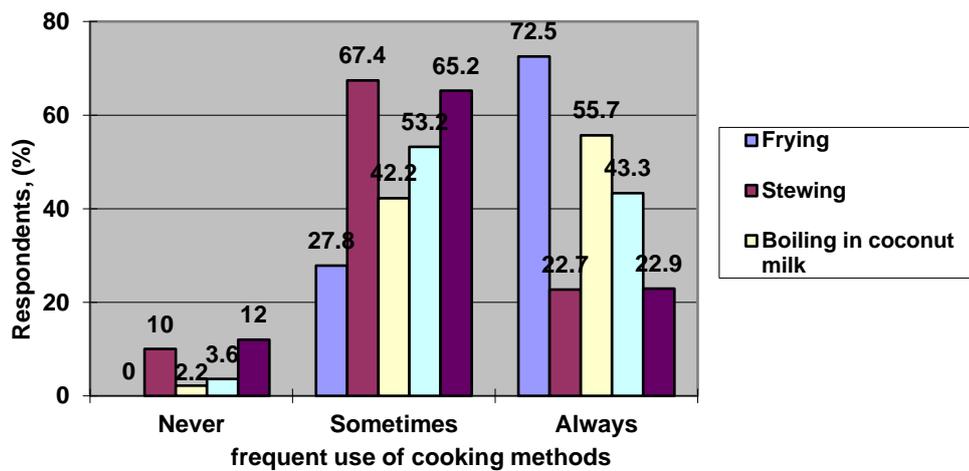


Figure 1. Cooking methods used in homestay food provision

Figure 2 revealed that *nasi lemak* (steamed rice in coconut milk), *nasi goreng* (fried rice), *mee* or *mihun goreng* (stir fried yellow or rice noodles) were popular breakfast dishes served by the respondents in both states. The popularity of rice dishes such as *nasi minyak* (ghee rice), *nasi kuning* (turmeric yellow rice) and *laksam* (rice noodle) were higher in Terengganu because these dishes reflected Terengganu region and food identity (Haji Hussain, 1992). Understandably, rice is less costly, easily available and more sustainable compared to Western style breakfast of bread, milk and cereals. The result confirmed rice as core foods and that complementary food pairings of coconut milk and herbs are the key in Malay cuisine structure to enhance the flavour as suggested by Kittler and Sucher's (2004) model.

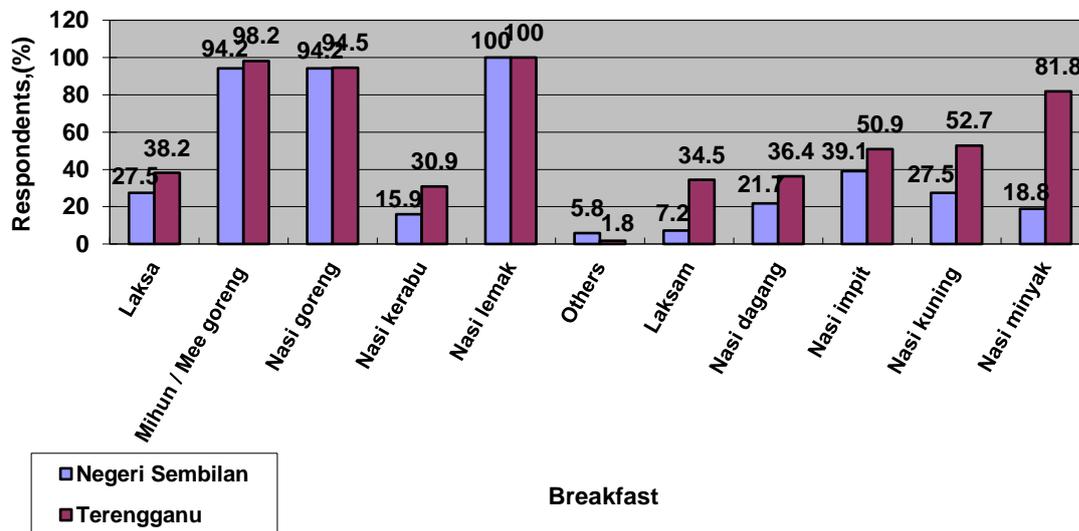


Figure 2. Types of breakfast dishes in Malay cuisine

The *nasi berlauk* or *nasi campur* (mixed rice) are widely consumed in Malaysia as a wholesome meal during lunch and dinner. *Nasi berlauk* consists of steamed white rice served with a selection of high protein dishes (chicken, meat, or fish), vegetables and condiments (MOTOUR, 2006). The results showed *gulai ayam* (chicken yellow curry) and *sup ayam*

(chicken soup) dominated the secondary food group in both study locations (Figure 3). This however, did not reflect Malay authentic cuisine, particularly Minangkabau which used generous amounts of birds' eye chilies and thick coconut milk (Haji Ismail, 1998; 1999). Respondents perceived that foreign tourists' palate may not accept very spicy food. As such, for convenience and economic reasons, both Minangkabau and Terengganu homestays offered less spicy menu.

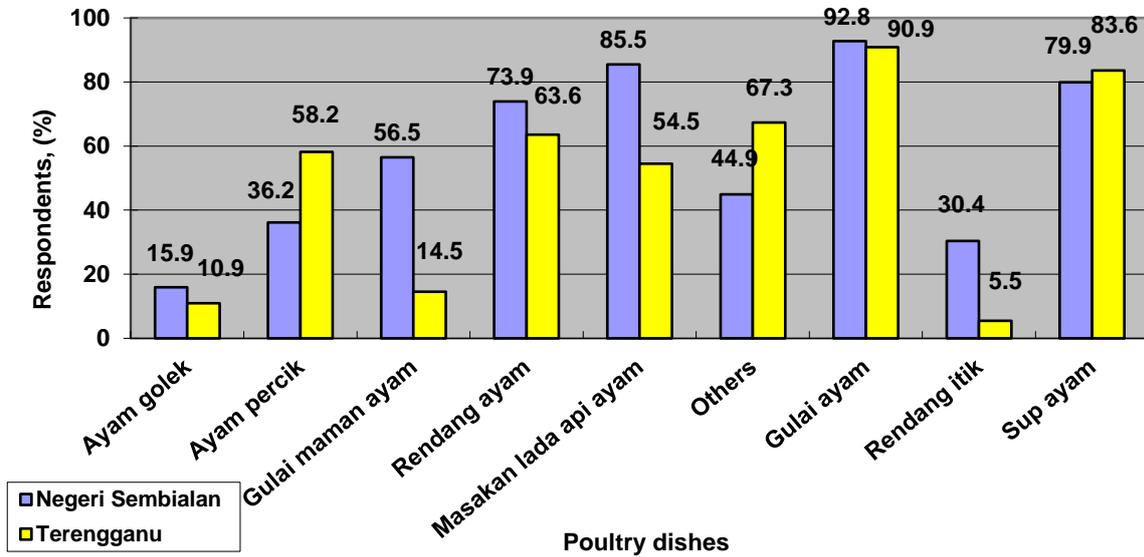


Figure 3. Types of chicken dishes in Malay cuisine

Despite this, Minangkabau respondents argued, *Minang* cuisine including slow-braised duck curry or *Rendang itik*, *lada api ayam* (spicy chicken curry) and *gulai maman ayam* (chicken yellow curry with maman herb) were still included at homestays in Negeri Sembilan, mainly to highlight their *Minangkabau* ancestral roots. Meat dishes (Figure 4) were also more popular in Minangkabau homestays, as dishes “*daging salai masak cili api*” (fiery smoked meat gravy), “*gulai daging*” (meat gravy) and “*gulai kuning daging*” (yellow hue meat gravy) reflected on unique Minangkabau’s identity (MKNNS, 2005).

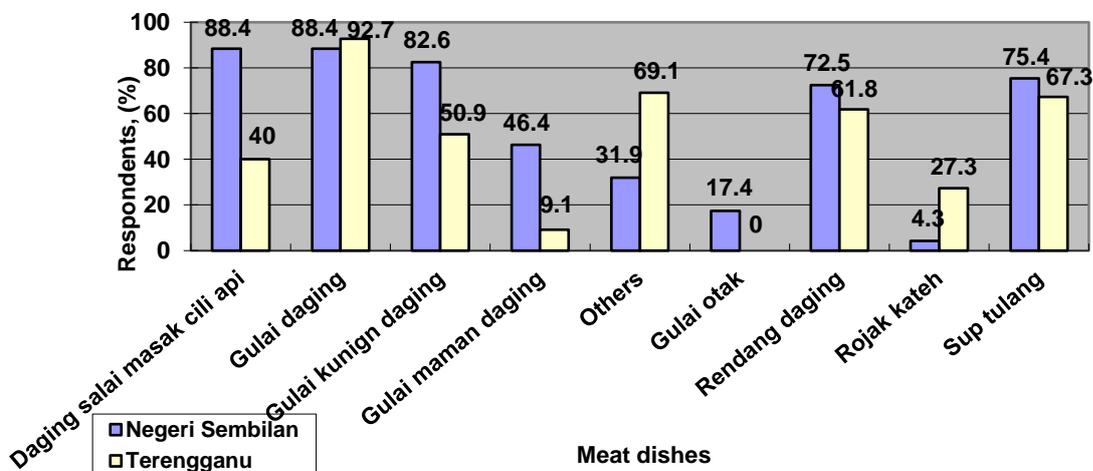


Figure 4. Meat dishes served during lunch

Personal Hygiene and Hygiene Practice

Results on Table 1 showed majority of respondents wore hair restraints and aprons when handling food. This is consistent with the recommendation from Ministry of Health Malaysia (MOH, 1996), whereby clean and protective clothing must always be available for food handlers. When it comes to wearing jewellery during food handling, Minangkabau respondents indicated good hygiene practice by not wearing any. This was consistent with hygienic practices among domestic food handlers in Italy (Sammarco *et al.*, 1997). Thus, the Minangkabau respondents appeared to take more precautionary steps in reducing cross-contamination through personal hygiene.

Table 1. Personal hygiene and hygiene practice

Personal hygiene and hygiene practice	Percentages of respondents		
	Minangkabau	Terengganu	Mean
1. Food handlers should:			
wear hair restraints and aprons	98.6	98.2	98.4
avoid jewellery as sources of contaminations	81.2	58.2	69.7
2. It's not acceptable to handle foods while			
diarrhea	81.2	87.3	84.3
hypertension	73.9	72.7	73.3
a cold	50.7	67.3	59.0
Some coughing but wearing face mask	81.2	54.5	67.9
fever	65.2	70.9	69.1
cut on myself	82.6	74.5	78.6
wounds on hands	85.5	83.6	84.6
3. Hand washing before food preparation:			
not necessary	0	0	0
depends on what I was previously doing	4.3	3.6	4.0
depends on the food I am going to prepare	2.9	0	1.5
always wash my hands	92.8	96.4	94.6
4. Hand washing steps after handling raw			
wipe hands with paper towel	0	0	0
wipe hands with kitchen cloth	1.4	0	0.7
using cold or hot water	10.1	23.6	16.9
using warm water and soap	88.4	72.7	80.6
did not wash hands while handling raw foods	0	3.6	1.8
5. Drying of hands:			
did not dry hands	0	3.6	1.8
using apron	0	0	0
using cloth for wiping dishes	2.9	1.8	2.4
using kitchen towel	97.1	94.5	95.8

The result suggests majority of respondents perceived one should not handle food when not in optimum health condition. Homestay food handlers appeared to have good knowledge when identifying potential health hazards and cross contamination through ill health. The result was consistent with Ministry of Health, Malaysia policy urging food handlers who are unwell to avoid from handling food (MOH, 1996).

The result contends that majority of respondents washed their hands prior to preparing food. This result was further supported by Jevšnik *et al.*, (2007). According to REHIS (2007), hands must be washed with water and plenty of liquid soap at regular intervals during the food operation, ensuring a good lather. Interestingly, the result from this study suggested nearly all respondents used kitchen cloth to dry their hands (Table 1). The result obtained on hygiene practice scored better compared to studies in Slovenia and Italy (Jevšnik *et al.*, 2007; Sammarco *et al.*, 1997).

Utensils and surfaces cleanliness

Results suggested less than 18% respondents did not use effective means of cleaning cooking utensils (chopping board and knives), which may lead to cross contamination (Table 2). Although the percentage was considered low, however respondents' had engaged with poor hygiene practices including cross contamination between raw and cooked food.

According to Haysom and Sharp (2005) key contamination areas included work contact surface area, chopping board and taps or sink, therefore must be clean immediately after food has been prepared. Good hygiene practice including thorough cleaning, ensuring the potential death of contaminants correspond with desiccation of their immediate environment. The results suggested a positive attitude towards food safety practices among Minangkabau and Terengganu respondents. However, no significant differences in levels of hygiene practice ($X^2= 19.045$, $p > 0.05$) between the two groups was found.

Table 2. Utensils and food contact surface (FCS) cleanliness

Cleanliness of cooking utensils and FCS	Percentages of respondents		
	Minangkabau	Terengganu	Mean
1. Using same knife to cut vegetables and			
Use same knife	11.6	3.6	7.6
wipe the knife with a damp cloth	0	0	0
wash the knife with water without detergent	17.4	5.5	11.5
wash the knife with water and detergent	26.1	5.5	15.8
use another knife	44.9	85.5	65.2
2. Using same cutting board to cut			
Use same cutting board to cut poultry	2.9	10.9	6.9
Wipe cutting board with damp cloth and use	0	0	0
Wash the cutting board with water	23.2	14.5	18.9
Wash the cutting board using hot water and	30.4	7.3	18.9
Use a new cutting board	43.5	67.3	55.4

Also, there was a negative and weak correlation between age and hygiene practice (Table 3). This indicate older respondents may have lower tendency towards hygiene practices. In terms of academic level and hygiene practice, the results suggested respondents with higher education levels tend to have higher awareness on hygiene practices.

Table 3. Correlations between hygiene practice and demographic data

Elements	Demographic data	r ^a	Sig.
Hygiene practice	Age	-0.051	0.571
	Academic level	0.145	0.108
	Length of involvement	-0.005	0.959
	Attended courses	0.085	0.347

^aCorrelation coefficient.

*Correlation is significant at the 0.05 level

Conclusion

This study had provided insights into homestay food provision in Negeri Sembilan (Minangkabau) and Terengganu. The results suggested keeping cost low, convenient to prepare, utilisation of local ingredients and modification of flavours to suit tourist's palate formed the Malay cuisine structure at homestay. Within this cuisine structure, both homestays managed to retain their unique food identity as a competitive advantage in tourism sector. Surprisingly, this study found high level of hygiene practice among domestic food handlers relating to personal hygiene and hand washing, consistent with academic level. However, risk of cross contamination remains evident.

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Leaf cutting of *Labisia pumila* (Kacip Fatimah)

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Abstract

Leaf cutting propagation trial on *Labisia pumila* was conducted in this study. Since the raw material supply of this herb are limited, this study aimed to identify feasible propagation methods for future planting production of this herb. These cuttings were propagated in sand beds (0.3m depth) containing 100% medium of fine sand and covered with plain white UV plastic sheet using closed capillary propagation system. Each leaf was divided into 3 (upper, middle and lower part namely Up-Leaf, Mid-Leaf and Low-Leaf respectively). All cuttings were treated with commercial hormone, IBA Rootmone. Each treatment consisted of 50 cuttings and replicated into 4 replicates. All types of cuttings used in this experiment begin establish root systems after 6 weeks after propagated. Based on the result the highest percentage of rooting was achieved by using cutting class of Mid-Leaf ($93.0 \pm 2.6\%$) and followed by cutting class of Low-Leaf ($86.0 \pm 10.5\%$). Cutting class of Up-Leaf gives the lowest percentage of $84.0 \pm 6.9\%$. There are no statistically significant differences between leaf parts in rooting ability as determined by one-way ANOVA ($F_{2,9} = 1.634, P = 0.248$). For shoot formation, all rooted cuttings part initiated new shoots after 6 months. The highest number of shoots (1.0 ± 0.2 shoots per cuttings) was observed on leaf cuttings from middle parts which significantly higher than upper part (0.8 ± 0.3 shoots) and lower part (0.4 ± 0.2 shoots) as determined by one-way ANOVA ($F_{2,9} = 6.471, P = 0.180$). This study shows that leaf cuttings of the species could be new a dimension for mass production of the species. Even though taking an extensive period to initiate shoot formation (up to 12 months), leaf cuttings have the ability to produce multiple propagules (up to 4 in this study), thus can produce more new plants.

Keywords: *Labisia pumila*; leaf cutting; propagation.

Introduction

Labisia pumila, commonly known as Kacip Fatimah is a plant from family Myrsinaceae. *L. pumila* is native to Southeast Asia and naturally found in the damp forest floor at elevations of between 80-100 m (Stone, 1988). Generally, the plant have been used traditionally to induce and ease childbirth and as post-partum medication to contract the womb, delay conception, regain body strength, firm up breasts and abdominal muscles, and also regulate and ease painful menstrual cycle among young women (Wan Ezumi *et al.*, 2007). It is also being proved to have positive value in regulating body weight (Fazliana *et al.*, 2009), preventing photoaging (Hyun-kyung *et al.*, 2010), possessing anti-bacterial and anti-fungal activities (Karimi *et al.*, 2011; Ali and Khan 2011) and also antioxidant activity (Mohd Hafiz and Hawa, 2012).

This plant is usually obtained from its natural habitat for pharmaceutical and medicinal purpose. Its availability in the forest is becoming scarce due to the increase in demand by the users (Mohd Satefarzi, 2001) and it has been reported that 50% of the raw materials used by the local herbal industry came from forests (Zurinawati, 2004). In addition, the plant have slow growth rate and take a long time to grow (Mohd Noh *et al.*, 2002).

The plant can be propagated by seeds or vegetative cuttings. Vegetative cuttings of this species can be done in multiple ways by using the shoot, stem or leaf cuttings. The success of vegetative cuttings from different parts of the species was already reported (Rozihawati *et al.*, 2004; Nurul Nahar, 2009; Syafiqah Nabilah *et al.*, 2014). Generally, vegetative cuttings using stem parts of the plants was chosen to propagate the plant to reduce reliability on planting material from natural habitat and can mass produce the plants systematically. However, the study on vegetative cuttings of *L. pumila* in previous study by Syafiqah Nabilah *et al.*, (2016) found the promising result in leaf cuttings as alternative methods for mass propagation of the species. Thus, the objectives of this study are to investigate the ability of rooted leaf cuttings to produce multiple shoots and to enhance a more successful method using leaf cuttings.

Table 1. Leaf cuttings preparation and experimental design.

Treatment	Block 1	Block 2	Block 3	Block 4
Low-leaf	50	50	50	50
Mid-Leaf	50	50	50	50
Up-Leaf	50	50	50	50

Materials and Methods

Plant materials

L. pumila variety of Alata was used in this study. The stock plants of *L. pumila* var. alata were sampled from matured wildings collected from Sg. Siput, Perak. Only healthy and disease-free plants will be used for leaf cuttings propagation in this experiment.

Leaf cuttings preparation and propagation

Each selected leaf was divided into 3 parts i.e. lower, mid and upper. These cuttings were propagated in sand beds (0.3m depth) containing 100% medium of fine sand and covered with plain white UV plastic sheet using closed capillary propagation system to keep and maintain optimum humidity surrounding the cuttings and medium (Mohd Afendi *et al.*, 1996). The system was reported potentially can increase rooting of cuttings (Ab. Kahar *et al.*, 2009) and used in other propagation study of the species (Nurul Nahar, 2009).

All of the leaf cuttings were treated with commercial hormone, IBA Rootmone. IBA was applied at the base of cuttings before planting. Each treatment consisted of 50 cuttings and replicated into 4 replicates (Table 1). The cuttings were maintained and raised in the nursery for up to 5 months and rooting and post-rooting activities were then recorded for each treatment.

Post-rooted of leaf cuttings

Once the root system established, 30 rooted leaf cuttings were transferred into 12.7”x17.8” black perforated polybag containing the mixture medium of top soil, peat and sand (3:2:1). The rooted cuttings were maintained and raised under the nursery. The cuttings were observed and recorded for the production of new shoot and the ability to produce multiple shoots for up to 12 months.

Statistical analysis

Data collected in all experiments were analyzed by SPSS and subjected to analysis of variance (ANOVA). The mean differences were tested using Tukey's honestly significant difference test (Tukey HSD) with significant value of $P = 0.05$.

Results and Discussions

Rooting ability of *L. pumila*'s leaf cuttings

Based on observation, leaf cuttings of *L. pumila* begin to establish root system after 6 weeks of propagation. The effect of different leaf parts used for *L. pumila* cuttings propagation showed in Table 2. Result shows that the middle part of leaves (class Mid-Leaf) gives highest percentage of rooting ($93.0 \pm 2.6\%$) followed by lower part ($86.0 \pm 10.5\%$) and upper part ($84.0 \pm 6.9\%$) for the period of 5 months after propagated. Most of the leaf cuttings rooted in the first 3 months after propagated and slowed afterward (Figure 1). There are no statistically significant differences between leaf parts in rooting ability as determined by one-way ANOVA. This finding was supported by Syafiqah Nabilah et al., (2016) although the study show the lower part produced the highest rooting percentage (90%). Leaf cuttings were reported have higher percentage of rooting compared to other plant parts of the species (Rozihawati et al., 2004), even though Nurul Nahar (2009) reported stem cuttings gives preferable survival percentage ($>80\%$) than leaf cuttings ($<15\%$).

Table 2. Rooting and shoot production ability of *L. pumila* leaf cuttings from different parts.

Leaf parts	Cuttings rooted	Cuttings	Cuttings with	Mean number of
Low-Leaf	86.0 ± 10.5^a	34.2	5.0	1.1 ± 0.1^a
Mid-Leaf	93.0 ± 2.6^a	72.5	17.5	1.3 ± 0.1^a
Up-Leaf	84.0 ± 6.9^a	66.7	10.8	1.2 ± 0.1^a

* Values represent mean \pm standard deviation of 4 replicates. Means followed by the same letter did not differ significantly ($P < 0.05$).

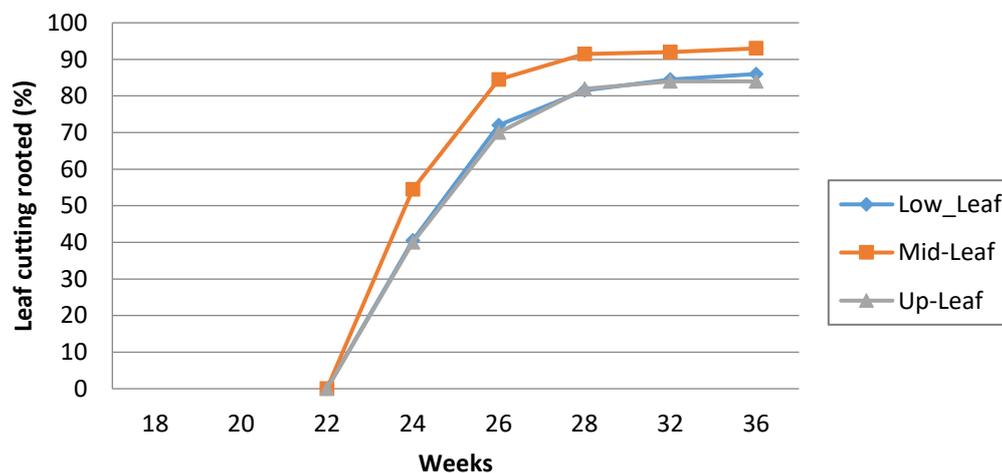


Figure 2. Percentage of rooted *L. pumila* leaf cuttings by date

3.2 Shoot production ability of *L. pumila*'s leaf cuttings

Rooted leaf cuttings initiated new shoots started 24 weeks after propagating although Syafiqah Nabilah et al., (2016) reported the first shoot emergence observed in week 19 in their study. After transferred into polybag and maintained in nursery until 12 months, based on results showed in Table

2, the highest percentage of cuttings produced new shoots (72.5%) was achieved using middle leaf parts followed by 66.7% and 34.2% using top and bottom parts of the leaves. The used of leaf cuttings from middle parts has significantly initiated new shoots formation with up to 17.5% of cuttings responded with multiple shoots whereas cuttings from lower leaf parts only 5.0%. The highest number of shoots (1.3 ± 0.1 shoots per cuttings) was also observed on leaf cuttings from middle parts but not significantly higher than upper part (1.2 ± 0.1 shoots) and lower part (1.1 ± 0.1 shoots) as determined by one-way ANOVA. There are no findings were available on the numbers of shoot production of leaf parts cuttings of this species for comparison purposes.

Conclusions

The species is known it can be propagated by using seeds or cuttings (Stone, 1988). The germination using seeds is a natural process of the species in its natural environment. However, its not efficient for commercial purposes as the percentage of germination using seeds was only 15% after germinated. Results of this study showed that the vegetative propagation using leaf parts of the species as cuttings can give higher percentage and success for mass production of the species for commercial planting. Leaf cuttings of the species could be new a dimension for mass production of the species. Even taking an extensive period to initiate shoot formation (up to 12 months), leaf cuttings have the ability to produce multiple shoots (up to 4 in this study), thus can produce more new plants. In addition, matured plants of the species have an average of 8 leaves and each leaf can be divided into 3 or 4 sections.

In conclusion, leaf cutting of *L. pumila* has been successfully carried out in this study and indicates that the used of certain different parts of leaf significantly could promote shoot formation with multiple numbers. Although leaf cuttings took longer time to establish as a new plant, the used of the leaf parts as the planting material for propagation method is an effective way by reduced waste of planting materials and in addition, it can be mass propagated in smaller area even from limited planting materials. Further observation and study should be carried out to refine and enhance the leaf cuttings techniques for this species to improve the propagation rate further.

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Potential of vermitower as a system to produce organic lettuce

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Abstract

Vermiculture of *Eudrillus eugeniae* is the cultivation of African nightcrawlers to produce organic fertilizer. A study was conducted to see the potential of *Eudrillus eugeniae* residual in assisting the growth of leafy vegetables, *Lactuca sativa*. A structure has been built by innovating components of the vermitower planting method to achieve the optimum condition to produce the vermicompost and it is where the lettuce fully utilized the compost to grow. Through the data obtained, the physicochemical factors and the earthworm survival were the interacting factors that affect the media condition where the organic fertilizer collected therefore determine the growth of lettuce. The pH range recorded was between 5.0 and 6.0, the temperature was between 27.1°C and 30.2°C, and the moisture was between 2 and 5. The lettuce could grow well through the seven weeks of observation with the final weight of total harvested lettuce reached 172.17g. In conclusion, this vermitower structure is suitable for the production of organic vegetables in a small scale by only utilized the vermicompost as fertilizer that produced within the system.

Keywords: *Eudrillus eugeniae*; *Lactuca sativa*; vermicompost; salad; organic vegetables.

Introduction

This study is about the potential of vermicompost for producing organic vegetables in a cultivation system, vermitower. The designed structures of this cultivation system were focused on the distribution of specific areas for the earthworm-eating process to happen. Optimizing the full use of earthworms in nourishing the medium is crucial in order to provide nutrients evenly throughout the planting area which can be utilized by vegetables roots.

Utilization of *Eudrilus eugeniae* in producing organic fertilizer and for soil conditioner is due to the species is capable of decomposing large quantities of organic wastes quickly and incorporating them into the top soil (Neuhauser et al., 1979, 1988; Edwards, 1988; Edwards et al., 2011). High rate of decomposition of organic wastes by *Eudrilus eugeniae* will give the plant continuous nutrient needed and the increasing in soil quality that provide optimum condition for plant to growth and produce high quality organic based vegetables.

Vermiculture concept is the concept in which earthworm wastage used as organic nutrient or organic fertilizer for plant. The earthworm wastage will supply nutrient required to plant such as macronutrient and micronutrient (Nandwani, 2016). Vermiwash is one of the materials produced by vermicomposting which primarily through the action of earthworm with the support of other microorganisms. According to Nandwani, (2016) organic fertilizer such as vermiwash provide a relatively cost-effective and safe alternative to chemical fertilizer.

Lettuce (*Lactuca sativa*) is produced commercially in many countries worldwide and is also widely grown as vegetable in home gardens (Rubatzky, and Yamaguchi, 1997; Kristkova et al., 2008).

Therefore this study was conducted in order to provide information about the implementation of vermitower structure for producing organic lettuce.

Materials and Methods

Vermitower preparation

Two containers in size of 60 cm x 44 cm were used in the system. The first container was used to place the peat moss as the medium and the second container was to collect the vermiwash, placed under the first container. Two parts of pipes were placed in the middle of the medium to provide the earthworm foods. *Eudrilus eugeniae* were given nutrients through foods waste such as old pumpkins and used tea bags. The two containers were put inside a structure that was built to protect the earthworm and vegetables from any pests attack. At the upper part of the structure, fluorescent light was placed with a timer to light the vermitower during night to prevent the earthworm crawling out from the medium. This is because of the African Night Crawler (*Eudrilus eugeniae*) were very sensitive towards the light.



Figure 1. Vermitower structure

Planting lettuce

Lettuce's seeds need to be germinate first in the seed tray until the seed germinate that indicated by the coming out of two to four leaves in a week time. Later, the transplanting process will take place. The germinated seeds will be transfer from the seed tray to the container with the peat moss. It took about seven weeks for the lettuce to grow maturely from the transplanting time to the harvesting time.

Data collection and analysis

Data were collected throughout the study for 37 days at the same time of the day, 5pm. The data collected were temperature, moisture content and pH at the part of the peat moss. At the end of this study, the weight of harvested lettuce was also documented. The data was analysed using Microsoft Excel and direct observation.

Results and Discussion

From the Figure 2, it could be seen that the pH recorded for the medium is in slightly acidic condition. The lowest pH recorded was 5 and the highest was 6.6. The consistent acidity environment in the medium is the indicator that the vermicompost has produced Plant Growth Regulators such as auxins, gibberellins and cytokinins that help to promote the plant growth (Edwards et al., 2011).

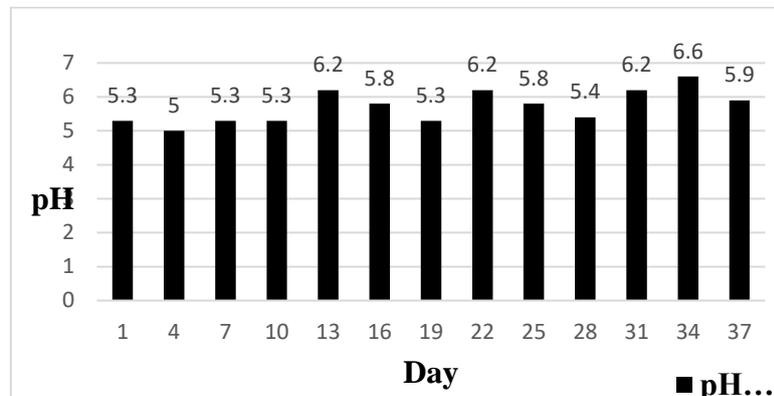


Figure 2. pH value for the peat moss until day 37

For temperature, the lowest temperature recorded was 27.1 °C while the highest was 30.2°C (Figure 3). Observation of the temperature was crucial in order to ensure the earthworms lived in optimum condition as it is very sensitive to the change of temperature (Edwards et al., 2011). According to Edwards et al., (2011) the medium temperature was influenced by the environmental temperature and the heat released by the earthworms during the bio-chemical process happened.

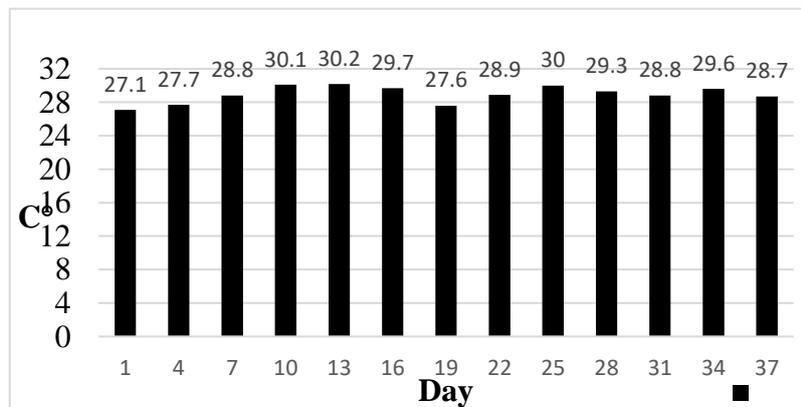


Figure 3. Temperature for the peat moss until day 37

Moisture content recorded in this study could be seen in the Figure 4. The lowest moisture recorded was at 2 and the highest was 5. It could be seen that the moisture content were not in stable state. This continuously changing in the moisture content of the medium might be due to the environmental factors, weather, biological process of earthworms and biological process of vegetables grown. As studied before, vermicompost is one of the medium that capable to hold water in large amount with good aeration (Aranda and Barois, 2000).

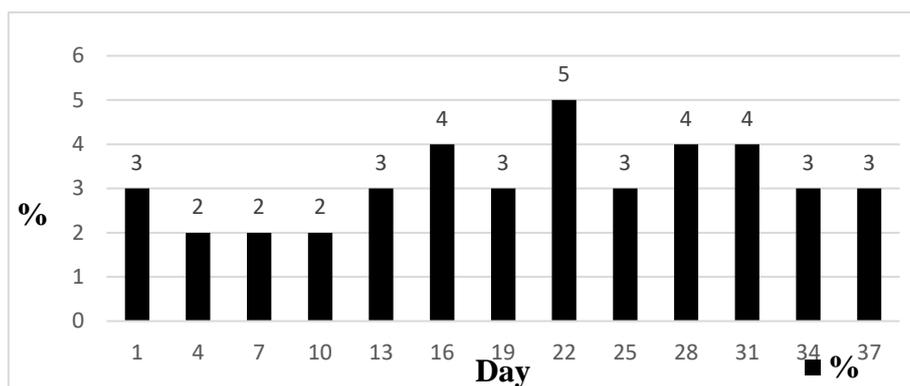


Figure 4. Moisture content for the peat moss until day 37

The total weight of 23 harvested lettuce was recorded to achieve 172.1 gram at the 7 weeks being in the vermitower. The vermitower had been shown to function for producing the organic lettuce. With the protection from the fiberglass net at the protection structure, it seems that the lettuce and earthworms were free from any pests attack. Anti-climbing technique was applied to the parts of vermitower structure by using grease oil to protect the plants from any pests that can climb into the medium particularly ants. Ants is one of the main predator for the African Nightcrawlers. On one hand, the vermiwash collected inside the container will be apply to the peatmoss for additional nourisher of the lettuce. The additional uniqueness of this vermitower is the structure was made portable. Thus, it can be moved to be placed in the right place especially during the day for the lettuce to receive sunlight.

Conclusion

This present study has shown that *Lactuca sativa* were able to grow by only using the vermicompost of *Eudrilus eugeniae*. The vermitower structure could be one of the alternative to produce lettuce by only utilized the vermicompost. Further study might focused on how to produce the vermicompost in large scale especially on how to separate the vermicompost and the earthworm without cause injury to the earthworm for production of organic fertilizer purposes.

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The growth of organic agriculture in Bhutan

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Abstract

The aim of the study was to comprehend the growth of organic agriculture in Bhutan since its inception (i.e. 2004). The growth was examined in terms of five dimensions: (1) production, (2) varieties of crops grown, (3) availability of organic fertilisers and pest control mechanisms, (4) technologies adopted, and (5) farmers' awareness. The analyses were carried out based on the secondary data. Agriculture statistics from Department of Agriculture starting 2004 to 2016 were used for determining crop production and variety. Relevant articles were referred for better comprehension of the study. The policy of 100 % organic by 2020 demands big responsibility for the country. Studies revealed that Bhutan was never into the practice of intensive conventional agriculture since there has been nil or minimal application of synthetic fertilisers. As per the data from two districts (Gasa and Samfrupjongkhar), the production is seen to remain steady since 2004 but crop variety has been increased to a large extent. The various documents and studies revealed the availability of the appropriate technologies, and the sustenance of sustainable cropping system in the country. Yet, farmers still lack the awareness on the benefits of organic agriculture. We found that National Organic Program and Department of Agriculture must implement practical strategies to encourage more organic producers. Thus, we recommend the concern agencies to encourage more research and development. Suggestions for future research includes research on the same topic using primary data; development of organic pest repellents, organic fertilisers and relevant technologies; assess production using Life Cycle Assessment approach; and impact of organic agriculture on environment. **Keywords:** Bhutan; organic agriculture; sustainable agriculture; production; mechanisms.

Introduction

Organic agriculture (OA) is one of the best modes of agricultural production system, embracing the basic principles of sustainable agriculture (Ahlem and Hammas, 2017; Tashi and Wangchuk, 2016; Thimmaiah, 2007). According to IFOAM (2005), OA is defined as “a production system that sustains the health of soils, ecosystems, and people relying on ecological processes, biodiversity, and cycles adapted to local conditions, rather than the use of inputs with adverse effects. OA combines tradition, innovation, and science to benefit the shared environment and promote fair relationships and a good quality of life for all involved”.

Bhutan is determined to practice OA due to a very low usage of chemical inputs (Duba *et al.*, 2008). Also, Tashi and Wangchuk (2016) revealed numerous contextual strengths and opportunities of OA. Sensing those benefits, Bhutan envisioned to become 100 % organic by 2020 (IFOAM, 2014). To start with, the Ministry of Agriculture (MoA) officially launched the policy National Framework for Organic Farming in Bhutan (NFOFB) for organic farming in 2007 (Department of Agriculture, 2007b).

The stated mission is to develop and promote organic farming that will enable Bhutanese farmers and traders to provide safe, quality food, produce and products for local consumers as well as for other markets (Duba *et al.*, 2008). Two districts Gasa in the northwest and Samdrupjongkhar in the southeast are declared as organic districts in 2004 and 2010 respectively (Tashi, 2015). Organic farming is also carried out in small pockets in other 18 districts. Thus, the objective of this study was to comprehend the growth of organic agriculture in Bhutan since its inception in terms of five dimensions.

Materials and Method

This study used secondary data to examine five dimensions: (1) production, (2) varieties of crops grown, (3) availability of organic fertilisers and pest control mechanisms, (4) technologies adopted, and (5) farmers' awareness. Data were collected from websites of International Federation of Organic Agriculture Movements (IFOAM), MoAF, National Organic Program (NOP), and Google Scholars. Agriculture statistics from Department of Agriculture (DoA) starting 2004 to 2016 were used for determining the trends for crop production and variety.

Results and Discussion

Production

Figure 1 shows the trend of organic production from two organic districts as per the data from DoA. The result showed that OA production remained almost constant within the time span of 11 year. Of six categories of crops, the production of cereals, vegetables, legumes and oilseeds were constantly higher than other categories (Figure 1). This shows the importance of those crops in Bhutanese food system. Cereals had been the stable crops for Bhutanese. Organic land comprises of 39,921 acres out of which 38,558 are certified/wild collection and 1,363 acres are cultivated in line with NOP guidelines (NOP, 2015).

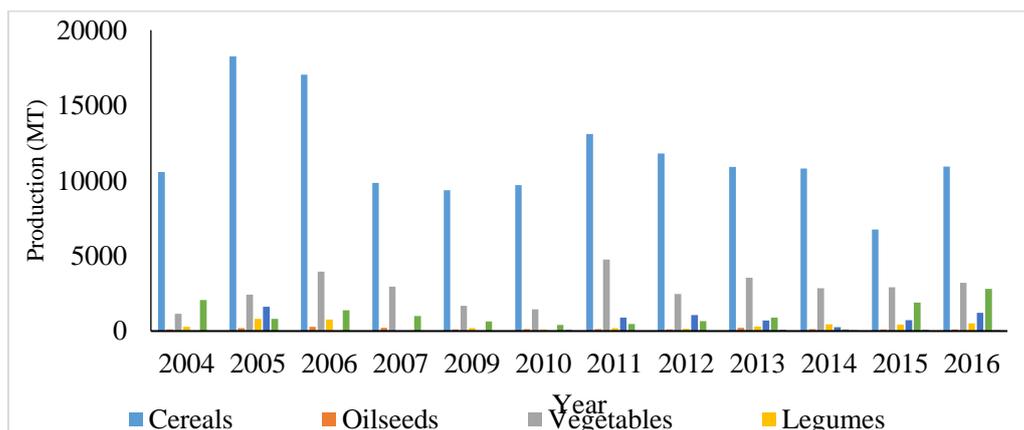


Figure 3. Organic crop production (MT) in Gasa and Samdrupjongkhar districts
Data source: (Department of Agriculture, 2004; Department of Agriculture 2005; Department of Agriculture, 2006, 2007a, 2009, 2010, 2011, 2012, 2014, 2015, 2016).

Many studies showed the reasons for slow growth of OA in Bhutan despite ambitious commitment (Feuerbacher *et al.*, 2018; McCrae-Hokenson, 2014; Tashi and Wangchuk, 2016). There are number of studies contesting the capability of OA system's production. Markuszewska and Kubacka (2017)

stated the inability of OA to fulfill food sufficiency. Contrary some studies attested that OA can produce enough quantity of food to meet the growing demand Badgley *et al.* (2007); Paull (2017); Rundgren (2002); Ton (2013).

Crop varieties

As depicted in Figure 2, the crop production has been more diversified. However, the variety in cereals and oilseeds have infinitesimally small variation since 2004. Unlike olden days, the diversification of crops have reduced the dependency on the production of cereals (Planning Commission, 1999). In the past, the farmers of Bhutan rely on cereals and very few cultivated other crops.

Now with the introduction of more crop variety, farmers could enjoy the change of the production system. New crops have provided farmers with a new source of income fundamentally changing the dimensions of subsistence agriculture as well as the lives of farmers (Planning Commission, 1999).

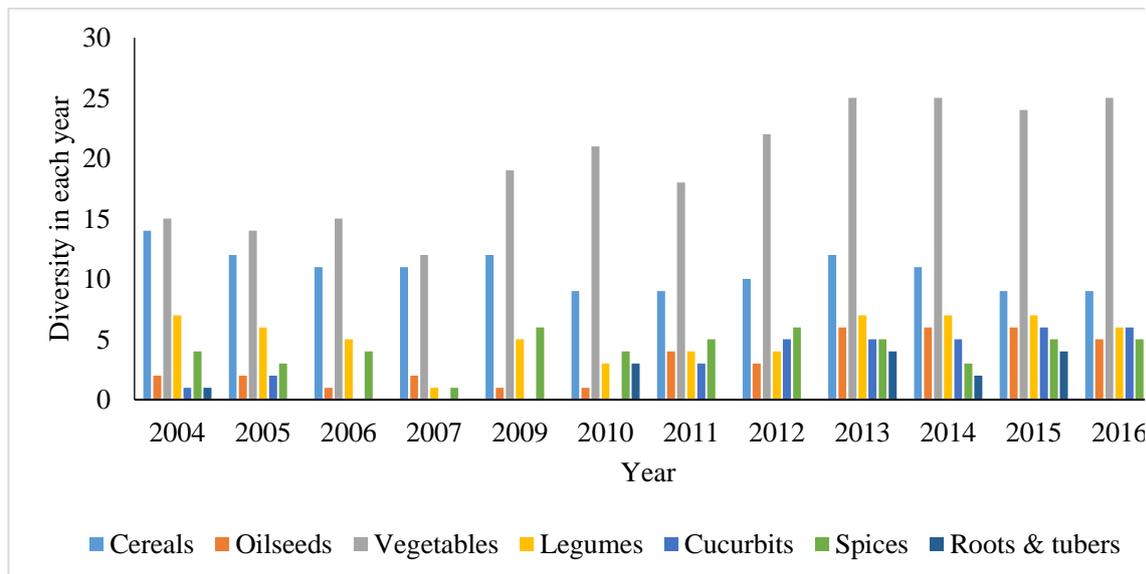


Figure 4. **Organic crop diversity in Gasa and Samdrupjongkhar districts**
 Data source: (Department of Agriculture, 2004; Department of Agriculture 2005; Department of Agriculture, 2006, 2007a, 2009, 2010, 2011, 2012, 2014, 2015, 2016)

OA contributes to regenerate ecosystem by providing species evenness to ecosystems (Crowder *et al.*, 2010). This is because diversification and maintaining species evenness are the paramount principle components of OA system. as revealed in the figure the species richness is steadily increasing in consecutive years.

Fertilisers and pests control mechanisms

Generally, the farmers had been relying heavily on traditional knowledge that uses organic materials such as cattle manure, leaf litter, and crop residues as fertilizers (Dorji, 2008; Tashi and Wangchuk, 2016). Leaf litters are used for soil fertility management (Kobayashi *et al.*, 2015). These had been the reason that agriculture in Bhutan is well-known for organic by default. The recent organic manure

used in the farms are vermicompost and liquid manure. National Organic Program provides guidelines on preparation of vermicompost, liquid manure, natural pesticides, panchgaviya, and pests and disease control measures.

They also practice some techniques such as crop rotation, intercropping and agroforestry for pest control mechanisms. Also plants repellent plants such as marigold flower, ginger and chrysanthemum are used. However, Bhutan still lacks the implementation of modern organic mechanism (Feuerbacher *et al.*, 2018).

Technologies

The farm road connecting the far-flung of the villages, mobile phones connectivity and hydroelectricity throughout the country had been a recent achievement. With the accessibility to all those mechanisms, the farmers got better opportunity to access for agricultural technologies ever than before (Dendup, 2018). Moreover, Agriculture Machinery Centre (AMC) was established in 1983 with the following objectives:

1. Creation and innovation of indigenous farming tools suitable to Bhutanese farms.
2. Promotion of improved agriculture technology in the country.
3. Effective and efficient distribution of agriculture equipments to the farmers.
4. Capacity development of the farmers in the use of improved technology and farming practices.(MoAF, 2018).

However, Bhutan managed to adopt very few technologies. According to Feuerbacher *et al.* (2018), OA in Bhutan is largely based on the phasing out of chemical inputs and does not properly integrate the improved organic farming practices. The commonly used technologies include electric fencing and power tiller. The recently few machines for weeding, transplanting, threshing and harvesting are also used by the farmers.

Farmers' awareness

The country's ambitious commitment to go fully organic by 2020 was announced in the opening session of International Federation of Organic Agriculture Movements Sustainable Development Learning Event at UNCSD Rio+ 20 in 2012 by former Prime Minister Jigme Y Thinley. The OA programme has been in place since 2004 during which the Gasa district was declared as 100 % organic district. Also, Ministry of Agriculture (MoA) officially launched the policy National Framework for Organic Farming in Bhutan (NFOFB) for organic farming in 2007 (Department of Agriculture, 2007b). NOP was formed as the focal agency for coordinating and implementing activities for promotion of OA which got its status in 2006.

However, the concept is yet to reach every farmer of the country. Many studies stated that farmers still has limited knowledge on OA (Feuerbacher *et al.*, 2018; Tashi and Wangchuk, 2016). Another study reveals that farmers of Bhutan had varied understanding on OA (Kobayashi *et al.*, 2015). They found that the farmers of Gasa district has the highest awareness level at 80 %. Feuerbacher *et al.* (2018) found out the reason for the failure of achieving the commitment. The author learned that 100 % OA policy is not the result of a bottom-up process involving farmers, but rather a top-down policy and concept has not reached every farmer.

Conclusions

The study found that Bhutan is trying to promote OA through various means. As of now two districts are declared organic and practice in small pockets in other 18 districts. However, OA has still not been so vibrant in terms of implementation due to mentioned reasons. It questions the ambitious commitment of becoming 100 % organic by 2020. The rigorous work needs to be done for the goal. We would suggest providing trainings to the extension officers to train farmers and to give awareness on the value of organic production to the farmers by NOP. More research and development should be done to introduce modern mechanisms of OA.

However, the findings of this study were limited to the secondary data. The production and variety trend were only from two districts since data was not available for other organic groups. Future researchers could explore more on these five dimensions through field visits and interviewing the farmers. The other scopes include development of organic pest repellents, organic fertilisers and relevant technologies; assess production using Life Cycle Assessment approach; and impact of organic agriculture on environment.

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Growth performance of climbing perch (*Anabas testudineus*) fingerlings fed at different feeding rate

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Abstract

A twelve-week study was conducted to determine the growth performance of *Anabas testudineus* fingerlings fed at different percentage body weight. The fish were fed with same commercial pellet in four treatments; 2% of body weight (Treatment 1), 4% of body weight (Treatment 2), 6% of body weight (Treatment 3) and at ad libitum (Treatment 4). Growth performance of the fishes was observed in terms of weight gain, body length, specific growth rate, food conversion ratio (FCR), survival rate, and protein efficiency ratio. Proximate analysis of the fishes also was conducted at the end of the experiment. Average body weight gained by *Anabas testudineus* was highest for Treatment 2 ($185.17 \pm 52.59\%$), followed by Treatment 3 ($176.51 \pm 10.68\%$), Treatment 4 ($91.44 \pm 27.72\%$) and Treatment 1 ($49.78 \pm 12.21\%$). Best FCR value also was obtained from Treatment 2 (1.63 ± 0.407). Fish from Treatment 2 has the highest lipid ($30.39 \pm 2.484\%$) and energy (6.07 ± 0.485 kcal/g) content compared to other treatments. It also possesses the lowest ash ($15.28 \pm 2.754\%$) level. Protein content of fish from Treatment 2 ($53.80 \pm 1.975\%$) has no significant differences with Treatment 4 ($55.31 \pm 1.185\%$) which possess the highest protein content. Thus, Treatment 2 where *Anabas testudineus* fed at 4% of body weight can be applied as the optimum feeding rate in *Anabas testudineus* farming due its best growth performance and body composition.

Keywords: *Anabas testudineus*; optimum feeding rate; growth performance; body composition; fingerlings, tropical

Introduction

Anabas testudineus (climbing perch) is an important air-breathing freshwater species widely distributed throughout India and also in other Southeast Asian countries. The species is considered as a valuable dietary item for sick and convalescents, as it contains a high amount of physiologically available iron and copper, which are essentially needed for haemoglobin synthesis. Over the years, this species was highly consumer demand due to its commercial value and medicinal value (Kamal et al., 2010).

Adhikary et al. (2009) stated that *A. testudineus* is a tropical fish that inhabit in fresh and brackish water throughout the world. It is one of the most economic and important fishes because of its high market demand, good nutritional value and delicious taste. They are hardy species and can tolerate extremely unfavourable water conditions and feed primarily on fish but also on macrophytic vegetation, shrimps, fish fry and insects. However, suitable feeding

strategy is not only important to improve fish growth, but it is also important to reduce the production costs and environmental pollution. The cost of feed is a major constrain in fish production. It minimizes the production cost and also ensures the proper utilization of the animal waste.

Fish feeding activity play a decisive role in growth and survival. Feeding practices such as feeding rate, feeding frequency, feed particle size, and methods of delivering feed to the fish will affect fish growth rates, food conversion rates, and uniformity of fish size within a pond. Feeding rate can be to satiety or at a fixed rate, expressed as a percentage of body weight per day (Hardy, 1989). Feeding rate means food intake per unit feeding time. It also stated that feeding rate can be affected by many factors such as morphological factor, physiological factor, social factor and scramble competition characteristics of animals (Cho et al., 2006; Nakagawa, 2008).

Presently, there is no published information on the specific effects of feeding rate on growth performance of *A. testudineus* fingerlings. Thus, the objective of this study is to evaluate the effect of feeding *A. tetudineus* fingerlings at different percentage of body weight towards their growth performance.

Materials and Methods

Fish and tank set up

Anabas testudineus fingerlings were bought from local farmers in Kajang, Selangor and brought to University Putra Malaysia Research Centre in Puchong, Selangor. Thirty fingerlings of *A. testudineus* with initial length of 3-3.5 inches and initial weight of 3-4.5 gram were stocked in (90 x 45 x 45) cm aquaria for four feeding rate treatment (2% of body weight, 4% of body weight, 6% of body weight, ad libitum) in triplicates. The experiment was in twelve weeks. All the aquaria arranged on rack in completely randomized design (CRD) and connected to the aeration system. The fingerlings were acclimatized to the culture condition for seven days prior to start the experiment. The fingerlings fed with commercial pellet *ad libitum*, twice a day during the entire period of the experiment. Quality of the water was maintained by daily water exchange at 5 – 50% rate. The excess feed and feces were siphoned out every day. Water was changed if the pH and dissolve oxygen level were in poor condition.

Experimental diets and feedings

The *Anabas testudineus* fingerlings was fed twice per day with starter commercial fish pellet. They were fed at 0900 h and at 1700 h. The feed contained a minimum 32% of crude protein, maximum 10% of moisture, minimum 5% of crude fat, maximum 5% crude fiber, and maximum 10% of crude ash. The total amount of feed given per day was divided into two parts; for morning and evening.

Data Collection

All the fishes from each treatment was sampled once per week to measure their growth performance in term of weight (g), and length (cm). The number of fish survive also recorded daily. Water temperature were taken daily in this study by using digital thermometer. Only four basic water parameter measured in order to maintain the water quality. Dissolved oxygen concentration and temperature was measured by dissolve oxygen meter, water pH was measured using pH meter weekly and ammonia level measured using ammonia test kit.

Analytical Methods and Statistical Analysis

The chemical analyses were carried to determine proximate composition such as moisture, protein, lipid, ash content and energy content. It was carried out according to the methods of AOAC (1993). Growth performance of these *A. testudineus* was evaluated in term of weight gain (%), length increment (cm), specific growth rate (SGR) (% day⁻¹) and survival rate (%) using these formulae (Sawhney and Gandotra, 2010; Muntaziana et al., 2013):

$$\text{Weight Gain (\%)} = [(W_2 - W_1)/W_1] \times 100$$

Where:

W_1 = Initial weight of fingerlings

W_2 = Final weight of fingerlings

$$\text{Length increment (cm)} = \text{final length (cm)} - \text{initial length (cm)}$$

$$\text{Specific Growth Rate (\% day}^{-1}\text{)} = [(W_2 - W_1)/t] \times 100$$

Where:

W_1 = Initial weight of fingerlings

W_2 = Final weight of fingerlings

t = time

$$\text{Food Conversion Ratio (FCR)} = \text{Total feed intake (g)/weight gain (g)}$$

$$\text{Protein Efficiency Ratio (PER)} = \text{weight gain (g)/protein intake (g)}$$

$$\text{Survival (\%)} = T_2 / T_1 \times 100$$

Where:

T_1 = Initial number of fingerlings

T_2 = Final number of fingerlings

The calculated results were subjected to one way analysis of variance (ANOVA) using SPSS software. The differences between treatment used in the experiment were compared using Duncan's multiple range test at significance level $p < 0.05$. All percentage data were arcsine transformed prior the analysis.

Results and Discussion

Water quality parameters measured throughout this study are summarized in Table 1. Water temperature are recorded in the range of 25.5 – 27.5 °C. The water pH was in range 5.5 – 8.0 for each treatment. The dissolve oxygen (ppm) was in the range of 3.67 – 5.01 by aeration system with slow air bubble. The variations of water parameters in this study were in the

suitable range and acceptable for *Anabas testudineus* culture (Habib et al., 2015; Uddin et al., 2016).

Table 1. Water parameters of treatment on *Anabas testudineus* fingerlings fed at different feeding rate

Water Parameter	Treatment 1 (fed at 2% of body weight)	Treatment 2 (fed at 4% of body weight)	Treatment 3 (fed at 6% of body weight)	Treatment 4 (fed at ad libitum)
Temperature ($^{\circ}\text{C}$)	25.5 – 27.5	25.5 – 27.5	25.5 – 27.5	25.5 – 27.5
pH	5.5 – 8.0	5.5 – 8.0	5.5 – 8.0	5.5 – 8.0
Dissolve Oxygen (ppm)	5.68 – 6.03	4.67 – 5.01	4.15 – 4.42	4.98 – 5.48
Ammonia (ppm)	1.5-2.0	1.5-2.0	1.5-2.0	1.5-2.0

Growth performance of *Anabas testudineus* fry fed with different feeding rate are presented in Table 2. The weight gain (g) of fish in the all treatment showed positive growth where *Anabas testudineus* in Treatment 2 showed the highest weight gain (185.17 ± 52.59). This result was different with Aryani et al. (2017) and Okorie et al. (2014) where fishes in their study showed highest weight gain in the treatment feeding at 6% of body weight and at satiation respectively. *Anabas testudineus* fed with 4% and 6% of body weight showed no significant different ($P > 0.05$) in fish length increment (5.00 ± 0.350 cm and 5.10 ± 0.450 cm respectively) which is significantly higher ($P > 0.05$) than fishes fed at 2% of body weight and fed at ad libitum (4.31 ± 1.069 cm and 4.45 ± 0.161 cm respectively).

Fishes in Treatment 2 possess the highest specific growth rate (SGR) (2.23 ± 0.214 % day $^{-1}$) which no significant different ($P > 0.05$) with fishes in Treatment 3 (2.21 ± 0.043 % day $^{-1}$). The value of SGR for the *Anabas testudineus* in this study were less than SGR of the *Anabas testudineus* studied by Kohinoor et al. (2007) and Hossain et al. (2012). This may due to the variety of climbing perch used in the experiment. The best food conversion ratio also found in Treatment 2 (1.63 ± 0.407) as well as the result of protein efficiency ratio (0.89 ± 0.100). Fishes in Treatment 2 possess $98.89 \pm 1.923\%$ of survival rate which is not significant different ($P > 0.05$) with Treatment 1 ($100.00 \pm 0.00\%$) and Treatment 4 ($100.00 \pm 0.00\%$). This may be due to water quality level such as range of dissolve oxygen in Treatment 2 is slightly lower compared to Treatment 1 and Treatment 4.

Table 2. Growth performance of *Anabas testudineus* fed at different percentage body weight during experimental period.

Parameters	Treatment 1	Treatment 2	Treatment 3	Treatment 4
IBW ¹ (g)	4.48 ± 0.376^a	4.08 ± 0.571^a	3.97 ± 0.058^a	4.61 ± 0.443^a
FBW ² (g)	6.74 ± 1.036^c	11.45 ± 0.463^a	10.97 ± 0.390^a	8.75 ± 0.580^b
WG ³ (%)	49.78 ± 12.21^b	185.17 ± 52.59^a	176.51 ± 10.68^a	91.44 ± 27.72^b
LI ⁴ (cm)	4.31 ± 1.069^b	5.00 ± 0.350^a	5.10 ± 0.450^a	4.45 ± 0.161^b
SGR ⁵ (% day $^{-1}$)	1.48 ± 0.100^c	2.23 ± 0.214^a	2.21 ± 0.043^a	1.76 ± 0.172^b
FCR ⁶	2.36 ± 0.931^b	1.63 ± 0.407^a	3.55 ± 0.303^b	1.87 ± 0.986^a
PER ⁷	0.76 ± 0.160^{ab}	0.89 ± 0.100^a	0.58 ± 0.030^b	0.87 ± 0.210^a
SR ⁸ (%)	100.00 ± 0.00^a	98.89 ± 1.923^a	97.78 ± 1.923^a	100.00 ± 0.00^a

Letters with same superscript in rows are not significantly different ($P > 0.05$)

¹ Initial Body Weight; ² Final Body weight; ³ Weight Gain; ⁴ Length Increment; ⁵ Specific Growth Rate; ⁶ Feed Conversion Ratio; ⁷ Protein Efficiency Ratio; ⁸ Survival Rate

Body composition of the *Anabas testudineus* in this study were analyzed in the laboratory as the result showed in Figure 1. Moisture content of all the fishes were in normal range as the fishes in Aryani et al. (2017). Protein content of the fishes in this study were very height compared to *Anabas testudineus* studied by Hossain et al. (2012) and Cho (2014). Lipid content of *Anabas testudineus* in Treatments 2 and Treatment 3 is very height $30.39 \pm 2.484\%$ and $29.02 \pm 1.43\%$ respectively while lipid content of *Anabas testudineus* in Treatment 1 and 3 were as the same range of fishes studied by Mizanur et al. (2014). Energy content of fishes in this study were slightly higher than *Anabas testudineus* studied by Hossain et al. (2012).

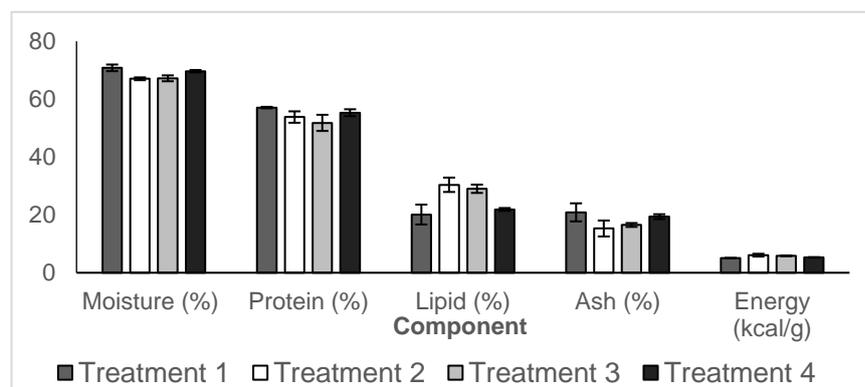


Figure 1. Body composition of *Anabas testudineus* from each treatment

Feeding rate used in this study were affecting the results of growth performance and body composition of *Anabas testudineus* where most all the parameters in Treatment 2 and Treatment 3 is not significantly different ($P > 0.05$). But the results of most parameters in Treatment 2 and 3 are better than treatment 1 and 4. In comparing of feeding *Anabas testudineus* at 4% of body weight (Treatment 2) and 6% of body weight (Treatment 3), the 4% of body weight is preferable since it can reduce cost of feed where smaller amount of feed used was gave the result of better fish growth performance and body composition. This was different from fishes studied by Aryani et al. (2017) where their fishes are increase in growth performance with increasing of the feeding rate applied. Thus, application of feeding fish according to the rate of body weight is practical since Okorie et al. (2014) stated that feeding trials that were studied showed the result of optimum growth performance for their fishes.

Conclusion

In conclusion, the best feeding rate for *Anabas testudineus* in this study is by feeding at 4% of body weight (Treatment 2). *Anabas testudineus* in this treatment possess highest weight gain, specific growth rate, feed conversion ratio, protein efficiency, and survival rate. From proximate analysis, Treatment 2 also show the good body composition. *Anabas testudineus* in this treatment possess high protein, lipid and energy content.

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Holistic agriculture as the model for understanding of sustainable society

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Abstract

Establishment of sustainable society is one of the most important global issues. Progress of technics for agriculture enabled us for mass production of agricultural products. This led to the convenient style of daily life and growth of population in the global level. However, sustainability of the social system has decreasing in the global level because of the limited land space and natural resources as well. Right observation of the current situation would lead to the right understanding of the law of nature of the global system. Alternatively, trial of natural farming would contribute on the holistic understanding of the natural law. At present, we have a limited opportunity to consider about the sustainability without the biased idea that would be taken as the common sense of the society during the process of social development. Appropriate scale of natural farming (holistic agriculture) likely to lead to coming up the idea for sustainable society.

Keywords: Sustainable society; Natural farming; Holistic agriculture; Global.

Introduction

In the modern agriculture, up to eight-fold increase of yield was performed mainly by four elements. However, it has problems in terms of sustainability. Firstly, improvement of seeds was done by cross-hybridization and artificial selection for open pollinated seeds. Major global companies are occupying the seed market for selling F1 hybrid seeds. F1 hybrid seeds grow better quality vegetables easily. However, this trend resulted in the loss of open pollinated seeds elected by local farmers themselves. Thus, major seed companies control most of the modern farming. Furthermore, spread of GMO seeds has a risk to contaminate local open pollinated seeds. The other three elements that contributed on the increase of yield are introduction of chemical fertilizer, pesticide and mechanization.

Practice of ESD in agriculture

The remarkable increase of yield of products in modern agriculture cannot solve the unequal problem such as poverty or starving in the global level. This indicates that the total amount of agricultural products do not promise the food supply and satisfactory. Thus, this situation is not sufficient for sustainable society even though this system may continue for a long time.

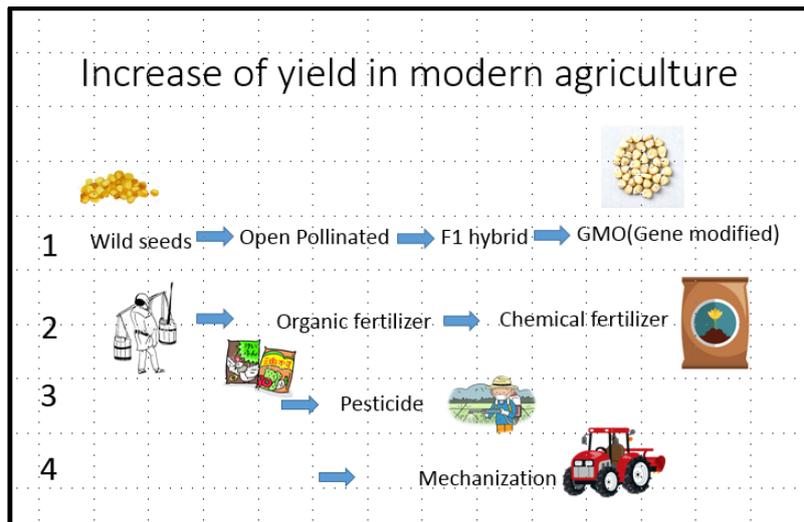


Figure 1. Factors for increasing yield in modern agriculture.

Education for Sustainable Development (ESD) is commonly understood as education that encourages changes in knowledge, skills, values and attitudes to enable a more sustainable and just society for all (Leicht et al. 2018). School education introduced ESD as a key element of quality education for sustainable development. Among many topics concerning ESD, agriculture seems to be one of the important topics to understand ESD. In the present study, I would like to propose that natural farming in the local community would contribute on the understanding of sustainability and this idea will be applicable to the global level in future.

Ecology, economy and society are considered as the major three elements of ESD. Ecology (ecosystem) is closely related to the understanding of interactions of natural events. The theory of natural farming is based on the natural events caused by the law of nature, and based on the ecology.

Thus, ecology can be understood as the result of the integration of each element of nature. Understanding of the various factors concerning interactions between different organisms is essential for the study of an ecosystem. The easiest way to understand the ecosystem would be to just take a look at nature around without a biased idea, and accept the current situation under the control of the law of nature. If needed, the introduction of a historical view will be helpful for understanding of an ecosystem in terms of the interaction of each other with surrounding other organisms.

Natural farming

Natural farming has a philosophy that we are just a part of nature. In order to understand the interaction of the agricultural plant with other surrounding organisms, we should keep a minimal reaction to nature by farming because nature will automatically give us fruitful products without any reaction to nature.

M. Fukuoka is the originator of natural farming with taking the philosophy of nature (Fukuoka 2009). He observed agricultural plants in terms of their interactions with other surrounding plants. For applying the theory into practice as natural farming, he tried

farming without pesticide nor fertilizer. Furthermore, he tried farming without cultivation in order to keep the agricultural field as a natural condition. He left even natural grasses in the field. Under these conditions, agricultural plants usually cannot grow up well by serious affection of surrounding wild grasses. Under these philosophy for natural farming, M. Fukuoka influenced many people who want to study the mechanism of interactions in nature as a whole system. They tried natural farming of various vegetables, fruits or rice by following Fukuoka's methods in their own fields.

The most difficult point is the competition of plant growth between agricultural plants and surrounding wild grasses. Rapid growing up of the wild grass usually suppress the vegetable plant grow in the natural farming. To avoid this negative selection by natural grass, he tried to seed several kind of vegetable plant seeds concomitantly in one place. Mixture of several kinds of seeds may be one of the possible ways to avoid the influence of surrounding wild grass by natural selection. For this purpose, he made soil cake containing several vegetable seeds and put it on the agricultural field. Variation of plants and microorganisms in soil enabled a selected plant growth in agricultural fields. Thus, Fukuoka's method may be useful for those who want to study the mechanisms of nature.

However, this variety of plants in agricultural field cannot promise the yield of farming. Furthermore, wide variety of plants is not a good condition for harvesting. Another problem of natural farming may be a variation of condition in each agricultural field. Setting up of the initial stage for farming will be essential for natural farming. Natural field where many natural leaves are spoiling is rich in organic fertilizer. This interaction between different kinds of plants seems be a model for natural farming.

Y. Kawaguchi and A. kimura are those who followed Fukuoka's method with several modifications in the procedure and got some successful results. They modified Fukuoka's method of natural farming for removing wild grass by hands if needed. A. Kimura use wood vinegar instead of pesticide for protection against insect attacks.

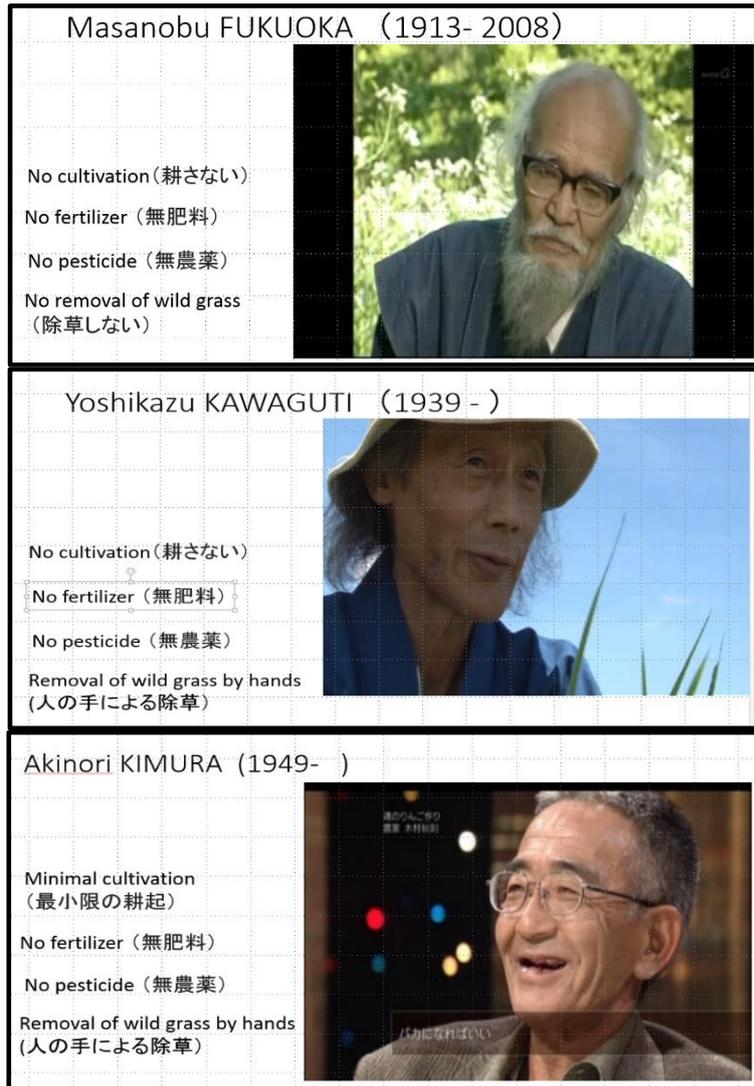


Figure 2. Frontiers of natural farming.

<Upper> M. Fukuoka is a philosopher for natural farming based on the law of nature. He tried minimal effort agriculture following the law of nature. <Middle> Y. Kawaguchi is a practical natural farmer organizing a natural farmer’s association. <Lower>A. Kimura applied M. Fukuoka’s philosophy for apple tree plantation. He took 8 years for getting the apple (Miracle apple) by natural farming (Ishikawa 2008). Some farmers followed Kimura’s method by applying to the vegetables, rice and peach tree plantation with slight modifications.



Figure 3. Traditional agriculture in the mountainous area in Tokushima Prefecture, Japan. Kaya (Japanese pampas grass) is used for natural fertilizer. Covering of the soil by Kaya have the role to protect flow away of soil in the rainy season in the sloped area.

Traditional agriculture using Kaya

Traditional agriculture using Kaya (Japanese pampas grass) was nominated as the world heritage for agriculture in 2018. This style of agriculture was typical natural farming in the mountainous area in Shikoku Island, Japan (Nagai 2018). Mixing up of soil with dried Kaya cut into small pieces was done before seeding of vegetable plants every year. The field where these kind of agriculture may be ready for farming without cultivation. Appropriate cultivation of the field may be needed depend on the condition of the field.

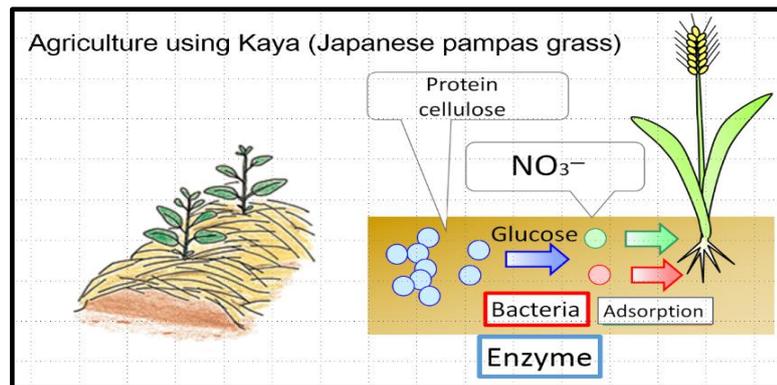


Figure 4. Theory of agriculture using Kaya.

Kaya-derived proteins and cellulose are digested into amino acids or glucose by bacteria-derived enzymes in the soil. Amino acids and glucose are used as the nutrients for bacterial growth, and NO_3^- for plant growth.

Reductionism and holism

We should remind that the usage of “sustainable” should focus not on only the total system but also on elements and interaction between the elements that compose the total system. If the total system of agriculture in a large scale may run in a sustainable condition depend on the large scale of modern agriculture, improvement of unequal situation in the global level will be hard because of the problem of capitalism exist in this modern agriculture.

Therefore, I may propose the term of “holistic” instead of “sustainable” for the agriculture as the sustainable style, because sum of such communities become sustainable and no starving nor poverty. Furthermore, the cost for transportation of agricultural product should be minimum. This means that the use of fossil energy will also be minimal in this holistic agriculture.

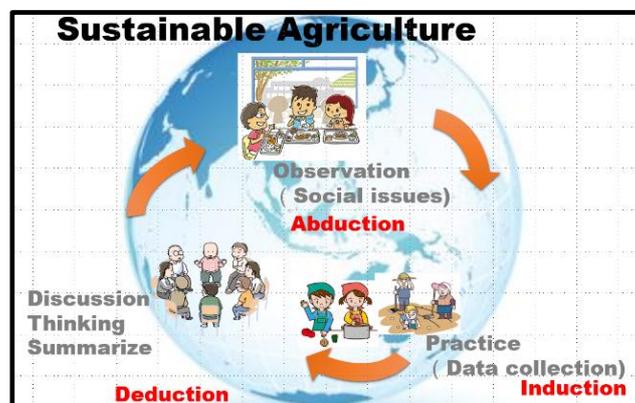


Figure 5. Theory for conceptualization for sustainable agriculture in global level.

The biased amount of agricultural production per person in each country will result in the large amount of waste of food as well as starvation in other countries.

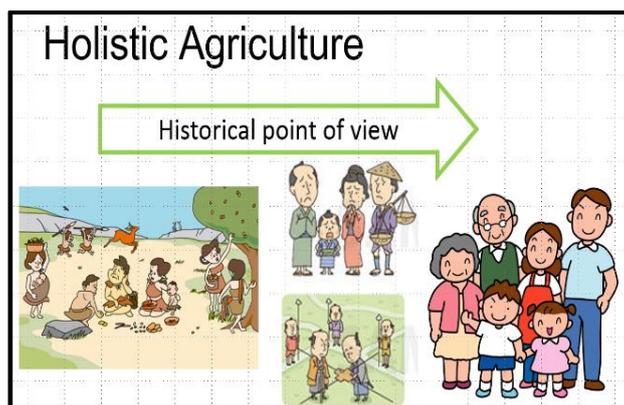


Figure 6. Holistic agriculture from the historical point of view.

Holistic agriculture will act as the model for understanding of the discrepancy of agricultural products supply (waste of food and starving). Introduction of historical point of view is helpful for understanding of the discrepancy.

A kind of virtual utopia was set up as the object of mass production by mono-agriculture. Enough amount of food produced by mass-scale agriculture should be supplied for all the people. However, food distribution is not going well and starvation is still remaining in all over the world except for several developed countries. Thus, mass production by mono-agriculture cannot solve the issues for unequal society. This may be caused by the discrepancy between the virtual utopia and achievement of equal society.

In the ancient period, people in the small community get all the food for all the people in the community. In general, the population has been controlled by the amount of food in the community. In this ancient society, sustainability of community was depending on law of the nature.

Trial of natural farming in the local community have a role to remind us the wisdom for the relationship between our activity for agriculture and the law of nature. This will act as the trial for social system of sustainable society in the local community in terms of the food supply.

Expansion of the successful cases of natural farming in the local community will contribute on the planning for the theory establishment for holistic agriculture.

Science of natural farming

Reductionism contributed on the development of modern science. Agriculture has been developed through many experiences. In these experiences, the continuous circuit of observation (Abduction), practice (Induction) and thinking (Deduction) contributed on the increase of yield. The cycle of these three elements is held in common with modern science. Thus, natural farming is an applied science for holism.

In the modern science, the main object was set to clarify the novel element(s) in some natural events. In this case, reductionism is useful for clarifying the novel element(s). So, most fields

of science have developed under the reductionism. Similar to the agriculture, observation, experiment and thinking are three major elements for the process of the research in scientific methods. Observation lead to the abduction that acts for establishing the hypothesis. Practice is the place for the possible application of the hypothesis as the induction. Thinking of the experimental result is the process that determines the next observation for the expansion of the deductive thinking way. Among three factors for scientific research, observation of the natural events is the first step for the start of the research work.

In the agriculture, observation of plant growth is the first step for understanding the control mechanism by nature collaborating with surrounding other plants. Trial for modification of the natural environment around the plants by a hypothesis will acts as the induction process. Speculation of the result will lead to the improved method based on the revised hypothesis for growing up the plants. This cycle of each process would be essential for the development of natural farming.

Holistic standpoint of view

Development of science has been performed by applying the reductionism. Understanding of this theory as law of nature is essential for the integration process of each element of natural phenomenon. In general, right observation of natural phenomena is the first step for right understanding of the law of nature. Thus, observation of natural phenomena is the start point for either science or natural farming. We have to remind that “the noble eightfold path” (Fig 8) also start from right view, followed by right intention. Gautama Buddha found that the right view in the real world as it is should be the first for the removal of suffering. The solution of the current problem is kept in common with removal of suffering and problems of natural farming, because results of practice are desired for improvement as soon as possible. Therefore, we can apply this theory not only for science but also for natural farming.

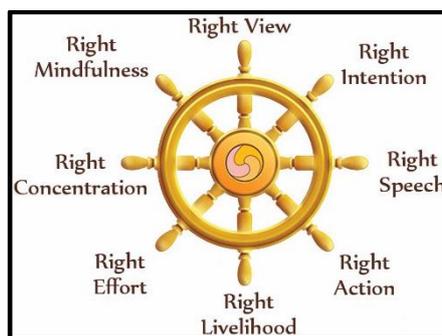


Figure 7. The Noble Eightfold Path.

The Noble Eightfold Path is an awakening of Gautama Buddha for leading to liberation from samsara (suffering).

Conclusion

In conclusion, trial of natural farming will be useful for ESD as the model of sustainability for local community. Furthermore, process of natural farming is the novel findings of law of nature. To solve problems in natural farming, continuous cycling of network of abduction, induction and deduction is essential akin to the scientific practice. In contrast to science, integration of the result of natural farming is required for establishment of sustainable society as the grovel level as the holistic agriculture. Introduction of historical point of view will be helpful for this integration process.

In contrast to the modern science, the final purpose of the development is the increase of yield of product. In the traditional agriculture, a population of the community determined the desired yield. Therefore, the maximum yield of agricultural products and population of the community is determined by the total system of nature in the local community. In this system, reductionism is not a main stream of wisdom for development of agriculture. Thus, development of agriculture in the local community has been done mainly by holism under the transforming process.

Thus, development of natural farming seems to be the ultimate model for understanding the holism and reductionism as well as sustainable society.

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Supporting activities and preservation of the traditional food culture for sustainable development in Fukushima, Japan

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Abstract

Magnitude 9.0 earthquake and huge tsunami struck northeast Japan on March 11, 2011, which lead to the Fukushima's nuclear power plant accident. Many people in Fukushima escape from their hometown because of pollution by radioactivity. After that, many communities including traditional culture were destroyed in Fukushima, and they have been suffering from reputational risk, prejudice, and bullying. For seven years, I have done some activities for supporting Fukushima where I was born, by telling current situation, radioactivity level, and correct knowledge about radiation to people including young students, and sending out questionnaire about safety and reputational risk to Fukushima. From these results, I have found that misunderstanding radioactivity and its effects on human might cause the prejudice against people, foods, and cultures in Fukushima. I also focused on the food cultures in Fukushima. In Fukushima, the production and consumption of cucumber are very high. Some are made into pickles such as the traditional Soma cucumber pickles (SP) produced at northern part of Fukushima, the production method is unique that whole cucumber preserved and fermented with salt, followed by soak into soy sauce seasoning liquid. Thus far, bacteria in SP have not been reported. In order to archive this traditional food culture as report, I investigated the bacteria in two kinds of SPs (SP1 and 2) by the clone library method. As results, it was elucidated that they contained three kinds of LABs (*Leuconostoc citreum*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*). Therefore, as compared with popular cucumber pickles in Japan, we found that SP are different in bacterial feature containing these LABs. To preserve traditional food culture in Fukushima, more studies on the isolation and preservation of useful LABs are greatly needed.

Keywords: Fukushima's nuclear power plant accident, Supporting activity, Cucumber pickles, Traditional food culture

Introduction

Magnitude 9.0 Great East Japan Earthquake and huge tsunami struck northeast Japan on March 11, 2011, and the dead and the missing persons were about 22,100 totally. Subsequently, Fukushima 1st nuclear power plant accident happened, followed by the proliferation of large amount of radioactive substances and radioactive contamination in East Japan, especially Fukushima prefecture (Figure 1). Minami-soma city (about 70,000 population) where I was born, is located in the northeast area of Fukushima prefecture, and

its distance from Fukushima 1st nuclear power plant is about 20~40 km (Figure 1). This city was severely hit by earthquake, Tsunami (about 1,100 persons dead, Figures 2&3), and the following the Fukushima's nuclear power plant accident. So many people must escape from their hometown because of radioactive contamination (Figures 4). After that, many communities including traditional culture were destroyed in Fukushima, and they have been suffering from reputational risk, prejudice, and bullying. Even after 5 years, over 90,000 people had been forced to live in temporary houses as refugees (Figures 5).

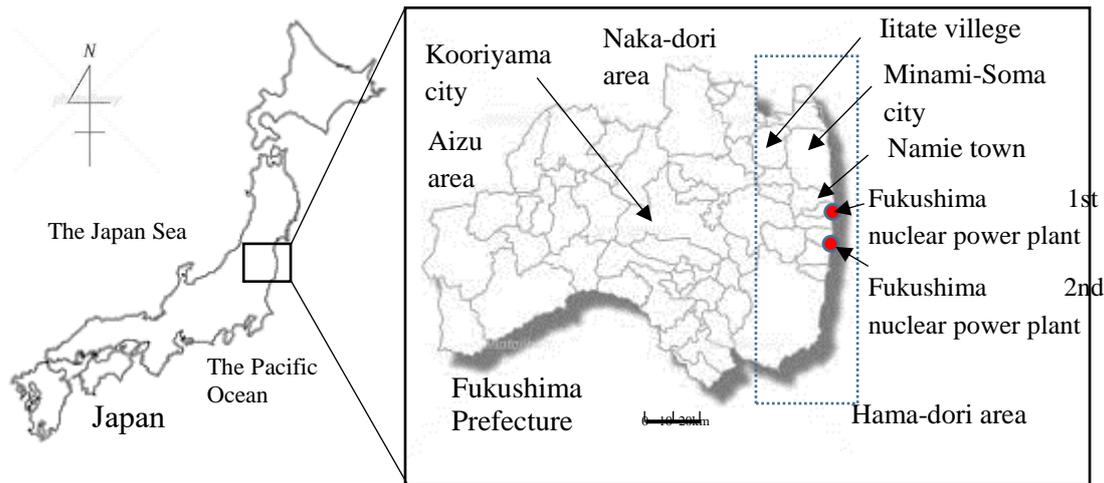


Figure 1. Location of Fukushima prefecture in Japan

Therefore, to explore clues for solution against above problems on Fukushima, I have done some activities for supporting Fukushima by telling current situation, radioactivity level, and correct knowledge about radiation to general people including young students, and sending out questionnaire about safety and reputational risk to Fukushima, and so on (Satoh et al., 2016, 2017). In this paper, I describe that the results of these supporting activities including radioactivity measurement on Minami-Soma city and Namie town (Figure 1), education for radiation, the conferences and lectures for telling their experiences and problems relating with disaster in Fukushima for seven years.

Furthermore, I focused on the agricultural products and food cultures in Fukushima, Japan. So many farmers had produced vegetables, fruits, rice in Fukushima. However, because of effects of high radiation dose and reputational risk, they could not produce agricultural products after above accident, but now they can do by carrying out an inspection for radioactivity level in food strictly. In Fukushima, the production and consumption of cucumber are very high. Some are made into pickles such as the traditional Soma cucumber pickles (SP) produced at northern part of Fukushima (Figure 1), the production method is unique as compared with generally produced pickles in Japan (Miyao, 2002), whole cucumber preserved and fermented with salt, followed by soak into soy sauce seasoning liquid. As mentioned above, it was possibility that this traditional food culture in Fukushima might disappear by disaster at that time. Hence, in order to archive and preserve this

traditional food culture, I investigated the features of bacteria in two kinds of SPs (SP1 and 2) by the clone library method, which bacteria have not been reported.



Figure 2. Lost community by Tsunami (2012).



Figure 3. Broken cars by Tsunami (2012).



Figure 4. Spoiled soils by decontamination at Minami-Soma city (2012).



Figure 5. Temporary houses at Minami-Soma city (2016).

Materials and Methods

Examination of situation in Fukushima for seven years

From the year 2012 to 2017, I visited to Kooriyama city (Naka-dori area), Iitate village, Minami-Soma city, and Namie town (Figure 1). I took photos, and interviewed some persons including my parents and brothers. The collected materials are used for some lectures and conferences. On the other hand, the investigations for the radioactivity level were carried out in above area, especially Minami-Soma city (the year 2012-2017) and Namie town (the year 2017), by using environmental radiation monitor Radi PA-1100 (Horiba Ltd.) for detecting ^{137}Cs . The environmental radiation dose was shown as micro-Sievert (μSv) per hour or mSv per year, which means the amount exposed to radioactivity for human.

Analysis for bacteria in Soma cucumber pickles

As mentioned above, Soma cucumber pickles (SP) are the traditional food at northern part of Fukushima including Minami-Soma city. I purchased two kinds of SP (SP1 and SP2) at Aug.2017 in Minami-Soma city. SP1 was produced at Kashima area, whereas SP2 at Haramachi area both in Minami-Soma city.

Firstly, to identify bacteria in each SPs, I extracted total genomic DNA from each seasoning solution (namely, SP1A and SP2A), and SP washing solution (SP1B and SP2B). In sample SP1B and 2B, each SP samples (SP1:4.86g, SP2:5.44g) were cut and washed by 10mL of

sterilized water for 1 hour. These solutions involving bacteria were harvested by centrifugation, and total bacterial genomic DNAs were extracted by Wizard SV genomic DNA purification system (Promega), followed by amplified 16S rRNA gene by PCR using each genomic DNA as template and PCR primers for 16S rRNA gene as follows (Beffa, 1996), upper primer: 5'-ATTCTAGAGTTTGATCATGG CTCA-3' (24mer, T_m=53°C), Lower primer: 5'-ATGGTACCGTGTGACGGGCGGTGTGTA-3' (27mer, T_m=67 °C). The amplified PCR products were cloned into pGEM-T vector (Promega), followed by DNA sequenced. The determined DNA sequences (totally 48 clones) were analyzed by Serial cloner software ver.2.6 (Serial Basics), Blast program (NCBI: National Center for Biotechnology Information), Clustal W program (DDBJ: DNA data bank of Japan).



Figure 6. Soma cucumber pickles used in this study.

Results and Discussion

Overviewing situation in Fukushima for seven years

As mentioned above, Great East Japan Earthquake, huge tsunami, and Fukushima 1st nuclear power plant accident made Fukushima's people escape from their hometown. My parents bred about 50 cattle until the disaster happened, but they and my brother must also be evacuated from Minami-Soma city to Kooriyama city (80 km distance, Figure 1) for one and half year, because of nuclear power plant accident and high level of radioactivity. At Jan. 2012, I visited Kooriyama city and also looked around Minami-Soma city (Figure 7), to meet them and archive their situation to tell so many people. Then, I explored the environmental radiation dose of ¹³⁷Cs in Fukushima for 2012-2017 (Table 1), and found that they must refuge under high radiation dose, even at Kooriyama city which is 60 km away from Fukushima 1st nuclear power plant. Then, Japanese government decided if radiation dose could be less than 20 mSv/y by decontamination, people can return and live in their hometown, though it is generally safe for human to be less than 1 mSv/y. After seven years, the radiation level decreased from 3.49 μSv/h (Maximum, 2012) to less than 0.1 μSv/h at the Minami-Soma city (Table 1), and half of people have come back to their home. On 2017, the restricted areas was lifted in the part of the Namie town (Figure 8). Thus, I investigated environmental radiation dose in the Namie town on June and August, 2017. As shown in Table 1, abnormally high values (7.66 μSv/h, Maximum, 2017) were detected in the mountain side of the Namie station (Figure 9). In spite of the evacuation released, only 2% residents could return to the Namie town, because of anxiety to radiation, effects on health, job and income, medical care, education, and so on. It is difficult to revert to the previous state once communities had broken, as exemplified in case of the Namie town.



Figure 7. Examination for environmental radiation dose in the barn of my parents (Minami-soma city, 2012).



Figure 8. Broken houses in Namie town (2017).

Table 1. Environmental radiation dose in Fukushima for 2012-2017

Place	Date	Radiation dose ($\mu\text{Sv} / \text{h}$)	Radiation dose (mSv / y)
Kooriyama	Jan.2012	0.12-1.31	1.05-11.5
Iitate	Jan.2012	3.63-4.00	31.8-35.0
Kawamata	Jan.2012	0.50-0.52	4.38-4.56
Minami-Soma (Haramachi)	Jan.2012	0.11-3.49	0.96-30.6
	Dec.2012	0.33-2.01	2.89-17.6
	Jan.2013	0.07-1.73	0.61-15.2
	Jan.2014	0.08-0.99	0.79-8.65
	Sep.2016	0.06-0.31	0.53-2.72
	Jun.2017	0.07-0.44	0.61-3.85
Minami-Soma (Odaka)	Aug.2017	0.07-0.12	0.61-1.05
	Jun.2017	0.04-0.41	0.35-3.59
Namie (Sea side)	Jun.2017	0.06-0.53	0.53-4.64
Namie (Mountain side)	Aug.2017	0.09-7.66	0.79-67.1



Figure 9. Closed Junior high school in Namie town, because of high level of radiation (2017).

Activities for telling the right information on the Fukushima

The misunderstandings of Fukushima's information lead to so many problems in Japanese society, such as reputational risk, prejudice, and bullying. For example, a student escaped from Fukushima was said "You are polluted by radiation, don't close to us". To let so many people understand Fukushima's situation, I and the collaborators have carried out the supporting activities for seven years, such as telling the right information on the current situation, radioactivity level, and correct knowledge about radiation to people including young students, citizens, and foreigners, and also sending out questionnaire about safety and reputational risk to Fukushima (Sato et al., 2016; 2017). From these results, I have found that misunderstanding radioactivity and its effects on human might cause the prejudice against people, foods, and cultures in Fukushima.

From these points of view, we held some conferences since the year 2014, which some speakers were invited from Fukushima. They told the experience of disaster, problems on refuge and shelter, and effects on health by radiation to students of Tokushima University, citizens, and foreigners (Figures 10&11). In addition, I have also taught what I have seen on my lectures. Some students said that "To have been turning our eyes to the Fukushima's victims by disaster is very important thing, so that we can understand these problems and I want to support just a little thing that I can from now", "I'm ashamed of not knowing the right information on Fukushima and radiation, and I must learn so much", "To listen to Fukushima's circumstances was useful for the our refuge and protection, if the Nankai great earthquake may struck on the Tokushima", and so on. Moreover, some newspaper company took up our activities, and so many citizens could know my activities for telling and supporting Fukushima (Figure 12). It was considered that these supporting activities were fruitful for not only understanding Fukushima's situation, but also giving opportunities to let the consciousness of young students change.



Figure 10. Conference "Learning from the disaster weak people like women and foreigners (2016).

Figure 11. Lectures about radiation for Japanese and foreign students (2017-2018).



Figure 12. Articles in newspaper for supporting activity to Fukushima (2016-2017).

Analysis for bacteria in Soma cucumber pickles

As described above, the communities and their traditional cultures might disappear in Fukushima after the disaster broke out. Thus, to preserve and archive them is crucial for sustainable development in Fukushima. From this point of view, I focused on the Soma cucumber pickles (SP) of traditional food culture in Fukushima. Then, I investigated the features of bacteria in two kinds of SPs (SP1 and 2) by the clone library method, which bacteria have not been reported.

Firstly, I prepared whole bacterial genomic DNAs from SP1 and 2 according to the methods described above. Each 16S rRNA gene was amplified by PCR using these genomic DNAs. As results, about 1.5 kbp of PCR product was found both in SP1 and 2 samples (Figure 13), and they were cloned into cloning vector, pGEM-T, and determined DNA sequences.

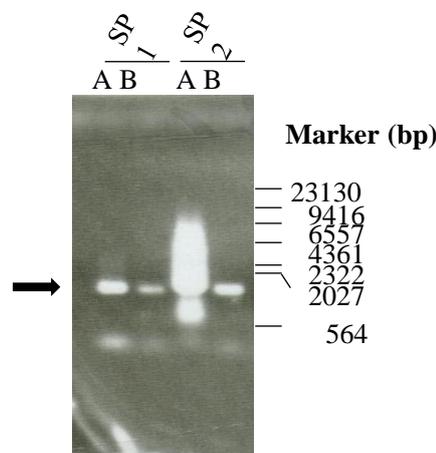


Figure 13. Agarose electrophoresis of amplified 16S rRNA genes of bacteria from soma pickles. Arrow shows PCR product (1.5 kbp). Template; A: DNA from the seasoning solution of SP products, B: DNA from the washing solution of SPs. Annealing for PCR primers are 55°C, and 5µl of PCR product was applied on 0.8% agarose gel.

As results, it was found that they contained three kinds of lactic acid bacteria (LABs), *Leuconostoc citreum* (*Lec*), *Lactococcus lactis subsp. lactis* (*Lcl*), and *Lactococcus lactis subsp. cremoris* (*Lcc*), with more than 99.6% of homology to known LABs except for SP2A27 clone (Table 2). In SP1, there were *Lcl* (7 clones) and *Lec* (1 clone) only in SP1A from the seasoning solution of SP1 product, whereas *Lcl* (5 clones), *Lcc* (2 clones), and *Lec* (1 clone) detected both in SP2A and SP2B. Miyao (2002) reported that LABs in popular pickles in Japan are composed of *Lactobacillus plantarum*, *Leuconostoc menteroides*, and *Enterococcus*, *Pediococcus* genus bacteria. On the other hand, Nakagawa *et al.* (2001) suggested *Lactococcus lactis* and *Leuconostoc menteroides* are predominant in some soy sauce pickles in Japan. This study is the first report on the bacteria in SPs, and found with interest that *Lactococcus lactis subsp. lactis* are predominant, and different in bacterial diversity as compared with popular cucumber pickles in Japan. To preserve traditional food culture in Fukushima, more studies on the isolation and preservation of useful LABs are greatly needed, and additional investigations are now taken.

Table 2. Clones corresponding to LABs in Soma cucumber pickles

Samples		Identification of bacteria by 16S rRNA sequence			
Clones	Determined length (bp) of 16S rRNA gene	Identified species	Length (bp) of 16S rRNA gene in database	Accession number	Homology (%)
SP1 (Kashima)					
SP1A32	1371	<i>Lactococcus lactis subsp. lactis</i>	1552	CP025500	99.9
SP1A34	1371	<i>Lactococcus lactis subsp. lactis</i>	1552	CP024958	99.6
SP1A36	1373	<i>Lactococcus lactis subsp. lactis</i>	1552	CP025500	99.9
SP1A38	1371	<i>Lactococcus lactis subsp. lactis</i>	1552	CP024958	99.8
SP1A44	1372	<i>Lactococcus lactis subsp. lactis</i>	1552	CP024958	99.7
SP1A46	1373	<i>Lactococcus lactis subsp. lactis</i>	1482	HM058555	99.9
SP1A50	1371	<i>Lactococcus lactis subsp. lactis</i>	1552	CP025500	99.7
SP1A48	1374	<i>Leuconostoc citreum</i>	1517	MG557903	99.9
SP2 (Haramachi)					
SP2A9	1373	<i>Lactococcus lactis subsp. lactis</i>	1552	CP024958	99.9
SP2A17	1371	<i>Lactococcus lactis subsp. lactis</i>	1552	CP024958	99.9
SP2A19	1377	<i>Lactococcus lactis subsp. lactis</i>	1552	CP024958	99.6
SP2A13	1373	<i>Lactococcus lactis subsp. lactis</i>	1547	CP015906	99.6
SP2A11	1371	<i>Lactococcus lactis subsp. cremoris</i>	1548	CP015899	100.0
SP2A27	1372	<i>Leuconostoc citreum</i>	1517	MG557903	94.8
SP2B3	1371	<i>Lactococcus lactis subsp. cremoris</i>	1572	AM944595	99.9
SP2B4	1372	<i>Lactococcus lactis subsp. lactis</i>	1552	CP024958	99.8

Conclusions

In this study, I reported some activities for supporting Fukushima, Japan. From these results, it was found that misunderstanding the radiation and its effects on human might cause the prejudice against people, foods, and cultures in Fukushima. I also examined the bacterial features of traditional food in Fukushima, Soma cucumber pickles (SP). As results, it was elucidated that SPs contained three kinds of LABs (*Leuconostoc citreum*, *Lactococcus lactis subsp. lactis*, *Lactococcus lactis subsp. cremoris*), and have bacterial feature rich in *Lactococcus lactis* especially. For sustainable development in Fukushima, continuous supporting activities and the preservation of traditional cultures in communities must be greatly needed.

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Intra-species space competition in cocoa ecosystem: invader ants' geovisualisation

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Abstract

Malaysian Cocoa Board (MCB) has conducted numerous research and development activities to ensure sustainability of the cocoa industry. One of the important aspects is the management of pests and diseases of cocoa. A qualitative study to detect relationship between presences of biological control agents, Cocoa Black Ant (CBA) against the major pest of cocoa (Cocoa pod borer, CPB) with other ants' intrusion was done with the aid of geospatial mapping and analysis approaches. The presence of other species of ants (ant invades) will affect the availability of CBA in controlling CPB infestation. Thirty months of data collection comprised of two different blocks; Blocks 10B and 18A were used as the study area. In order to understand the CBA's behavior and pattern distribution, analysis of the differences and similarity of other ant's intrusion for each block and Plots were performed. Four different artificial nests were introduced; Plot A was augmented with one artificial ant nest for every two cocoa trees, Plot B had one nest on every single cocoa tree, Plot C had two nests on every single cocoa tree and Plot D without artificial nest. The study reveals that the highest or lowest in CBA populations and distribution could induce a strong reasonable relationship against other ant types. Plots with artificial ant nests (A, B and C) showed low rate of intrusion with ratio of 1:1 for the Plot B indicated the best result, whereas, Plot without artificial ant nest (Plot D) highest rate of intrusion during the months and years of observation.

Keywords: Cocoa; Cocoa black ant; *Dolichoderus thoracicus*; *Theobroma cacao*; geovisualisation.

Introduction

Cocoa crop is one of the national commodities and plays an important role for the economy in Malaysia. These plants can bear fruit throughout the year, so it can be a source of daily or weekly income to growers. Cocoa like other plants also has problems with diseases and pests wherever it is grown. Some pests can not only reduce the yield but also inhibits the growth of trees. In addition to cocoa pests are also useful insects as parasites, predators and insect pollinators. In Malaysia, the cocoa pod borer (CPB), *Conopomorpha cramerella Snellen* is a major pest of cocoa today. The effects of CPB attack resulted in quality cocoa beans to be low, a small seed and sometimes stick to each other. CPB if not controlled afford cause yield losses of up to 100% (Saripah, 2012).

Various efforts have been undertaken to control insect pests of cocoa since it was found attacking cocoa. These include biological control and chemical control. Nowadays, chemical control is widely practiced method for controlling insect pests. This effectiveness will be increased if coupled with a spraying pesticide frequency shortly before the harvesting stage. However, this technique has relatively high cost. Besides cost, the use of chemical pesticides with high doses instead of making pests of plants disappear, it will cause other effects on the environment such as non-point source pollution. Alternative method such as biological control has yet to be widely used despite the potential to use it properly. One of the natural enemies that are proven effective in controlling fruit pests are ants known as Cocoa Black Ants (CBA) acted as an effective biological control (Babin, 2010; Saripah, 2010). CBA will eat the larvae of CPB and thus reduce the rate of attacks. However, farmers should always check whether the black ants on a tree are still a lot, especially in the rainy season (Saripah, 2003; Saripah, 2014).

In agriculture application, GIS data, tools and techniques are very useful for predicting the area and production of agricultural commodities, mapping of agriculture land resource potential and the prediction of the spread of pests and diseases of plants such as CPB (Adnan et al., 2018; Ayorinde et al., 2014; De Caires et al., 2015). GIS technique able to be used on a large scale (detailed) incorporated high-resolution input data hence, produce an accurate results (product). Furthermore, GIS can also be based on a small scale with low-resolution input data and produce lower accuracy in a wide area mapping. In this paper, two objectives of the study are i) to generate the other ants' intrusion distribution map for four Plots within two blocks (i.e. 18A and 10B) based on monthly and yearly conditions and ii) to assess qualitative relationship between CBA augmented nest and other ant's intrusion category.

Materials and Methods

The methods involving three stages known as data collection stage, data conversion and processing in GIS software and data analysis and findings. Thirty months (with biweekly data collecting activities) database of CBA and other ants' species data population were collected by the Malaysian Cocoa Board (MCB) researchers from February 2007 and ended in July 2009 as primary data. However, the other ant species data represented by either notation of present or absent, in contrast to CBA data with number of ants counted for each cocoa tree. Subsequently, position of each cocoa tree was collected using handheld Global Positioning System (GPS) to record the x and y coordinates location to make the data compatible with the GIS software of ArcGIS in the projection system of Malaysia Rectified Skew Orthomorphic (MRSO). The coordinate of tree combine with attribute of number of ants were converted into shapefile in ArcGIS software. There are four Plots of cocoa area known as Plot A (one artificial ant nest on two cocoa trees or symbolized as 1N:2T), Plot B (one artificial ant nest on a single cocoa tree or symbolized as 1N:1T), Plot C (two artificial ant nests on a single cocoa tree or symbolized as 2N:1T) and Plot D (none artificial nest or symbolized as 0N:1T) and each Plot represented by 50 tree points and accumulated to 200 sample cocoa trees in total each for every block (Block 18A and 10B). Figure 1 shows a cocoa tree position of the Block 18A.

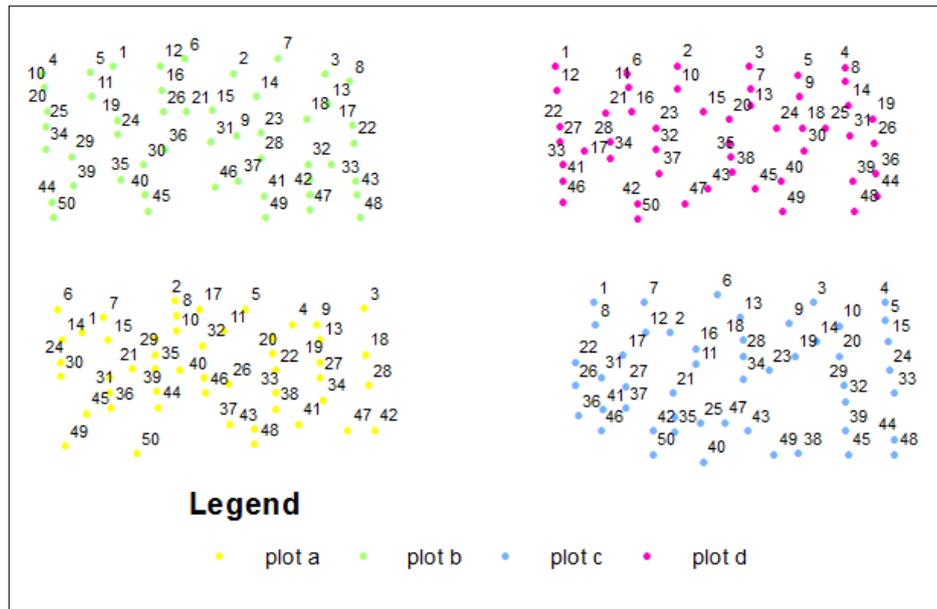


Figure 1. Position for each cocoa tree within four Plots (A, B, C, D)

The highest CBA population counted as total number per tree could make reasonable relationship against other ant types (Saripah, 2014). The weightage for other ants is used to determine level of intrusion and treat for the CBA. A value of zero (0) weighting category indicated the present of CBA and classified as no intrusion or healthy condition if there are no disturbance from the other ants. Otherwise, a value of one (1) weighting category given to indicate the present of the other ant species and classified as intermediate intrusion (unhealthy condition) and a value of one (2) weighting category given to indicate the present of the other ant species and classified as heavy/bad intrusion (unhealthy condition) as shown in Table 1

Table 1. Classification of weightage for other ants' data collection

Data observation (1 ST and 2 nd week data)	Weighting Categories	Description
0 + 0 = 0	0	No intrusion
1 + 0 = 1	1	Intermediate intrusion
0 + 1 = 1	1	Intermediate intrusion
1 + 1 = 2	2	Heavy/ Bad intrusion

In the ArcGIS data processing the widely used interpolation techniques known as Kriging spatial data were incorporated to produce population distribution map for entire block. Kriging produces an estimate of the core surface by a weighted normal of the information, with weights declining with separation between the point distance of the information points (Junita, 2012; Adnan et al., 2018; Briggs, 2010). Therefore, kriging interpolation will apply to the CBA population and other ant type species to visualize both ants' distribution. For each cocoa tree, the CBA population was classified as highest population when every tree has

more than 500 and reach to 1000 ants and however when less than 500 cocoa black ants it is low population (Saripah, 2014; Adnan et al., 2018). Furthermore, methods to classify high, moderate or low categories of CBA and other ants' distribution for each block were divided into four quadrants visualization. If any quadrant has a CBA above 500 is considered high and less than 500 is considered as low. When the ratio is $\frac{3}{4}$ or all quadrants covered by CBA above 500 is considered as high. While the moderate when the ratio of half or $\frac{1}{2}$ and cocoa black ants are lower at a ratio of $\frac{1}{4}$ or quarter (Adnan et al., 2018).

Results and Discussion

Individual Other Ants' distribution according to Block for four Plots (A, B, C, D)

The data that used in this study comprised of two different blocks, Block 18A and 10B with four different Plots as explained earlier in the Methods section. In a cocoa-coconut plantation system, there are many other types of ants' species apart from beneficial ant species as CBA. The presence of the other species of ants will affects the availability of the CBA. The information on the presence of other ant species is taken but the actual count numbers is not made into consideration. The individual other ants' intrusion according to the three categories as classified earlier (i.e. no intrusion, intermediate and heavy/bad intrusions) as discussed further below.

The Plot A of the Block 10B according to years of 2007 until 2009 represented by the heavily/bad intrusion on November and December (2007), May and September (2008) and March and April (2009). For the Plot B represented by the heavily/ bad intrusion on July 2007, September 2008, April and May 2009. For the Plot C again represented by the heavily/ bad intrusion on February 2008 and May 2009. For the Plot D heavily/bad intrusion as indicated by the months of May, June, November and December in 2007, June in 2008 and March in 2009.

For the Plot A of the Block 18A represented by the heavily/bad intrusion on May and June 2008. For the Plot B, heavily bad intrusion as indicated by the months of May and June 2008. Meanwhile, for the Plot C represented by heavily/ bad intrusion on March until July and October (2008) and February (2009). For the Plot D the heavily bad intrusion happened in the months of March until July and September until December 2008 and January until June 2009.

Geovisualization Comparison of Other Ants' Distribution for Two Different Blocks

To further understand the CBA's behavior and pattern, analysis on the differences and similarity of other ant's intrusion for each blocks and Plots were performed. The comparison was done by investigating each Plot for the same year starting from 2007 to 2009 (i.e. 30 months). Only summary result (Table 2) for each Plot and years of CBA and the other ants' intrusion were presented in this paper since extensive figures need to be included for every Plot and year in these two different blocks. Several examples of other ants' intrusion maps presented to elaborate the findings. The findings discussed in this section assessed qualitative

relationship between CBA augmented nest and the other ant's intrusion conditions. Overall the result in Table 2 revealed that if the CBA dominated or highly populated inversely, the other ants' intrusion will be lower and vice-versa. A negative qualitatively interaction relationship between these two ants type existed in a cocoa plantation ecosystem.

Table 2. Summary results CBA and the other ant's intrusion.

	PLOT A 1N:2T		PLOT B 1N:1T		PLOT C 2N:1T		PLOT D 0N:1T	
	10B	18A	10B	18A	10B	18A	10B	18A
High CBA Population								
2007	Sept	Aug	Sept	Aug	Sept	Aug	Sept	Aug
2008	Apr Oct	-	May Dec	Mar	Apr	Mar	Apr Dec	-
2009	Jun	-	-	-	-	-	July	-
High Intrusion								
2007	Nov Dec	-	July	-	Oct	-	Nov	-
2008	May Sept	May June	Sept	<i>May June</i>	Jun	Apr May Jun July	May	Except Aug
2009	Mar Apr	Feb	May	-	May	Jan	Mar	All

Figure 2 shows comparison maps of the other ant's intrusion for the Plot A in the year 2008. Block 10B shows that the heavy/bad intrusions classification occur on September whereas, the Blocks 18A have other ant's intrusion the heavy/bad intrusions classification in June. Figure 3 shows comparison maps of the other ant's intrusion for the Plot B in the year 2008. Block 10B shows that the heavy/bad intrusions classification occur in March and in September, whereas, the Blocks 18A have other ant's intrusion the heavy/bad intrusions classification in May.

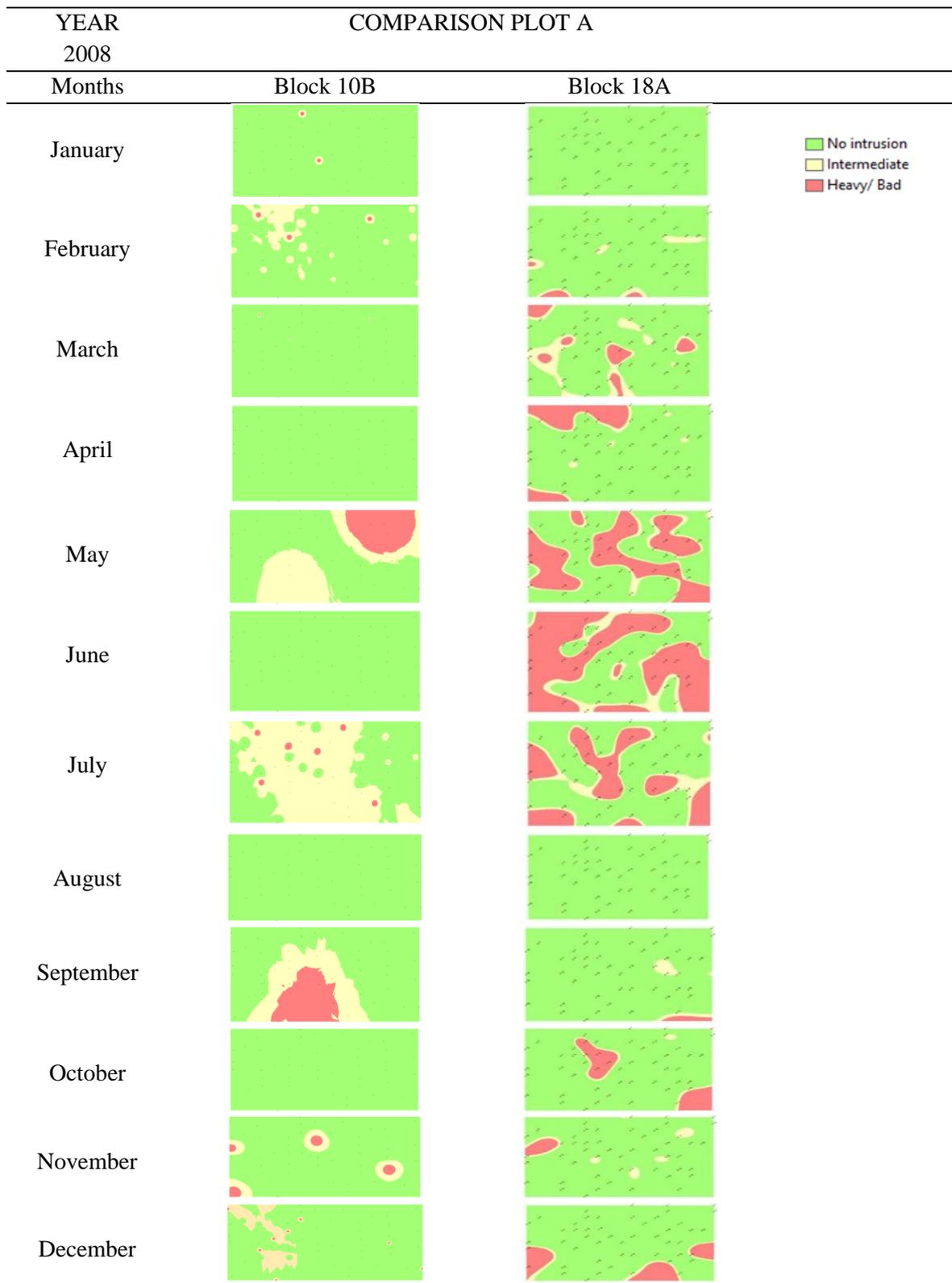


Figure 2. Comparison maps of other ant's intrusion distribution for the Block 10B and 18A for Plot A in the year 2008.

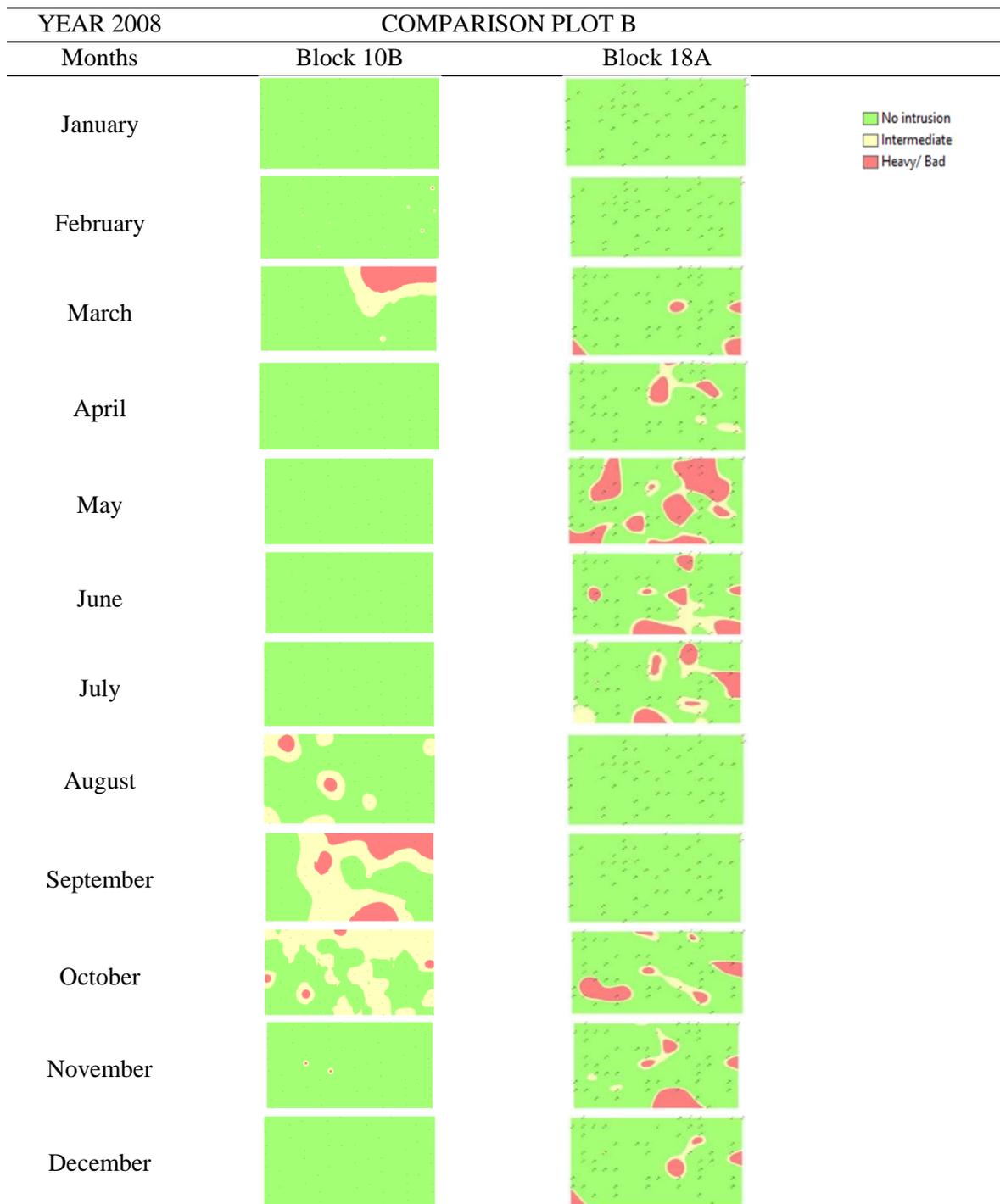


Figure 3. Comparison map of other ant's intrusion distribution the Block 10B and 18A for Plot B in the year 2008.

Figure 4 shows comparison maps of the other ant's intrusion for the Plot C in the year 2008. Block 10B shows that the heavy/bad intrusions classification occur in June, whereas, the Blocks 18A have other ant's intrusion the heavy/bad intrusions classification in May until July. Finally, Figure 5 shows comparison maps of the other ant's intrusion for the Plot D in the year 2008. Block 10B shows that the heavy/bad intrusions classification occur in May, whereas, the Blocks 18A have other ant's intrusion the heavy/bad intrusions classification in in all months except for August. This indicated that no similar pattern of ants' intrusion

happened from these two blocks and generally it's happened by randomly manner. However, both blocks showed similar pattern with indicator if the CBA is higher than the other ants' intrusion will be lower.

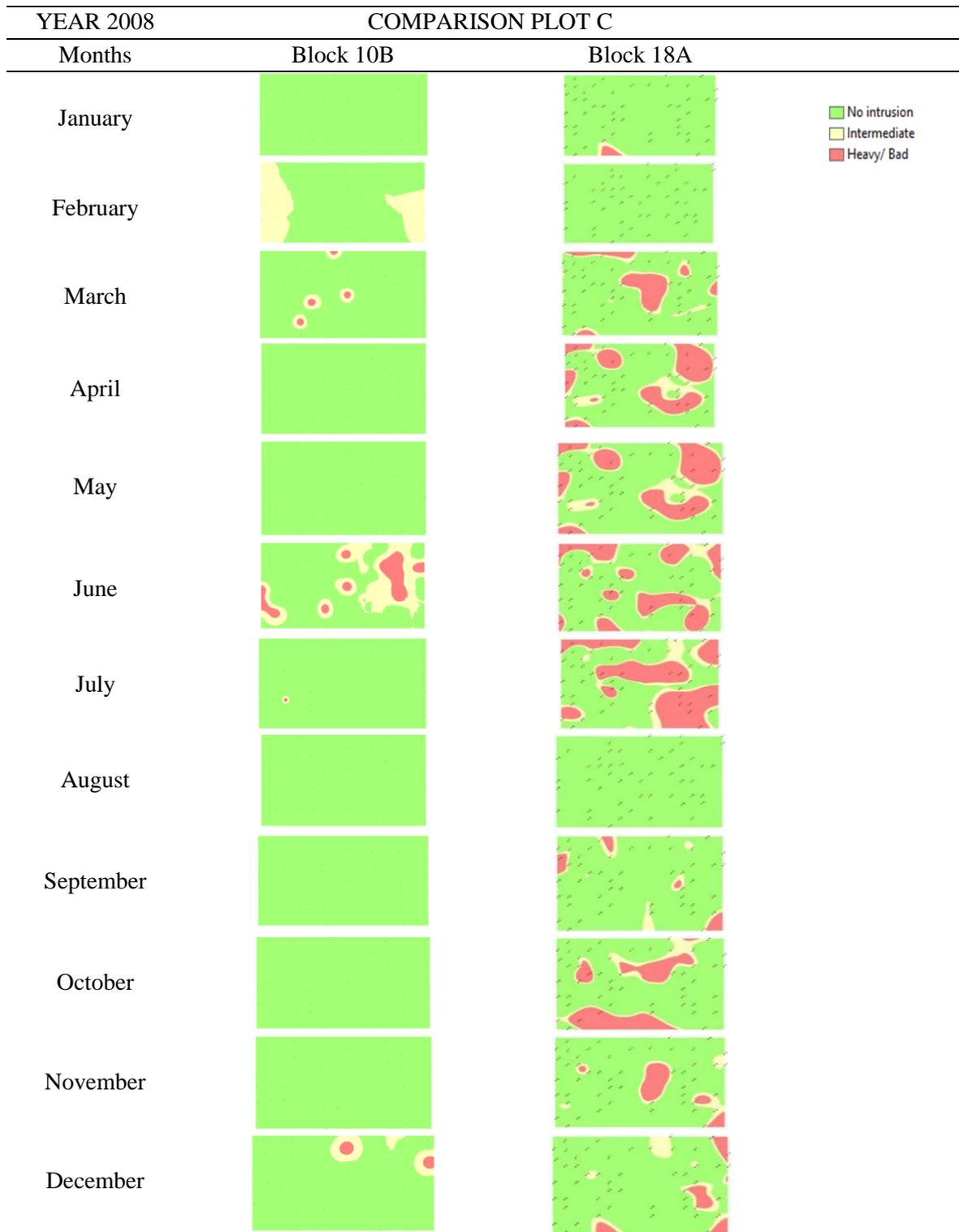


Figure 4. Comparison map of other ant's intrusion distribution the Block 10B and 18A for Plot C in the year 2008.

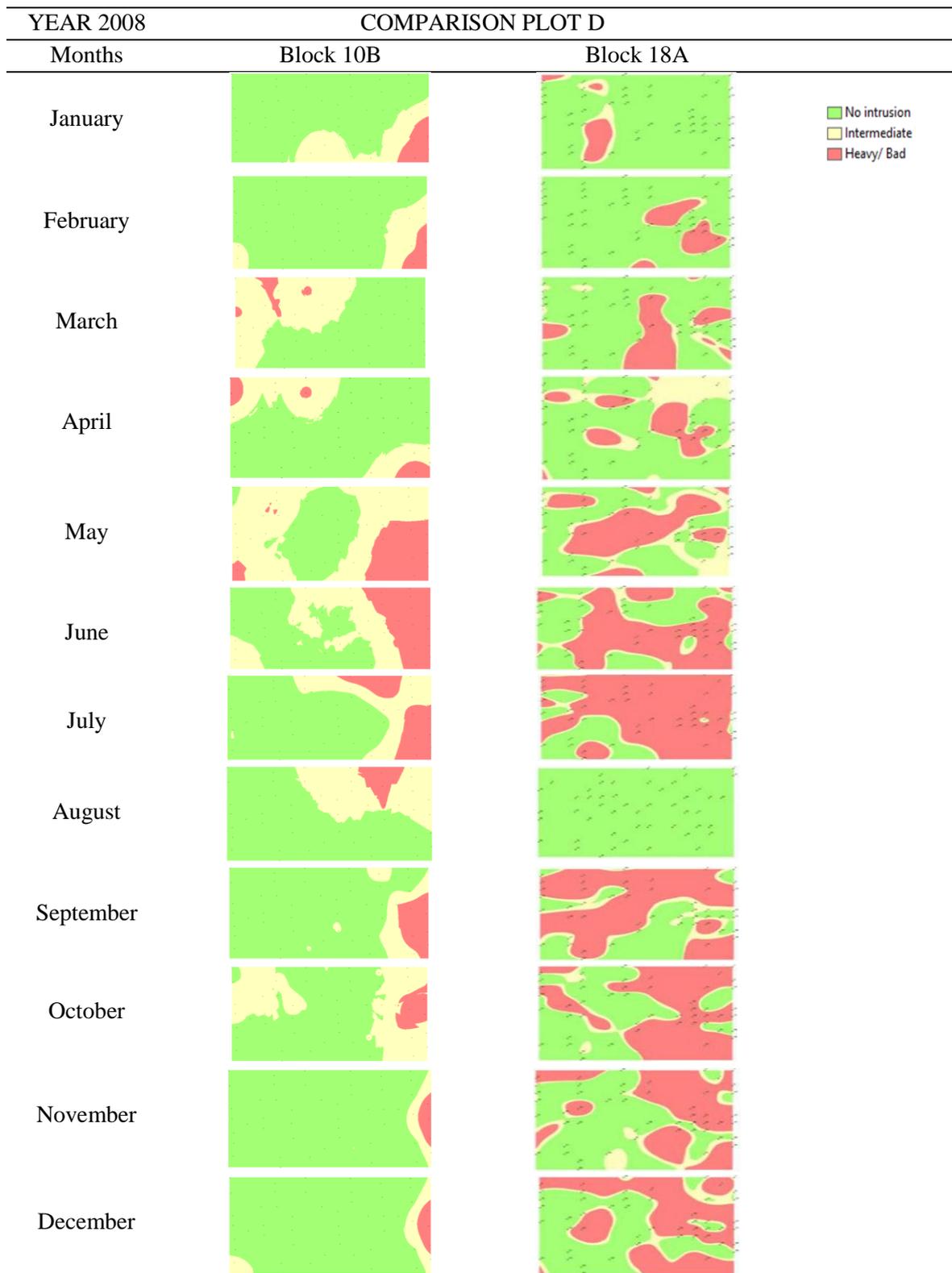


Figure 5. Comparison map of other ant's intrusion distribution the Block 10B and 18A for Plot D in the year 2008.

The previous study indicated that other ant's intrusions often dominantly in each month with lower CBA distribution that varies according to fruit cocoa season cycle (Adnan et al., 2017). This analysis revealed that the higher amount of artificial ant nest presented does not really implies the population condition of the Plots will turn out to be better. Higher amount of artificial ant nest might have complex competitive level among beneficial biological control of CBA (Saripah, 2014). The best number of artificial ant nest to be implemented is the Plot B with one 1:1 as opposed to Plot A (1:2) and Plot B (2:1) as discussed in this section. Therefore, to increase the number of CBA population in combating Cocoa Pod Borer (CPB) infestation that destroy the quality of cocoa beans inside a cocoa pod, one artificial nest for each cocoa tree suitable to be implemented by the cocoa growers in Malaysia.

Conclusion

The highest in CBA population could induce a strong reasonable relationship against the other ant types. Artificial ant nest was introduced to increase CBA population in controlling the infestation by the CPB for any cocoa trees. The GIS techniques used in this study able to revealed negative geovisualization relationship between existence of CBA with the other ants' intrusion (i.e. if the CBA is higher than the other ants' intrusion will be lower). Four study plots were investigated with different ratio artificial ant nest to the other ants' intrusion category. The study concluded that the plots with artificial ant nests (A, B and C) showed the lower rate of intrusion, with ratio of 1:1 for the Plot B indicated the best result, whereas, plot without artificial ant nest (Plot D) highest rate of intrusion detected during the months and years of observation.

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Adult emergence inhibition of Zingiberaceae essential oils against the *Conopomorpha cramerella* (Snellen) Lepidoptera: Gracillariidae

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Abstract

Zingiberaceae (Order: Zingiberales) is recognized as the most important herbaceous species in the tropics. Several species of Zingiberaceae have shown a potential in managing some Lepidopteran and Dipteran pests. Currently, there is no report in Malaysia explores the potential use of Zingiberaceae in managing cocoa pests, particularly the Cocoa pod borer (CPB), *Conopomorpha cramerella*. The study was conducted to evaluate the potential of Zingiberaceae as an adult emergence inhibition of *C. cramerella* using three locally grown species in Malaysia; *Alpinia galanga* (galanga), *Curcuma longa* (turmeric), and *Zingiber officinale* (ginger). Different ages of the CPB pupa (Days 1, 2, 3, 4 and 5) were selected and were sprayed with Zingiberaceae essential oils (EOs). Pupa was sprayed with EOs at four different concentrations; 100 ppm, 200 ppm, 400 ppm and 800 ppm. Observations were carried out after 5 to 7 days, where the percentage of the unhatched pupa and healthy adult was recorded. The percentage of the deformed adults were examined based on the abdomen, the antenna, the wings, and overall deformities. The study was repeated four times for each EOs. Results denoted that the percentage of the unhatched pupa was the highest at *C. longa* (49.750% a) and significantly different ($p < 0.05$) with *A. galanga* (48.000% b), *Z. officinale* (40.976% c) and control treatment (13.592% d). The highest healthy adult emergence was recorded under the control treatment (75.777 a %), and significantly different with all Zingiberaceae EOs. The overall results denoted that most of the deformities occurred after pupa were treated with the Zingiberaceae EOs at high concentrations (400 and 800 ppm), and engaged with the body and wing deformities. The symptom of adult deformities after treating with EOs may indicate the potential of Zingiberaceae as an adult emergence inhibitor of *C. cramerella*, and can be possibly used for the development of Zingiberaceae based botanical pesticide in the future.

Keywords: Cocoa pod borer; *Alpinia galanga*; *Curcuma longa*; *Zingiber officinale*; Zingiberaceae.

Introduction

Cocoa, *Theobroma cacao* (Linnaeus) (Malvales: Sterculiaceae) is an important crop that widely planted in the humid tropical regions, and subjected to be infested by a wide range of pests. Number of fifteen insects (mosquito bug, bee bug, cockchafers beetles, branch borer,

mealy bug, bagworms, aphids, grasshopper, shot-hole borer, cocoa white grub, husk borer, shoot borer, cocoa pod borer and thrips) were recognized as an important pest infested cocoa plantation either at the nursery, young or mature tree (Lee et al. 2013). Among all pests, the Cocoa pod borer (CPB), *Conopomorpha cramerella* Snellen (Lepidoptera: Gracillariidae) a tiny moth becomes a major threat to the Southeast Asia cocoa growers. *C. cramerella* female is capable of laying eggs from 40 to 100, and has the potential to lay more than 300 eggs during their maturity stage (Lee et al. 2013). With the ability to regenerate 10 to 12 times a year, crop loss to farmers can be substantial. Continuous infestations may cause an unacceptable level of damage, influenced by the feeding and oviposition preferences for pods (Saripah, 2014). The infestation of *C. cramerella* occurred where developed cocoa beans were hardened and clumped together. Losses expected to be greater in smallholder's crops, due to a lack of knowledge about the pest management, finance, as well as the manpower (Alias, 2011).

A diverse control attempts have been undertaken to manage the infestation of *C. cramerella*. Three decades after the first arrivals, biweekly prophylactic treatment with chemical insecticides was considered as one of the most effective approaches; even the cost can be considered high (Alias, 2011). However, recent studies conducted denoted that the combination of complete management practices was proven effective in reducing *C. cramerella* infestation. The complete management practices, including schedule insecticide spraying, pruning, fertilization and weekly pod harvesting (Saripah and Alias, 2016). Mitigation technique must be promoted, however the management must be feasible, easy to understand, less technical, safe to the environment and health, yet efficient must be taken into consideration.

The risk of resistance mechanism towards insecticide due to prolonged use in the cocoa plantation is greater with excessive usage of chemicals. Chemical insecticides must be suggested as a last resort, therefore an effort is warranted to find alternatives to currently used insecticides. There is a need to assess some indigenous botanical plants, especially those with a presumably better potential for extension (Tounou et al. 2011). Many plant products and chemicals may play a role as oviposition deterrent, insect repellent, antifeedant either as larvicidal, pupicidal or adulticidal activities. Such plants, in addition to their inherent pesticide effectiveness must be rustic, perennial and easily cultivated. Parkash & Rao (1997) cited more than 800 species pose insecticidal and repellent, anti-feeding effects were used in agriculture, and this will offer more variety for plant selection in the future.

With more than 1,000 species recorded, Zingiberaceae (Order: Zingiberales) is one of the largest families in the plant kingdom. There are approximately 1,000 of the known species of the Zingiberaceae, obtained from 52 genera (Wohlmuth, 2008). Zingiberaceae plant will bear several horizontal or curved rhizomes when mature, which are again branched. *Alpinia galanga* (galanga), *Curcuma longa* (turmeric), and *Zingiber officinale* (ginger) were reported to have an effect on insect pests via oviposition deterrent, insect repellent and antifeedant activities (Suthisut et al. 2011). These three species were common in Malaysia and widely planted especially as cooking condiments and medicinal use.

Most Zingiberaceae studies were demonstrated on several insect orders from Diptera, Lepidoptera, and Coleoptera. Zingiberaceae was tested in several forms, such as raw plant materials, plant extract, and essential oils (EOs). EOs are complex mixtures of volatile organic compounds produced as secondary metabolites in aromatic plants. EOs is basically volatile, natural and characterized with strong odor (Bakkali et al. 2008). Insecticidal plants usually contain EOs with specific odor and flavor, like secondary metabolites that beneficial as a defense mechanism against pests. Pure compounds or blends of several major chemical components in EOs found to be toxic to insects. Although EOs performed lesser than insecticides, herbicides or chemical repellents (Isman, 2008); they have been managed to execute insecticidal actions such as negative developmental and reproductive effects, repellent, and feeding deterrence. Currently, there is no report in Malaysia on the potential use of Zingiberaceae plants in managing cocoa pests, particularly the *C. cramerella*. Therefore, the objective was to focus on the effects of three Zingiberaceae EOs as an adult emergence inhibition of the *C. cramerella*. The inhibition was determined by the percentage of the unhatched pupa, and adult deformities at four body parts; abdominal, antenna, wings and overall body.

Materials and Methods

The study was conducted at the Malaysian Cocoa Board, Cocoa Research and Development Center (CRDC) Bagan Datuk (3.906 N, 100.866 E), Perak, Malaysia. Zingiberaceae EOs were purchased from a local authorized dealer in Malaysia. Bioassay test was performed where EOs were diluted into four concentrations (100, 200, 400 and 800 ppm). Nonionic surfactant, Polyoxyethylene (20) sorbitan monooleate (Tween 80) was added, and the concentration was vortexed for 1-2 minutes at 1800 rpm. Control was prepared using the same amount of Tween 80 with addition water up to 100 ml. Concentrations were prepared 24 hours in advance, stored in dark amber glass bottle at 4°C prior to the experiment.

Mature cocoa pods with the symptoms of *C. cramerella* infestation was harvested, and destructive samplings were performed using approximately 200 to 300 cocoa pods to obtain a sufficient number of pupa. Pods were placed as a single layer in a large container and covered with dry cocoa leaves. Pupa that pupates on the leaves were divided into an even number for each treatment, and EOs were sprayed at the distance of 15 cm using the hand sprayer. Pupa was left air-dried for two hours and kept in a transparent container. The number of successful emerging adults, deformed adult and pupa were observed after 96 hours and onward. Pictorial data of the deformed adults and pupa were recorded using the Dino-Lite Digital Microscope. The experiment was repeated four times for each EOs. Data were arranged separately in Microsoft® Excel 2007, and were subjected to statistical analysis using Analysis of Variance (ANOVA) and PROC GLM, SAS software from the SAS® Software Version 8.

Results and Discussion

Effect of EOs on the developmental stages of *C. cramerella* denoted that regardless of different concentrations, *Z. officinale* shows the best performance when sprayed to Day-4 pupa (54.667% a) and significantly different (One-Way ANOVA; $p < 0.05$) compared to other days (Table 1). Meanwhile, for *C. longa*, the highest mean of unhatched pupa was recorded at Day-3 (68.333% a) and significantly different from Day-1 (21.000% e), Day-2 (42.000% c), Day-4 (60.000% b) and Day-5 (23.333% d). Days-1 (48.000% a) and 2 (48.000% a) performed at the best age to spray with *A. galanga*. For all EOs, Days-5 recorded the lowest percent of the unhatched pupa, and this might suggest that older or matured pupa has high potential to survive, compared to the younger age of pupa. This study was not undertaken to more than Days-5 old pupa, because the probability of pupa emergence of an adult will be higher. At different concentrations, all EOs performed better than control (Table 2), which resulted in a significant percentage of the unhatched pupa. The best concentration was obtained from 400 ppm for *Z. officinale* (49.476% a), *C. longa* (55.000% a) and *A. galanga* (52.000% a) compared with other concentrations. Fluctuated results were obtained for the highest concentrations (800 ppm) for all EOs.

Table 1. Percentage of unhatched pupa of *C. cramerella* after treated at different age of pupa

Age of pupa	n	Unhatched pupa of <i>C. cramerella</i> (%)		
		<i>Zingiber officinale</i>	<i>Curcuma longa</i>	<i>Alpinia galanga</i>
Day-1	20	43.111 b	21.000 e	48.000 a
Day-2	20	33.905 d	42.000 c	48.000 a
Day-3	20	33.333 d	68.333 a	40.000 b
Day-4	20	54.667 a	60.000 b	36.000 c
Day-5	20	20.000 e	23.333 d	24.000 d

Prior to ANOVA analysis, means followed by the same letter at vertical rows shows no significant difference at $p \geq 0.05$.

Table 2. Percentage of unhatched pupa of *C. cramerella* after treated at different concentrations of Zingiberaceae EOs

Treatment	n	Unhatched pupa of <i>C. cramerella</i> (%)		
		<i>Zingiber officinale</i>	<i>Curcuma longa</i>	<i>Alpinia galanga</i>
T1 (100 ppm)	20	44.048 b	48.333 c	48.000 b
T2 (200 ppm)	20	33.000 d	48.660 b	40.000 c
T3 (400 ppm)	20	49.476 a	55.000 a	52.000 a
T4 (800 ppm)	20	37.381 c	47.000 d	52.000 a
T5 (Control)	20	21.111 e	15.666 e	4.000 d

Prior to ANOVA analysis, means followed by the same letter at vertical rows shows no significant difference at $p \geq 0.05$.

In agreement as in the observation on the unhatched pupa (Table 1), Day-5 old pupa recorded the highest percentage of the successful emergence of a healthy adult (61.778% a) after

treating with Zingiberaceae (*Z. officinale*, *C. longa* and *A. galanga*) treatments, and significantly different to other days (Table 3). Meanwhile, observation of the deformed adult, spraying of treatments at the young age of the pupa (Day-1) may result in the highest percentage of deformed adults (19.962% a). This might occur during early development of cocoon, this newly established cocoon was still fragile, soft and the most exposed to the treatments applied or any environmental factor. Control treatments recorded highest percent of healthy adults (75.777% a), and significantly different (One-Way ANOVA; $p < 0.05$) compared to other EOs (Table 4). The lowest percentage of healthy adult emergence was recorded at *A. galanga* (34.000% d), followed by *C. longa* (35.083% c) and *Z. officinale* (46.982% b). *A. galanga* performed as the best EOs which recorded the highest percentage of deformed adults (18.000% a) and significantly different (One-Way ANOVA; $p < 0.05$) with *C. longa* (15.167% b), *Z. officinale* (12.042% c) and the control treatment (10.630% d).

Table 3. Percentage of unhatched pupa and adult emergence of *C. cramerella* after treated at different age of pupa using Zingiberaceae essential oils

Age of pupa	n	<i>C. cramerella</i> after treated with treatments (%)		
		Unhatched pupa	Healthy adult	Deformed adult
Day-1	60	37.370 b	42.667 c	19.962 a
Day-2	60	41.302 b	45.587 b	13.111 b
Day-3	60	47.222 a	41.944 d	10.833 b
Day-4	60	50.222 a	38.555 d	11.222 b
Day-5	60	22.444 c	61.778 a	15.778 ab

Prior to ANOVA analysis, means followed by the same letter at vertical rows shows no significant difference at $p \geq 0.05$.

Table 4. Percentage of adult emergence of *C. cramerella* after treated with different Zingiberaceae EOs

Treatment	n	<i>C. cramerella</i> after treated with treatments (%)		
		Unhatched pupa	Healthy adult	Deformed adult
<i>Zingiber officinale</i>	80	40.976 c	46.982 b	12.042 c
<i>Curcuma longa</i>	80	49.750 a	35.083 c	15.167 b
<i>Alpinia galanga</i>	80	48.000 b	34.000 d	18.000 a
Control	80	13.592 d	75.777 a	10.630 d

Prior to ANOVA analysis, means followed by the same letter at vertical rows shows no significant difference at $p \geq 0.05$.

Adult deformities were observed at four different parts; the body (abdominal parts), the antenna, the wings and the overall deformed (Figures 1A and 1B). Deformity of the abdominal part of *C. cramerella* was commonly observed and recorded the highest for all treatments (Figure 2). All four types of deformities only can be observed from *Z. officinale*, whereas no overall deformities observed for *C. longa* and control. Even the percentage of *C. cramerella* adult deformities were lower compared to the healthy adult, but all EOs shows promising results, where managed to deter the emergence of an adult from pupa stages as recorded in this study.



Figure 1.A Examples of un-hatched pupa after the cocoon were removed (30x)



Figure 1.B Example of deformed adults a) abdomen b) wings c) antenna d) overall

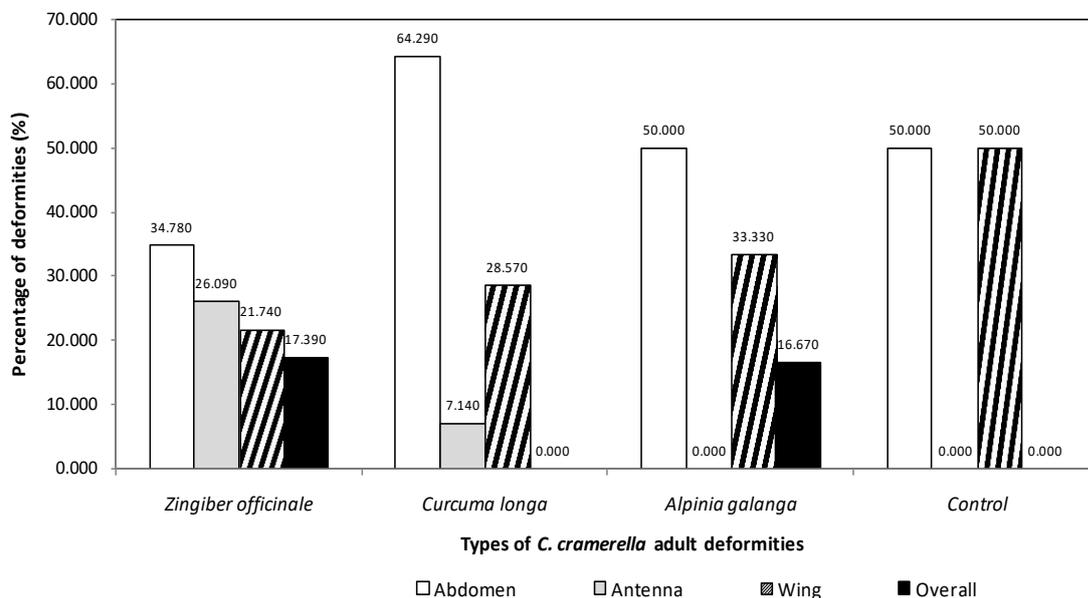


Figure 2. Percentage of adult deformities of *C. cramerella* adult deformities after treated with different Zingiberaceae EOs

Application of Zingiberaceae as potential botanical pesticides is an advantage because they are not labeled as poisonous, as well as dangerous to the environment and human health (Isman, 2008). Several species of Zingiberaceae produce EOs with chemical components that

lead to pesticide mode of action. The mode of action of EOs is either in a form of direct contact or through ingestion. Insidious characteristics of *C. cramerella* larva inside the pod for their entire instars leaving only egg, pupae, and adult as targeted life cycle to be controlled.

During pupa developmental stages, the pupae will change color from an initial light green to dark gray as they mature which takes six to eight days. In most cases, the deformed pupa can be seen even without removing the cocoons, when the pupa turned to entire dark gray or black in color. In our study, the percentage of the unhatched or the deformed pupa were higher for all Zingiberaceae EOs, with the range from 33.000% to 55.000% (Table 2) of the deformed pupa were observed. Unhatched pupa can be observed higher in concentration more than 400 ppm. This result shows that all EOs have the potential to disrupt the development process of adult emergence. The highest percentage of the healthy adult with more than 75% was observed emerging from pupa at control treatment (75.777%; Table 4) comparisons to pupa exposed to EOs. Healthy adult of *C. cramerella* was characterized by a live adult emergence, which usually with a wing expanse of 10-14mm. The fore wing of a healthy adult has distal third except for costa pale orange-yellow. The basal marked with a zigzag pattern of six irregularly space transverse white lines partially edged with black. *C. cramerella* head is white with the crown roughened posterior and mixed or overlaid with fuscous. The antenna is white, scape suffused with fuscous dorsally, flagellum with indistinct fuscous annular bars dorsally to near apex (Bradley, 1986). The antenna is about one and three-fifths length of the fore wing. This small, complete and delicate structure of this gracillariid micro-moth usually unseen in deformed adults. The percentage of adult deformities were the lowest at control (10.630%), and significantly different compared to other EOs. *A. galanga* recorded the highest percentage of adult deformities (18.000%) with the abdominal deformity at 50.000% (Figure 2). Percentage of abdominal deformities were the highest for all Zingiberaceae EOs, compared to wings, antenna and overall deformities.

Conclusion

The study has demonstrated the potential use of Zingiberaceae EOs as an adult emergence inhibition from a pupae to an adult will reduce the number of healthy adults, number of ovipositing eggs later on. Deformities and adult emergence inhibition have strongly suggested that the mode of action of EOs could be through contact as an application of EOs was carried out at pupa stages. Selected Zingiberace species, *Z. officinale*, *C. longa* and *A. galanga* that used in this study are safer for both human and environmental health. Thus, spraying with biopesticide formulation derived from Zingiberaceae EOs could help in reducing the population of *C. cramerella* in the near future, either as a single tool or as a part of an integrated pest management strategy.

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Genetic diversity of common and rarely occurring *Carica papaya* L. plants

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Abstract

Papaya (*Carica papaya* L.) belongs to the family of Caricaceae is an economically important crop of the tropical regions of the world. This plants are exhibiting considerable phenotypic variation in morphological and horticulture traits. Thus, studies on genetic diversity of *C. papaya* plant forms are essentially important as it provides the basis for varietal improvement. A field survey was conducted along the Kuala Tatau local farming area (3° 4' 48.54" N to 112° 47' 57.9" E) at Bintulu, Sarawak. Based on the survey conducted, a total of 240 papaya plants have been recorded and mainly comprised of female plants (48%) and least population was recorded for rarely occurring papaya form (5%). The common papaya plant forms were female, hermaphrodite and male plants. In addition two rarely occurring papaya plants; subandroecious and trimonoecious plant forms were also observed in the present study. The trimonoecious plant also known as "papaya bertali" where the female or hermaphrodite flowers present in the terminal of peduncle surrounded by male flowers producing fruits at the edge of terminals besides its stems that could increase the production. While the subandroecious plant form undergo sex switching from male to hermaphrodite thus producing fruits. Genetic variation was distinguished using microsatellite (SSR) markers that clearly separate the plant forms into two different clades without overlapping characters. The pairwise distance of *C. papaya* measured by Tamura 3-parameter model ranged 0.02-0.86 included the outgroup and aligned sequences showing there were 58 informative parsimony sites from 265 base pairs. This research enhancing the informations on genetic diversity among common and rarely occurring papaya forms which are potential source for improvement of papaya production and also for plant breeding.

Keywords: *Carica papaya*; SSR markers; subandroecious; trimonoecious

Introduction

Papaya (*Carica papaya* L.) is a tropical fruit having commercial importance because of its high nutritive and medicinal values. Papaya originated from tropical region of America and it is an economically important crop at Southeast Asia including Malaysia (Ming et al., 2007).

According to Sudha et al. (2013), papaya is the fourth most traded tropical fruit followed by bananas, mangoes and pineapples.

Papaya is polygamous which have three common plant forms; male, female and hermaphrodite or bisexual flower. Hermaphrodite plants are the major type for commercial cultivation due to its high productivity and pear-shaped fruits with a lower ovarian cavity (Magdalita and Mercado, 2003). Interestingly, based on the field observed apart from the common papaya forms there are two other rarely occurring papaya plants forms have been recorded; i.e., subandroecious and trimonoecious plants. Subandroecious plant known when there are female or hermaphrodite flowers surrounded by the male flowers while trimonoecious are plants that exhibited female and hermaphrodite flowers are surrounded by many male flowers on the same plants (Deghan and Webster, 2011).

A loose linkage between floral morphology and plant forms has been identified by Storey (1969) and plant forms determination based on floral morphology is not possible until after four months of cultivation. Plant forms determination in papaya is particularly challenging not only because it has three different forms or more within the species, and also it showed frequent form switching caused by environmental factors (Storey, 1969). Thus, this led to interest in resolving the genetic diversity of various papaya plant forms through morphological and molecular approaches.

Materials and Methods

Diversity of *Carica papaya* Plant Forms

The present study was performed at local farming area in Kuala Tatau (3° 4' 48.54" N to 112° 47' 57.9" E) Bintulu, Sarawak. There were 51 sampling sites were chosen for evaluate the papaya plant diversity. The sampling points were marked using GARMIN GPS64s. Total number of *C. papaya* plants at the Kuala Tatau were count during field visits to assess the diversity. The papaya plants' forms evaluated based on inflorescences and floral morphology.

Sample Collection

The local farming area was visited for *in-situ* observation to record flowering, non-flowering or fruiting plants. Inflorescences bearing flowers and fruits were freshly picked from individual plants and placed in separate zip-locked plastic bags for morphometric analysis. Young fresh leaf were sampled for genetic analysis and stored in silica gel prior to analysis.

Morphological Analysis

Qualitative and quantitative variables of arils parts of papaya plant; leaves, stems, petioles, inflorescences, flowers and fruits were measured and recorded. The variables were recorded and measured using ruler and Mitutoya Digimatic Vernier Caliper.

Polymerase Chain Reaction (PCR) Amplification

Genomic DNA was isolated from 200 mg of fresh leaves based on the standard procedure provided by NucleoSpin® Plant II. The microsatellite (SSR) marker was selected for PCR amplification and sequence analysis (F: TCAACTATTTCCCCGCATA and R: CACCTCCTTGTCCTCAAAGGTT) were chosen based on Oliveira et al. (2010). The PCR amplification for SSR method was followed the protocol by Oliveira et al. (2010) and Warnakula et al. (2017).

The PCR was performed in total of 25 µL reaction mixture containing 17.5 µL ultra-pure H₂O, 2.5 µL PCR buffer, 1.5 µL 2.5 mM MgCl₂, 0.5 µL 0.2 mM dNTP, 0.25 µL of each primer, 0.025 µL of Taq polymerase and 2.5 µL DNA template. The PCR reaction using SSR primer was performed in S1000 Thermal Cycler (BioRad, USA) using the following condition: initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 40 sec, annealing at 60°C for 40 sec, extension at 72°C for 1 min and 2 min final extension at 72°C. The end reaction was set at 4°C for forever to maintain the stability of PCR product. The obtained PCR product were sent for DNA sequencing at First BASE Laboratory Sdn. Bhd. in Seri Kembangan, Selangor.

Statistical Analysis

For morphometric analysis, single-factor analysis of variance (ANOVA) with post hoc Tukey's test ($p < 0.05$) was used to compare the mean values using SAS 9.4 Software. Discriminant analysis (DA) based on linear combinations of the predictor variables was used to find the maximum separation between types of papaya plants using XLSTAT 2013. For genetic analysis the electropherograms of the sequence fragments were inspected and assembled using Phred, Phrap, and Consed software in MacPro. Only good-quality fragments (sequence quality > 20) were chosen. MEGA 7.0 program was used to construct the phylogenetic tree and estimate sequence divergence of *C. papaya* plant forms. Maximum likelihood is a method that applies an explicit criterion-the log-likelihood to compare the models of nucleotide substitution in the presence of a large number of short sequences.

Results and Discussion

Diversity of Carica papaya Plant Forms

The sampling area covered the stretch along the roadside of Kuala Tatau, Bintulu, Sarawak comprised of 51 sampling points. Based on survey conducted a total of 240 papaya plants were assessed and they were classified into 5 different forms based on their reproductive morphological variability (Figure 1). Out of 240 papaya plants, the higher population of papaya form 115 plants (48%) was female type, followed by 77 plants (32%) were male, 22 plants (9.2%) were hermaphrodite, 10 plants (4.2%) were subandroecious, two plants (0.8%) were trimonoecious and 14 plants (5.8%) were unknown papaya forms (no flowers were found during sampling period).



Figure 1. Commonly occurring papaya plants; (a) male papaya forms, (b) female papaya forms, and (c) Hermaphrodite papaya forms. Rarely occurring papaya forms; (d) Subandroecious papaya plant form where the male flowers undergo sex switching and bore the fruits and (e) Trimonoecious plant form where a peduncle consists of one or two female or hermaphrodite flowers (produced fruits) and surrounded by 20-30 male flowers.

*Morphological Variation and Discriminant Analysis (DA) of *Carica papaya* Plant Forms*

Variations in the quantitative morphological characteristics of the papaya plant forms are presented in Table 1. The male flowers only possessed stamen that are not bearing fruits thus these trees are being chopped off. Female flowers have pistil and as the flower is pollinated from surrounding male pollen, it will bear fruits. As for hermaphrodite flower, it consists both male and female, then can enable to cross-pollinate and able to produce fruits.

In addition, there are two rarely occurring papaya plant forms; subandroecious and trimonoecious observed to continuously producing fruits throughout the field visits. During the first sampling, we observed the subandroecious plant producing many male flowers and developing fruits. No female or hermaphrodite flowers have been recorded. The male flowers do not look like typical male flowers but they are similar to hermaphrodite flower shape and sizes. This conflict arises in classifying the plants species because fruits were being observed on the trunk. During the subsequent samplings, it was observed the male flowers undergo sex switching and developing ovary. From this observation, it can be stated that male flowers reflect toward the environment surrounding and also undergo sex switching. According to Odu et al. (2006) and Ming et al. (2007), the sex determination in papaya showed frequent sex reversal caused by the environment factors and if the conditions are favourable it will result in true hermaphrodite flowers as observed in the present study.

Meanwhile, the trimonoecious plant forms possess male, female and hermaphrodite flowers on the same plants (Deghan and Webster, 2011). In common papaya the fruits are

being produced on the stem. But in this plant from the fruits are being produced on the stems and also at the terminal of the peduncle. This could increase the production of fruits per plant. Trimonoecious observed to have fruits that hanging at the end of male inflorescence stalks. A peduncle of trimonoecious consists one or two female or hermaphrodite flowers and surrounded by 20-30 male flowers. This unique form also has been recorded in India (Chávez-Pesqueira et al. 2014). Trimonoecious female and hermaphrodite flowers are smaller in size as its male flower. This make the conflicts arises where the male plant producing fruits but in reality the fruits are developed from female or hermaphrodite flowers that borne on the terminal of the peduncle.

Table 1. Quantitative floral morphological variables of *Carica papaya* plant forms

Variable s	Male	Female	Hermaphrodit e	Subandroecious		Trimonoecious		
				Male	Hermaphrodit e	Male	Female	Hermaphrodit e
Flower size (cm)	1.97±1.61 ^b (1.20-2.70)	2.34±1.61 ^b (1.00-5.14)	2.51±0.13 ^{ab} (1.90-4.50)	3.58±0.13 ^a (2.00-3.80)	2.57±0.11 ^{ab} (2.00-4.00)	2.15±0.11 ^b (1.30-2.70)	1.95±0.15 ^b (1.00-3.20)	2.09±0.14 ^b (1.30-3.00)
Petal length (cm)	1.14±0.05 ^c (0.50-1.50)	2.23±0.18 ^a (1.00-4.89)	1.92±0.13 ^{ab} (1.00-3.30)	1.34±0.15 ^b ^c (0.70- 2.80)	1.70±0.16 ^{abc} (0.80-3.10)	1.22±0.05 ^c (0.70-1.50)	2.05±0.16 ^a (1.00-3.30)	2.21±0.20 ^a (1.20-3.00)
Petal width (cm)	0.25±0.02 ^a (0.10-0.40)	0.24±0.03 ^a (0.10-0.64)	0.23±0.02 ^a (0.10-0.64)	0.25±0.03 ^a (0.10-0.62)	0.22±0.03 ^a (0.10-0.64)	0.28±0.02 ^a (0.10-0.40)	0.23±0.03 ^a (0.10-0.62)	0.22±0.06 ^a (0.10-0.64)
Anther length (cm)	0.23±0.20 ^b ^c (0.10- 0.40)	na	0.22±0.20 ^{bc} (0.10-0.50)	0.21±0.02 ^c (0.10-0.30)	0.17±0.02 ^c (0.10-0.35)	0.30±0.03 ^a ^b (0.10- 0.52)	na	0.30±0.03 ^a (0.18-0.45)
Filament length	1.46±0.09 ^a (1.00-3.00)	na	1.46±0.90 ^a (1.00-3.00)	1.63±0.13 ^a (0.50-2.70)	1.22±0.06 ^{ab} (1.00-2.00)	0.85±0.11 ^b (0.25-1.70)	na	0.94±0.20 ^b (0.30-2.27)
Stigma length (cm)	na	0.28±0.04 ^a (0.10-1.34)	0.36±0.09 ^a (0.20-3.00)	na	0.25±0.02 ^a (0.10-0.40)	na	0.22±0.03 ^a (0.10-0.40)	0.22±0.03 ^a (0.10-0.35)
Ovary length (cm)	0.61±0.02 ^c (0.40-0.82)	0.55±0.06 ^c (0.20-1.70)	0.71±0.11 ^c (0.30-2.10)	0.72±0.03 ^c (0.50-1.00)	1.21±0.13 ^{ab} (0.70-2.27)	0.65±0.02 ^c (0.50-0.81)	1.13±0.17 ^b (0.20-2.10)	1.54±0.12 ^a (1.00-2.00)
Ovary width (cm)	0.10±0.02 ^c (0.05-0.10)	0.35±0.04 ^a ^b (0.08- 1.00)	0.39±0.04 ^{ab} (0.10-0.70)	0.13±0.01 ^c (0.09-0.18)	0.40±0.06 ^a (0.10-1.00)	0.09±0.04 ^c (0.05-0.11)	0.02±0.02 ^b ^c (0.10- 0.40)	0.18±0.02 ^c (0.10-0.23)

Mean values in the same row with different alphabets (a>b>c) are significantly different at p<0.05 (ANOVA, Tukey' test). Values are given in means±standard error and values in bracket are the range. na- not available.

The morphological data were analyzed for discriminant analysis and based on linear combinations of the variables produced better discrimination of the papaya plant forms. Biplots of the morphological dataset for discriminant functions (DF) one and two are shown in Figure 2. The discriminant function analysis accounted for 78.48% of the total variance. Among the variable flower size and ovary width two factors that highly discriminated the papaya plant forms. Plant forms are classified into three main groups.

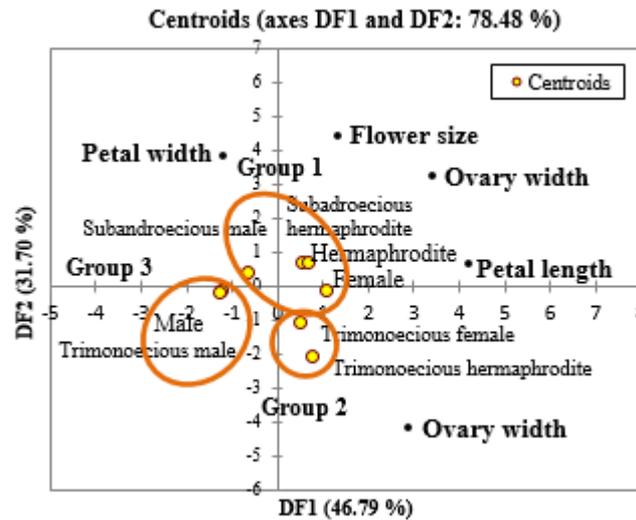


Figure 2. Plot of the quantitative morphological parameter of the five papaya form

Based on DA, common hermaphrodite, female, subandroecious hermaphrodite and subandroecious male were classified in same Group 1. These three forms has bigger flower size, petal width and ovary width compared to other flowers forms. Among the *C. papaya* forms, flower size of subandroecious male (3.58 ± 0.13 cm) and subandroecious hermaphrodite (2.57 ± 0.11 cm) was not significantly different and similar as common hermaphrodite flowers (2.51 ± 0.13 cm) compared to the others form of *C. papaya* that smaller than these three forms. Subandroecious male (3.58 ± 0.13 cm) is two times larger than typical common male (1.97 ± 1.61 cm). Trimonoecious female and trimonoecious hermaphrodite were classified as in Group 2 because the have the similar ovary length. Meanwhile, Group 3 consist of typical male and trimonoecious male flowers. Trimonoecious male flower size (2.15 ± 0.11 cm) was similar common male (1.97 ± 1.61 cm).

Phylogenetic Relationship of Carica papaya Plant Forms

The PCR sequences for *C. papaya* were approximately 250 base pairs for SSR *Vasconcellea* sp. was used as an outgroup. There is no reference sequences for papaya assession has been published in NCBI blast for SSR primer sets for comparison. The pairwise distance of *C. papaya* ranged between 0.02 to 0.86 included outgroup and the average intraspecific pairwise distance was 0.12. For SSR marker the aligned sequneces yielded a most parsimonious tree with high proportion informative sites; 58 informative parsimony from the 265 base pairs.

The Maximum Likelihood (ML) analysis using the Tamura 3-parameter model with gamma distributions (T92+G) was selected as the best-fitting substitution model (Hall, 2011). The

results of algorithms applied showed that papaya accessions examined were distributed into two distinct well-supported clades. All of the samples occupied separate topological positions; that is, no overlap among the papaya plant forms was observed. The resultant ML tree (Figure 3) exhibited bootstrap value 87% between Clade 1 and Clade 2. Clade 1 comprised of fruit producing papaya plants; i.e., hermaphrodite, subandroecious, female and trimonoecious plant forms. Whereas the non- fruit producing male papaya is the only plant form in Clade 2. *Carica papaya* might have wide range of vegetative characteristics, however SSR marker in the present study was able to distinguish related species of *C. papaya* into two different clades. The elevated amounts of inconstancy related with microsatellites, speed of handling, and the possibility to detach extensive number of loci give a marker framework fit for distinguishing among closely related species (Selkoe and Toonen, 2006).

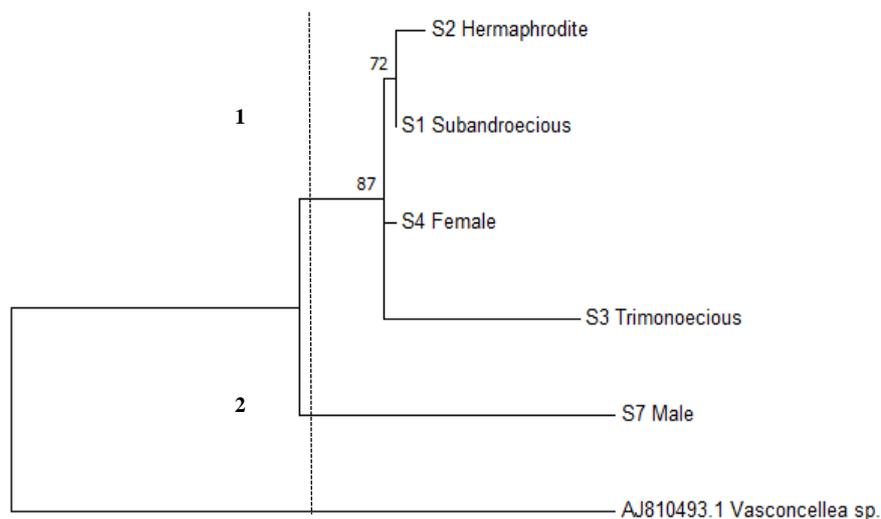


Figure 3. Phylogenetic tree of *Carica papaya* plant forms generated from Maximum Likelihood (ML) analysis with 500 bootstrap of SSR regions using Tamura 3-parameter model

Conclusion

Beside of three common papaya including male, female and hermaphrodite, there were detected two rare forms which were subandroecious and trimonoecious. In common papaya plant form; female and hermaphrodite fruits are only produced at the stem. Whereas the rarely occurring trimonoecious and subandroecious plant forms could increase the papaya production. Besides, the current work also provided the better integration of floral quantitative morphological data and SSR sequences to understand the relationship between the various papaya plant forms. This research enhancing the informations on genetic diversity among common and rarely occurring papaya forms which are potential source for improvement of *C. papaya* production and also for plant breeding.

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Effect of basal media, plant growth regulators and complex additives on asymbiotic seed germination and shoot induction of *Coelogyne pulverula*

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Abstract

Coelogyne is one of the largest epiphytic and wild orchids among the family Orchidaceae. *Coelogyne pulverula* can be found in Borneo, Sumatra, Java, and Thailand. One of the unique characteristics of this species is it produces light and aromatic fragrance from the full bloom flower. Apart from a slow growth rate of seed germination, wild orchids are also difficult to be propagated through traditional methods. *In vitro* culture techniques are considered an effective method for mass production of *Coelogyne* sp and conserving the wild orchids. Therefore, the present study focused on the effects of two different basal media, plant growth regulators (PGRs) and complex additives on asymbiotic seed germination subsequent shoot induction. Immature seeds from a five-month old capsule were used as plant materials. All cultures were grown under 24h light at $25\pm 2^{\circ}\text{C}$. The result showed that seeds cultured on half strength MS medium were germinated after 30 days with $98.1\pm 0.21\%$ germination rate, followed by MS medium ($4.40\pm 0.02\%$). Addition of 20% (v/v) coconut water in MS media has significantly enhanced ($11.10\pm 0.05\%$) germination within 60 days. Based upon the results, it was found that half strength MS medium was more effective than full strength MS medium for germination. For shoot induction, half strength MS medium supplemented with 1.5 mg/L BAP and 0.5 mg/L NAA was found to be more efficient for shoot regeneration within 60 days of culture with $100\pm 0.22\%$ and mean number of shoot at 88 ± 0.18 , followed by half strength MS with 1.0 mg/L BAP and 1.5 mg/L NAA ($96.4\pm 0.05\%$) and mean number of shoot at 60 ± 0.48 . We have established an efficient protocol of *in vitro* propagation of *C. pulverula*. Our results can be used to determine the best condition for the acclimatization process.

Keywords: Asymbiotic; *Coelogyne pulverula*; basal media; complex additive; plant growth regulator

Introduction

The Orchidaceae is one of the largest families in the plant kingdom. It is site specific and can act as a good indicator of a healthy ecosystem. They are well known for their exquisite and long-lasting flowers which have made them the doyen among ornamentals (Gupta, 2016). Recently, researchers had revealed that the *Coelogyne* orchid has become a rare, endangered and threatened species all over the world (Kaur and Bhutani, 2014). Besides being an ornamental treasure in the commercial market, orchids have their own medicinal properties that have been widely applied in traditional uses. Wild orchids are nowadays experiencing a problem of a steady decrease in quantity due to the over-collection of orchid hunters and

forest logging activities which nearly caused extinction for many orchid species. Thus, plant tissue culture and micropropagation are the options to reduce pressure from illegal collection, meet commercial needs and re-establish threatened species back into the wild. Hence, *in vitro* propagation technique is considered as an effective technique or strategy to multiply this orchid species, either for conservation purposes or for horticultural activities. Since then, *in vitro* seed germination protocols have been established for many orchid species by using various types of media and plant growth regulators (Arditti and Ernst, 1993). This has proven to be an important tool for orchid propagation, as well as one of the efforts to conserve any rare or endangered orchid species (Utami, Hariyanto and Manuhara, 2017; Zeng *et al*, 2013).

The epiphytic or lithophytic genus *Coelogyne* consists of 190 species which are distributed from Borneo, Sumatra, Java and Thailand. Two subspecies and 12 varieties have been recorded in the Borneo Island, of which the epiphytic *Coelogyne pulverula* is endemic to Borneo. Several orchids like *Coelogyne cristata*, *Coelogyne nervosa*, *Coelogyne sandariana*, *Coelogyne flaccida* and *Coelogyne swaniana* have currently been successfully propagated through *in vitro* tissue culture (Naing *et al*, 2011; Sathiyadash *et al*, 2013; Sachdev and Kulshreshtha, 1986 and Chen *et al*, 2013). *In vitro* propagation of *Coelogyne* species through asymbiotic seed germination technique could further multiply the cultures using the protocorm-slicing method. This technique could ensure better germination percentage as well as saving the time lapse between pollination and *in vitro* propagation of orchids via multiple shoot formation (Kaur and Bhutani, 2013). *C. pulverula* can easily be found on large tree trunks and branches of trees, especially at the lowland rainforests and lower montane forests (Chan *et al*, 1994). *C. pulverula* has the potential to be commercialized as a plant producing fragrance for the cosmetic industry.

Therefore, this study was carried out to develop an efficient and effective *in vitro* propagation protocol, as well as one of the efforts to conserve this orchid species. Determination of the best basal media for asymbiotic seed germination, production of healthy plantlets of this species as well as the establishment of *in vitro* protocol has been carried out.

Materials and Methods

Plant Material

A five-month old green immature capsule of *C. pulverula* was collected at Poring Orchid Center, Ranau, Malaysia. The orchid pod was immersed in 1% fungicide solution to reduce the risk of contamination and cleaned by using a toothbrush to remove any dirt or small particles trapped in between the orchid capsule.

Surface sterilization

The capsule of *C. pulverula* was immersed in a 0.5% fungicide solution for 10 minutes and gently scrubbed with soap solution for another 10 minutes. The capsule was then rinsed and left under running tap water. The capsule surface was sterilized by immersing in pure Clorox added with two drops of Tween 20 (Sigma Aldrich, USA) for 20 minutes. It was then rinsed with sterilized distilled water and repeated three times to cleanse the capsule from any impurities followed by dipping it in 95% ethanol before passing it briefly through the flame (David *et al*, 2015). The capsule was cut longitudinally before being inoculated on half strength and full strength Murashige and Skoog (MS) media respectively. The selected basal

medium was later supplemented with various concentrations of complex additives such as coconut water and potato (5% v/v, 10% v/v, 20% v/v, and 30% v/v) as well as PGRs with both single treatment and combinations for the shoot and root induction.

Data analysis

All the experiments were performed in a Completely Randomized Design (CRD) and were repeated in five replicates. Also, all of the cultures were observed under Carl Zeiss microscope and DinoLite Capture™ (UK) starting from the seed germination until root formation. Parameters observed in this study were the percentage of seed germination, percentage of shoot development, protocorm proliferation, percentage of shoot forming leaves and roots, length of leaves and roots and the percentage of necrosis for each treatment. The percentage of germinated seeds was calculated by dividing the number of germinated seeds by the total number of cultured seeds (Shimora and Koda, 2004). Analysis of variance (ANOVA) was performed on the data and mean values were compared by using the Duncan Multiple Range Test (DMRT) at $p < 0.05$. The process of seed germination was divided into the following six categories according to developmental stages of embryos, which were modifications of those given by Miyoshi and Mii (1995):

Stage 0: 'No germination' stage. No growth of embryo occurs

Stage 1: 'Pre-germination' stage. Embryo swells to fill the seed coat

Stage 2: 'Germination' stage. Embryo emerges from the seed coat

Stage 3: 'Protocorm' stage. The embryo is completely discharged from the seed coat

Stage 4: 'Rhizoid' stage. Rhizoids are formed on the protocorm surface

Stage 5: 'Shoot stage'. The shoot is differentiated from the protocorm

Results and Discussion

In this present study, MS basal media had recorded the highest percentage of protocorm proliferation. Two types of MS basal media which are half strength and full strength MS media were used in this study. MS media had proven to be the best basal media for the development of orchid species namely *Dendrobium tosaense* and *Coelogyne nervosa* (Lo *et al.*, 2004). As for the study that involved complex additives, half strength MS media supplemented with 20% coconut water was the best media as compared to potato extract. Previous studies had proven that coconut water is not only high in glucose and fructose, but also contains various amino acids, fatty acids, and minerals (Jualang *et al.*, 2015).

Effect of basal media on seed germination and development of C. pulverula

Basal media are a key factor in the success of plant tissue culture protocol (Shekarriz *et al.*, 2014). In the present study, two types of basal media were assayed for their effectiveness on enhancing the seed germination of *C. pulverula*. The effect of basal media on seed germination was tested by using half strength (1/2) MS and full-strength MS media. After 6 weeks of culture, half strength MS ($100 \pm 0.21\%$) media performed better than MS media ($4.40 \pm 0.02\%$) (Table 1). Seeds were aseptically inoculated on both basal media and maintained under conditions at $25 \pm 2^\circ\text{C}$. Observations were made from time to time for any changes on the seed. Germination is considered to occur only if the seed coat bursts and

embryo emerges from the seed coat. The percentage of seed in different developmental phases was calculated by dividing the number of seeds in each phase by the total amount of seeds and multiply it by 100% (Utami *et al.*, 2017). The first seed to be germinated was from half strength MS media which showed higher germination rate as compared to MS media (Figure 1A). After 3 weeks of culture, the seed has swollen, and germination rate increased $100\pm 0.21\%$ and eventually formed protocorm-like bodies (PLB) (Figure 1B). Most of the seeds of *C. pulverula* started to germinate after three weeks of culture. This suggests that growth and development of protocorm of *C. pulverula* preferred lower concentration and simple nutrient complexity as a formulated for $\frac{1}{2}$ MS basal medium.

Table 1. Effect of two different basal media on seed germination of *C. pulverula* after 6 weeks of culture.

Media	Percentage of Germination (%)	Development stages
$\frac{1}{2}$ MS	100 ± 0.21^{bcd}	Most of the protocorm were at stage 4 (Protocorm with the emergence of the first leaf)
MSO	4.40 ± 0.02^{abcd}	Most of the seed reached stage 2

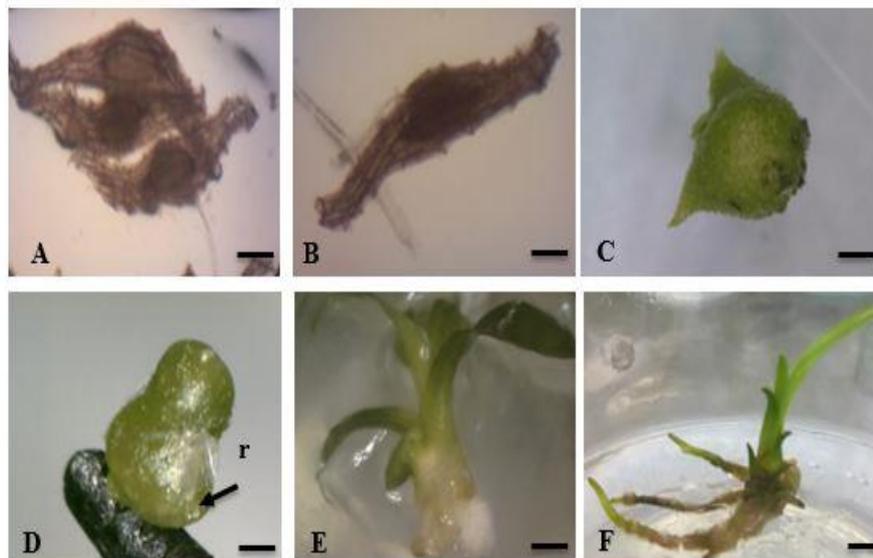


Figure 1. Developmental stages of *C. pulverula* from seed germination to shoot formation in asymbiotic culture *in vitro*.

(A) Original seed with an embryo (B) Close up of viable seed (C) Protocorm stage, embryo discharged from seed coat (D) Rhizoid formed on the surface of protocorm (E) Shoot stage, the shoot is differentiated in protocorm (F) Mature seedlings with roots. Scale bars = 1 mm. r= rhizoid (pointed arrow)

A study was done by Zhang *et al.* (2013) also proved that any terrestrial orchids usually require media with only low salt concentrations for their seed germination. Many orchid species prefer a medium with low salt and nitrogen for seed germination and PLB formation. Similar results can be seen in the previous study which had been conducted on *Renanthera imshootiana* (Wu *et al.*, 2012). The results clearly showed that $\frac{1}{4}$ MS basal medium which content low salt concentration successfully increased percentage seed germination of *R.*

imshootiana compare to the other basal medium. Also, another study reported that seed germination of *Paphiopedilum wardii* on ½ MS was significantly higher than on ¼ MS or MS media. This indicates that the effect of basal media on seed germination is likely to be genotype-dependent in orchids (Zeng *et al*, 2012).

Effect of basal media supplemented with complex additives on seed germination of C. pulverula after 6 weeks of culture

The basal medium of ½ MS and MS were used to test the effect of organic additives on seed germination of *C. pulverula*. In general, the success of tissue culture as a means of plant propagation is greatly influenced by the choice of nutritional components and growth regulators. Nutrients from the addition of complex additives have greatly influenced the germination of *C. pulverula*'s seed (Bakar *et al*, 2014). Complex additives are needed in the propagation of orchids besides salts compositions, carbon source and growth regulators (Murdad *et al*, 2010). Also, complex additives could also have helped in producing more protocorm-like bodies (Akter *et al*, 2007) as well as promoting the growth and development of asymbiotic seeds and regeneration of plantlets (Al-Khateeb, 2008). MS media were individually tested by using coconut water and potato extract at the concentrations of 5%, 10%, 20%, and 30% respectively. Several reports have shown that coconut water has cytokinin function that could enhance the growth of protocorm-like bodies of this orchid species (Table 2).

Table 2. Effect of basal media supplemented with complex additives after 6 weeks of culture

Basal media	Treatments	Percentage of Seed Germination (%)
½ MS	10% PE	2.20±0.63 ^{abcd}
	20% PE	4.40±0.24 ^{abcd}
	30% PE	4.40±0.01 ^{bcd}
	10% CW	6.70±0.06 ^{cd}
	20% CW	11.10±0.05 ^{ab}
	30% CW	No germination
MSO	10% PE	2.20±0.15 ^{abc}
	20% PE	No germination
	30% PE	No germination
	10% CW	6.70±0.05 ^{abcd}
	20% CW	No germination
	30% CW	No germination

*PE (Potato extract) *CW (Coconut water); *Data are means of 10 replicates. Mean followed by the same letter (column) did not differ significantly at $p \leq 0.05$ according to Duncan's Multiple Range Tests. SD-Standard deviation

In this study, coconut water media showed a significant effect on the development of *C. pulverula* seed as compared to potato extract. Previous studies also proved that coconut water helped to increase the number of shoots and length as well as protocorm development (Nasib

et al, 2008). Half strength of MS media supplemented with 20% coconut water showed higher percentage with (11.10±0.05%) after six weeks of culture as compared to other treatments (Table 2). Only full MS media supplemented with 10% v/v coconut water shows sign of germination (76.70±0.05%) after 6 weeks of culture but none for any concentrations of potato extract where most of the seeds died and failed to germinate after 6 weeks of culture.

Effect of basal media supplemented with plant growth regulators on seed germination of C. pulverula after 6 weeks of culture

Most orchid cultures require plant growth regulators (PGRs) for growth, callus and PLB formation, proliferation and development (Hossain *et al*, 2013). PGR had been widely used to obtain the maximum or higher response towards seed germination. Plant growth regulators were proven to enhance shoot proliferation as well as induction of the protocorm-like bodies of *C. pulverula* in this study (Table 3).

Table 3. Effect of half strength MS media supplemented with plant growth regulators on protocorm and shoot induction after 3 weeks of culture

Treatments	Percentage of Shoot Induction (mean, % ± SD)				No. of Leaves (mean,% ±SD)	Leaf Length (%)
	3	6	9	12		
1.0 mg/L BAP+ 0.5 mg/L NAA	60.00±0.14 ^{bc}	86.00±0.02 ^{abcd}	91.10±0.035 ^{ab} _c	96.4±0.05 ^{bc} _d	3.03±0.18 ^{de}	86±0.12%
1.5 mg/L BAP+0.5 mg/L NAA	77.00±0.02 ^a _b	88.90±0.08 ^{bcd} _e	95.50±0.04 ^{cde}	100±0.22 ^a	5.00±0.42 ^{ab}	92.2±0.03 %
2.0 mg/L BAP+0.5 mg/L NAA	64.40±0.54 ^c _d	77.63±0.74 ^{abc}	88.90±0.03 ^{abe} _d	92.2±0.04 ^{ab} _c	2.00±0.36 ^{ab} _c	82.6±0.40%

^{abcd} means with different superscript in the same column differ significantly (P<0.05) SD-Standard deviation (T-test value, p=0.00).

After nine weeks of culture, protocorm-like bodies (PLBs) started to proliferate in half strength MS media. Hence, half strength MS media were the best basal media for seed germination and development. For shoot formation, half strength MS media supplemented with PGR 1.5 mg/L BAP+0.5 mg/L NAA had shown the highest percentage for shoot induction of *Coelogyne pulverula*, 100±0.22% as well as having the highest number of leaves (5.00±0.42) and having the longest leaf length (92.2±0.03%) followed by treatment 1.0 mg/L BAP+0.5 mg/L NAA (96.40±0.05%) and 2.0 mg/L BAP+0.5 mg/L NAA (92.20±0.04%) (Table 3). The protocorm development was observed for six weeks of culture. The effect of various combinations or concentrations of BAP was found to be more effective in boosting the elongation of shoots as a previous study done by Shatnawi (2013) which highlighted the *Vanilla planifolia*. Application of auxin to microshoots is stated to intensify the number of adventitious roots. The role of NAA in stimulating root formation of *Dendrobium* sp. was highlighted in a previous study (Hajong *et al*, 2013).

Hormones NAA and BAP had successfully formed healthy plantlets and reached the maximum or highest response towards seed germination and shoot formation. The protocorms formed responded variably when cultured on different concentrations of PGR after six weeks of culture. First shoot formation was formed in half strength ($\frac{1}{2}$) MS media supplemented with PGR media of 1.5 mg/L BAP + 0.5 mg/L NAA. This result has been proven and supported by similar research done by Novak *et al.* (2014) in which a lower ratio of cytokinins (BAP): NAA promoted protocorm-like bodies (PLBs) formation from seedling nodal explant, but a higher ratio induced shoot formation of *Spathoglottis*. After nine weeks of culture, PLBs were present in $\frac{1}{2}$ MS media supplemented with 1.5 mg/L NAA+ 0.5 mg/L BAP which has the fastest response of shoot formation (Figure 2). Similarly, studies conducted by Parthibban *et al.* (2015) revealed that NAA with $\frac{1}{2}$ MS medium at any concentration able to produce multiple shoots and roots of *Dendrobium aqueum*. Also, in other species such as *Dendrobium longicornu*, NAA was reported to produce multiple numbers of shoots and stimulate seedlings growth (Dohling *et al.*, 2012; Parvin *et al.*, 2009).

Shoot formation of C. pulverula

The growth of protocorm was better in half strength MS media compared to full strength MS media. As a result, five-week old protocorm formed shoots in half strength MS media supplemented with 1.5 mg/L BAP + 0.5 mg/L NAA (Figure 2; G-I) whereas in full strength MS media, there was no obvious growth can be seen. Eventually, after five weeks of protocorm formation, shoots had formed in three types of PGR media with different concentration of NAA and BAP: 1.0 mg/L BAP+0.5 mg/L NAA, 1.5 mg/L BAP+0.5 mg/L NAA and 2.0 mg/L BAP+0.5 mg/L NAA, each supplemented with half strength MS media. In a previous research done by Nongdam and Tikendara (2014), it took five to six weeks for protocorm formation. Green protocorm indicates that the protocorm grew healthily which was due to the presence of magnesium and potassium that stimulated the development of chlorophyll (Birhalawati *et al.*, 2014).



Figure 2. Growth and development of shoot in PGR media treated with different concentrations of PGR. (A-C) Shoot formation from ½ MS media supplemented with 2.0 mg/L BAP+0.5 mg/L NAA (D-F) Shoot formation from PGR 1.0 mg/L BAP+0.5 mg/L NAA (G-I) Shoot formation from PGR 1.5 mg/L BAP+0.5 mg/L NAA. Scale bars = 1 mm

Conclusion

The *in vitro* propagation protocol has been established in this study. It can be concluded that half strength MS media was the best media for seed germination and growth development. The highest rate of germination and shoot development were observed in plant growth regulators media 1.5 mg/L BAP+0.5 mg/L NAA with 100% germination; roots were also formed from this media. Thus, *in vitro* propagation is the best method for plant regeneration and to produce healthy plantlets of any orchid species in large-scale.

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Molecular Phylogeny of *Renanthera bella* J.J Wood using Internal Transcribe Spacer (ITS)

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Abstract

Renanthera bella is one of the unique species that are endemic in Sabah, Malaysia. This species now is classified in Appendix I as endangered in Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The aim of the study is to identify the phylogenetic relationship of *R. bella* from 3 different locality which were Greenhouse, Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah (IBTP) (06°03'33" N, 116°12'29"E, elevation: 110 m), Poring Conservation Orchid Centre, Ranau (Poring) (06°02'20.7"N, 116°06'38.2"E, elevation: 540 m) and Pekan Nabawan (PN) (05°03'93"N, 116° 43' 72"E, elevation: 1200 m). The phylogeny of *R. bella* from three different geographical regions reconstructed based on the analysis of ITS in 53 taxa and analyzed using maximum parsimony analysis. The phylogenetic analysis suggests that *R. bella* from IBTP and *R. bella* from Poring are poorly supported with 60% bootstrap value may due to uncertainty of nodes or short sequence. Furthermore, *R. bella* from PN were form well supported monophyletic group with *R. bella* from IBTP and Poring. The little ITS divergence exists within the sequence of *R. bella* from PN compared to *R. Bella* from IBTP and Poring clade. Phylogenetic patterns revealed by this study imply 60% close relationship between the clade of *R. bella* from IBTP and Poring. The sequence of ITS region obtained is the initial step to build of the molecular database of *R. bella*. Shared pattern among the three locations showed *R. bella* from PN form sister taxa with *R. bella* from IBTP and Poring with 62% bootstrap value and this contributed better understanding on phylogenetic relationship of *R. bella*. Furthermore, the ITS sequence is informative enough to infer the phylogenetic relationship of *Renanthera* genus and further research are needed to clarify their taxonomic status.

Keywords: Phylogenetics; Internal Transcribe Spacer; *Renanthera bella*; amplification; maximum parsimony

Introduction

Orchidaceae represent the large and diverse of flowering plants with covering of 800 genera and 26000 species (Govaerts *et al.*, 2014) and can be found all over the world. They are particularly diverse in every habitat except in the coldest and driest regions (Seidenfaden and Wood, 1992). *R. bella* is one of the unique species of orchid that only endemic in Sabah, Malaysia and they had distribution within locality of Gunung Kinabalu to Lahad Datu district. This species now has been classified in Appendix I as endangered in Convention on

International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Chan *et al.*, 1994). *R. bella* had stunning flower that give an attraction not only to the insect but also to the group of orchid collectors which made them become popular as ornamental plant and lead to the over collection to occur. Besides that, the habitat destruction by human development also causes them to facing with high risk of extinction.

The *Renanthera* genus having immense floricultural potentials displaying deeply coloured and also had long lasting flowers (Rajkumar and Sharma, 2010). Many of *Renanthera* genus are not easy to identify and recognised without their flowers. This causes difficulties in identifying them in monitoring the orchid trade and in conservation activities. Basically, the orchid species has been identified based on morphological and cytological characteristics. However, these methods are limited by environmental effects and diagnostic resolution. Molecular markers may be a feasible method to distinguish the related plants (Kress *et al.* 2005; Hossain *et al.* 2013). In order to build the molecular database for the conservation and molecular identification, we have been investigating genetic diversity of this genus from different locality.

The internal transcribed spacer (ITS) region of 18S-26S nuclear ribosomal DNA has been popularly used in plant systematic to reconstruct phylogenetic relationships at different taxonomic levels (Baldwin, 1992; Sun *et al.*, 1994; Markos, 2001; Kocyan *et al.* 2004; Yang and Pak, 2006). The ITS region is claimed to be useful for low-level phylogenetic analysis, such as infra-generic level, due to its relatively fast rate of evolution (Bayer *et al.* 1996 and Tsai *et al.* 2006). On the other hand, ITS is one of the most extensively used molecular markers for angiosperm phylogenetic inference and genetic relatedness and is a proven useful source of character for phylogenetic studies in many orchids (Sun *et al.* 2011; Takamiya *et al.* 2011 and Parveen *et al.* 2012). Thus, internal transcribe spacer region are widely used around the world and they were universal marker that popularly used in plant phylogenetic inference and genetic relatedness and is proven as useful sources of characters for phylogenetic studies in orchids (Trung *et al.*, 2013). Specifically, ITS sequences have shown as molecular evidence to evaluate the phylogeny of several taxonomic groups, e.g. in the studies of the subfamily Cypridioideae (Cox *et al.* 1997; Chochai *et al.* 2012) and genus *Paphiopedilum* (Rodrigues *et al.*, 2009). The aim of the present study was to investigate genetic variability and relationship among three *Renanthera bella* species from different locality by sequencing the internal transcribed spacer (ITS) region.

Materials and methods

Plant Materials

Fresh leaves sample of *Renanthera bella* were obtained from three different location in Sabah which is Greenhouse, Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Poring Orchid Centre, Ranau and also Pekan Nabawan. Figure 1 show the map of sampling location of *R. bella*.

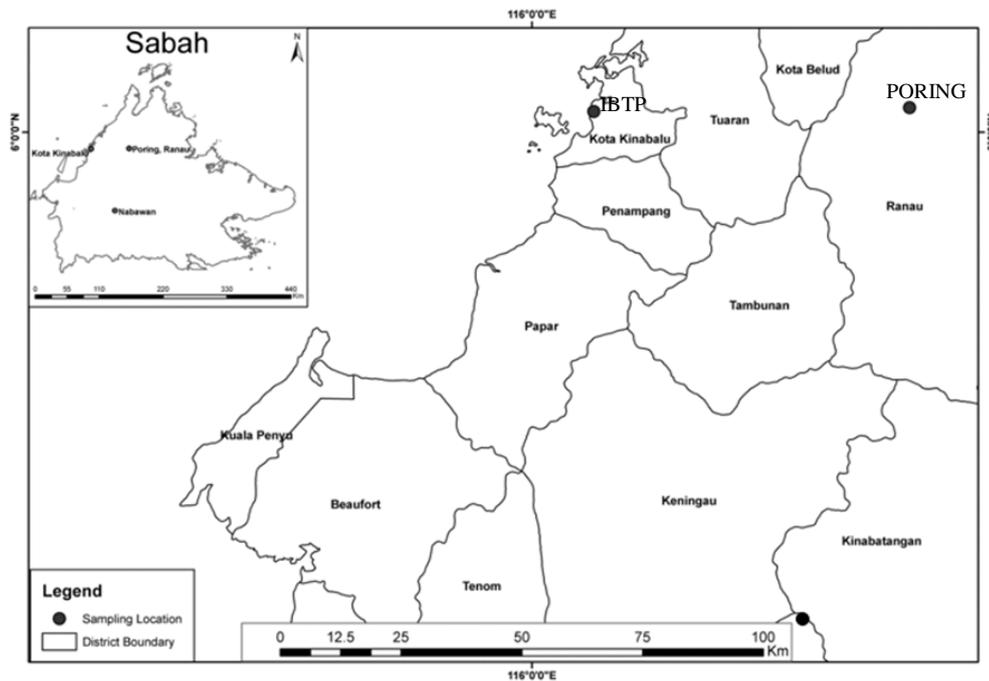


Figure 1. Map of sampling location

Genomic DNA Isolation

Genomic DNA of *R. bella* was extracted from fresh leaf tissue by using Wizard Genomic DNA extraction kit (Promega, USA). The extracted DNA was multiply by polymerase chain reaction (PCR) amplification of ITS 1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS 4 (5' TCCTCCGTCTATTGATATGC 3') intergenic spacer as described by (Cox *et al*, 1997). The existence of genomic DNA was examined in electrophoresis gel and visualized under UV light using a Vilber Lourmat UV scanner.

PCR Amplification

The internal transcribed spacers were amplified from total genomic DNA by using PCR amplification. The reaction was performed using PCR kit provided by Promega Corporation with 25 μ L total volume for each PCR reaction. Each of the PCR reaction contained 12.5 μ L of GoTaq Green Master Mix (2X), 0.5 μ L upstream primer (ITS1) (10 μ M), 0.5 μ L downstream primer (ITS4) (10 μ M), 2 μ L DNA template and also 9.5 μ L of Nuclease-free Water. The amplification of ITS region was carried out using Bio-Rad T100TM thermal cycler with thermal profile of primary denaturation for 1 minutes 30 second for 94°C, followed by 32 cycles of 45 seconds at 94°C, 30 seconds for 57°C and followed by final extension of 72°C at 2 minutes.

PCR Product Purification, Cloning and Sequencing

PCR products were checked on 1% TBE agarose gel with 1 Kb DNA ladders. The PCR product are stained using GelRed and visualized under Vilber Lourmat UV scanner (UK) to observe the existence band. The positive PCR products included two upstream and

downstream primers (ITS 1 and ITS 4) were send for purified and sequenced to MyTACG Sdn. Bhd (Kuala Lumpur, Malaysia)

Data Analysis

The sequences obtain were aligned using BioEdit Sequence Alignment Editor version 7.0.5. The maximum parsimony analysis was selected for construction of phylogenetic tree. The sequence output was analyze using MEGA version 7 to draw the phylogenetic tree. The consistency index (CI), homoplasy index (HI), retention index (RI) were calculated with most parsimonous tree.

Result and Discussion

Sequence alignment

The DNA sequences were compared and aligned using Bioedit version 7.2.1 and MEGA 4 software version 7.0.26 and further verified by comparing with the sequences of other species by BLAST search in the National Center for Biotechnology Information (NCBI database, <http://www.ncbi.nlm.nih.gov/blast.cgi>). The BLAST analysis was shown in Table 1.

There were many studies indicated that the variation in nucleotide sequences among the highly conserved regions or coding sequences might be a valuable source for genetic diversity analysis and molecular phylogeny study. The BLAST algorithm calculated the similarity of scores for alignment between the query sequence and subject sequences using scoring matrices and give the best matches of database. The BLAST taxa are selected based on the identity and query cover value. DNA sequence BLAST database shows that the ITS sequence identifies as *Renanthera matutina* (AY912263.1) with highest identity value which is 99% and 90% query cover.

Moreover, *Renanthera imschootiana* (DQ091710.1, KJ733442.1) and *Renanthera citrina* (KJ733440.1) matches the sequence with identity of 96%. Besides that, the result show the same species with different accession number such as *Vandopsis lissochiloides* (DQ091689.1, AY217597.1, AY912267.1 and EF670377.1), *Esmeralda Clarkei* (DQ091711.1 and MG820621.1), *Vandopsis undulata* (JN114732.1, JN114731.1, MG786550.1 and KJ733463.1) and *Rhynchostylis retusa* (JN114709.1, JN114711.1, JN114708.1 and EU558933.1) with identity of 96% respectively. The same species had different accession number because they obtain in different locality and it give specific information for record of DNA sequence.

After the default alignment in ClustalW and manual alignment, lengths of aligned sequences varied from 20 bp to 750 bp inclusive of internal gaps. Phylogenetic methodology is depending on the assumption of the characters that used to generate the tree are homologous. Figure 2 show the alignment of nucleotide sequence of *R. bella* with other sequence. This study showed the molecular tree generated based on multiple sequence alignment which were

edited manually since the automatic alignment may fail to correctly identify the conservation region within a gene. The 720 bp region between base pairs 20 and 750 of ITS gene sequence was used for the phylogenetic analysis since there are some species that have short base pair sequence length.

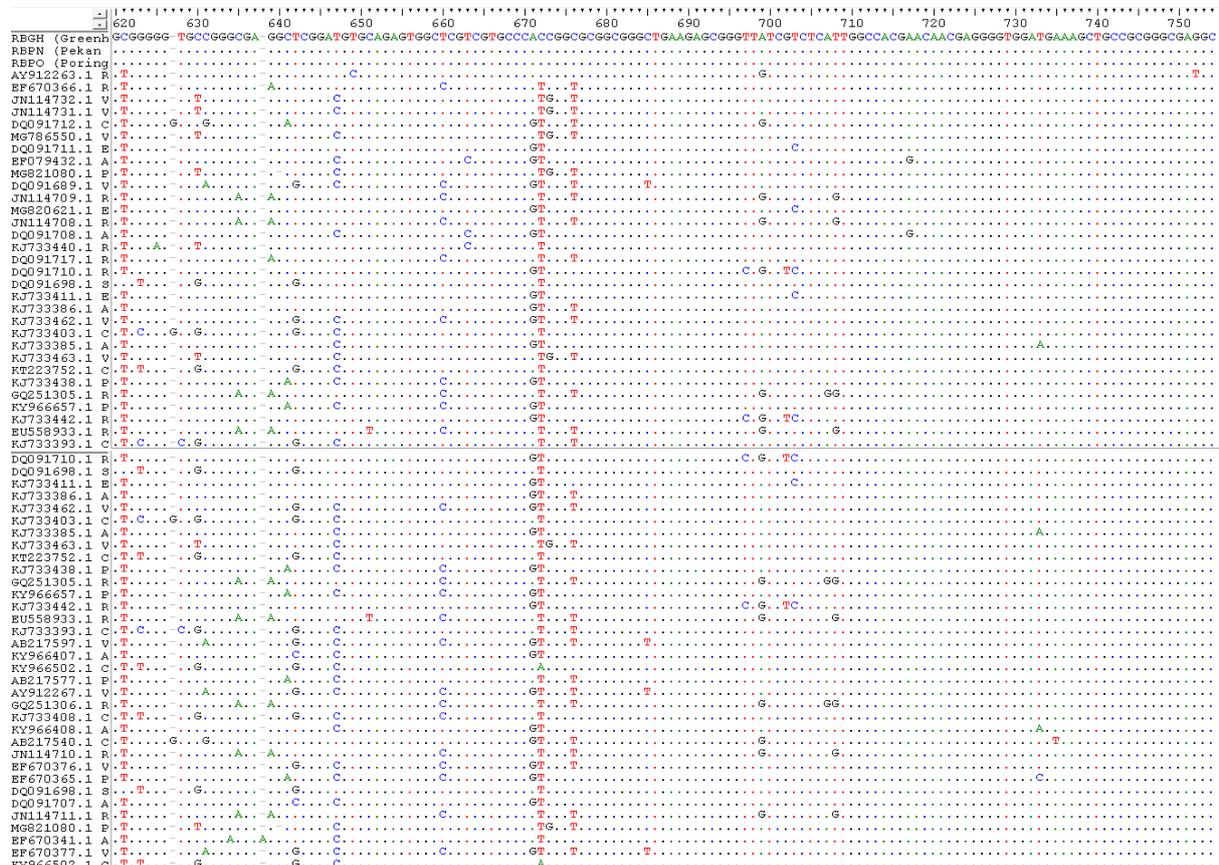


Figure 2. Alignment of ITS nucleotide sequence

Analysis of Phylogenetic tree

The analysis used was based on character based-method which is maximum parsimony. Maximum parsimony method takes the information and use it to recreate the series of nucleotide change that can result a pattern of variation revealed by multiple alignments (Brown, 2002) and mostly used to infer the evolutionary history (Moudi and Go, 2016). Furthermore, the parsimony principle states that the simplest explanation that explain the greatest number of observation is preferred over more complex explanation (Kannan and Wheeler, 2012). Other than that, it also help in minimize the number of changes on phylogenetic tree by assigning character states to interior nodes on the tree (Yang and Rannala, 2012). Fifty three taxa was chosen from blast analysis of *R.bella* sequence in all three different geographical region. The consistency index is 0.581152, the retention index is 0.872000 and the composite index is 0.582539 for all sites and parsimony informative sites and there were total of 601 positions in final dataset.

Figure 3 showed one single parmonious tree of *R. bella* from Internal Transcribed Spacer (ITS) sequence data. The construction of maximum parsimony showed *R. Bella* from Pekan Nabawan were form well supported monophyletic group with *R. Bella* from IBTP and Poring. There is a little divergence exist within the sequence of *R. Bella* from Pekan Nabawan compares to *R. bella* from IBTP and Poring clade. Furthermore, the constructed phylogenetic analysis suggests that *R. bella* from IBTP and Poring form supported with 69% bootstrap value.

Shared pattern among three localities of *R. Bella* from Pekan Nabawan forming sister taxa with *R. bella* from IBTP and Poring with 70% bootstrap value. *R. matutina* (AY912263.1) form a basal root to *R. bella* from IBTP greenhouse and *R. bella* from Poring with bootstrap support of 71%. Moreover, *R. bella* from IBTP greenhouse and *R. bella* from Poring form a relationship as sister taxa with 69% bootstrap value. Other than that, they also form a lineage with *R. bella* from Pekan Nabawan with 70% bootstrap value. Furthermore, *R. imschootiana* (DQ091710.1 and KJ733442.1) form sister taxa supported by 55% bootstrap value and it form a lineage with *R. citrina* (KJ733440.1) with 80% bootstrap value. So, from the findings it is confirm that the *R. bella* from three localities were from the same species. But, *R. matutina* (AY912263.1) were excluded from the clade because it found in different locality and widespread from Thailand, Sumatra, Java and also Malaysia (Tsai *et al*, 2006). Other than that, it can be seen that *R. imschootiana* (DQ091710.1 and KJ733442.1) and *R. citrina* (KJ733440.1) exist in different clade because they shared certain same trait with *Aerides* clade (Zou *et al*, 2015).

Moreover, *Vandopsis gigantea* (KJ733462.1) form a basal root to *Vandopsis lissochiloides* (DQ091689.1, AY217597.1, AY912267.1 and EF670377.1) with bootstrap support value of 60%. Two species of *Ceratochilus biglandulodus* (DQ091712.1 and AB217540.1) form sister taxa with bootstrap value of 54%. Meanwhile *Rhychostylis gigantea* (EF670366.1 and DQ091717.1) form sister taxa with bootstrap value of 58% and they form lineage to *Vandopsis undulata* (KJ733453.1, MG786550, JN114732.1 and JN114731.1) and *Papilionanthe vandarum* (MG821080.1) with 76% bootstrap value. Other than that, *Renanthera citrina* (KJ733440.1) strongly supported with 80% bootstrap value and form lineage to *Renanthera imschootiana* (DQ091710.1 and KJ733442.1) with 55% bootstrap value. Furthermore, *Micropera pallida* (AB217562.1) form a basal root to *Acampe papillosa* (DQ091708.1) and *Acampe sp. Szlachetko* (EF079432.1) with bootstrap support of 99% while *Acampe papillosa* (DQ091708.1) and *Acampe sp. Szlachetko* (EF079432.1) form relationship of sister taxa with 61% bootstrap value. ITS sequence data may continue to provide insights into phylogenetic history and genomic relationship and data generated will have most value if issue such as those raised are done experimentally (Wissemann, 2002).

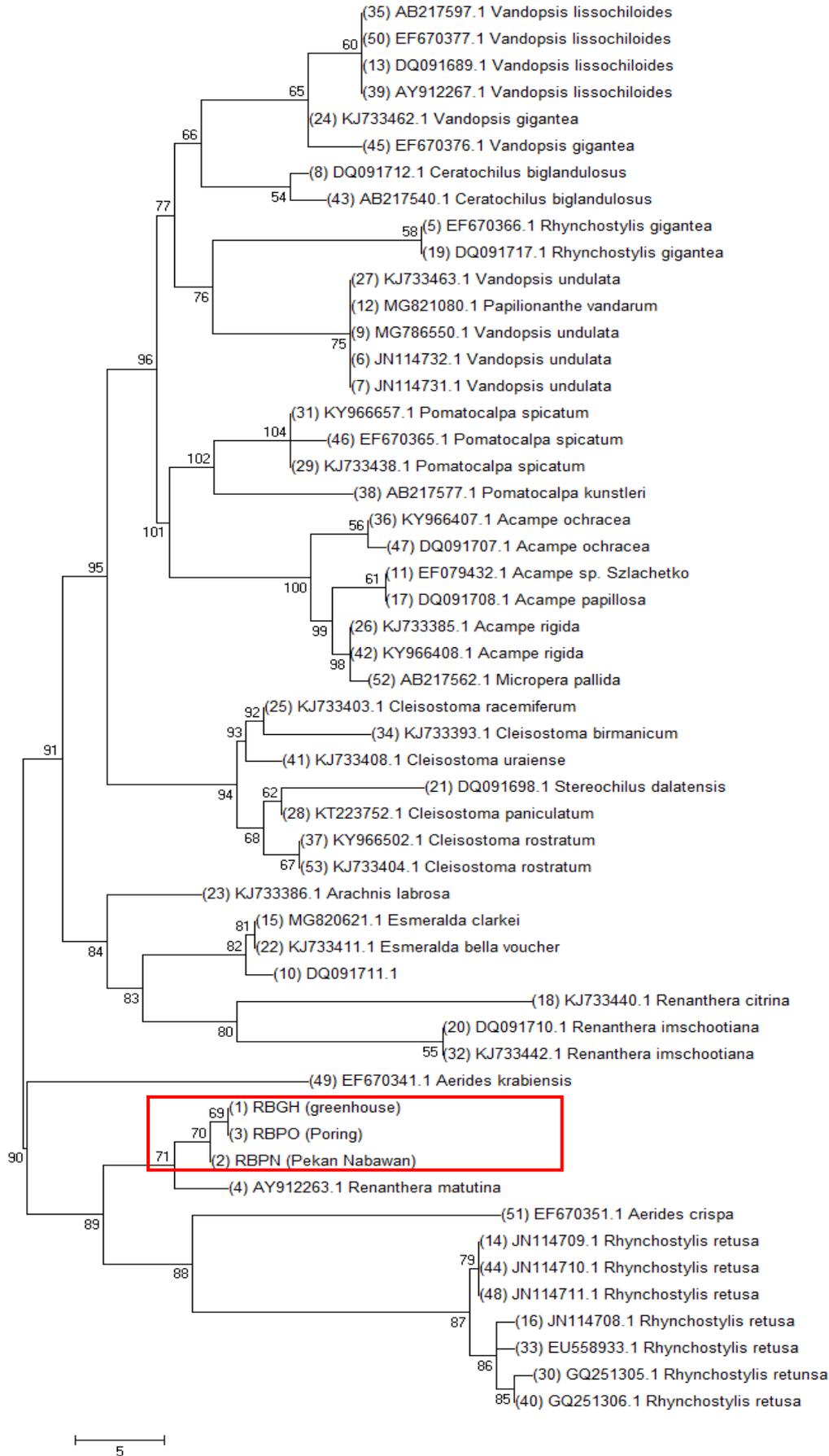


Figure 3. Maximum parsimony tree of *R. bella* from ITS sequence

Renanthera genus had lack of identification based on ITS rDNA sequence data and they have significantly low level of nucleotide variation between species that closely related species and the presence of small amounts of variation between different accession of the same species (Rajkumar and Sharma, 2010). Optimal tree recovered from maximum parsimony analysis produce suitable results and similar overall topology.

Conclusion

The ITS region is the possible marker to evaluate the relationships of *Renanthera bella* species from different area. However, this paper has only established the first steps in constructing a molecular database for future assessment of inter- and intraspecific molecular diversity of *Renanthera* species from Sabah. Our subsequent studies to find specific markers to fill the need of a molecular tool in identifying the varieties of *R. bella* species from different locality that are difficult to be determined without morphology characteristics such as flowers.

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Distribution and adaptability of *Neptunia oleracea* Lour in Sarawak

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Abstract

The invasion and fast growth of aquatic macrophyte, *Neptunia oleracea* Lour. caused problems in rice fields and drainage systems. However, they are also marketed and consumed as leafy vegetables among local urbanites mainly in the central region of Sarawak, Malaysia. This aquatic weed are harvested from the wild, and there had been no attempt to cultivate them. Hence, this research evaluated its distribution and ecology, i.e., habitats and adaptability. This information can be used for further observational and detailed studies on growth development and yield patterns. *Neptunia oleracea* were growing in the ditches of Kg. Medong, Kg. Kekan and Kg. Penipah in Mukah District. The plants showed two life forms as adaption to the environments, i.e., terrestrial (at Kg Medong and Kg Kekan) and floating (at Kg Penipah). However, the terrestrial life form is less common. The terrestrial plants had woodier stem than the floating plants that covered by thick white aerenchyma tissues formed when stem is in contact with water within 6 to 8 days. It grew densely in the ditch which had comparatively high concentrations of dissolved NO_2^- , NO_3^- and NH_3^- and total N and available Na in the substrate, e.g., at Kg Penipah.

Introduction

Aquatic plants occur in permanently or seasonally wet environments and they include a diverse group of macrophytic, aquatic plants, including angiosperms, ferns, mosses, and liverworts, and some freshwater macroalgae (Lacoul and Freedman, 2006). They are noticeable plants dominating diverse natural and man-made wetlands from small ditches, ponds, irrigation canals, sewage lagoons, streams, rivers, water reservoirs, shallow lakes, and marshes to swamps (Muta Harah *et al.*, 2005; Suzalina Akma, 2008). These diverse plant groups can be separated into four categories based on their habit of growth, i.e., floating unattached or attached, submersed, and emergent (Cronk and Fennessy, 2001). Based on their behavior, colonization characteristic and effects to the natural and man-made water bodies, they are often considered as noxious plants or weeds. Despite being noxious aquatic plants, a number of studies (Nather Khan, 1990; Lim *et al.*, 1998; Muta Harah *et al.*, 2005; Suzalina Akma, 2008) have reported several of them as being useful. A study conducted by Muta Harah *et al.* (2005) in Sarawak, East Malaysia recorded 43 species belonging to 29 families and of which 19 species are used as vegetables. Aquatic plants, e.g., water mimosa, *Neptunia*

oleracea Lour. grow in profusion in wetland habitats although considered weeds are gathered from the wild by the local people (Saupi *et al.*, 2009).

Neptunia oleracea is distributed in warm, slow-moving and frequently stagnant fresh waters in Central and South America (Windler, 1966; Forno *et al.*, 2000), Southern India (Shah and James, 1968; Sharma *et al.*, 1984); Burkina Faso and Central Africa (Wittig, 2005); North Central Namibia (Burke, 2000); West tropical Africa (Windler, 1966; Forno *et al.*, 2000); Madagascar (Sculthorpe, 1967); Southern Taiwan (Chou *et al.*, 2007); tropical region of Australia (Forno *et al.*, 2000); Thailand (Edwards, 1980; Sripen *et al.*, 1991; Paisooksantivatana, 1994; Suppadit *et al.*, 2005); Singapore (Holtum and Ivan, 2002); West Malaysia (Ridley, 1967; Henderson, 1974; Kamarudin and Latiff, 2002).

Neptunia oleracea is normally found in constructed and natural wetlands such as ponds, garden pools, swamps, canals, irrigation schemes, reservoir, ditches and rivers (Mashhor, 1988; Sripen *et al.*, 1991; Burke, 2000; Holtum and Ivan, 2002; Peter *et al.*, 1991; Paisooksantivatana, 1994; Forno *et al.*, 2000; Kamarudin and Latiff, 2002; Samy *et al.*, 2005).

In Sarawak, East Malaysia, the distribution of *N. oleracea* were recorded in Miri and Bintulu Districts and the environmental factors gathered only focusing on the parameters such as day time water temperature, salinity and water pH (Muta Harah *et al.*, 2005; Suzalina Akma, 2008). For that reason, the present study was undertaken to evaluate the distribution of *N. oleracea* in central region of Sarawak (Mukah, Bintulu, Sibuan and Sarikei District), to collect detailed environmental factors and habitat characteristics including water and soil chemistry parameters such as concentration of nitrate, phosphate, total organic carbon and available macro and micro nutrients.

Materials and Methods

Distribution and habitat characteristics of Neptunia oleracea

Field surveys were carried out in the central region of Sarawak from August 2008 until December 2011 covering three sites of *N. oleracea*. The cover of these species was determined by employing three randomized quadrat, i.e., 100 cm x 100 cm subdivided into 25 cm x 25 cm *N. oleracea* in the subquadrats were recorded and counted (Saito and Atobe, 1970). The drainage width and water depth were also measured. Temperature and pH in relation to *N. oleracea* were recorded in three readings using a pH meter (Mettler Toledo® SevenGo). Type of water flow, i.e., stagnant and slow moving and area, i.e., residential and agricultural system were also recorded. The occurrences of other aquatic macrophytes were also recorded and identification of the plants was done following the taxonomic references of aquatic weeds by Kostermans *et al.* (1987), Lim *et al.* (1998) and Samy *et al.* (2005).

Water and soil sample collection

The water and soil chemical status were determined at the three sites of *N. oleracea* i.e., Kg Medong, Kg Kekan and Kg Penipah, Mukah. Five hundred milliliter of water from these sites were collected in the bottle and placed in an ice chest before being transported to the laboratory to determine the concentration of ortho-phosphate, ammonia, nitrate and nitrite. Three replications of water sample were collected from each site. 2 kg of soil samples were collected from rooting depth (up to 30 cm) of *N. oleracea* using plastic auger following the method by Abhilash *et al.* (2008). The samples were then placed in labeled plastic bags for transportation back to the laboratory to determine the concentration of total organic nitrogen and carbon, available macro and micro nutrients. Three replications of soil sample were also collected from each site.

*Chemical variables in habitats of *Neptunia oleracea**

In the same three sites as mentioned above, water samples were collected and in the laboratory they were filtered through Whatman filter paper No.1, Whatman glass microfiber filter GF/C and Whatman cellulose nitrate membrane filter 0.45 μm by using Rocker® 600 filter vacuum set following the method of EPA (2009). The absorbance of nitrate (NO_3^-), nitrite (NO_2^-), ammonia (NH_3^-), and orthophosphate (PO_4^{3-}) content of water samples were analyzed by using a portable spectrophotometer-DR/2400 with a 2.5 cm cell following Hach DR/2400 Spectrophotometer Procedure Manual 2002.

*Substrates of *Neptunia oleracea* habitats*

The analysis of soil pH followed the procedures of Gupta (2004). Total organic nitrogen (N) in the substrates was determined by using semi-micro Kjeldahl described in Bremner (1965) and total organic carbon (TOC) was determined from a modified procedure by Chefetz *et al.* (1996) based on the loss weight on ignition at 550°C for 8 hours in furnace. Sample preparation of availability of cations in the substrates were determined by using ammonium acetate method described in Schollenberger and Simon (1945). Determination of macro and micro cations were following the method 975.03 of AOAC (1990) for K, Na, Mg, Ca, Cu and Zn, while available P was determined colorimetrically using UV-VIS (Lambda 25 Perkin Elmer, Germany) spectrophotometer as described in Murphy and Ridley (1967).

Statistical analysis

Analysis of variance (ANOVA, $p \leq 0.05$) and LSD ($p \leq 0.05$) were used to compare water and soil chemistry between growing sites of *N. oleracea* including water pH, nitrate, nitrite, orthophosphate, ammonia, soil pH, total organic carbon and nitrogen, available K, Na, P, Mg, Ca, Cu and Zn. Statistical analysis was conducted by using Statistical Analysis System (SAS) version 9.2 for Windows 2008.

Results and Discussion

Distribution and habitat characteristics of Neptunia oleracea

Neptunia oleracea is distributed only in Mukah District of Sarawak in three locations which are Kg Medong, Kg Kekan and Kg Penipah (Figure 1). The information on the location and longitude positions, habitat characteristic, associated plants and environmental parameters in different sites of *N. oleracea* are given in Table 1. The water temperature during the day at sites ranged from 27.82 to 32.28°C and water pH ranged from 5.63 to 6.52 (Table 1). *Neptunia oleracea* was found associated with other floating aquatic e.g., *I. aquatica*, *E. crassipes*; emergent e.g., *M. vaginalis*, *L. adscendens*, *C. malaccencis* and terrestrial plants e.g., *I. muticum*. The plant grew floating in water channel of Kg Penipah, Kg Kekan and Kg Medong. It can also adapt to dry terrestrial condition and creeping on the roadside at Kg Kekan and Kg Medong. Plants were exposed to the sunlight in open area, floating on water surface with branching stems. The plant found floating in water channel was comparatively larger in leaf size and the stem was tender and stout. The plant growing in terrestrial environment was smaller in leaf size and the stem was woody (Figure 2). This species generally can be found in man-made wetlands such as rice fields, ponds and constructed dams. According to the Windler (1966), this species inhabit warm, slow-moving and frequently stagnant water and thrive well in effluent shrimp pond where the pH in alkaline with range of 7.80 - 8.10 (Suppadit *et al.*, 2005).

Chemical variables in Neptunia oleracea habitats

Based on concentration criteria as reported by Boyd (2000), *N. oleracea* grew well in eutrophication water systems mainly those from residential drainage system. The water pH varied with sites (Table 2, ANOVA, LSD, $p \leq 0.05$). The present study showed *N. oleracea* grew in acidic condition (pH 5.63 – 6.52) while in Thailand this species grew in alkaline (pH 7.70 – 8.10) shrimp farming effluent (Suppadit *et al.*, 2005) and also marsh areas (pH 7.00 – 8.00) of Keoladeo National Park, India (Prusty *et al.*, 2007).

Additionally, *N. oleracea* thrived in the water with high NO_3^- and with PO_4^{3-} , NH_3^- and NO_2^- and with variable the concentrations (Table 2, ANOVA, LSD, $p \leq 0.05$). The content of NO_3^- in *N. oleracea* habitats is comparatively higher than recorded in other aquatic plants habitats and this might be due to the nitrification of root nodules in *N. oleracea*. Kg Penipah ditch was recorded having the most concentration of NO_3^- , NO_2^- and NH_3^- among the sites. The mean PO_4^{3-} varied among sites. In wetlands of Keolado National Park, this species inhabited area with more concentration of PO_4^{3-} up to 3.50 ppm (Prusty *et al.*, 2007).



Figure 1. The habitat of *N. oleracea* in Mukah, Sarawak. (a) The plant growing in the ditch bank of Kg Medong. (b) The plant floating on the water surface of Kg Medong ditch. (c) The plant can adapt to terrestrial condition and creeping on the roadside of Kg Kekan and (d) the dense *N. oleracea* floating in the ditch of Kg Penipah.



Figure 2. *Neptunia oleracea* in Mukah. (a) The woody stem with small leaves of terrestrial plant at Kg Kekan and (b) fleshy stem covered by spongy layer and big size leaves of plant at Kg. Penipah.

Table 1. Location, latitude and longitude, and environmental parameters of *N. oleracea* in central region of Sarawak. *Different alphabets indicate significant differences at $p \leq 0.05$ (ANOVA, LSD), i.e., $a > b > c$. All values are given as mean \pm s.e., $n=5$ and value in parenthesis is the range.

Location	Latitude N and Longitude E	Environmental condition	Form and association	Species percentage cover (%)	Depth range (m)	Width range (m)	*Water Temp. (°C)	*Water pH
1. Kg Medong, Mukah	N 02° 41.205' E 111° 56.122'	Floating in slow moving peat water residential ditch and also creeping on the ditch bank.	<i>Ipomoea aquatica</i> , <i>Eichhornia crassipes</i> , <i>Monochoria vaginalis</i> and <i>Ischaemum</i> <i>muticum</i> .	60.00	0.1 - 0.5	1.5 - 2.0	32.28 \pm 0.18 ^a (31.60 - 32.60)	6.00 \pm 0.02 ^b (5.95 - 6.08)
2. Kg Kekan, Mukah	N 02° 42.398' E 111° 56.963'	Floating in slow moving peat water residential ditch and also creeping on the ditch bank.	<i>Mimosa pudica</i> , <i>Cyperus malaccensis</i> and <i>I. muticum</i> .	50.63	0.1 - 0.5	1.0 - 1.5	27.82 \pm 0.14 ^b (27.60 - 28.20)	6.52 \pm 0.07 ^c (6.32 - 6.73)
3. Kg Penipah, Mukah	N 02° 57.213' E 112° 14.145'	Floating in stagnant peat water roadside ditch and also creeping on ditch bank.	<i>C. malaccensis</i> , <i>Ludwigia adscendens</i> and <i>I. muticum</i> .	93.75	0.1 - 0.5	0.1 - 0.5	29.02 \pm 0.12 ^c (28.80 - 28.90)	5.63 \pm 0.04 ^a (5.47 - 5.74)

Table 2. Concentration of NO_2^- , NO_3^- , NH_3^- and PO_4^{3-} (given in mean \pm s.e) in water of freshwater macrophyte habitats. Different alphabets indicate significance differences of pH and water nutrients in *N. oleracea* habitat at $p \leq 0.05$ (ANOVA, LSD) i.e., $a > b > c$, $n=3$.

Freshwater aquatic macrophytes	Location	pH	NO_3^- (ppm)	NO_2^- (ppm)	NH_3^- (ppm)	PO_4^{3-} (ppm)	References
<i>Neptunia oleracea</i>	Kg Medong, Mukah	6.00 \pm 0.02 ^b	0.6600 \pm 0.0678 ^b	0.0046 \pm 0.0002 ^b	0.0860 \pm 0.0025 ^b	0.2000 \pm 0.0063 ^a	Present study
	Kg Kekan, Mukah	6.52 \pm 0.07 ^a	0.4200 \pm 0.0583 ^c	0.0032 \pm 0.0002 ^c	0.0520 \pm 0.0058 ^c	0.0840 \pm 0.0081 ^c	Present study
	Kg Penipah, Mukah	5.63 \pm 0.04 ^c	1.7400 \pm 0.0872 ^a	0.0106 \pm 0.0002 ^a	0.2800 \pm 0.0032 ^a	0.1480 \pm 0.0102 ^b	Present study
	Pichet Farm, Prachinburi Province, Thailand	7.70 - 8.10	0.01- 1.03	-	-	-	Suppadit <i>et al.</i> (2005)
	Keoladeo National Park, Bharatpur, India	7.00 - 8.80	-	-	-	0.10 - 3.50	Prusty <i>et al.</i> (2007)

Table 3. Comparison of the different chemical variables (given in mean \pm s.e) in substrates across sites of freshwater macrophytes among sites. Different alphabets indicate significance differences of TON, TOC, C:N and available nutrients in *N. oleracea* habitats at $p \leq 0.05$ (ANOVA, LSD) i.e., $a > b > c$, $n=3$. *** total nutrient (ppm).

Freshwater aquatic macrophyte	Location	pH	TON (%)	TOC (%)	C:N	Available nutrient (ppm)								References
						P	K	Na	Mg	Ca	Mn	Zn	Cu	
<i>Neptunia oleracea</i>	Kg Medong, Mukah	5.38 \pm 0.04 ^b	0.0813 \pm 0.0129 ^b	0.0565 \pm 0.0026 ^b	0.74 \pm 0.08 ^a	224.77 \pm 10.69 ^a	88.60 \pm 3.84 ^a	48.46 \pm 1.24 ^b	48.16 \pm 1.74 ^a	54.38 \pm 0.83 ^b	0.38 \pm 0.03 ^b	0.17 \pm 0.04 ^b	0.26 \pm 0.24 ^a	Present study
	Kg Kekan, Mukah	3.64 \pm 0.02 ^c	0.1358 \pm 0.0019 ^a	0.0940 \pm 0.0004 ^a	0.69 \pm 0.01 ^a	78.78 \pm 0.23 ^b	31.91 \pm 1.68 ^b	29.49 \pm 1.17 ^c	34.63 \pm 0.59 ^b	83.64 \pm 5.66 ^a	0.86 \pm 0.12 ^a	0.44 \pm 0.02 ^a	0.00 ^b	Present study
	Kg Penipah, Mukah**	5.69 \pm 0.08 ^a	0.1483 \pm 0.0129 ^a	0.0121 \pm 0.0002 ^c	0.08 \pm 0.01 ^b	86.44 \pm 0.57 ^b	29.76 \pm 3.43 ^b	62.41 \pm 1.73 ^a	15.33 \pm 0.48 ^c	80.89 \pm 3.16 ^a	0.48 \pm 0.02 ^a	0.48 \pm 0.02 ^a	0.00 ^b	Present study

Substrate in Neptunia oleracea habitats

The root zone soil pH varied (Table 3, ANOVA, LSD, $p \leq 0.05$) across sites and Kg Kekan's substrate was the most acidic (pH 3.64). Both total organic carbon and nitrogen also varied among the sites. Total organic nitrogen in *N. oleracea* substrates was higher. This may be attributed to nitrogen fixing *Rhizobium leguminosarum* in *N. oleracea* root nodules (Suppadit *et al.*, 2005). The low value of C:N ratio in Kg Penipah indicated that high amount of dissolve nitrogen i.e., NO_3^- , NO_2^- and NH_3^- are released in water. The concentration of available K, Na, P, Mg, Ca, Zn and Cu content across sites were different. The trend of concentration also varied i.e., $\text{P} > \text{Ca} > \text{Na} > \text{Mg} > \text{Mn} > \text{Zn}$ (Kg Medong), $\text{Ca} > \text{P} > \text{Mg} > \text{K} > \text{Na} > \text{Mn} > \text{Zn}$ (Kg Kekan) and $\text{P} > \text{Ca} > \text{Na} > \text{K} > \text{Mg} > \text{Mn} > \text{Zn}$ (Kg Penipah). However, the total organic N and C in *N. oleracea*'s habitats are relatively lower than in the habitats of other freshwater macrophytes i.e., *Lunorium natans* and *Myriophyllum spicatum* (Table 3).

Conclusion

As for *N. oleracea*, the plant grew floating in water channel of Kg Penipah, Kg Kekan and Kg Medong in Mukah District. However, it can also adapt to dry terrestrial condition and creeping on the roadside at Kg Kekan and Kg Medong. The plant found floating in water channel was comparatively larger in leaf size and tender and stout stem. It grew well in acidic condition of Kg Penipah ditch with high concentration of dissolved NO_3^- , NH_3^- and NO_2^- . The ditch also has high in total organic N and available Na, Ca, Mn, and Zn. The content of N in *N. oleracea* habitats is comparatively higher and this might be due to the nitrification of root nodules of rhizo-bacteria.

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Soil macronutrients and abundance of soil bacteria in two rubber plantations and a forest area in the Chee watershed area

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Abstract

Rubber plantation has been increasing continuously over time in Northeastern region of Thailand. Expansion of rubber plantations in the Chee watershed area has been a land use transformation trend leading to losses of forest area cover in this region. This expansion could influence soil microorganisms which are the key factor of transformation of soil organic matter, nutrients and of most soil processes. Therefore, this study aimed to investigate the influence of soil macronutrient contents on abundance of soil bacteria under rubber plantation area compared to forest area. Some soil macronutrient contents i.e. total nitrogen, available phosphorus and exchangeable potassium were investigated under different management of rubber plantation area compared to forest area in the Chee watershed area along with pH, electrical conductivity, cation exchange capacity and organic matter content. The study areas included: 1) a rubber plantation area (13-year-old), 2) a rubber plantation area (13-year-old) with *Calopogonium caeruleum* and 3) a forest area. The results indicated that the highest total nitrogen, available phosphorus and exchangeable potassium contents were shown under forest area. The same trend reflected in the dataset of bacterial abundance. The highest soil bacterial abundance was found in forest area (5.41E+11 copies g⁻¹ soil dry weight) followed by the rubber plantation (4.2E+10 copies g⁻¹ soil dry weight) and the rubber plantation with *C. caeruleum* (3.69E+10 copies g⁻¹ soil dry weight). However, there was no significant difference of soil microbial abundance between the rubber plantation and the rubber plantation with *C. caeruleum*. Interestingly, the total nitrogen content was positively related to soil bacterial abundance. The findings of this study also suggested that soil macronutrients and bacterial abundance were responsive to land conversion from forest to rubber tree.

Keywords: forest, rubber tree, nitrogen content, bacterial abundance

Introduction

The rubber tree (*Hevea brasiliensis*) is an important economic crop in Thailand through the production of latex and wood. Nowadays, land conversion from forest to rubber plantations are rapidly extended to northeast Thailand. In 2018, suitable rubber plantations in northeast Thailand covered more than 0.6 million ha (Rubber Authority of Thailand, 2018). Several reports showed that land conversion can modify the soil microbial biomass, structure and

activities (Abraham and Chudek, 2008; Krashevskaya et al., 2015; Schneider et al., 2015). Moreover, various agricultural practices may also play vital role on soil microbial abundance, structure and activities. Among all microorganisms, bacteria are the most abundant and diverse group (Gans et al., 2005). They contribute to key functions in terrestrial ecosystems through mineralization, cycling of carbon and nitrogen, and the macronutrients turnover for plant growth (Chatterjee et al., 2008; Madsen, 2011; Nacke et al., 2011; Koranda et al., 2013). Therefore, concerns about the impact of land conversion under various agricultural practices are very important to understand. Thus, the purpose of this study was to investigate the influence of soil macronutrient contents on abundance of soil bacteria under rubber plantation area compared to forest area.

Materials and Methods

Study site and sampling

The experimental site is located in the Chee watershed area, Non Sang district, Nong Bua Lamphu province, Thailand (17° 0' 7'' N; 102° 39' 39'' E). This area is characterized by a tropical climate with a rainy season and dry season. The mean rainfall was 4.21 mm. The mean annual temperature was 27°C. The study areas included: 1) a rubber (clone RRIM 600) plantation area (13-year-old), 2) a rubber plantation area (13-year-old) intercropping with *Calopogonium caeruleum* and 3) a forest area. Each site was sampled once in dry season (February 2016). Soil samples were taken from randomly selected locations in each plot, at depths of 0-15 cm with four replications of each site.

Soil Physicochemical property analysis

Particle-size distribution and soil texture by hydrometer method. Chemical properties studied include soil reaction by pH meter method in ratio of 1:2.5 with water, electrical conductivity (EC) by EC meter, cation exchange capacity (CEC) by the 1.0 M ammonium acetate (NH₄OAC) method (Chapman, 1965), total nitrogen (Total N) by micro Kjeldahl method (Bremner, 1960), available phosphorus concentration by colorimetric method (Bray and Kurtz, 1945), exchangeable potassium level measured by flame photometer, organic matter in soil by wet oxidation method (Walkley and Black, 1934).

Soil bacterial abundance

Quantification of bacterial copy numbers in soils was performed by real-time polymerase chain reaction targeting (Real-Time PCR) the 16S rRNA gene using the primer pair Eub338 (5' CCTACGGGAGGCAGCAG) - Eub518 (5' ATTACCGCGGCTGCTGG) (Muyzer et al., 1993). Each reaction (20 µl) contained 5 ng DNA template, 10 µl of SYBR® green (Applied Biosystems, Foster City, CA, USA), 0.2 µl T4 gene 32 protein (500 ng µl⁻¹, MP Biomedicals), and 0.2 µM of each oligonucleotide. Cycling started with initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 secs, annealing at 55 °C for 35 sec, and polymerization at 72 °C for 45 sec.

Data analysis

Statistical analysis of the result was carried out by two-way analysis of variance (ANOVA) with least significant differences (LSD) for multiple comparisons among treatments using SPSS, a statistical program (SPSS, version 17.0). The Pearson correlation analysis was also conducted to study relationships between soil bacterial abundance and soil macronutrients.

Results and Discussion

Soil physicochemical analysis and bacterial abundance data

The data of physicochemical property of soil and bacterial abundance were shown in Table 1. Being very rich in organic matters in forest area creates organic fertilization in the soil which raises the pH of the soil and increases availability of soil macronutrients. The bacterial populations can change due to land conversion from forest to rubber plantation. The highest bacterial abundance was found in soil sample that taken from a forest area for 5.41×10^{11} copies/g soil dry weight and decrease of bacterial abundance was found in rubber plantations. The significant decrease of bacterial abundance may originate from a change in intensity of agricultural practices, which is a key driver of microbial activities in soil (Creamer et al., 2016). In addition, the bacterial abundance may be affected by changing in soil physicochemical properties and some certain soil nutrient such as nitrogen, which are recognised as the main drivers of the soil biodiversity (Birkhofer et al., 2015; Ruiz et al., 2011; Thomson et al., 2015). The results revealed that significantly higher in total N were found in forest soil when compared to rubber plantation area (Table 1). These findings suggested that land conversion under various agricultural practices had effects on bacterial abundance due to the changes in soil physicochemical properties such as total nitrogen content.

Table 1. Physicochemical property of soil and bacterial abundance

Treatment	1	2	3	SEM ¹
Soil Texture	Sand	Sand	Sand	-
Organic matter; OM (%)	0.81 ^b	0.70 ^b	3.26 ^a	0.37
Total N (%)	2.27 ^b	1.95 ^b	9.11 ^a	1.03
Available phosphorus (mg/kg)	2.47 ^a	2.14 ^a	4.75 ^a	2.14
Exchangeable potassium (mg/kg)	75.37 ^a	49.79 ^a	104.28 ^a	10.25
Soil pH	5.35 ^{ab}	4.87 ^b	5.90 ^a	0.15
Electrical conductivity (dS/m)	0.007 ^c	0.010 ^b	0.019 ^a	1.62
Cation exchange capacity (cmol/kg)	4.43 ^b	3.89 ^b	8.37 ^a	0.69
Soil bacterial abundance (copies/g soil dry weight)	4.20 x 10 ^{10b}	3.69 x 10 ^{10b}	5.41 x 10 ^{11a}	7.87 x 10 ¹⁰

Study area: 1) a rubber plantation area 2) a rubber plantation area with *C. caeruleum* 3) a forest area. Means values followed by different letters are significantly different (LSD test, $p < 0.05$). ¹ standard error of the mean

Relationships of soil bacterial abundance and soil macronutrients

The results showed that only total N was significantly positively correlated with soil bacterial abundance (Table 2). Available phosphorus was negatively correlated with soil bacterial

abundance but showed no significantly correlation. Our results were agreed with previous studies that bacteria exhibited significant responses to the nitrogen addition (Mueller et al., 2015). In addition, soil properties, including pH, soil organic matter and total N are all correlated with bacterial community structures and diversity (Smith et al., 2010; Lee et al., 2012; Van Horn et al., 2013).

Table 2. Correlation (Pearson correlation coefficients (r)) between soil bacterial abundance and soil macronutrients

Soil macronutrients	Soil bacterial abundance (copies/g soil dry weight)
Total nitrogen (%)	0.851**
Available phosphorus (mg/kg)	- 0.740
Exchangeable potassium (mg/kg)	0.435

Significance levels: ** ($p < 0.01$), ns ($p > 0.05$)

Conclusion

The study of soil macronutrients and abundance of soil bacteria in two rubber plantations and a forest area in the Chee watershed area has shown that the highest bacterial abundance was found in soil sample that taken from a forest area and then bacterial population tend to decrease when land use was changed from forest to rubber plantation. Moreover, a correlation proved that there was the influence of the soil total nitrogen on the bacterial abundance. This finding suggested that bacterial abundance was affected by land conversion and some certain soil macronutrient such as nitrogen.

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Horizontal and Vertical Emissions of Methane from Peat Soils

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Abstract

Methane emission depends on the rates of methane production, consumption and ability of soil and plants to transport the gas to the soil surface and also within soil particles. The objective of this study was to determine CH₄ fluxes horizontally and vertically from the floor and wall of the pit of a tropical peat soil. The horizontal emissions in the dry and wet seasons were 2.96 t CH₄ ha⁻¹yr⁻¹ and 4.27 t CH₄ ha⁻¹yr⁻¹, respectively and the vertical emissions were 0.36 t CH₄ ha⁻¹yr⁻¹ and 0.51 t CH₄ ha⁻¹yr⁻¹, respectively. The total amount of the horizontal and vertical emissions in the dry and wet seasons were 3.32 t CH₄ ha⁻¹yr⁻¹ and 4.78 t CH₄ ha⁻¹yr⁻¹, respectively. Horizontal emission was higher in the wet season due to an increase in the water table which resulted in an increase of CH₄ emission. Thus, there is a need for direct CH₄ measurement from cultivated peat soils to ensure that CH₄ emission is neither underestimated nor overestimated.

Keywords: Emissions; Horizontally; Vertically; Methane fluxes; Tropical peatlands.

Introduction

Globally, agriculture is considered to be responsible for about two-thirds of the anthropogenic sources of greenhouse gas emissions (Melling et al., 2005). Agriculture, forestry, and peat extraction for fuel and horticultural purposes are major causes of peatland disturbance because when peats are disturbed, the alteration in their hydrology results in the peat's oxidation which alters the greenhouse gases balance (Maria, 2008). Concerns on the role of peatlands as a main carbon sequester started because greenhouse gases (GHGs) emission contributes to global warming (Daud, 2009). Cultivation of different crops has varying impacts on the environment (Azqueta and Sotelsek, 2007). Currently, there is lack of information on soil CH₄ emissions horizontally and vertically from pineapple cultivation on drained tropical peatlands. The objective of this study was to quantify horizontal and vertical CH₄ emissions on a drained tropical peatlands cultivated with pineapple.

Materials and Methods

Site description

Malaysia Agricultural Research and Development Institute (MARDI) Peat Research Station Saratok, Sarawak, Malaysia has a total area of 387 hectares located on a logged-over forest. The von Post Scale of Humification shows that the peat soils are sapric peat (well decomposed peat) with humification (Ahmed and Liza, 2015). The mean temperature of the peat area ranges from 22.1 to 31.7 °C with relative humidity ranging from 61 to 98% (Ahmed and Liza, 2015). The annual mean rainfall of the area is 3749 mm during the wet season.

Greenhouse gases emission measurements

Methane emission measurements were carried out at 0-5 cm and 5-10 cm peat soil depths, respectively. Measurements of the CH₄ emission were carried out in 10 m x 10 m drained peat soil plots cultivated with pineapple. Methane flux sampling was carried out for 24 hours at every 6 hours interval (between 0600 hr to 0600 hr) and were monitored once throughout the dry (July 2015) and wet (December 2015) period.

Soil CH₄ horizontal emission measurements

The horizontal movement of CH₄ emissions from the peat soil surface was measured using the closed chamber method (Norman et al., 1997; Crill et al., 1988). The fabricated I-shaped chamber was gently pressed vertically on the surface of the soil pit at of 3-5 cm depth. The chamber was equilibrated for 30 minutes. The headspace samples of 20 mL were extracted from the chamber every minute for 6 minutes starting at 1 minute using a 50 mL syringe. The extracted gas was then transferred to 20 mL vacuum headspace vials using a disposable syringe needle. Methane concentration was measured using a Gas Chromatography (GC-Agilent 7890A) equipped with a thermal conductivity detector (TCD) (Ahmed and Liza, 2015).

Soil CH₄ vertical emission measurements

The vertical CH₄ movement was measured at 5 cm depth interval (0-5 cm, 5-10 cm), from the surface to 10 cm above water table (saturated zone). The L-shaped chamber was installed horizontally to the wall of the peat soil at 20 cm. The end of the chamber was covered and sealed with steel cap and parafilm, respectively. For each depth, peat soil was manually removed to a suitable working size pit. The open cylinder was left standing for approximately 30 minutes to establish an equilibrium state. Methane concentration was measured using the method described previously.

Methane flux calculation

The CH₄ results were based on the measurement of CH₄ from the five durations using two methods (I-chamber and L-chamber) in the dry and wet seasons. The values of CH₄ emitted were averaged and converted into units of t ha⁻¹ yr⁻¹. The CH₄ fluxes were then calculated using the following equation (Zulkefli et al., 2010; Widen and Lindroth, 2003; IAEA, 1992).

$$\text{Flux} = [(\text{CO}_2/\text{CH}_4) \text{ dt}] \times \text{PV ART}'$$

Where $(CO_2/CH_4)/(dt)$ is the evolution rate of CO_2/CH_4 within the chamber headspace at a given time after which the chamber were placed into the soil, P is the atmospheric pressure, V is the volume headspace gas within the chamber, A is the area of soil enclosed by the chamber, R is the gas constant, and T is the air temperature.

Statistical analysis

Analysis of variance (ANOVA) was used to detect treatment effects whereas treatments means were compared using Duncan's New Multiple Range Test (DNMRT) at $P \leq 0.05$. The statistical software used for this analysis was Statistical Analysis System (SAS) version 9.3.

Results and Discussion

Soil CH_4 horizontal emission

Methane emission was significantly affected by season (Figure 1). The seasonal variation in CH_4 flux was higher in the wet seasons due to rainfall which might have increased the water table of the peat soil. According to Farmer et al., (2011), during wet season, CH_4 is emitted in the form of bubbles which are transported by molecular diffusion through the aerobic layer of the peat. Soil CH_4 emission was not significantly different regardless of sampling period (Figure 1). Methane emission in the morning ($1.02 \text{ t ha}^{-1}\text{yr}^{-1}$) was the highest during the wet season followed by early morning ($0.99 \text{ t ha}^{-1}\text{yr}^{-1}$). The lowest CH_4 emission occurred at noon ($0.72 \text{ t ha}^{-1}\text{yr}^{-1}$). However, in the dry season, CH_4 emission in the early morning ($0.68 \text{ t ha}^{-1}\text{yr}^{-1}$) was highest in the dry season followed by at noon ($0.64 \text{ t ha}^{-1}\text{yr}^{-1}$). The lowest CH_4 emissions occurred in the morning ($0.51 \text{ t ha}^{-1}\text{yr}^{-1}$). The differences in the day and night temperature of the experimental site in the dry and wet seasons might have also inhibited the photosynthetic activity of the *Ananas comosus* (L.) Merr. plants. Furthermore, the root respiration and decomposition of the pineapple plants might have contributed to the emission of CH_4 in the wet season (Ahmed and Liza, 2015).

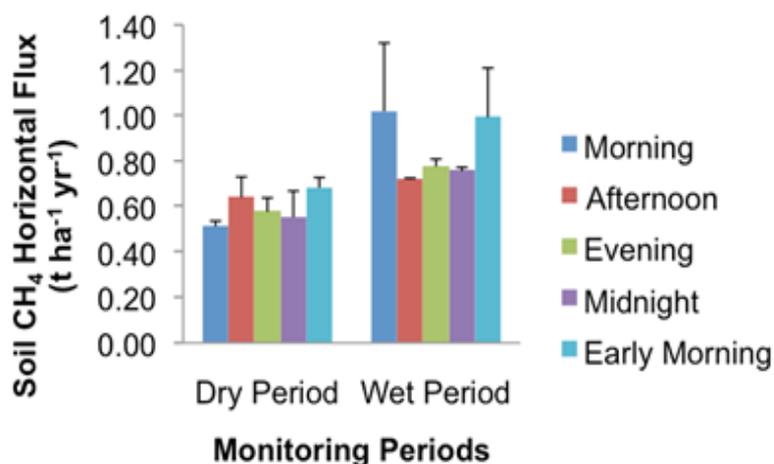


Figure 1. Horizontal emission of methane from peat soil cultivated with pineapple at different sampling periods (Error bars represent standard error).

In the dry and wet seasons, there was distinct seasonal variation in the vertical emission of CH_4 (Figure 2). Vertical emission of CH_4 was higher in the wet season due to the water table level of the peat soil. In the wet season, higher CH_4 emission occurred because of favourable conditions essential for methanogenesis which increases oxidation of CH_4 (Melling et al., 2005). The CH_4 emission is also related to the microbial population and the availability of adequate substrate for microbial metabolism, but not for plant root activities (Kechavarzi et al., 2010; Kuzyakov, 2006). According to Melling et al., (2005), a thick aerobic layer with higher temperature increases CH_4 oxidation thus, resulting in higher CH_4 uptake as shown in CH_4 emission during the wet season (Figure 2). The CH_4 emissions were statistically similar across all the five sampling periods however, there was higher CH_4 emission in the early morning ($2.73 \text{ t ha}^{-1}\text{yr}^{-1}$) during wet season followed by at noon ($2.59 \text{ t ha}^{-1}\text{yr}^{-1}$) and morning ($2.06 \text{ t ha}^{-1}\text{yr}^{-1}$) in that order. The CH_4 emitted regardless of time of sampling was not significantly different (Figure 2).

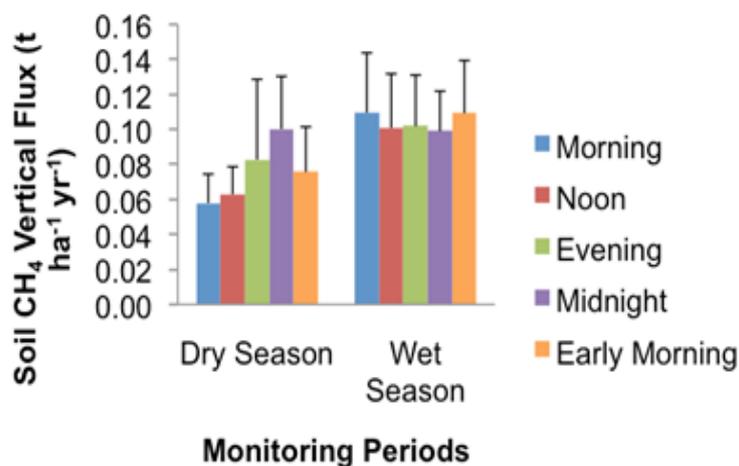


Figure 2. Vertical emission of methane from peat soil cultivated with pineapple at different sampling periods (Error bars represent standard error).

Conclusion

In tropical peat soils cultivated with pineapples, horizontal emission of CH_4 is higher than vertical emission. However, to avoid underestimation of CH_4 emission from pineapples cultivation on tropical peats, both horizontal and vertical emissions of this gas must be considered regardless of season.

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Seed priming of Okra (*Abelmoschus esculentus* L. Moench)

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Abstract

Seed priming is an efficient method for synchronizing germination and increasing seed vigor. The study was carried out to study the effect of various priming treatments to overcome germination hindrance of fresh or stored seeds of okra. There were five treatments with five replications. The experiment were laid out in a Completely Randomized Design (CRD). Seeds were germinated using between paper method. The treatments were T1: control; T2: soaking in water for 24 hours; T3: soaking in 80% H₂SO₄ solution for 3 minutes; T4: soaking in 60°C hot water for 40 minutes; and T5: soaking in 0.3% KNO₃ solution for 1 hour. Germination of okra seeds were carried out under alternating temperature regimes of 30/20°C with 8 hours light and 16 hours darkness. Analysis of the data revealed that accession and treatment significantly affected germination percentage of okra while the interaction of accession x treatment did not. T3 marked the highest germination percentage for both accessions. For germination index, the interactions of accession x treatment, main effect of both accession and treatment showed significant differences. Both accessions showed interesting different germination index results. The results obtained indicated that different accessions showed different trend in the germination rate of okra seeds.

Keywords: *Abelmoschus esculentus*; okra; priming; germination percentage; germination index.

Introduction

Germination is a critical stage in the life cycle of weed and crop plants. It often controls population dynamics with major practical implications (Keller and Kollmann, 1999). Seed priming is an efficient method for synchronization of germination and increasing seed vigor as well as allowing the growth of seedlings under stressful conditions (Bajehbaj, 2010). Heydecker and Coolbear (1977) noted that one of the main merits of priming treatments was to increase germination and emergence rate.

Okra (*Abelmoschus esculentus* L. Moench) of the family Malvaceae is a vegetable widely distributed in Southeast and South Asia, Tropical Africa and Brazil. It is a popular crop that is widely cultivated under tropical and subtropical conditions and in warm temperate regions. Young fruits are rich in mucilage and possess high antioxidative activities (AVRDC, 2009). The okra seed germination is reported to be impacted by genotype, position of pods on the

plant, time of pod harvest, seed moisture content and micronutrient applications (Purquerio *et al.*, 2010; El Balla *et al.*, 2011; Mohammadi *et al.*, 2012).

Hardness is one of the most important and commonly considered mechanical properties of seeds. Morris and Massa (2003) stated that hardness is one of the important factors in quality of seeds. Okra seeds show a particular kind of hardseededness and restricted permeability. Nada *et al.* (1994) noted that restricted permeability can be disadvantageous because the lack of simultaneous germination causing uninformed seeds germination. They germinate very slowly and unevenly although they are viable seeds. The germination percentage of certain okra accessions are relatively low, due to occurrence of hardseededness in this plant (Luis-Felipe *et al.*, 2010). Poor and delayed seed germination due to hardseededness is one of the major challenges.

There are a few priming methods that have been used to increase the rate of germination and improve the uniformity in emergence of problematic seeds. Hydropriming i.e. soaking in water (Afzal *et al.*, 2002), osmopriming i.e. soaking in a solution of osmoticum (Rouhi *et al.*, 2011), halopriming i.e. soaking in salt solution (Nawaz *et al.*, 2011) and solid matrix priming (SMP) i.e. priming using solid carriers (Khan, 1992) are most commonly used. Even though the mechanism behind the techniques of priming the seeds are still not fully understood, but certain physiological and beneficial changes have been observed that were associated with priming of seeds (Basra *et al.*, 2005; Hu *et al.*, 2006).

Objective

The aim of this experiment was to study the effect of various priming treatments to overcome germination hindrance of okra seeds.

Methodology

Seed Materials

The priming of seeds and subsequent germination test experiments were carried out from April to May, 2017, at Genetic Resources and Seed Unit, World Vegetable Center, Taiwan. Two different accessions of okra seeds VI046536 (from Thailand) and VI050958 (from Zambia) were used in this experiment. Both accessions were stored at 5°C for 30 months before the experiment.

Seed Priming Treatments

The priming treatments used in this experiment were as follow:

Treatment ID	Priming Method
T1	Control
T2	Soaking in water for 24 hours
T3	Soaking in 80% H ₂ SO ₄ solution for 3 minutes
T4	Soaking in 60°C hot water for 40 minutes
T5	Soaking in 0.3% KNO ₃ solution for 1 hour

Laboratory Germination Test

For germination test, 200 seeds were randomly taken from each treatment and between-paper (BP) method was used. Five replicates of 50 treated seeds were germinated between double layered rolled towel papers and moistened with distilled water. Treated okra seeds were placed in an incubator (model ST3, Saint Tien Co. Ltd.) at alternating temperature regimes of 30/20°C with 8 hours light and 16 hours darkness. The germination of seeds was monitored daily over a period of 21 days. Germination was considered to have occurred when the radicles reached 2mm long.



Figure 1. Rolled papers with seeds were placed into a tray filled with deionized water and placed in a germination chamber. The submerged level was lower than the last row of seeds in the rolled paper.

Data Collection And Analysis

All the paper rolls were arranged in Completely Randomized Design (CRD). Evaluation of germinated seeds was done according to ISTA Rules 2015. The final number of germinated seeds were collected as germination percentage (GP). Germination index (GI) was calculated as described in the Association of Official Seed Analysis (2002) as the following formula:

$$GI = \frac{\text{No.of germinated seeds at first count}}{\text{Days of first count}} + \dots + \frac{\text{No.of germinated seeds at final count}}{\text{Days of final count}}$$

Data obtained from this experiment was subjected to analysis of variance (ANOVA), using SAS software version 9.4. The means were compared using Duncan's Multiple Range Test (DMRT).

Results And Discussion

The main effect of both accessions ($F= 8040.78$, $p<0.001$) and priming treatments ($F=5.84$, $p<0.001$) showed highly significant differences in germination percentage, while no significant interaction among accessions x priming treatments ($F=1.12$, $p=0.36$). Figure 2 shows that soaking in 80% H_2SO_4 for 3 minutes (T3) resulted in the highest germination percentage for the both accessions, which $8.00\pm 1.41\%$ for VI046536 and $89.50\pm 4.56\%$ for VI050958. For germination index, all the interactions of accessions and priming treatments ($F=5.53$, $p=0.0012$), main effect of both accessions ($F= 8439.12$, $p<0.001$) and priming treatments ($F=4.94$, $p=0.0025$) showed significant differences. Both accessions showed interesting different germination index results. For accession VI046536, the germination

index ranged between 0.17 until 0.22 among all treatments, while the highest index marked under treatment of soaking in 80% H₂SO₄ solution as showed in Figure 3. These findings were similar to those reported by Pahla *et al.* (2014) who observed that H₂SO₄ promoted highest germination rate in *Acacia angustissima*. High concentration of H₂SO₄ allowed the removal of several lignified layers in seeds, which are packed tightly together and contained water repelling compounds (Baskin, 2003). These layers could act as a physical barrier for water absorption and gaseous exchange in seeds (Colling, 2009). Removal or reduction in thickness of the seed coat allowed the seed to take up water and respiratory gases thus the germination process could be initiated (Musara *et al.*, 2015). For accession VI050958, the germination index ranged between 9.29 until 10.62 where the highest germination index resulted when the seeds were primed by soaking in 60°C hot water for 40 minutes. Even though accession VI050958 produced the highest germination percentage in T3, it did not show the same trend in germination index. However, H₂SO₄ succeeded to promote the highest germination percentage and rate for accession VI046536. The results obtained indicated that different accessions showed different trend in the germination rate of okra seeds.

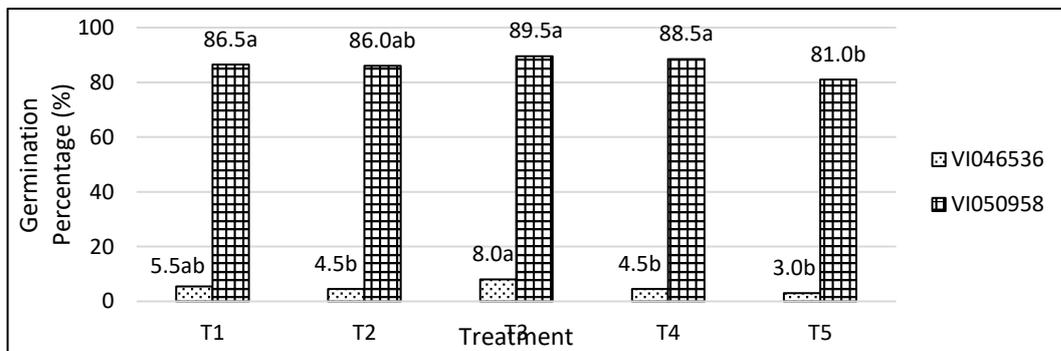


Figure 2. Effect of various priming treatments on germination percentage (GP) of okra seeds. *T1=Control; T2= Soaking in water for 24 hours; T3= Soaking in 80% H₂SO₄ solution for 3 minutes; T4= Soaking in 60°C hot water for 40 minutes; and T5=Soaking in 0.3% KNO₃ solution for 1 hour. *Mean values followed by same letter on bars are not significantly different at p=0.05 by Duncan’s Multiple Range Test.

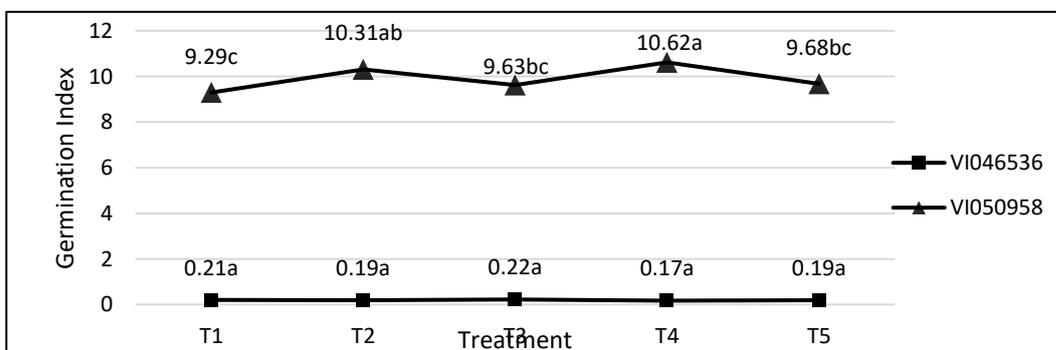


Figure 3. Effect of various priming treatments on germination index (GI) of okra seeds. *T1=Control; T2= Soaking in water for 24 hours; T3= Soaking in 80% H₂SO₄ solution for 3 minutes; T4= Soaking in 60°C hot water for 40 minutes; and T5=Soaking in 0.3% KNO₃ solution for 1 hour. *Mean values followed by same letter on bars are not significantly different at p=0.05 by Duncan’s Multiple Range Test.

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Effect of different types of organic materials and clones on survival of grafted cocoa (*Theobroma cacao* L.) seedlings in nursery

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Abstract

A study has been carried out to determine the effects of different sources of organic materials on the survival rates of cocoa grafted seedlings at the nursery. The treatments listed as: T1: - conventional fertilizer (5 gram / NPK Yellow 12:12:6:4) for cocoa nursery standard, T2: - Chicken manure (CHM), 1.8:1.6:4 (33 gram), T3 - Cocoa pod husk (CPH), 1.7:0.04:3.1 (35 gram), T4 - Cow manure (CM), 1.9:0.9:2.9 (31 gram) and T5 - Empty fruit bunch (EFB), 1.7:0.2:2.2 (35 gram) were applied once a month and grafted with two selected clones; C1 - PBC 123 and C2 - KKM 22. The design of the trial was Randomized Complete Block Design (RCBD) in three replicates with two factors studied; Fertilizers and Clones. From the result, it can be concluded that clone KKM 22 has the highest rate of survival in comparing with clone PBC 123, while all fertilizer treatments have given none effect on the success rate of survival of the grafted seedlings. Thus, it can be concluded that clone was correlated with success rate of survival of grafted seedling, whereas fertilizer treatment is merely on the growth and development of the grafted seedlings in the nursery.

Keywords: Cocoa, Fertilizer, Organic materials, Growth Development, Nursery

Introduction

Normally cocoa is planted as seedlings in the nursery. Seedlings are easy and cheap to raise, and they develop into trees with a uniform growth. In modern cocoa cultivation, the aim is to maximize early growth, obtain high early yields, and sustain peak yields subsequently. An essential ingredient in most cocoa growing situations is high fertilizer input. However, it is generally known that cocoa seedlings need great care as they are very sensitive to fertilizer toxicity. Therefore, organic materials as fertilizer would be an option.

Organic materials are naturally occurring materials of biological or mineral origin (Allen, 2010). Even though organic materials are low in nutrient content or solubility or both, but the slow release of nutrients makes the nutrient availability for a longer period. There are plenty of waste by-products (biomass and manure) available in plantation and livestock farming that can be used as a source of nutrients. The application of these organic materials is essentially important to supply various kinds of plant nutrients, including micronutrients, improve soil physical and chemical properties, nutrient holding and buffering capacity, and enhance microbial activities (Suzuki, 1997). Besides that, recent study by Si et. al. (2016) also stated that organic materials not only improved soil conditions, but also promoted the growth of plant seedlings in the nursery.

Vegetative propagation has an important role where it is necessary or desirable to produce trees true-to-type. Propagation through grafting and stem cutting methods were techniques used in propagating superior commercial clones (Wood and Lass (1985). However, grafted seedlings always have been used to supply planting materials from selected trees. The success of this method depends on several factors related to the plant and surrounding environmental conditions, include the toxicity of fertilizer that could affect the success rate of grafting seedling. Therefore, the objective of this study was to determine the effects of different sources of organic materials on the rates of survival of grafted cocoa seedlings in the nursery. The result from this study was expected to be positively contributing to the survival rate of grafted seedlings.

Materials and Methods

The trial was conducted at the Malaysian Cocoa Board Research and Development Centre nursery in Tawau, Sabah. The design for the trial was Randomized Complete Block Design (RCBD) with two (2) factors; Fertilizers and Clones on survival of grafted cocoa seedlings. The treatments were:

T1 – Conventional fertilizer (5 gram / NPK Yellow 12:12:6:4)

T2 – Chicken manure, 1.8:1.6:4 (33 gram),

T3 – Cocoa pod husk, 1.7:0.04:3.1 (35 gram),

T4 – Cow manure, 1.9:0.9:2.9 (31 gram) and

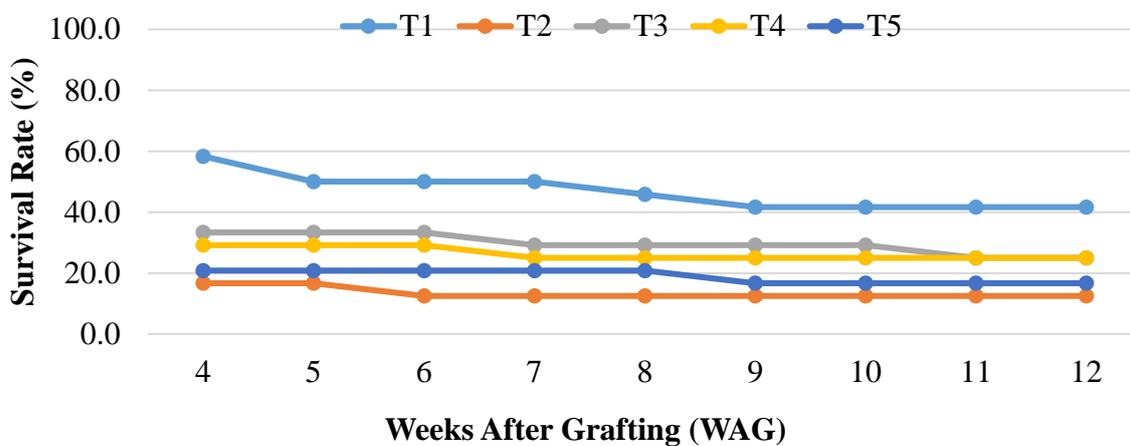
T5 – Empty fruit bunch, 1.7:0.2:2.2 (35 gram)

All seedlings were treated with composted organic materials and inorganic fertilizer when first leaf had hardened, then the application was postponed one (1) month before grafting, and resumed after bud eye has sprouted, for the next three months of study. This study used sixteen (16) of three (3) months old seedlings as rootstock and grafted with selected budwood from two (2) of Class I Clones; PBC 123 (C1) and KKM 22 (C2) in three (3) replicates, with a total of 240 seedlings. All seedlings were grafted according to the MCB (2013) method where the top of rootstock was cut at the middle stem, placed the scion and wrapped firmly the entire union and scion with parafilm or biodegradable plastic. This method is also known as whole-wrapped top grafting. Once the bud eye has sprouted and developed at the fourth week, observation on survival of the grafted seedling was recorded. At the end of study, observation data obtained from all treatments were analysed statistically using computer software package SPSS to determine the survival rate of the grafted seedling and its interaction effect.

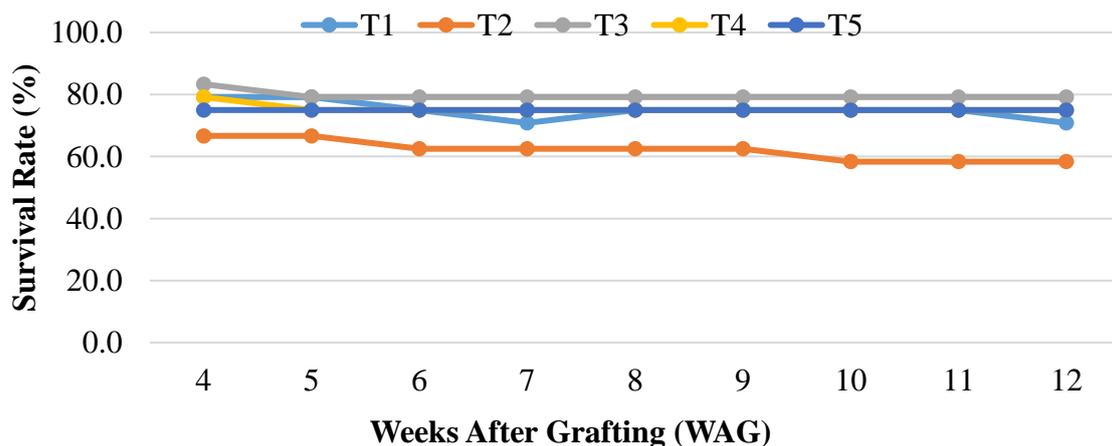
Results and Discussion

Effect of Treatments and Clones on Survival Rate of Grafted Seedlings

Observation completed at the twelfth (12) WAG had indicated that all treatments has a similar downward trend of survival rate on both clones. For clone PBC 123, the percentage of survival rate had decreased since the fourth (4) WAG (Figure 1 (a)), while for clone KKM 22 the percentage of survival were more consistent until the end week of study (Figure 1 (b)). The result for the percentage of survival for each treatment on both clones also suggested that there were no significant differences ($p>0.05$) between the treatments (Figure 2). The figure indicated that the percentage of survival rate of grafted seedlings for clone KKM 22 was higher compared to clone PBC 123 at the last week of observation, whereas the figure also shown that there were no significant differences between treatments on both clones.



(a)



(b)

Figure 1. The survival rate of grafted seedlings on (a) clone PBC 123 and (b) clone KKM 22 for twelve (12) WAG for all treatments

Note: T1 – NPK Yellow, T2 - Chicken Manure, T3 – Cocoa pod husk, T4 – Cow manure & T5 – Empty fruit bunch (EFB) (\pm S.D)

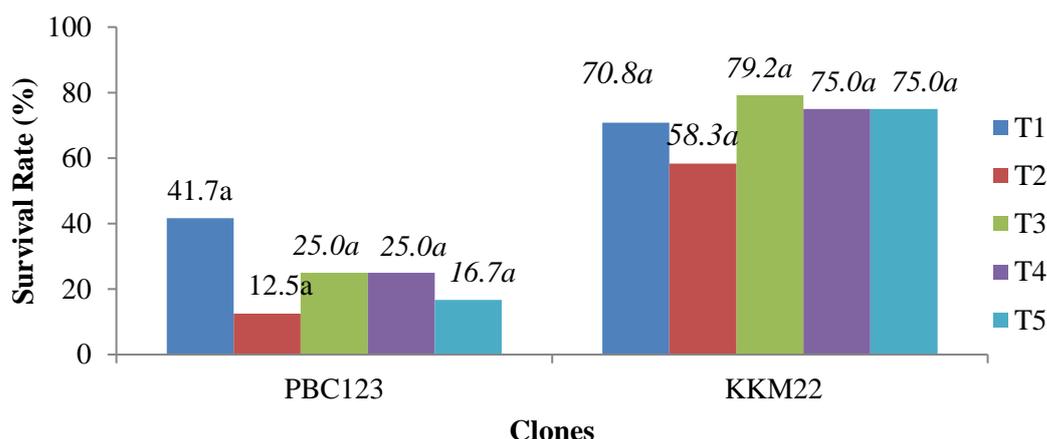


Figure 2. The survival rate of grafted seedlings of different clones at twelve (12) WAG for all treatments

Note: T1 – NPK Yellow, T2 - Chicken Manure, T3 – Cocoa pod husk, T4 – Cow manure & T5 – Empty fruit bunch (EFB) (\pm S.D)

Fertilizer particularly organic materials had been known to promote growth and development of plant seedlings (Murmu et al. (2013), but for this study result had shown that all treatment fertilizers had no effect on the survival rate of grafted seedlings. Despite that, there was a significant difference ($p < 0.05$) between clones that had been determined through a two-way ANOVA statistical analysis.

Table 1. Main and interaction effects of survival rate of grafted seedlings

	Source	df	F	Sig.	Partial Eta Squared
Survival rate (%)	A. Clone	1	42.195	.001*	.678
	B. Treatment	4	.942	.460 ns	.158
	A*B	4	.474	.754 ns	.087
	Error	20			

ns – not significant

* mean difference is significant at the .05 level

A two-way between-groups analysis of variance had been conducted to explore the impact of clone and treatment on survival rate of grafted seedlings (Table 1). The survival rate results indicated that there were significant difference ($p < 0.05$) in the effect of clone [$F(1,20)=42.19$, $p=.001$] on grafted seedlings, as the effect size had also been large (partial eta squared =.678) meaning that clone affected the survival rate of grafted seedlings by 67.8%. Nevertheless, there was no significant difference ($p > 0.05$) indicated in the main effect for treatments [$F(4,20)=.94$, $p=.46$] and the interaction effect [$F(4,20)=.47$, $p=.75$] on the survival rate. Again, results on the grafted seedlings showed that the application of all treatments had not affected the survival rate of grafted seedlings, but has affected on the survival rate by the clones.

Table 2. The Pearson Correlation between survival rate of grafted seedlings, fertilizers treatments and clones

	Survival rate	Treatment	Clone
Survival rate			
Treatment	-0.029		
Clone	0.789**	0.00	

** Correlation is significant at the 0.01 level (2-tailed).

The correlation analysis of survival rate, fertilizer treatments and clones indicated that Clone a has large correlation coefficient (0.789) to Survival Rate as stated in Table 2. It is significant at the 1% level of probability. However, there was no significant difference in the correlation between Survival Rate and Treatment (-0.029). Therefore, this study had clearly identified that treatments applied include organic and inorganic fertilizer had given no effect on the survival rate of cocoa seedlings, while clones might have influenced the success of grafted seedlings.

Conclusion

As for conclusion, clone KKM 22 has shown the highest rate of survival in comparing with clone PBC 123, while fertilizer treatment has given none effect on the success rate of survival of the grafted seedlings. Clone also was correlated with success rate of survival of grafted seedling, whereas fertilizer treatment is merely on the growth and development of the grafted seedlings in the nursery.

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Effect of early feed restriction and yeast supplementation on sensory quality of chicken breast meat

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Abstract

Sensory quality of breast meat from broiler chicken fed under diet supplemented with yeast and feed restriction was investigated. Parameters of sensory analysis were tenderness, juiciness, aroma, flavor, and overall acceptability. Three dietary treatments with three replicates were assigned completely randomized where eleven chicks were placed in each replicate. The treatments were: fed with commercial pellet diet (T1); commercial pellet mixed with 0.3% yeast (T2); commercial pellet mixed with 0.3% yeast with feed restriction at 7 to 14 days of age (T3). Thirty-five untrained respondents from Universiti Putra Malaysia Bintulu Sarawak Campus were asked to evaluate their impression based on the aroma, tenderness, juiciness, flavor, and overall acceptability of the sample using a nine-point Hedonic Scale. Present study found that four parameters such as tenderness, flavor, aroma, and overall acceptability of breast meat from broiler chicken fed 0.3% supplement with (T3) and without restriction (T2) were significantly ($p>0.05$) no different over normal commercial diet (T1). However, the difference can only be observed on juiciness aspect where the yeast supplement with and without feed restriction significantly ($p<0.05$) different over birds fed normal commercial diet. In conclusion, present study recommends that the supplementation of yeast and employing feed restriction in broiler chicken production have the potential to optimize feed consumption and may also reduce feeding cost without reducing sensory quality of broiler meat product.

Keywords: chicken meat; feed restriction; sensory quality; yeast.

Introduction

Baker's yeast (*Saccharomyces cerevisiae*) is extensively used in livestock as feed additive. Several digestive enzymes are also excreted by the yeast that help the gastrointestinal tract to boost the nutrient digestibility, growth rate and feed conversion ratio (Nawaz *et al.*, 2016). Recently, it has been reported that yeast has a probiotic effect in diets, and can become an alternative to antibiotic-based drugs in feeds of broiler chick (Morales-López *et al.*, 2010).

Periodic restriction of the daily feed offered to simulate compensatory growth is a mean to reduce the feed cost. Feed restriction during the growing period in broiler chicken lowers body weight and carcass fat, while improves feed efficiency with compensatory growth

during re-feeding (Jahanpour *et al.*, 2015). In addition, over-feeding often leads to a reduction in the quality of carcass and increase in fat deposition (Fernandes *et al.*, 2018). Therefore, early feed restriction can improve the carcass quality of chicken (Jahanpour *et al.*, 2015).

Meat quality products are those that meet some need or expectation from consumers and are safe. One of the main aspects that affecting the meat produce is the sensory quality. Several aspects in sensory evaluation are usually examined such as on aroma, tenderness, juiciness, flavor, and acceptability. The inclusion of certain new or modified ingredients in animal diets may change the nutrients and biochemical composition of meats concomitantly may affect its sensory characteristics (Chamorro *et al.*, 2015). Therefore, the present work aimed to examine the effect of early feed restriction and yeast supplementation on sensory quality of chicken breast meat.

Materials and Methods

From day 1 to 42, a total of 99 broiler chickens (Ross) were sheltered in cages and ready for experimentation. Broilers were randomly assigned into nine different divisions cages with dimension of each cage at 3m² and heated with 24 hours lighting (for seven days) by two-incandescent light bulb (60 watts) and the temperature at 32 °C. The starter (from day 1 to 21), grower (from 22 to 36) and finisher (day 37 to 42) basal diets of commercial pellet were given. The chicks were randomly divided into three groups of treatments (33 chicks/treatment). Each treatment had 3 replicates containing 11 broilers in each replicate.

The treatments were as follows and were laid out in completely randomized: Treatment 1 (control) – commercial pellets (*ad-libitum*) starting day 1 until day 42. Treatment 2 – commercial pellets (*ad-libitum*) with addition of 0.3% yeast (*Saccharomyces cerevisiae*; Mauri-pan[®] instant yeast) starting day 14 until day 42. Treatment 3 – commercial pellets 50% (feed restriction) with addition of 0.3% yeast (*Saccharomyces cerevisiae*) starting day 14 until day 42, and feeding was restricted from day 7 until day 14 at 50% reduction of total amount of normal diet rate. At the end of the experiment, two birds (at day-42) per replicate were randomly selected, slaughtered by severing the carotid artery and jugular veins (Halal method). The procedures were according to the guidelines set up by Department of Standard Malaysia (Malaysian Standard, MS1500: 2004). At slaughtering, fresh boneless and skinless chicken breast meat were obtained and cut into cubes and weighing about 10 grams each.

In sensory evaluation, breast chicken meat samples were cooked in a boiling water bath for 15 to 20 min or until the temperature reached 80°C. The samples were cut into small cubes, placed onto a white plate and allowed to rest for 2 min prior to tasting. A panel of thirty five untrained students from Universiti Putra Malaysia Campus Bintulu were asked to evaluate their impression in term of aroma, tenderness, juiciness, flavor and overall acceptability using a nine-point Hedonic Scale (Score 1= strong dislike; 5= neither like nor dislike; 9= strong like) (Peryam and Pilgrim, 1957). Panelists were required to rinse their mouth with water before tasting the next sample. The sample were coded and presented on a white plate and

the order of presentation for the different treatments were randomized. The mean scores for all the sensory attributes were calculated.

All data were subjected for analysis of variance (ANOVA). Duncan's test was performed to determine the differences among treatment means at 5% level of probability. Statistical Analysis Software (SAS) versions 9.4 was used to perform the data analysis.

Results and Discussion

Meat from broiler chicken after fed on diet supplemented with yeast and with restricted feeding were evaluated for sensory quality (Table 1). Present study found that the treatments were statistical different significantly ($p < 0.05$).

Table 1. Tenderness, juiciness, flavor, aroma, and overall acceptability of boiled meat from broiler chicken fed normal diet, diet with yeast and feed restriction

Sensory Parameters ^b	Score		
	T1 ^a	T2	T3
Tenderness	4.17 ± 0.20 ^b	6.21 ± 0.21 ^a	5.73 ± 0.19 ^a
Juiciness	4.33 ± 0.18 ^c	6.14 ± 0.20 ^a	5.37 ± 0.20 ^b
Flavor	5.11 ± 0.18 ^b	6.44 ± 0.17 ^a	6.00 ± 0.15 ^a
Aroma	5.37 ± 0.18 ^b	6.31 ± 0.13 ^a	6.03 ± 0.15 ^a
Overall acceptability	4.86 ± 0.25 ^b	6.43 ± 0.23 ^a	6.29 ± 0.24 ^a

Means in the same column with the different superscript (a-c) were statistically significant (Duncan's test, $p < 0.05$) by using ANOVA (Duncan Multiple Range).

^aTreatment 1 (T1) = Control (Commercial pellet); Treatment 2 (T2) = Commercial pellet + 0.3% yeast without feed restriction; Treatment 3 (T3) = Commercial pellet + 0.3% yeast with feed restriction.

^bTenderness, juiciness, flavor, aroma, and overall acceptability (6 = like slightly and 1 = dislike extremely)

Boiled meat sampled from broiler chicken fed on 0.3% yeast and feed restriction during day 7 till day 14 were significantly improved ($p < 0.05$) on all sensory parameters such as tenderness, juiciness, flavor, aroma, and overall acceptability. Between meat samples from yeast supplementation and feeding restriction, both were no different significantly ($p > 0.05$), except, on juiciness where meat sample from broiler chicken fed diet supplemented with yeast was better than meat sample from broiler chicken with feed restriction. The positive effect of supplementing yeast in diet as demonstrated in this study was in contrast with Perenlei *et al.* (2014) where compound rich in yeast was not altered flavor score of meat soup. Smaller or fewer amounts of yeast added in animal diet may be important to positively change the sensory quality of broiler meat. However, Kunal *et al.* (2017) reported that feed restriction could improve color, texture, juiciness, and overall acceptability of chicken meat which was consistent with this study.

Conclusion

Adding yeast at 0.3% in diet and employing restriction during animal feeding may improve sensory quality of meat product and consequently increasing consumer acceptance and consumption. Moreover, feed restriction in dietary scheme could possibly improving feeding cost efficiency.

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Effects of NPK fertilizer on growth and yield of several rice varieties grown in Sabah

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Abstract

Traditional rice varieties are believed to be less responsive with increasing level of NPK fertilizer which may cause lodging and result in low rice yield. A pot experiment was carried out to assess the influence of various levels of NPK fertilizer (F1: 60-30-30, F2: 90-30-60, and F3: 120-30-90 kg ha⁻¹) on the rice growth and yield of four Sabah's local rice varieties (V1: Tadong, V2: Filipin, V3: Serendah Merah and V4: TR8 (High yielding varieties)), arranged in a Completely Randomized Design (CRD) with three replications. Urea, triple super phosphate (TSP) and muriate of potash (MOP) were used as source of nitrogen, phosphorus and potassium, respectively. Our results revealed no significant effects between the interaction of NPK levels and different rice varieties on physical characteristics, lodging incidence characteristics and yield components of rice. Application of F3 significantly increase the grain yield (4.58 t ha⁻¹) and this in line with thousand grain weight value of 24.69 g. Serendah Merah variety (V3) recorded the heaviest thousand grain weight (29.98 g) which in turn having higher grain yield (4.39 t ha⁻¹). Tadong variety (V1) recorded larger culm diameter (0.58 cm) with shorter internode length (8.11 cm) compared to other traditional varieties. Sabah's local traditional varieties may achieve higher grain yield with least application of NPK fertilizer. Traditional rice variety also showed better results in resistance towards lodging incidence.

Keywords: Sabah rice varieties; high yielding variety; NPK; lodging; rice yield.

Introduction

Tadong, Filipin, Serendah Merah and TR8 are among the rice varieties widely grown in Sabah. Tadong, Filipin and Serendah Merah are local traditional rice varieties cultivated in Sabah especially by smallholders. Some local farmers prefer to cultivate traditional rice varieties due to their good grain characteristics and, also a better tasting. Moreover, traditional rice varieties have a high quality of rice seeds with scented aroma and less susceptible to pests and diseases such as fungus diseases, bacterial leaf blight and brown plant hopper.

TR8 (Seri Aman) is a high yielding rice variety (HYV) with good eating quality, highly resistance towards disease and has a potential of producing higher yield up to 7 t ha⁻¹ (Jabatan Pertanian Sabah, 2009). Furthermore, this variety is moderately resistance towards lodging incidence and has a maturity period of 130-135 days with fertilizer rate application of 170-80-150 kg ha⁻¹ of N, P₂O₅ and K₂O, respectively.

The high yielding variety is more responsive to fertilizer and has greater potential in producing higher yields compare with the traditional varieties. Therefore, the current practice of applying high rates of nitrogen fertilizer by Malaysian rice farmers in targeting high grain production with the use of HYV has reduced the cultivation of traditional varieties. When nitrogenous fertilizer is applied to traditional varieties, they will response to it with an increase in vegetative growth development. However, the tall stature of the traditional varieties may lead to lodging incidence of paddy plants due to the weakening of culm causing yield to decrease. Berry *et al.* (2004) stated that lodging may cause losses of yield by up to 80% and can cause severe knock-on effects, including reduced grain quality and greater drying cost. The improvement in the stem strength of rice plants will definitely reduce losses due to lodging. The application of potassium can increase the strength of stems in which higher potassium content in the internodes may prevent the plant from lodging-off.

Nitrogen is mostly needed during early and mid-tillering, panicle initiation, booting and ripening phases of grain development. According to Dobermann and Fairhurst (2000), the application of nitrogen fertilizer can increase the plant height, panicles number, spikelets number and number of filled spikelets which largely determine the yield capacity of rice.

Phosphorus plays an important role in root growth, promote early flowering and ripening stage and resistance towards disease and drought conditions. Deficiency of phosphorus may delay the maturity period of rice plant (Fageria, 1980) and increase susceptibility to rice disease (Fageria *et al.*, 2003).

Appropriate application of potassium is closely related to lignification of sclerenchyma cells, vascular bundles and culm strength which enhance lodging resistance (Kong *et al.*, 2014). Rice plants having potassium deficiency will commonly have high instances at disease infestation that may lead to lodging incidence. Thus, this study was carried out to assess the influence of various level of NPK fertilizer on rice growth and yield of Sabah's local varieties.

Materials and Methods

A pot experiment was carried out to assess the influence of various level of NPK fertilizers (F1: 60-30-30, F2: 90-30-60, and F3: 120-30-90 kg ha⁻¹) on rice growth and yield of four Sabah's local rice varieties (V1: Tadong, V2: Filipin, V3: Serendah Merah and V4: TR8 (HYV)) under the rain shelter condition at Faculty of Sustainable Agriculture, Universiti Malaysia Sabah. The experimental design used was Completely Randomized Design (CRD) with three replications.

The seeds used were pre-soaked in distilled water for 24 h before planted in the plastic nursery pots (50% Silabukan soil: 33% topsoil: 17% sand). The transplanting of rice seedlings into planting pots was carried out on 15 DAS in the nursery pots (Silabukan soil) and two hills of seedlings were planted in each pot with planting depth and distance of 2 cm and 20 cm, respectively. Cultural practices such as weeding, irrigation, pest control etc. were done as and

when necessary. Regular flood irrigation was applied in pots throughout the vegetative stages and left completely dried upon reaching the grain filling stages.

Single fertilizer namely urea, triple super phosphate (TSP) and muriate of potash (MOP) fertilizer were used as source of nitrogen, phosphorus and potassium, respectively. Split application of NPK fertilizer (Sariam, 2008) was applied based on the rice varieties growth phase, day after sowing (DAS) due to their different maturity period. Urea fertilizer was applied at split application of 20%, 31%, 35% and 14% during vegetative, active tillering, booting and heading phase, respectively. Meanwhile, TSP was applied at 100% during vegetative phase. MOP fertilizer was applied at split application of 35%, 33% and 32% during vegetative, active tillering and booting phase, respectively.

Parameters measured for physiological characteristics of rice were: (i) plant height (cm); (ii) tillers number; and (iii) percentage of productive tillers. Lodging incidence of rice crops were measured during the flowering stage; (i) culm diameter (cm); and (ii) internodes length (cm). Harvesting were carried out when 90% of the grains had turned hard, clear and free from greenish tint (Panda, 2010). The following yield components analysis were determined: (i) panicle number per hill; (ii) determination of grains per panicle was done by threshing the grains from the samples and unfilled grains were separated manually from filled grains; (iii) percentage of filled grains was calculated using a formula (filled grains per panicle/ total grains per panicle) x 100 (Yoshida *et al.*, 1976); and (iv) 1,000 grain weight (g). Grain yield ($t\ ha^{-1}$) was calculated using this equation proposed by Yoshida (1981):

$$Y = N * W * F * 10^{-5}$$

Where Y = grain yield ($t\ ha^{-1}$)

N = spikelet number m^{-2}

W = 1,000 grain weight (g) and

F = filled spikelet (%)

Data collected were statistically analysed using two-ways analysis of variance (ANOVA), and Duncan's new multiple range test (DMRT) was employed to determine the mean differences between the treatments using the statistical package, Statistical Analyses Software (SAS) v9.4.

Results and Discussion

The results revealed no interaction effects of NPK fertilizers and rice varieties on the physiological characteristics, lodging incidence characteristics and yield component. There were significant different observed on the plant height, tillers number, percentage of tillers number, culm diameter, internodes length, panicle number, percentage of filled grains and 1,000 grains weight of different rice varieties. Different levels of NPK exerted significant effect on culm diameter, internodes length, 1,000 grains weight and grain yield.

Filipin variety (V2) recorded the highest plant height (145.75 cm), tillers number (19) (Table 1) and percentage of filled grains (80.45%) (Table 2) compared to other traditional varieties. Most of the local varieties used, recorded higher plant height compared to HYV (TR8), and this finding is similar with Souki (2015), who stated that traditional varieties can reach about 120-160 cm in height. According to Jahan *et al.* (2014) and Hosain *et al.* (2008), varietal variation in plant height and tillers numbers may due to their genetic make-up. Similar findings reported by Roy *et al.* (2014), stated that rice varieties may varies in filled grains percentage due to their genetic make-up. Most of the traditional varieties recorded lower panicle numbers compared to HYV and this was similar to findings by Gagandeep *et al.* (2016), who stated the variation may due to their difference in genetic make-up.

Lodging incidence in rice plants is mainly influenced by weakening of culm, related with culm diameter and thickness and internodal length. Plants having larger culm diameter with shorter internodal length are less susceptible to lodging incidence. Tadong (V1) resulted in larger culm diameter (0.58 cm) with internodes length of 8.11 cm (Table 1). Serendah Merah variety (F3) produced the heaviest 1,000 grains weight (29.98 g) (Table 2).

From Table 1, NPK rate of 120:30:90 kg ha⁻¹ (F3) produced the largest culm diameter (0.51 cm) and shortest internodes length (8.90 cm). A larger culm diameter and shorter internode length increase proportionally with increasing level of NPK, which related to potassium elements, important in strengthening the culm of rice plant. Sufficient amounts of potassium in the stem helps in synthesizing high molecular weight compounds like cellulose which plays an importance role in strengthening the stem (Marschner, 2012). Thus, aids in preventing the lodging incidence in rice plant.

Table 1. Mean physiological characteristics of rice

Factors	Plant height (cm)	Tillers number	Productive tillers hill ⁻¹ (%)	Culm diameter (cm)	Length of internode (cm)
Rice varieties					
Tadong (V1)	127.19 ^b	15 ^b	85.78 ^a	0.58 ^a	8.11 ^c
Filipin (V2)	145.75 ^a	19 ^a	83.64 ^a	0.44 ^c	9.88 ^b
Serendah Merah (V3)	137.38 ^c	13 ^b	93.17 ^a	0.44 ^c	17.55 ^a
TR8 (HYV) (V4)	100.13 ^d	18 ^a	92.04 ^a	0.53 ^b	6.41 ^d
NPK rates (kg ha⁻¹)					
60:30:30 (F1)	126.02 ^a	16 ^a	86.02 ^a	0.48 ^b	11.39 ^a
90:30:60 (F2)	128.81 ^a	16 ^a	89.40 ^a	0.51 ^a	11.17 ^a
120:30:90 (F3)	128.02 ^a	16 ^a	90.56 ^a	0.51 ^{ab}	8.90 ^b

*means with different superscript in the same column differ significantly (P<0.05)

Table 2. Mean yield attributes of rice

Factors	Panicle number	% Filled grains	1,000-grain weight (g)	Grain yield (t ha ⁻¹)
Rice Varieties				
Tadong (V1)	12 ^b	76.51 ^{ab}	25.80 ^b	3.67 ^b
Filipin (V2)	16 ^a	80.45 ^a	18.78 ^d	4.23 ^{ab}
Serendah Merah (V3)	12 ^b	76.61 ^{ab}	29.98 ^a	4.39 ^a
TR8 (HYV) (V4)	17 ^a	71.47 ^b	20.22 ^c	4.02 ^{ab}
NPK rates (kg ha ⁻¹)				
60:30:30 (F1)	13 ^a	74.14 ^a	22.46 ^b	3.31 ^b
90:30:60 (F2)	14 ^a	76.18 ^a	23.93 ^a	4.35 ^a
120:30:90 (F3)	15 ^a	78.47 ^a	24.69 ^a	4.58 ^a

*means with different superscript in the same column differ significantly (P<0.05)

Application of F3 also recorded the heaviest 1,000 grains weight (24.69 g) which in line with grain yield value of 4.58 t ha⁻¹ (Table 2). Fageria (2009) stated that increase in 1,000-grain weight may due to increase in N-absorption by the plant and advanced photosynthetic rates. Large amounts of phosphorus derived mainly from that accumulated in leaves before the flowering stage begins may aid in increasing the 1,000 grains weight (Yoshida, 1981). The increase in NPK fertilizer rate may have increased the grain yields which may be due to the adequate supply of nutrients to the crops to produce higher panicle numbers associated with higher percentage of productive tillers. Further, the increase in NPK rate may aid in higher spikelet sterility thus contributing to higher rate of grain filling that may increase yields.

Conclusion

Some of the Sabah local traditional varieties have potential to be cultivated because they can contribute to higher rice yield production and their lodging incidence can be minimized by the application of NPK. Serendah Merah variety (V3) received with F1 had no significant different between NPK fertilizer applied, therefore it can be recommended to farmers. The amount of fertilizer used in treatment F1 (60:30:30 kg ha⁻¹) is the least, thus, it gives an economical advantage as low fertilizer cost is required to achieve higher yield and better grain quality. Moreover, this variety also have shorter maturity period of 100-110 DAS which may enable farmers to cultivate it twice a year, thus, Serendah Merah can be recommended to farmers. Meanwhile, Tadong variety (V1) gave the best results in vegetative growth related to lodging incidence characteristics compared to other local traditional varieties.

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The indirect toxicity of selected insecticides to the pollinator *Heterotrigona itama*

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Abstract

Stingless bees (Hymenoptera: Apidae) are being developed locally for their commercial, environmental, educational and eco-tourism values. In Malaysia, one of the most popular species being cultivated for its honey, propolis and pollinator services, is *Heterotrigona itama*. Stingless bees are great pollinators that pollinate valuable crops such as fruit trees, vegetables and ornamental plants. However, the application of insecticides to the field to suppress the insect pest population had indirectly affected the population of beneficial insects including stingless bees. The toxic effect of commercial insecticide had contributed to the shortage of pollinators which significantly result in a poor quality of the fruit set. Hence, a study of the toxicity was carried out toward *H. itama* at Agricultural Research Station Tenom, Sabah. A single colony of *H. itama* was used in the study of toxicity of commercial insecticides used. For bioassay tests (topical and residual), foragers bees were collected from the colony and tested on two selected insecticides for five different concentrations. Each concentration was replicated three times. The objective of this study is to identify the lethal concentration of 50% (LC₅₀) and 95% (LC₉₅) of Deltamethrin and Chlorpyrifos against stingless bee forager workers using contact and topical bioassay tests. The result showed that deltamethrin and chlorpyrifos were indicated harmful to *H. itama* and susceptibility varied significantly with the type of exposure both residual and topical.

Keywords: Stingless bee, Pollinator, Insecticide, Toxicity, Bioassay

Introduction

Stingless bee that lacking functional stings is the largest group of eusocial bees on earth. More than 500 species were identified and about 20 to 30 species can produce honey (Chuttong et al., 2016). In Malaysia, the meliponiculture was started in 2012 (Mohd Fahimee, et al., 2016). Meliponiculture is the cultivation of stingless bees on a commercial scale for honey production or pollination had gained popularity among the farmers. According to Mohd Fahimee and Rosliza (2016), Malaysian Agriculture Research and Development Institute (MARDI) had promoted two species of stingless bees namely *Heterotrigona itama* and *Geniotrigona thoracica* as an additional pollinating agent for many crops including cash crops and commodity crops. Pollinator such as *H. itama* was found in the area of crops which present nectar, resin, and pollen.

The application of insecticides to the field is to suppress the population of insect pests. The uncontrolled insect pest populations will affect the yield production and reduce the survival rate of the crops. The farmers commonly tend to use insecticide to manage insect pest, because it showed a faster result in controlling pest and easy to apply. Unfortunately, the toxic effect also affects the beneficial insects that result in decreasing population and crop yield quantity (Valdovinos-Núñez et al., 2009).

Although stingless bee is not the target of insecticide compounds, they may have foraged in the contaminated area and highly vulnerable to the contamination. Thus, the objective of this study was to identify the lethal concentration (LC₅₀) and LC₉₅ of deltamethrin and chlorpyrifos on *H. itama*.

Materials and Methods

Location

This study was conducted at the Agriculture Research Station (ARS) Laboratory, Tenom with a controlled room temperature of 27±2°C and 70% relative humidity.

Preparation of test insect

The *H. itama* colony was reared in ARS bee breeding yard before being examined in the laboratory. The colony hive was examined for the presence of diseases and pest during routine colony maintenance to ensure the tested stingless bees are free from diseases. Only adult forager workers (age between 26 – 70 days) were chosen for insecticide treatments.

Collection and inactivation of H.itama

Adult forager workers of stingless bees were collected from their hive during morning hour by using a modified sweeping net. The collected stingless bees were transported instantly to the laboratory. The stingless bees were anaesthetised by chilling for 40 seconds at 3 °C to facilitate easy handling. The chilling method was used with slight modifications as recommended by Thomas and Phadke (2017) and no chemical involved in anaesthetised process. They are temporarily inactive after being chilled for 40 seconds. During the inactive period of the stingless bee, only healthy, uninjured and uniform sizes were selected for the further experiment.

Insecticides

Two formulated insecticides were tested in this experiment. The selection of insecticide is based on the common insecticide used by the farmer in controlling agricultural pests. Insecticides that were assessed in this study were Deltamethrin (1.4% w/w) and Chlorpyrifos (21.2% w/w) as summarised in Table 1.

Table 1. Information of test insecticides

Common name	Active ingredient(s)	Class	Mode of action	Group of insecticide
Decis®	Deltamethrin	III	Contact and stomach	Pyrethroid
Agr Fipro® 21.2 EC	1.4% w/w Chlorpyrifos 21.2% w/w	II	Contact, stomach, and respiratory action	Organophosphate

Preparation of stock solution

The desired concentrations of insecticides as treatments in the bioassay were made from original stock solution. The selected insecticides were examined at five dose concentrations which prepared using distilled water as a solvent to obtain mortality in the range of 50-100% (Table 2). The control treatment was served with distilled water alone. Replications were made three times for each treatment with ten stingless bees each to obtain accurate mortality data.

Table 2. Five different concentrations of insecticides tested against *Heterotrigona itama*

Insecticides	Recommended concentration (ml/10L of water)	Concentration (ml/L of water)				
		C ₁	C ₂	C ₃	C ₄	C ₅
Deltamethrin	22.0	3.30	2.80	*2.20	1.66	1.12
Chlorpyrifos	6.0	*0.60	0.30	0.15	0.08	0.04

Note: *Recommended rate in unit of ml/L of water.

Laboratory bioassay

Determination of LC₅₀ and LC₉₅ Topical Bioassay

The determination procedure for topical bioassay was based on the OECD (1998) which initially developed for *Apis mellifera*. Thus, the method was copied with slight modification to suit with stingless bees. The insecticides were initially diluted with distilled water and prepared the desired five concentrations. The stingless bees were anaesthetised for easily handling of bioassay and placed in the petri dish. In this experiment, micropipette (Nichipet EX made in Japan) was used to apply 1µl of diluted insecticide to the pronotum area of stingless bee. Control treatment only received 1µl of distilled water. The tested stingless bees were transferred to the post cage (9x9x9 inches) and provided with 50% sucrose solution as a diet substituted. The mortality was determined after 24 hours of treatment. The dose-mortality relationship was determined by Probit analysis (SPSS Statistic 21).

Determination of LC₅₀ and LC₉₅ Residual Bioassay

In this method, the insecticide was diluted with distilled water and the dilution was coated inside the glass jar. The jar was left to dry at room temperature for at least 2 hours. After the jar completely dried, 10 selected stingless bees from the anaesthetised procedure were introduced in it for about 1 hour. The control jar was coated with distilled water and allowed to dry before introducing the stingless bees. Five dosages of diluted insecticide were tested with three replications each. After 1 hour, the stingless bees were transferred into the post

cage (9x9x9 inches) with 50% sucrose solution diet. Mortality was observed for 24 hours and data was analysed using Probit analysis (SPSS Statistic 21).

Statistical analysis

Mortality data obtained from the bioassay tests both topical and residual were analysis using Probit Analysis (SPSS Statistic 21). The analysis was calculated the LC₅₀ and LC₉₅ values.

Result and Discussion

Results from the studies indicated that deltamethrin and chlorpyrifos were harmful to the *H. itama* forager workers. Since both of these insecticides are differ in group and class, the LC₅₀ results gained were varied. In this experiment, two routes of exposure (topical and residual) were used to assess the toxicity responses of deltamethrin and chlorpyrifos against *H. itama*. This is justified when the stingless bees foraged in insecticide-treated areas, they will be exposed topically when chemicals suspended in the air come into contact with the stingless bee body (Johnson et al., 2010 and Mullin et al. 2010). Meanwhile, for residual, it occurs when stingless bee had a contact with contaminated plant surfaces such as leaves, flower, stem etc.

Toxicity of deltamethrin using topical and residual exposure methods

Table 3 shows, the LC₅₀ and LC₉₅ values of deltamethrin against *H. itama* using topical and residual exposure methods. The values were gained after 24 hours of insecticide exposure. Deltamethrin's mode of action is through contact and stomach (Table 1) which the death of insects was due to irreversible damage to the nervous system.

The LC₅₀ results showed significant differences in susceptibility between the two exposure mode (topical and residual) against *H. itama*. *H. itama* was more susceptible to topical exposure (LC₅₀ topical = 1.41 µl a.i./bee; LC₅₀ residual = 2.37 µl a.i./bee) in which deltamethrin was almost two times more toxic when applied topically than residual. The results for LC₉₅ valued revealed that *H. itama* was more susceptible to deltamethrin by topical exposure and moderately on residual (LC₉₅ topical = 2.83 µl a.i./bee; LC₉₅ residual = 5.14 µl a.i./bee).

The toxicity effect of deltamethrin can occur through cuticular penetration which quickly penetrates the nerve system of the insect (Hasibur et al. 2014). In addition, deltamethrin also has a low vapour pressure and considered to be non-volatile, thus the inhalation risk was low.

Table 3. Acute topical and residual toxicity of Deltamethrin to *H. itama*

Insecticide	Exposure mode	No. of samples	Time (hours)	LC50 ¹ (95% CI) ² (µl a.i./ml)	LC95 (95% CI) (µl a.i./ml)	X ² ³	D.F ⁴
Deltamethrin	Topical	10	24	1.41 (0.987-1.698)	2.83 (2.236-5.462)	2.549	3
	Residual	10	24	2.37 (1.942-3.048)	5.14 (3.685-14.706)	1.430	3

Note: ¹mean lethal concentration, ²confidence interval 95%, ³chi-square, ⁴degree of freedom

Toxicity of chlorpyrifos using topical and residual exposure methods

Table 4, showed the LC50 and LC95 of chlorpyrifos against *H. itama* adult forager workers. The results were obtained after 24 hours of exposure against the tested stingless bee. The insecticide mode of actions was through contact, stomach and respiratory system.

In *H. itama*, the LC50 for both exposures were LC50 topical = 0.14 µl a.i./bee; LC50 = 0.13 µl a.i./bee, there was no huge different of LC50 result for both exposures. LC95 of chlorpyrifos (LC95 topical = 0.39 µl a.i./bee; LC95 residual = 0.77 µl a.i./bee). However, the result of LC95 showed that topical was more toxic to *H. itama*, LC50 from topical and residual exposure were below the recommended rate that shown in Table 2. Previous research showed, the acute exposure to chlorpyrifos was harmful to bees via dietary and contact exposure from flowers that were sprayed during application and remain available to bees after application (Culter et al., 2014).

Table 4. Acute topical and residual toxicity of insecticide Chlorpyrifos (commercial formulation) to *Heterotrigona itama*

Insecticide	Exposure mode	No. of samples	Time (Hours)	LC50 (95% CI) (µl a.i./mL)	LC95 (95% CI) (µl a.i./mL)	X ²	D.F
Chlorpyrifos	Topical	10	24	0.14(0.096-0.189)	0.39(0.257-1.016)	2.071	3
	Residual	10	24	0.13(0.77-0.210)	0.77(0.387-5.171)	1.705	3

Based on both of the insecticides result, chlorpyrifos was highly toxic than deltamethrin to the stingless bee. To justify from the conducted experiment, it showed that only the tiny amount of chlorpyrifos can harm *H. itama* than deltamethrin. Both of these insecticides were from a different group: Chlorpyrifos which belong in organophosphate group is synthetic organic compounds meanwhile deltamethrin belong in pyrethroid group that is an organic compound similar to the natural pyrethrins produced by the flowers. However, they have the similarity mode of action to suppress the population of insect pests with different dosage level which showed in Table 1. Previous studies revealed that both of these insecticides were harmful towards several Hymenoptera insects which revealed by Dorneles et al., (2017) that

chlorpyrifos is hazardous to bee health which tested (topical and oral) against two species of stingless bee *Scaptotrigona bipunctata* and *Tetragonisca fiebrigi*. As for deltamethrin, it been tested on *Melipolina quadrifasciata* and *A. melifera* through ingest, topical and contact, the results showed that ingested was highly toxic to the tested bees followed by topical and contact (Sarto et al., 2014).

Another factor affecting the result is the different age of the adult forager workers, the workers started the foraging activity when they reach 26 days (Mohd Fahimee et al., 2016). The stingless bee workers' lifespan was an average of 70 days. The immature forager workers were likely to be more sensitive with the application of insecticide (Ruvolo-Takasusuki, 2015). In addition, different insecticide susceptibility may result from insecticide formulation or insect-defense system in detoxification of chemicals.

Conclusion

In conclusion, the study showed that *H. itama* was sensitive to the application of insecticides (deltamethrin and chlorpyrifos) in both exposures topical and residual. Thus, we suggest that further analyses of the toxicity effect of other insecticides including botanical insecticide be carried out on stingless bee species.

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Selecting the most effective plant growth-promoting bacteria from oil palm (*Elaeis guineensis* Jacq) roots

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Abstract

A total of 30 bacterial isolates were successfully isolated from soil, rhizoplane, and internal tissue of oil palm roots. The isolates were qualitatively tested for their potential to fix N₂, solubilize inorganic P and K, and produce phytohormone indole-3-acetic acid. Of the 30 isolates, six isolates were able to exhibit multiple beneficial traits. All six isolates were then identified based on fatty acid methyl esters profile as *Escherichia coli* strain EX2, *Serratia* sp. strain EN1, *Pantoea ananatis* strain EN3, *Bacillus* sp. strain EN5, *Pantoea ananatis* strain EN8 and *Pantoea* sp. strain EN9. Subsequently, all shortlisted isolates were evaluated for plant growth-promoting potential by using shallot as a test plant. The plant test showed that no significant difference ($p>0.05$) between inoculated and uninoculated plants except for *Pantoea* sp. strain EN9 inoculation which increased significantly ($p<0.05$) in total root length over uninoculated control. Host specificity and IAA capacity of the isolates may be among the important factors that affect their effectiveness in plant growth promotion. In conclusion, *Pantoea* sp. strain EN9 with multi-beneficial traits has the potential to be formulated and used as an inoculum for sustainable agriculture.

Keywords: *Elaeis guineensis*; oil palm, plant growth-promoting rhizobacteria; rhizosphere

Introduction

Chemical fertilizer is widely used in agricultural crop production. However, improper use can cause negative effects to environment such as soil acidity and groundwater pollution. Use of certain fertilizer products can cause addition of heavy metals in plant and soil (Gall *et al.*, 2015). In recent years, various studies have been conducted to overcome the negative impact of chemical fertilizer to the environment (Gupta *et al.*, 2015). One of the approaches is using plant growth-promoting rhizobacteria (PGPR). PGPR which act as biofertilizer and biostimulant is safer for the environment and cost-effective as compared to chemical fertilizer. Srivastava and Singh (2017) stated that PGPR technology is a method used to dismiss the use of chemical fertilizers and pesticides by utilizing the microorganisms in soil. PGPR is referred to the bacteria found in soil that attach to roots of plant or rhizosphere. There are growing numbers of isolates, collected from various host plant. PGPR can be isolated from agricultural crops such as oil palm (Azlin *et al.* 2005), rice (Tan *et al.*, 2014), and wheat (Majeed *et al.*, 2015). Various successful application of PGPR on agricultural crops such as oil palm (Zakry *et al.*, 2012), rice (Singh *et al.*, 2016), wheat (Kumar *et al.*,

2017), maize (Sood *et al.*, 2017) and bell pepper (Tariq *et al.*, 2014) were reported. PGPR exhibit several beneficial mechanisms such as biological nitrogen fixation, P, and K solubilization and growth hormone IAA production (Zakry *et al.*, 2010). Isolation of PGPR is a continuous process with aimed to search for new strains with superior activity in plant growth promotion. Therefore, present study was aimed to isolate, screen and select for the most promising isolates in promoting plant growth.

Materials and Methods

Root samples was collected from the Oil Palm Plantation of Taman Pertanian Universiti, Universiti Putra Malaysia Bintulu Sarawak Campus. The soil and roots of the oil palm were collected and stored in sterile plastic bags. The plastic bags were placed in an ice box and brought to the Microbiology Laboratory for further analysis which was conducted within 48 hours.

To collect isolates from rhizospheric soil, the adhered soil from roots was removed and 10.0 g of soil was weighed. Then, the soil was mixed with 100 ml of NaCl into 150 ml conical flask. The solution was shaken using orbital shaker for one hour at 80 rpm. One ml from the solution was pipetted into 9 ml of NaCl inside falcon tube using micropipette. It was serially diluted up to 10^{-8} and plated onto nutrient agar medium. The inoculated plate was incubated at $28\pm 2^{\circ}\text{C}$ for up to 48 hours.

For collection of root surface, the roots were washed thoroughly using sterile distilled water two to three times. The roots were cut into small pieces (2 to 3 cm). Ten g of the roots were weighed and mixed with 90 ml of NaCl. The solution was shaken using orbital shaker for 30 minutes at 80 rpm. One ml from the solution was pipetted into 9 ml of NaCl in falcon tube. It was serially diluted and pipetted onto nutrient agar medium.

For endophyte isolation, the roots were soaked in 70% ethanol for 3 minutes to disinfect them. They were then washed thoroughly using sterile distilled water before soaked in NaOCl for 10 minutes. Next, the roots were washed thoroughly again using sterile distilled water for two to three times. Ten grams of the roots were weighed and placed into mortar. The roots were macerated using mortar and pestle with few drops of NaCl. 1 g of the macerated tissue was placed into 9 ml of NaCl. The serial dilution was prepared up to 10^{-7} . Then 0.1 ml of each dilution from 10^{-1} to 10^{-7} was pipetted and plated on nutrient agar medium. The plate was incubated at $28\pm 2^{\circ}\text{C}$ for up to 48 hours.

Screening was conducted qualitatively to determine Nitrogen (N)-fixing activity, qualitative assay for phosphorus (P)-solubilizing bacteria, potassium (K)-solubilisation activity and Indole Acetic Acid (IAA)-producing activity.

Jensen's medium was used for detection and cultivation of N-fixing bacteria. Jensen's medium containing 20 g of sucrose, 1 g of dipotassium phosphate, 0.5 g of magnesium sulphate, 0.5 g of sodium chloride, 0.1 g of ferrous sulphate, 0.005 g of sodium molybdate, 2

g of calcium carbonate and 2 g of bacteriological agar. The procedure was carried out in accordance with Ram *et al.* (2017). The isolates were considered as N-fixing bacteria when pellicle formed.

National Botanical Research Institute's Phosphate (NBRIP) growth medium was prepared by weighing 10 g of glucose, 5 g of tricalcium phosphate, 5 g of magnesium chloride hexahydrate, 0.25 g of magnesium sulphate heptahydrate, 0.2 g of potassium chloride, 0.1 g of ammonium sulphate and 15 g of bacteriological agar. The isolates were characterized as P-solubilizing bacteria based on the clear zone formation (Liu *et al.*, 2015).

Aleksandrow Medium (AM) was prepared by weighing 5 g of glucose, 0.005 g of magnesium sulphate, 0.1 g of ferric chloride, 2 g of calcium carbonate, 3 g of mica powder, 2 g of calcium phosphate and 20 g of bacteriological agar. The isolates were characterized as K-solubilizing bacteria based on the clear zone formation (Verma *et al.* 2016).

Determination of indole acetic acid producing ability was conducted according to Zakry *et al.* (2010). One loopful of respective bacterial culture was inoculated into 50 ml of nutrient broth using sterile inoculating loop. Then incubated at 28°C for four days. After incubation, 30 ml of bacterial culture was transferred into 50 ml sterile falcon tube. It was centrifuged at 4000 rpm for 10 minutes to separate the supernatant from bacterial cell. Then 2 ml from supernatant was pipetted into sterile test tube continued by 4 ml of Salkowski reagent and two drops of 85% orthophosphoric acid. Salkowski reagent was prepared by mixing 50 ml of 35% sulphuric acid (H₂SO₄) and one ml of 0.5M iron trichloride (FeCl₃). The test tube was then incubated in dark for 30 minutes. The isolates were characterized as IAA-producing bacteria based on changes of the solution into pink colour.

Selection from the total collection was made based on isolate that exhibit multiple traits or three or more with positive traits. Shortlisted PGPR isolates with multiple traits were characterized morphologically. Colony morphology was characterized based on colour, shape, size, and texture. Cell morphology was identified through Gram staining for determination of cell arrangement, cell shape and Gram reaction.

The shortlisted isolates from prior screening of PGPR were selected for gas chromatography analysis to determine the fatty acid methyl esters profile of the shortlisted bacteria. GC-FAME began by harvesting 40 mg of the bacteria cells from NA media plate using sterile inoculating loop. The bacteria cell was placed in a sterile test tube. The procedure then involved saponification, methylation, and extraction of fatty acid of cells prior to GC-FAME analysis.

Shallot (*Allium ascalonicum*) was used as a test plant for inoculation with the shortlisted PGPR isolates. The shallots were cleaned by removing one or two outer scales. The clean shallots were cut horizontally into half before surface sterilized by soaking into 70% ethanol around 30 seconds. Then, it was soaked in 10% NaOCl for 30 minutes. Next, rinsed thoroughly using sterile distilled water for five times. The base of the shallots was dipped for

5 minutes into the broth culture of the selected PGPR isolates which contains more than 10^8 CFU in one ml of nutrient broth. The shallots were then transferred to disposable clear plastic cups containing sterilized white sand. There were four replications of shallots for each treatment and one shallot for one replication. Shallots treated with fresh sterile nutrient broth served as control. The experiment was assigned in completely randomized design under ambient temperature of laboratory. After 17 days, the number of roots, total length of roots, shoot length and dry weight of roots and shoots were calculated, measured, and recorded.

Data on plant test were analysed statistically using the Statistical Analysis System (SAS version 9.4). Data was subjected to analysis of variance (ANOVA) and mean values between treatments were compared using Duncan's New Multiple Range test at p value at 0.05.

Results and Discussion

A total of 30 isolates were successfully isolated from rhizosphere of oil palm. The collection was then coded and sequentially numbered as RHI (rhizospheric soil), EX (rhizoplane) and EN (endophyte) (Table 1). The isolates were tested for their biological nitrogen fixation, phosphate and potassium solubilisation and IAA production abilities. The isolates with at least three multiple traits were shortlisted for further testing in plant test. The present study showed that EX2, EN1, EN3, EN5, EN8 and EN9 exhibited multiple plant growth-promoting traits.

Colony and cell morphology were identified, and the results was shown in Table 2. A total of six PGPR isolates were analysed by GC-FAME (Table 2). Isolate EX2 was identified as *Escherichia coli* while EN1 as *Serratia* sp. EN3 and EN8 were identified as *Pantoea ananatis* followed by EN5 as *Bacillus* sp. EN9 was identified as *Pantoea* sp.

Most of the rhizobacteria did not promote the growth of shallots even though they were able to exhibit potential multiple traits. This phenomenon occurred probably due to the host specific characteristic that was exhibited by the certain rhizobacteria isolated from the oil palm roots. Failure of some rhizobacteria to promote plant growth occurred when inoculated with non-host plant. Thokchom *et al.* (2017) reported that inoculation of mandarin by host strain enhanced plant growth compared to non-host strain. Moreover, the interaction between soil bacteria and plants can be either beneficial, harmful, or neutral depending on the species of the bacteria and types of plant (Turan *et al.*, 2016) thus demonstrated variable plant responses.

Table 1. Qualitative screening of PGPR isolates from oil palm (*Elaeis guineensis* Jacq.) for biological nitrogen fixation, phosphate and potassium solubilisation and IAA production

Beneficial traits	Isolates
Fix nitrogen	RHI1, RHI3, RHI7, EX3, EX9, EN5, EN9
Solubilize phosphate	EX2, EX6, EN1, EN2, EN3, EN5, EN8, EN9
Solubilize potassium	RHI5, RHI6, RHI8, EX2, EX7, EN1, EN3, EN5, EN8, EN9

Produce indole acetic acid RHI1, RHI2, RHI6, RHI7, RHI8, RHI9, EX1, EX2, EX6, EX9, EN1, EN2, EN3, EN4, EN7, EN8, EN9, EN10

Note: RHI = isolates from rhizospheric soil; EX = isolates from rhizoplane; EN = isolates from internal root tissue

Table 2. Colony, cell morphology and identity of PGPR isolated from oil palm (*Elaeis guineensis*)

PGPR	Colony		Cell			GC-FAME identification			
	Colour	Shape	Size	Texture	Gram colour	Cell shape	Name	Sim index	Similarity (%)
EX2	Colourless	Circular	Small	Smooth	Pink	Bacilli	<i>Escherichia coli</i>	0.633	96.2
EN1	Red and wh	Circular	Moderate	Smooth	Purple	Cocci	<i>Serratia</i> sp.	0.449	91.4
EN3	Yellow	Drop-shaped	Small	Shiny	Pink	Bacilli	<i>Pantoea ananatis</i>	0.568	98.2
EN5	White	Filamentous	Large	Rough	Purple	Bacilli	<i>Bacillus</i> sp.	0.156	54.8
EN8	Yellow	Circular	Small	Smooth	Purple	Bacilli	<i>Pantoea ananatis</i>	0.500	98.0
EN9	Yellow	Circular	Small	Smooth	Pink	Bacilli	<i>Pantoea</i> sp.	0.498	97.2

Table 3. Effect of PGPR inoculation on root number, total root length, shoot length, root dry weight, and shoot dry weight of shallot (*Allium ascalonicum*) plants.

Treatments	Root number	±	Total root length (cm)	±	Shoot length (cm)	±	Root dry weight (mg/plant)	±	Shoot dry weight (mg/plant)	±
EX2	12.00 ^{bc}	±	38.07 ^{bcd}	±	2.17 ^a	±	26.03 ^{ab}	±	27.33 ^a	± 9.36
	5.03		17.04		0.67		11.03			
EN1	19.67 ^{abc}	±	72.47 ^{cd}	±	1.63 ^a	±	37.37 ^{ab}	±	50.60 ^a	±
	9.94		9.43		1.20		17.78		43.90	
EN3	7.00 ^c	±	15.17 ^d	±	0.67 ^a	±	12.83 ^b	±	12.50 ^a	± 0.81
	2.65		5.36		0.07		3.94			
EN5	26.33 ^{ab}	±	83.10 ^{ab}	±	3.40 ^a	±	49.17 ^{ab}	±	35.07 ^a	±
	0.67		10.74		2.50		2.62		10.75	
EN8	21.33 ^{abc}	±	63.57 ^{bc}	±	0.63 ^a	±	46.93 ^{ab}	±	42.27 ^a	±
	1.86		12.82		0.09		8.52		33.46	
EN9	32.00 ^a	±	120.13 ^a	±	3.87 ^a	±	61.47 ^a	±	60.30 ^a	±
	2.31		12.28		1.33		7.97		24.10	
Uninoculated	18.33 ^{abc}	±	51.73 ^{bcd}	±	1.83 ^a	±	36.83 ^{ab}	±	32.67 ^a	±
	5.21		24.08		1.48		20.76		25.81	

Note: Different letters within column indicate significant differences between means using Duncan's New Multiple Range test at P value 5%. ±: Standard error of mean

Conclusion

In this study, 30 isolates were successfully recovered from the oil palm roots. The selection of the most promising isolates was made based on isolate that exhibit multiple beneficial properties and producing significant improvement in plant growth promotion. *Escherichia coli* EX2, *Serratia marcescens* EN1, *Pantoea ananatis* EN3, *Bacillus cereus* EN5, *Pantoea ananatis* EN8, *Pantoea ananatis* EN9 were having multiple traits while *P. ananatis* EN9 was the most promising in plant growth promotion. Therefore, the strain EN9 has the potential to be formulated as biofertilizer and biostimulant. However, the other five shortlisted strains may be effective in its original host, oil palm.

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Effect of different rates and frequencies of rejuvenator application to improve yield of *Hevea brasiliensis*

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Abstract

Brown bast is one of the most serious threats to natural rubber production. It is estimated that brown bast leads to approximately 15-20% decrease in latex yield. Thus, the formulation of Rejuvenator is created based on macro- and micro- nutrients to treat this problem. A study was carried out to evaluate the effect and suitable rate and frequency of Rejuvenator application to improve latex yield. The Rejuvenator treatment was applied to the selected tree with two different treatment frequencies (every 3 days and every 6 days) and three different Rejuvenator concentrations (5 g/L, 10 g/L and 15 g/L). Split Plot Design was used as the experimental design with 3 replications. Total number of Rejuvenator applications were 5 times. Data of latex yield were recorded 1 month after the last treatment application. The treatments used were T1: No Rejuvenator, T2: 5 g/L of Rejuvenator, T3: 10 g/L of Rejuvenator, T4: 15 g/L of Rejuvenator. Treatment T2 (5 g/L) resulted a higher production of latex yield (g/t) than control. It also gave the highest nutrient content in the bark tissue. Findings of this study revealed that the optimum concentration and frequency of Rejuvenator application were established at 5 g/L in every 6 days.

Keywords: *Hevea brasiliensis*, *Hevea*, Rubber, Brown Bast, Tapping Panel Dryness

Introduction

Rubber industry is the second most important commodity in Malaysia after oil palm and it provides direct employment to more than 200,000 smallholder families while the downstream manufacturing sector provides direct employment to more than 64,000 workers. However, competition among the main rubber producers like Malaysia, Indonesia and Thailand has placed the industries downstream under strain and this has led to a reduction in the price of rubber. Malaysia is undertaking necessary approach to position itself among the competitors by targeting a total Gross National Income from the industry of RM 3.11 billion in the year 2020. Consequently, rubber has been recognized among the Eight Entry Point Projects (EPPs) in the national key economic area (NKEA). One of the EPPs is increasing average national rubber productivity.

There are various pathogenic diseases and plant physiological conditions influence rubber production. Brown bast or also known tapping panel dryness is one of the most serious threats

to rubber production. Nandris *et. al.*, (2004) reported that brown bast contributes to 15-20% loss of the annual rubber production with an incidence of 20-50% of productive trees affected by brown bast in almost all rubber growing regions. In addition, around 14.75% of rubber trees were harmed due to tapping panel dryness (Qi *et.al.*, 2014). Interestingly, some researchers have assigned the syndrome to a disease, however no pathogen has been detected so far in association with brown bast condition (Liyanage *et.al.*, 1983). Thus, it is more generally recognized as a physiological disorder of rubber tree bark.

One of the factors causing the brown bast syndrome in rubber tree is over exploitation. The repeated removal of latex could cause nutrient stress on the tissue of the bark that is exploited. In 1982, de Fay has conducted the experiment by inducing dryness in clone GT1 rubber trees, which normally has been tapped twice a week but tapping them 6 times a day. He observed that continued tapping of rubber tree shows symptoms of exhaustion. According to Seetha (as cited in Schwelzer, 1936), some workers have suggested that the water and nutrient stresses are important in the development of brown bast. Currently, there is no research work and there is a lack of literature on the cure of brown bast syndrome to improve rubber production. Therefore, the formulation of Rejuvenator is created based on macro- and micro- nutrients to treat this phenomenon. Thus, the objective of the study was to evaluate the potential effect and most suitable rate and frequency of Rejuvenator application to improve yield of *Hevea brasiliensis*.

Materials and Methods

Experimental design and treatments

This study was carried out at rubber smallholder field in Kuala Nal, Kuala Krai, Kelantan (5.595077°N, 102.1564°E). Matured rubber tree of clone RRIM 2002 exposed to brown bast disease was obtained as plant material. Bark sample from healthy and brown bast affected trees were collected. There were six treatments with three replicates and arranged in Split Plot design. The treatment consists of treatment frequencies and rates of Rejuvenator concentration.

The different rates coded as (T1=control, T2= 5 g/L, T3= 10 g/L, T4= 15 g/L) of Rejuvenator concentrations were prepared. The Rejuvenator crystal were weighed and place in container and made up to 1 L by adding distilled water. The mixture was stirred to make sure Rejuvenator crystal completely dissolved. Rejuvenator solution was applied on the bark of brown bast affected tree from 1 m above the ground. The treatment was carried out at every 3 days and every 6 days. The treatment duration was 5 weeks and treated trees was tapped one week after a total 5 applications of Rejuvenator treatment. Data of latex yield were recorded from treated tree for 6 months. NPK Blue with formulation ratio (12N: 12P₂O₅: 17K₂O: 2MgO: 8S) was carried out manually every 3 months to all the trees as the maintenance process. Sample of bark from treated trees were collected for nutrient analysis. Percentage of brown bast cured was measured by using a measurement tape on the tapping panel.

$$\% \text{ of cure} = \frac{\text{total length of tapping panel} - \text{length of cured tapping panel}}{\text{Total length of tapping panel}} \times 100$$

Nutrient analysis

Bark tissue analysis was carried out to determine the nutrient element concentration. The bark samples were oven dried at 65°C for 48 hours until its reading constant weight. The sample was finely ground using a tissue grinder (3-120 mesh). A 0.25 g of the weighed ground dried sample was put into digestion tube. Then 5 ml concentrated sulphuric acid (H₂SO₄) was added, shaken and left for about 2 hours to absorb moisture. Subsequently, the mixture in digestion tubes were placed in the digestion block at the temperature 450°C in a fume chamber for approximately 45 minutes. The digestion tubes were removed and allowed to cool and then 2 ml of hydrogen peroxide (H₂O₂) were added and the heating process was repeated until the sample turned into a clear solution. The solution was left to cool and later diluted with distilled water to make up 100 ml. The samples were analysed for N, P, and K with Auto Analyzer, while Mg, Ca and Fe were analysed with Atomic Absorption Spectrometer (AAS) (Perkin Elmer, Model AAS 3110). All the data were subjected to analysis of variance (ANOVA) using statistical analysis system (SAS Ver 9.4). The mean separations were compared using Least Significant Difference (LSD) at 5% level.

Results and Discussion

Effect of different rates and frequencies of Rejuvenator application on latex yield of brown bast affected trees

There was no significant difference among different rates and frequencies of Rejuvenator application on latex yield of brown bast affected trees. Tree with 0 g/L of Rejuvenator was a healthy tree which produce latex normally as a treatment control. Noticeably the result showed that yield from different rates and frequencies of Rejuvenator application was similar to the yield of control. Thus, this indicated that Rejuvenator treatment at the optimum rate (T2: 5 g/L) can improve the latex yield of brown bast affected trees because the treated trees gave the same result as the healthy tree which is control. This supported by Lalani (2000), the increase in the latex yield due to fertilizer application could be direct effect on girdling, growth of bark, bark renewal and canopy maintenance. This is similar result of another study conducted by Lim Khan Tiva *et. al.*, (2016), the improvement of latex physiological condition after fertilization could be the main driver of the effect of the fertilizer on latex production. Furthermore, Lim Khan Tiva *et. al.*, (2016) had reported than after 4 years of fertilizer application, the treatment with fertilizer did show significant difference in rubber yield (g/tree) but could be increased by 10%. This is in line with Lalani (2000) which stated that rubber yields could be increased by 15-20%.

Based on table 1, the latex yield from March to May were not recorded due to wintering period however, the latex yield form June to September were recorded and the result showed there were improvement in production of latex due to completeness of leaf formation. During wintering period, most of the trees lost their leaves and have minimal photosynthetic activity,

hence low latex production. Nevertheless, the rubber trees were reopened for new tapping after resting period, some lapse of time is needed before the tree's metabolism was fully activated to give optimal yield.

Table 1: Effect of frequency and concentration of Rejuvenator application on latex yield for duration of 6 months

Latex yield	Jan	Feb	Jun	July	Aug	Sept
Frequency						
Three days	2.613 ^a	4.108 ^a	1.263 ^a	1.267 ^a	2.908 ^a	2.467 ^b
Six days	4.046 ^a	4.819 ^a	2.264 ^a	2.415 ^a	1.966 ^a	3.592 ^a
Critical value (LSD)	1.722	1.493	0.782	1.201	0.629	1.084
Rejuvenator concentration (g/L)						
T1: 0 (healthy tree)	3.330 ^a	4.462 ^a	1.763 ^a	1.840 ^a	2.438 ^a	3.030 ^a
T2: 5	1.923 ^a	3.418 ^a	0.932 ^a	1.042 ^a	1.113 ^a	1.430 ^a
T3: 10	4.297 ^a	5.017 ^a	2.347 ^a	2.385 ^a	3.187 ^a	3.995 ^a
T4: 15	3.768 ^a	4.958 ^a	2.347 ^a	2.097 ^a	3.008 ^a	3.663 ^a
Critical value (LSD)	7.954	5.710	4.694	4.961	5.940	7.278
Variable	Probability					
Freq (F)	0.464 ^{ns}	0.611 ^{ns}	0.388 ^{ns}	0.350 ^{ns}	0.518 ^{ns}	0.528 ^{ns}
Conc (C)	0.833 ^{ns}	0.828 ^{ns}	0.830 ^{ns}	0.868 ^{ns}	0.729 ^{ns}	0.734 ^{ns}
F*C	0.731 ^{ns}	0.483 ^{ns}	0.787 ^{ns}	0.846 ^{ns}	0.731 ^{ns}	0.698 ^{ns}

For each frequency and concentration, means values followed by the same letter in the same column are not significantly different at $P < 0.05$, based on the least significant different test (LSD).

Notes: * = Significant difference at $\alpha = 0.05$; ^{ns} = No significant difference at $\alpha = 0.05$

Effect of different rates and frequencies of Rejuvenator application on nutrient contents in bark sample.

Nutrient analysis showed percentage of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca) and ferum (Fe) over a period of 5 months after Rejuvenator treatment was applied. There was no interaction between different rates and frequencies of Rejuvenator application on percentage of nutrient contents. However, different rates of Rejuvenator concentration on percentage of N, P, K, Mg, Ca, and Fe varied greatly among the treatments (Table 2). The treatment T2 (5 g/L) for all percentage of nutrient contents were significantly different to the control (T1: 0 g/L) which recorded the lowest mean values. This indicated that Rejuvenator treatment has positive impact to brown bast affected tree compared to control which is tree without any treatment.

The Rejuvenator treatment application probably can cure brown bast affected tree due to availability of micro and macro nutrient in the treatment to the bark. This is in line with (Anonymous, 2010) who found the physiological disorder of brown bast is due to the deficiency of nutrients or trace elements. It has been reported that the effect of fertilizer application could be seen in short time that is one year or less for nutrient deficient in rubber trees (Ismail, 1981). Optimum nutrients are essential during mature phase to sustain health of trees and also to maintain production of latex yield (Noordin and Chan, 1987; Haridas, 1978).

Table 2: Effect of frequency and concentration of Rejuvenator application on nutrient contents in bark sample

Factors	N (%)	P (%)	K (%)	Mg (%)	Ca (%)	Fe (%)
Frequency						
Three days	0.054 ^a	0.173 ^a	0.545 ^a	0.112 ^a	1.111 ^a	0.039 ^a
Six days	0.055 ^a	0.179 ^a	0.553 ^a	0.122 ^a	1.076 ^a	0.038 ^a
Critical value (LSD)	0.016	0.0182	0.131	0.039	0.177	0.011
Rejuvenator concentration (g/L)						
T1: 0 (brown bast affected tree)	0.036 ^b	0.139 ^b	0.414 ^b	0.072 ^b	0.897 ^b	0.032 ^b
T2: 5	0.060 ^a	0.183 ^a	0.553 ^{ab}	0.121 ^{ab}	1.158 ^a	0.046 ^a
T3: 10	0.064 ^a	0.188 ^a	0.651 ^a	0.137 ^a	1.176 ^a	0.038 ^{ab}
T4: 15	0.059 ^a	0.193 ^a	0.579 ^{ab}	0.137 ^a	1.143 ^a	0.038 ^{ab}
Critical value (LSD)	0.008	0.013	0.195	0.049	0.188	0.012
Variable	Probability					
Freq (F)	0.712 ^{ns}	0.184 ^{ns}	0.865 ^{ns}	0.413 ^{ns}	0.456 ^{ns}	0.673 ^{ns}
Conc (C)	>.0001*	>.0001*	0.023*	0.005*	0.002*	0.043*
F*C	0.255 ^{ns}	0.133 ^{ns}	0.930 ^{ns}	0.421 ^{ns}	0.837 ^{ns}	0.788 ^{ns}

For each frequency and concentration, means values followed by the same letter in the same column are not significantly different at $P < 0.05$, based on the least significant different test (LSD).

Notes: * = Significant difference at $\alpha = 0.05$; ^{ns} = No significant difference at $\alpha = 0.05$

Effect of different rates and frequencies of Rejuvenator application on percentage of brown bast cured

The percentage of brown bast cured after different rates and frequencies of Rejuvenator treatment application are shown in Table 3. There was no interaction between different rates and frequencies of Rejuvenator treatment application since $P > 0.05$. However, there was significant difference between treatment control and other rates of Rejuvenator treatment application. Noticeably, there was no significant differences among different Rejuvenator concentration rates T2 (5 g/L), T3 (10 g/L) and T4 (15 g/L). This indicated that treatment Rejuvenator concentration at rates 5 g/L (T2) positively cured the brown bast affected tree.

According to Thomson *et. al.*, (2005), the number of trees having more than 75% of brown bast intensity because of time as the age of the tree and tapping progress. Symptoms of brown bast in terms of morphological features and nature of latex production are complicated and shown as partial dryness, full dryness, dryness with bark out-growth and bark cracks (Senevirathna, 2006). Therefore, the fluctuation of percentage of brown bast cured with time is expected, because of the differences in rates of onset and recovery of dry trees with time. Probably the high intensity of brown bast need more period of time in Rejuvenator treatment to full recover.

Table 3: Effect of frequency and concentration of Rejuvenator application on percentage of brown bast cured

Factors	Cure of brown bast (%)
Frequency	
Three days	41.667 ^a
Six days	59.167 ^a
Critical value (LSD)	6.2103
Rejuvenator concentration (g/L)	
T1: 0	0.000 ^b
T2: 5	51.670 ^a
T3: 10	86.670 ^a
T4: 15	63.330 ^a
Critical value (LSD)	43.088
Variable	Probability
Freq (F)	0.114 ^{ns}
Conc (C)	0.005 [*]
F*C	0.240 ^{ns}

For each frequency and concentration, means values followed by the same letter in the same column are not significantly different at $P < 0.05$, based on the least significant different test (LSD).

Notes: * = Significant difference at $\alpha = 0.05$; ^{ns} = No significant difference at $\alpha = 0.05$

Conclusion

Optimum rates and frequencies of Rejuvenator treatment on brown bast affected trees appeared to have similar production of latex yield from healthy rubber trees. Application of Rejuvenator treatment demonstrated good performance for percentage of nutrient content in bark of rubber tree and increase percentage of cure. The experiment revealed the suitability of Rejuvenator application at different rates and frequencies whereby 5 g/L of Rejuvenator concentration with 6 days application could be considered as optimum rates and frequencies. Though higher rates (10 g/L and 15 g/L) could be used but the former was sufficient as there was no significant difference in production of latex yield with both rates. The brown bast syndrome on rubber tree is considerable economic importance as it very affects the latex yield production. Therefore, application of Rejuvenator treatment on brown bast affected tree could be used by smallholders due to its potential effect as shown in this study. It has profoundly increased percentage of nutrient level in bark of rubber tree which could produce healthy bark and equally enhance production of latex yield.

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Evaluation of ovaries collected from slaughterhouse in relation to the cow's breed

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Abstract

Implementation of animal biotechnology including *in vitro* embryo production (IVEP) could be an alternative way to enhance the genetic improvement of livestock. However, one of the main obstacle to produce embryos from IVEP is recovery of low quality oocytes. Thus, a preliminary evaluation study of ovaries could provide the vital information needed for IVEP process. This study aimed to evaluate the morphology of ovaries and also quantity of ovarian surface follicles in relation to breed of the cows. Twenty (20) ovaries from Kedah-Kelantan breed and 20 ovaries from crossbreed cows aged between 3 and 5 years were collected from slaughterhouse in Taiping, Perak and Lahad Datu, Sabah. The left and right ovaries were collected immediately after slaughter, followed by measuring of the weight, length and width of the ovaries and also counting the number of visible follicles on the ovarian surface. Crossbreed cows were found to have significantly ($P < 0.05$) higher weight, length and width (9.20 ± 1.02 g, 3.37 ± 0.14 cm and 2.46 ± 0.11 cm, respectively) of ovaries compared to Kedah-Kelantan cows (3.45 ± 0.41 g, 2.56 ± 0.11 cm and 1.88 ± 0.08 cm, respectively). Crossbreed cows were also found to have significantly ($P < 0.05$) higher number of surface visible ovarian follicles (34.55 ± 18.39) than Kedah-Kelantan (22.10 ± 2.20). The mean weight, length, width and number of surface visible follicles between right and left ovaries in Kedah-Kelantan as well as in crossbreed cows were almost same and there was no significant difference. It is possible to conclude that breed of cows had statistically significant influence on the ovarian parameter and surface follicles and would be considered in the selection of ovaries for the use in IVEP.

Keywords: breed of cow; cattle ovary; follicles; slaughterhouse.

Introduction

Livestock industry in Malaysia consists of two sectors, namely ruminant and non-ruminant sectors. Non-ruminant sector, especially poultry sector had reached 100% self-sufficiency (Ariff *et al.*, 2015) but, the sufficiency level is still below 30% in the ruminant sector (Fadhilah, 2015). The local production of beef and milk is still inadequate to meet the increasing demand and lead to massive importation of beef, milk and live animals from other countries like India, Thailand, New Zealand and Australia to fulfill the demand. The low beef and milk production in Malaysia is associated with the low reproduction rate and slow

progress in the genetic improvement of the locally ruminant animals. Thus, implementation of animal biotechnology could be one of alternative ways to enhance the reproductive performance of livestock animals as to increase the low livestock production.

In vitro embryo production (IVEP) is one of the popular techniques in animal biotechnology to produce embryos from slaughterhouse ovaries (Hoque *et al.*, 2011). According to Freitas and Melo (2010), IVEP include four steps, namely (a) the collection of oocytes from follicles; (b) the *in vitro* maturation (IVM) of oocytes; (c) the *in vitro* fertilization (IVF) of the matured oocytes; and (d) the *in vitro* culture (IVC) of the resulting embryos. Follicles contain the oocytes and in recent years, the percentage of oocytes developing into blastocysts by *in vitro* technique is still low. Only 30-40% of oocytes reaching blastocysts stage through *in vitro* process (Ayman *et al.*, 2016). Therefore, preliminary studies need to be carried out in order to acquire the necessary knowledge in the IVEP process. The initial and crucial step in IVEP is the selection of ovaries, oocytes collection, and quantity and quality of ovarian follicles and oocytes harvested for IVM (Kouamo and Kharche, 2014, Kouamo *et al.*, 2014 and Hoque *et al.*, 2011).

Breed of cattle is one of factors that could be considered during the selection of ovaries for oocyte harvesting for IVEP. Reports on the influence of cow breed on the quantity of ovarian follicles and oocytes have been studied by various researches from different countries (Kouamo *et al.*, 2014; Silva-Santos *et al.*, 2011; Fihri *et al.*, 2004). Therefore, the present study was carried out to evaluate the breed of cattle in Malaysia that has potential for IVEP. The specific objective of this study was to evaluate the morphology of the ovaries and also quantity of ovarian surface follicles in relation to the breed of the cows.

Materials and Method

Collection of Ovaries

Twenty (20) pairs of ovaries from cyclic (corpus luteum present) cows aged between 3 and 5 years with a body condition score (BCS) between 3 and 4 (5-point scale) were used in this study. The ovaries collected from 10 heads Kedah-Kelantan breed and another 10 heads from crossbreed cows. Those ovaries were collected at local slaughterhouse in Taiping, Perak and Lahad Datu, Sabah. Both the left and right ovaries were collected from each cow soon after the slaughter, labeled and put in separate vials.

Evaluation of Ovaries

The collected ovaries were carefully trimmed to remove surrounding excessive tissues. Each ovary was weighed using electronic weighing balance and the ovarian length and width were measured using vernier calipers. The total number of visible follicles on the surface of each ovary was then counted and the data were recorded

Statistical Analysis

The data obtained was recorded and statistically analysed by the analysis of variance (ANOVA) using SAS (1998, Version 6.12) software. Results are presented as means \pm SEM. A probability of $P < 0.05$ considered statistically significant.

Results and Discussion

In this study, it was found that the breed of cattle slaughtered in Taiping and Lahad Datu were mostly Kedah-Kelantan (KK) and crossbreed cattle (Brahman cross and European-KK-crossbreed). The mean weight, length, width and number of visible ovarian follicles between KK breed and crossbreed cows are summarised in Table 1.

Table 1. Mean \pm SEM weight, length, width and number of visible follicles of cow ovary between different cow breed

Ovary	Kedah-Kelantan (KK)	Crossbreed
Weight (g)	3.45 \pm 0.41 ^a	9.20 \pm 1.02 ^b
Length (cm)	2.56 \pm 0.11 ^a	3.37 \pm 0.14 ^b
Width (cm)	1.88 \pm 0.08 ^a	2.46 \pm 0.11 ^b
Number of visible follicles	22.10 \pm 2.20 ^a	34.55 \pm 18.39 ^b

^{ab} means with different superscripts in the same row differ significantly ($P < 0.05$)

Between these two breeds, the weight, length and width of the ovaries from crossbreed cows were significantly ($P < 0.05$) higher than KK breed cows. The average weight of KK ovaries in this study is comparable to that of Uganda Ankole zebu (4.6 \pm 2.3 g) reported by Natumanya *et al.* (2008), might be because of both breeds are from the same group, *Bos indicus*. However, the KK ovarian weight was lighter than the weight of Friesian (*Bos taurus*) ovaries (10-19 g) reported by Pierson and Ginther (1987) which comparable to that of the crossbreed ovaries found in this study. The length and width of the ovaries from this study is comparable with the finding of Kouamo *et al.* (2014) where they found the mean length and width of Gudali breed cows were 2.77 \pm 0.05 cm and 1.90 \pm 0.03 cm, respectively, which were lower than those of European breeds. From the findings, we can consider that the weight and size of the ovaries of *Bos indicus* cattle were lighter and smaller compared to that of *Bos taurus* and crossbreed cattle. The difference of the ovarian weight, length and width between breeds may be due to the breed effect. In fact, KK cross cows were reported to have significantly heavier live body weight compared to pure KK cows (Ariff *et al.*, 1993).

Table 1 also shows that crossbreed cow ovaries have significantly ($P < 0.05$) higher number of visible follicles than ovaries of KK cows. The result supported by a study by Fihri *et al.* (2004) where less ($P < 0.05$) ovarian follicles were found in below 3 years Oulmes-Zaers breed cows compared to its crosses. Fihri *et al.* (2005) also indicated that Oulmes-Zaers has the lowest ($P < 0.05$) performance where the mean number of follicles is only 18.96 \pm 6.52 compared to crossbreed and European cows (24.71 \pm 8.94 and 25.19 \pm 8.33, respectively). Average number of preantral follicles were also found higher in the ovaries of *Bos taurus* than *Bos indicus* females, according to the study of Silva-Santos *et al.* (2011). These findings

suggested that pure *Bos indicus* females have lower number of visible ovarian follicles compared to pure *Bos taurus* and crossbreed females.

Table 2. Mean \pm SEM weight, length, width and number of visible follicles of right and left ovaries of Kedah-Kelantan (KK) and crossbreed cows

Ovary	Kedah-Kelantan		Crossbreed	
	Right	Left	Right	Left
Weight (g)	3.70 \pm 0.52 ^a	3.20 \pm 0.65 ^a	9.60 \pm 1.35 ^b	8.80 \pm 1.58 ^b
Length (cm)	2.60 \pm 0.18 ^a	2.52 \pm 0.13 ^a	3.47 \pm 0.18 ^b	3.27 \pm 0.21 ^b
Width (cm)	1.93 \pm 0.13 ^a	1.83 \pm 0.11 ^a	2.52 \pm 0.17 ^b	2.39 \pm 0.13 ^b
Number of visible follicles	24.50 \pm 3.61 ^{ab}	19.70 \pm 2.47 ^a	36.00 \pm 5.87 ^b	33.10 \pm 6.04 ^{ab}

^{ab} means with different superscripts in the same row differ significantly (P<0.05)

By comparing between left and right ovaries in KK and crossbreed cows, the mean weight, length, width and number of surface visible ovarian follicles was slightly higher in right ovaries than left ovaries in both breeds but there was no significant difference (Table 2). The finding of ovarian weight, length and width was similar to that of Islam *et al.* (2007) in goat ovaries and Alasafy and El-Shahat (2011) in sheep ovaries.

The same trend of finding was also reported by Kouamo *et al.* (2014) where no significant difference of the average number of follicles per ovary between right and left ovaries (16.95 \pm 0.86 and 16.57 \pm 0.88, respectively). Similar finding also found in sheep by Alasafy and El-Shahat (2011) where the number of small (>2mm) and medium (2-4mm) follicles were almost similar with no significant difference between right and left ovaries, but the number of large follicles (>4mm) was significantly (P<0.05) higher in the right ovary (1.0 \pm 0.14) than in left ovary (0.6 \pm 0.10). The difference between right and left ovaries might be due to right ovaries are more active than the left ones, but there is no clear reason for this ovarian asymmetry (Alosta *et al.*, 1998).

Conclusion

In conclusion, breed of cows had an influence on the ovarian parameter and surface follicles. The results from this study demonstrate that the weight, length, width and number of visible follicles on the ovarian surface were higher in crossbreed cows compared to pure *bos indicus* cows. This study indicated that for the practice of IVEP in the future, the breed of cows can be considered in the selection of females' oocytes donors. Although this study suggested that both right and left ovaries from crossbreed cows are suitable to be selected for oocytes collection to initiate an IVEP experiment, further research studies is required to evaluate the quality of oocytes and quantity of oocytes reaching blastocyst stage between those different breeds of cows for successful IVEP in Malaysia.

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Effect of the addition of seaweed *Sargassum polycystum* on the physicochemical properties of chicken sausage

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Abstract

This research was conducted to determine the physicochemical properties of chicken sausage incorporated with *Sargassum polycystum* (SP) seaweed powder. Four formulations including sample control (without seaweed), 2%, 4% and 6% of seaweed powder were produced. The result obtained from the research shows that the addition of seaweed into the chicken sausage lowered both moisture and fat content and increased the ash content ($p < 0.05$). Other than that, the addition of seaweed into chicken sausage reduced the L^* value and increased a^* value ($p < 0.05$). The sample containing SP reduced the percentage of expressible water and cooking loss of chicken sausage ($p < 0.05$). Study on the textural of the chicken showed SP seaweed increased the hardness and chewiness properties compared to control ($p < 0.05$). The sensory analysis showed that sample control of chicken sausage is more acceptable followed by SP2 (2% of seaweed) and SP4 (4% of seaweed) ($p < 0.05$). In conclusion, the addition of SP seaweed improved the textural properties of chicken sausages and the sensory acceptable up to 4%.

Keywords: seaweed, physicochemical, chicken sausage, *Sargassum polycystum*.

Introduction

For a decade, meat product had been under scrutiny by medicinal, nutritionist and consumer due to fat content and cholesterol that contribute to the several risks of chronic diseases such as ischemia, cancer, hypertension, and obesity (Cox and Abu-Ghannam, 2013; Freire *et al.*, 2017). Meat and meat product is the part of nutrient that provided protein, fatty acid, vitamin, mineral, and others bioactive component (Cofrades *et al.*, 2017; Cofrades *et al.*, 2008; López-López *et al.*, 2009). However, when consumes in large quantity, high chronic diseases could occur. Meat is one of the popular food consumed by many people. One of the ways of meat can be consumed without any radical changes are by addition of functional ingredient (Cofrades *et al.*, 2008).

A healthy meat product was produced using a variety of additives such as soybean, olive oil, rice, wheat, carrot, and others to increase the content of fat, antioxidant, prebiotic and dietary fiber (Jiménez-Colmenero *et al.*, 2010; Olmedilla-Alonso *et al.*, 2013). Meat-based functional are produced by the addition of ingredients which promotes health such as dietary, protein, unsaturated fatty acid (PUFA) and antioxidant (Biswas *et al.*, 2010). The addition of seaweed into meat product enhance the texture of the product in term of hardness, chewiness

and fat holding capacity (Jiménez-Colmenero *et al.*, 2010). Other than that, functional product improves hydration properties, oil binding capacity, viscosity and sensory attribute (Elleuch *et al.*, 2011).

Seaweed contained bioactive components such as protein, lipid, a polyphenol with other bacteria properties and anti-virus (Kolb *et al.*, 2004; Kumar *et al.*, 2014). In Sabah, Semporna is the main producer for seaweed (Sade *et al.*, 2006). Genus *Sargassum* is brown seaweed which classified as Phaeophyta and reported for a variety of bioactive (Marimuthu *et al.*, 2012) such as antioxidant, anti-cancer and anti-diabetic (Wijesinghe and Jeon, 2012). Tropical seaweed such as *Sargassum polycystum* (SP) contained high total dietary fiber which is 39.67% (Matanjun *et al.*, 2009). Therefore, the objective of this study was to investigate the effect of the addition of seaweed *Sargassum polycystum* into meat product towards the physicochemical properties of chicken sausage.

Materials and Methods

Seaweed preparation

Seaweed *Sargassum* was supplied by the Seaweed Research Unit, University Malaysia Sabah. The seaweed is then washed to remove the epiphytes and sand dirt. After that, the seaweed was cut into 3 until 4 cm then undergoes drying processes using a cabinet dryer (Thermoline, Australia) at 45°C until the weight is consistent. Seaweed was then grounded using the warring blender (WSG 30E, China) for 5 minutes. Seaweed powder was sieve passing through a screen with an aperture of 125 µm. Seaweed powder was then put inside the airtight plastic and was kept at -20°C until use again (Pindi *et al.*, 2017).

Preparation of chicken sausage formulated with Sargassum polycystum (SP)

Four formulations of chicken sausage containing 0%, 2%, 4% and 6% of SP seaweed was produced. The composition for all formulation was chicken meat (65%), chicken fat (10%), soy protein isolate (5%) and ice water (14.90%). The following were also added to all samples: 1.5% NaCl, 0.5% Sugar, 0.05% black pepper, 0.05% chicken patty flavour and 3% potato starch. The preparation for chicken sausage was prepared as described by Pindi *et al.*, (2017). The chicken meat was thawed at 4°C for six hours before used. The chicken meat was homogenized and comminute for 1 minute in cutting meat mincer (HOBART VCB 61, Sweden). After that, salt was dissolved in ice water and the seaweed powder was added to the mixture and mixed for 1 minute. For another 1 minute, all the fats, sugar, potato starch, soy isolate and other additives were mixed with the chicken meat and again was homogenized. All the chicken meat with additives was homogenized for 3 minutes. After that, the meat batter was stuffed into cellulose casing (diameter 2.5 cm). The sausage product was cooked using a water bath (DAIHAN SCIENTIFIC Wb-6, Korea) at 80 ± 5°C for 30 minutes. Then, the cooked sausage sample was cooled in cool water (8°C) for 30 minutes before kept in 4 ± 1°C overnight for further analysis.

Proximate analysis

The moisture content was analyzed by oven method at 105°C; AOAC, 934.01, crude protein was analyzed by micro-Kjedahl, N x 6.25; AOAC, 981.10, ash was analyzed by using method AOAC, 935.05, 550°C overnight, fat was analyzed by Soxhlet extraction with diethyl ether; AOAC, 991.36 and total dietary fibre was analyzed using enzymatic-gravimetric method; AOAC, 991.42 (AOAC, 2002).

Determination of water holding capacity (WHC)

Water holding capacity was analyzed using the method of Jin et al., (2007) with some modification. Sample sausage 5.0 g was weighed into centrifugation tubes covered with Whatman No.1 filter paper and thereafter centrifuged at 5°C at low speed 1, 000 x g for 15 minutes. The WHC was determined as a liquid loss and expressed as a percentage of the weight of liquid loss. Where a is the weight before the centrifuged process (g); and b is the weight after centrifuge process (g).

$$WHC (\%) = \left(\frac{a-b}{a} \right) \times 100\% \quad (1)$$

Determination of cooking loss (CL)

Cooking loss was determined using a method of Pindi et al., (2017). Sausage samples were cooked in a water bath for 30 minutes at 80°C and then cooled for 30 minutes. The weight of the sausage sample were measured before and after cooking. CL was calculated from the differences in the weight of uncooked and cooked samples, expressed as the percentage of initial weight:

$$Cooking\ loss (\%) = \left(\frac{weight\ before - weight\ after}{Weight\ before} \right) \times 100 \quad (2)$$

Determination of color

Eight samples from each formulation were evaluated for colour analysis using a HunterLab Colorimeter (HunterLab, USA). CIE L* (Lightness), a* (redness) and b* (yellowness) (Pindi et al., 2017).

Texture analyzer

Texture profile analysis was performed in a CT3 texture analyzer (TA.XT Plus, Stable Micro System, United Kingdom) at room temperature (Pindi et al., 2017). Eight determinations per formulation of cooked samples (diameter 2.0 cm, height 2.0 cm) were compressed to 40 percent of their original height with a cylinder probe with a diameter 35 mm. Force-time deformation curves were derived with 5 kg load cell applied at a constant crosshead speed of 1.5 mm/s. The parameters derived from the force-deformation curves in a two-cycle test were; Hardness, Hd (g); Cohesiveness, Ch (dimensionless); Springiness, Sp (mm) and Chewiness, Cw (g.mm).

Sensory analysis

Sensory analysis was conducted by 40 untrained panels from a student of Faculty of Food Science and Nutrition, UMS. A sample size of 2 cm height and 2 cm diameter was prepared

on a plate. The sensory form contained six attributes namely appearance, color, aroma, taste, hardness, juiciness and overall acceptability (Aminah, 2000).

Statistical analysis

Data collected were analysed by using SPSS 21.0 software. Data were represented as a mean value \pm standard deviation and analysed using analysis of variance (one-way ANOVA) with Tukey's test at a significance level of $p < 0.05$.

Results and Discussion

Proximate composition

The proximate compositions of the chicken sausage containing SP powder are shown in Table 1. The SP powder was analyzed for content of moisture (15.93%), ash (13.65%), protein (5.31%), fat (0.51%) and total dietary fibre (40.73%). The moisture content of all sample ranged from 66.81 – 68.98% ($p < 0.05$). López-López et al., (2010) stated that sausage that contained Wakame seaweed had lower moisture because the seaweed replaced the water. Cofrades et al., (2008) obtained a similar result in gel/emulsion system added with a variety of seaweed. In the present study, the ash content increased with the addition of seaweed powder ($p < 0.05$). This was due to a high content of ash in seaweed SP powder. The same result was obtained by Choi et al., (2012) and Kim et al., (2010) for the same reason. Fat and protein content were significantly different with the control ($p < 0.05$). The addition of seaweed lowered the fat content of chicken sausage due to lower fat and high protein content from the seaweed SP. The total dietary fibre ($p < 0.05$) increased as the seaweed powder increased. Choi et al., (2012) also stated that the addition of dietary fibre into patty increased the content of dietary fibre.

Table 1 Proximate composition and dietary fibre of the sample sausage

	SP0 ¹	SP2 ²	SP4 ³	SP6 ⁴
Moisture (%)	68.98 \pm 0.05 ^a	68.76 \pm 0.03 ^b	66.95 \pm 0.07 ^c	66.81 \pm 0.03 ^d
Ash (%)	5.58 \pm 0.37 ^c	6.53 \pm 0.02 ^b	6.92 \pm 0.05 ^b	9.38 \pm 0.23 ^a
Fat (%)	17.39 \pm 0.33 ^a	13.73 \pm 0.68 ^b	12.73 \pm 0.19 ^{bc}	12.27 \pm 0.00 ^c
Protein (%)	59.22 \pm 0.76 ^c	61.52 \pm 0.64 ^b	62.43 \pm 0.30 ^{ab}	62.95 \pm 0.60 ^a
Dietary Fibre (%)	0.07 \pm 0.05 ^c	1.28 \pm 0.01 ^b	1.28 \pm 0.39 ^b	2.68 \pm 0.17 ^a

^{abcd} means different superscript within the same row differ significantly ($p < 0.05$)

¹chicken sausage with 0% SP; ²chicken sausage with 2% SP; ³chicken sausage with 4% SP; ⁴chicken sausage with 6% SP

Color

Table 2 showed the color value of chicken sausage formulated with different percentage of seaweed powder. Generally, the addition of SP powder affected the lightness (L^*), redness (a^*) and yellowness (b^*) of the chicken sausage ($p < 0.05$). According to Choi et al., (2012), *Laminaria japonica* powder lowered the lightness of pork patties. This is due to the attribute color of brown seaweed which contained a yellow pigment such as chlorophyll, phycopine, and xanthophyll. This was supported also by Kim et al., (2010) for the same reason. The changes in color are due to different seaweed species and the quantity which affected the redness value and also the pigment content in the sausage (Cofrades et al., 2008).

Table 2. The color value of chicken sausage with different levels of seaweed

	SP0 ¹	SP2 ²	SP4 ³	SP6 ⁴
L* (Lightness)	65.44 ± 1.45 ^a	42.86 ± 1.88 ^b	37.54 ± 1.44 ^c	33.53 ± 0.60 ^d
a* (redness)	0.66 ± 0.11 ^d	1.03 ± 0.12 ^c	1.34 ± 0.12 ^b	1.61 ± 0.07 ^a
b* (Yellowness)	13.18 ± 0.47 ^a	10.82 ± 0.44 ^b	10.87 ± 0.32 ^c	11.95 ± 0.14 ^c

^{abcd} means different superscript within the same row differ significantly (p<0.05)

¹chicken sausage with 0% SP; ²chicken sausage with 2% SP; ³chicken sausage with 4% SP; ⁴chicken sausage with 6% SP

Cooking loss and water holding capacity

Table 3 showed the percentage of cooking loss and water holding capacity of a chicken sausage formulated with seaweed SP. The addition of seaweed was affected by the cooking loss and water holding capacity of the chicken sausage (p<0.05). As the amount of seaweed added increased, the percentage of cooking loss and the water holding capacity was decreasing. This result was indicated that the dietary fibre of seaweed SP was affected the cooking quality and water expressible of the sausage. The same results were obtained by Pindi et al., (2017), MDCM incorporated with seaweed lowered the cooking loss whereas Kim et al., (2010) stated that water content increased as the seaweed *Laminaria japonica* was added into the meat product.

Table 3. Cooking loss and water holding capacity of chicken sausage formulated with different levels of seaweed

Sample	Cooking loss (%)	Water holding capacity (%)
SP0 ¹	5.82 ± 0.12 ^a	8.61 ± 0.25 ^a
SP2 ²	4.55 ± 0.44 ^b	6.50 ± 0.03 ^b
SP4 ³	3.68 ± 0.11 ^c	5.56 ± 0.18 ^{bc}
SP6 ⁴	2.50 ± 0.15 ^d	5.06 ± 0.93 ^c

^{abcd} means different superscript within the same row differ significantly (p<0.05)

¹chicken sausage with 0% SP; ²chicken sausage with 2% SP; ³chicken sausage with 4% SP; ⁴chicken sausage with 6% SP

Texture

Texture profile analysis indicated that the addition of the seaweed SP into chicken sausage was significantly (p<0.05) increased the hardness and chewiness compare to control sample as shown in table 4. As expected, the chewiness was directly proportional to the hardness parameter. The same result was obtained by Pindi *et al.*, (2017) which stated that the addition of *Kappapycus* seaweed at 2, 4 and 6 percent increased the hardness of the sample sausage. This was due to high dietary fibre content in seaweed SP. Generally, the seaweed improved the water and fat holding capacity for meat matrix and forming a harder and chewier texture (Cofrades *et al.*, 2017).

Table 4. Texture analyzer for sample sausage formulated with seaweed.

	Hardness (g)	Springiness	Cohesiveness	Chewiness
SP0 ¹	2451.08 ± 157.24 ^c	0.93 ± 0.01 ^a	0.78 ± 0.00 ^a	1788.61 ± 118.81 ^c
SP2 ²	5573.75 ± 215.21 ^b	0.88 ± 0.01 ^b	0.74 ± 0.04 ^a	3616.59 ± 100.78 ^{ab}
SP4 ³	6273.30 ± 281.94 ^b	0.86 ± 0.01 ^b	0.64 ± 0.04 ^b	3430.66 ± 209.51 ^b
SP6 ⁴	8079.36 ± 176.17 ^a	0.83 ± 0.02 ^c	0.62 ± 0.04 ^b	4179.21 ± 742.00 ^a

^{abc} means different superscript within the same column differ significantly (p<0.05)

¹chicken sausage with 0% SP; ²chicken sausage with 2% SP; ³chicken sausage with 4% SP; ⁴chicken sausage with 6% SP

Sensory acceptability

The addition of seaweed SP into chicken sausage influenced the sensory acceptability (Table 5). The results of the overall acceptance showed that sample control had the highest acceptability compare to sausage with seaweed (p<0.05). For the appearance and color attribute, panelist indicated the highest score for sample control and SP2 with 7.49 and 5.60 respectively. The L* value for sausage treatment and control decreased as the seaweed powder added increased (p<0.05). The panelist was more attracted to control compared to sausage with seaweed. There were significant differences among all sample for taste attribute (p>0.05). For hardness and juiciness attribute, panelists indicated the lowest score for SP6 and SP4 (p<0.05). Overall, chicken sausage with 0% of the seaweed is acceptable followed by 2% and 4% (p<0.05).

Table 5. Sensory attributes of chicken sausage with the addition of seaweed SP.

	Appearance	Colour	Taste	Hardness	Juiciness	Overall acceptance
SP0 ¹	7.49 ± 0.62 ^a	6.72 ± 1.29 ^a	7.21 ± 0.72 ^a	6.19 ± 1.31 ^a	6.52 ± 1.12 ^a	7.49 ± 0.91 ^a
SP2 ²	5.60 ± 0.64 ^b	4.94 ± 0.90 ^b	6.05 ± 0.77 ^b	6.40 ± 0.55 ^a	4.60 ± 0.47 ^b	5.92 ± 0.60 ^b
SP4 ³	4.44 ± 1.23 ^c	3.74 ± 1.19 ^c	4.68 ± 0.98 ^c	4.90 ± 0.96 ^b	4.63 ± 0.92 ^b	5.32 ± 0.50 ^c
SP6 ⁴	3.85 ± 1.22 ^d	3.60 ± 1.37 ^c	3.04 ± 1.07 ^d	3.50 ± 0.88 ^c	3.01 ± 0.92 ^c	3.46 ± 1.10 ^d

^{abcd} means different superscript within the same column differ significantly (p<0.05); ¹chicken sausage with 0% SP; ²chicken sausage with 2% SP; ³chicken sausage with 4% SP; ⁴chicken sausage with 6% SP

Conclusion

The result of this research indicates that seaweed *Sargassum Polycystum* affected the nutrient composition of chicken sausage. The lightness was affected by the presence of the seaweed *Sargassum*. Other than that, the addition of seaweed *Sargassum* influenced the water holding capacity and cooking loss of chicken sausage. Moreover, formulation of a higher percent of seaweed *Sargassum* gave a harder texture in term of hardness and chewiness compared the sample without seaweed. Sensory characteristics showed that sample control had the highest overall acceptability.

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Effect of the addition of banana peel powder (*Musa balbisiana*) in nutritional value and lipid oxidation of fish patty

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Abstract

This study was conducted to determine the nutritional value, dietary fibre content and lipid oxidation of fish patties added with banana peel powder (BPP) *Musa balbisiana* (MB). Four different fish patties formulations were produced with different concentration of BPP which composed of 0% (BPP0) (Control), 2% (BPP2), 4% (BPP4) and 6% (BPP6). In terms of nutritional values, fish patties added with BPP have shown an increase in the moisture content, fat, ash composition and crude fibre content ($p < 0.05$). However, the protein contents of fish patties were inversely proportional to the addition of BPP ($p < 0.05$). The finding also found that the dietary fibre content of fish patties was increase as the level of BPP added increased ($p < 0.05$). Nevertheless, the addition of BPP has increased the lipid oxidation in fish patties when it was stored in a 4°C refrigerator for 30 days. Overall, there are positive effects of the addition of BPP in a fish patty product in term of its nutritional values. The finding of this study can be modified for further investigations to prevent the decreasing of protein composition and reduce the occurrence of lipid oxidation in fish patties added with BPP.

Keywords: fish patty; banana peel powder; nutritional value; dietary fibre; lipid oxidation.

Introduction

Fish and fish products are one of the important sources of nutrients in the human diet and become one of the primary sources of animal protein. The increasing trend of eating away from home triggers the growth of fast food industry. Fish patty is one of the fish-based products and is acceptable fast food products by the consumers.

Banana is one of the world's highest grown fruit and is widely cultivated in tropical countries. Available data showed that in 2015, the global production of the banana record of 117.9 million tonnes (FAO, 2017). Banana peel represents 40% of the fruit is usually underutilize and end up as a waste product (Anhwange *et al.*, 2008). This becomes one of the environmental issues of the industry concern.

The recommendation of dietary fiber (DF) intake varies in different countries and agencies. In Malaysia, Ng *et al.* (2010) recommended the DF intake should be 20-30g per day. However, a recent report showed that more than 50% of Malaysian failed to reach the recommended level of DF intake (Ng *et al.*, 2010).

Lipid oxidation is one of the most important factors that limiting shelf life and threatening the quality of food products especially of those containing highly unsaturated fats. Fish

supply the polyunsaturated fatty acids, however, are very susceptible to lipid oxidation. Some of the extensive consequences of lipid oxidation in foods are a production of unpalatable flavour, shortening of shelf life, reduction of nutritional values (e.g loss of PUFAs) and risk production of unhealthy molecules (Secci and Parisi, 2016).

Meanwhile, there are increasing in the interest of using natural sources of nutritional value compound from fruit peels along with the popularity of the functional foods, as well as the food products enriched with fruit peels are been developed (Babiker *et al.*, 2013). Emaga *et al.* (2007) stated that high dietary fiber content in banana peel (about 50g/100g) is indicative of a good source of dietary fiber. Meantime, there are several claims that banana peels are rich in natural antioxidants, especially the phenolic compound (Kondo *et al.*, 2005). Hence, it can be a good source of natural antioxidants in food products.

Development of functional fish product with the addition of banana peel powder could potentially increase the value of the product in term of economic and nutritional. Ultimately, it is very cost effective because natural by-product is used as the functional ingredients. Therefore, in this study, we capitalized the use of banana peel powder to improve the nutritional properties, especially dietary fiber content and its capability to reduce lipid oxidation in a fish patty.

Materials and methods

Preparation of banana peel powder

Saba banana (*Musa balbisiana*) was purchased from the market around Kota Kinabalu, Sabah, Malaysia. Ripe bananas (from stage 5 to 7 of ripening: yellow with green tips, all yellow and all yellow with brown flecks) were chosen (Fernandes & Bonaldo, 2011). The fruits washed and the peels were separated. Peels slices were then dipped in 0.5% (w/v) citric acid solution for 10 minutes, drained and dried in drying cabinet at 60°C overnight (Alkarhi *et al.*, 2011). The dried peels were grounded and pass through 60 mesh screen to obtain banana peel powder. Then, it was stored in airtight plastic packs in cold storage (15±2°C) until analyses.

Preparation of fish patty

Four different fish patties formulations were prepared with the addition of banana peel powder at a different level (0%, 2%, 4% and 6% dry matter) as shown in Table 1. Fish surimi was purchased from Bismaraya Sdn. Bhd., Kota Kinabalu, Sabah, Malaysia. Other ingredients used were procured from local markets of Kota Kinabalu. First, surimi and salt were mixed together in a bowl mixer for 1 min then ice water was added to ensure the mixture was maintained below 15°C. The banana peels powder and other ingredients were added and mixed until homogenous. Then, the fish batter of 50g each was shaped with hamburger maker (Hamburgatrice model SA 100 Manual, Italy) and individually packed, labelled and stored at 4(±1)°C until analyses (Vanitha *et al.*, 2015).

Table 1. Fish patty formulations with different levels of banana peels powder

Ingredients	Quantity (%)		
Fish surimi	65.00		
Ice water	15.00		
Soy protein isolate	6.00		
Sunflower oil	5.00		
Potato starch	3.50		
Sugar	1.50		
Salt	1.00		
White pepper powder	0.85		
Ginger powder	0.85		
Cummin powder	0.45		
Black pepper powder	0.30		
Chicken stock powder	0.30		
Cinammon powder	0.25		
Total	100		
Banana peels powder	BPP2	BPP4	BPP6
	2%	4%	6%

Note: Formulations modified from Bainy *et al.* (2014)

¹Samples: BPP0, control (without banana peels powder); BPP2, added 2% banana peels powder; BPP4, added 4% banana peels powder; BPP6, added 6% banana peels powder.

Proximate and total dietary fibre (TDF) analysis

Compositional properties of the samples were obtained using methods by AOAC (2000). Moisture content was determined by weight loss after drying at 105°C for 12h in air drying oven. Protein content was determined by the Kjeldahl method with an automatic Kjeldahl nitrogen analyzer. Fat content was determined by Soxhlet method using solvent extraction system. Ash content was determined using a muffle furnace at 550°C overnight. Crude fibre was determined using fibre bag technique. Carbohydrate was calculated by difference. Meanwhile, TDF was determined on a sample by using enzymatic-gravimetric as outlined by AOAC (2005).

Oxidative stability (Thiobarbituric acid reactive substances)

The methodology of Ilyasoglu *et al.* (2014) was used to evaluate oxidative stability of the fish patties formulations. The measurement was carried out on the day of patty production (0) and after 7, 14, 21 and 30 days of storage at 4°C. For evaluation of malondialdehyde, 5g of a sample in 20 mL of trichloroacetic acid (TCA) diluted with ethylenediaminetetraacetic acid (EDTA) were homogenized for 2 minutes with a blender. After filtration, the supernatant was reacted with a thiobarbituric acid (TBA) solution at 95°C in a water bath for 35 min. Substance reacted with TBA were measured spectrophotometrically at 532nm. The TBARS value was expressed as mg of malonaldehyde (MDA)/kg of samples. Lipid oxidation analysis was done in triplicate.

Statistical analysis

Data were analysed using Statistic SPSS software version 20.0. One-way Analysis of Variance (ANOVA) was carried out and the Tukey HSD test was used to identify significant differences ($P < 0.05$) among formulation and storage time.

Results and Discussion

Proximate composition

The proximate composition and total dietary fiber of fish patties with the addition of banana peels at different concentration are described in Table 2. The moisture content of control patty (61.28%) was lower compared to BPP2, BPP4 and BPP6 with moisture content at 63.71%, 63.71% and 63.90%, respectively. The increment in moisture content possibly related to the presence of fiber in the product, providing high water retention to the patties (Selani *et al.*, 2016). Ash content in BPP2, BPP4 and BPP6 also shown to be higher ($p < 0.05$) than the control patty.

The protein content of the fish patty added with BPP showed to have a significantly different ($p < 0.05$). BPP6 showed a minimum protein content which is only 9.92% compared to control (16.11%). A similar result was observed by Mahmoud *et al.* (2017) in which the protein content of beef burgers decreased as it added with 7.5% orange peel powder.

Table 2. Proximate composition and total dietary fiber of fish patties with different levels of banana peels powder

Composition (%)	Mean±Standard Deviation			
	BPP0	BPP2	BPP4	BPP6
Moisture	61.28±0.35 ^b	63.71±0.01 ^a	63.71±0.21 ^a	63.90±0.04 ^a
Ash	3.03±0.05 ^b	3.17±0.04 ^b	3.61±0.21 ^a	3.83±0.06 ^a
Protein	16.11±0.22 ^a	13.41±0.18 ^b	11.18±0.01 ^c	9.92±0.06 ^d
Fat	10.98±0.40 ^a	11.03±0.94 ^a	11.70±0.23 ^a	12.12±0.13 ^a
Crude fibre	3.06±0.55 ^c	3.87±0.11 ^{bc}	4.76±0.04 ^{ab}	5.04±0.10 ^a
Carbohydrate	4.56±0.67 ^a	4.82±1.18 ^a	4.55±1.33 ^a	5.20±1.49 ^a
Total dietary fibre	8.98±0.50 ^b	9.98±0.23 ^b	12.41±0.71 ^a	13.88±0.30 ^a

¹Samples: BPP0, control (without banana peels powder); BPP2, added 2% banana peels powder; BPP4, added 4% banana peels powder; BPP6, added 6% banana peels powder

²Same superscript alphabets indicates that there is no significant different ($p > 0.05$) within same row

The crude fiber content of the control sample was found significantly ($p < 0.05$) lower in comparison to fish patties added with 4% and 6% banana peels powder. This could be possibly due to the high fiber content in BPP incorporated in the fish patties.

The fat content of fish patty was increasing with the addition of BPP but there is no significant differences shown between the treatments ($p > 0.05$).

Oxidative stability

TBARS values (mg of MDA/kg sample) as a criterion of oxidation of lipid content of fish patties during refrigerated storage for 30 days are presented in Table 3. During the storage period, a significant difference ($p < 0.05$) was observed for TBARS values of all formulations of fish patty samples with a gradual significant increase in TBARS values for all prepared fish patty samples. In the same time, an addition of banana peels powder increased the rate of TBARS values. This contrast with the report by Devatkal *et al.* (2014) that stated the banana peels extract reduced TBARS value and inhibited the oxidation in chicken patties.

There are several factors that cause the increasing of TBARS with the addition of BPP in a fish patty. According to the previous study, all drying techniques that involve heat caused 15-58% loss in phenolic compounds in comparison with freeze-dried peel (Vu *et al.*, 2018). The phenolic content also found to decrease with the ripening process (Fatemah *et al.*, 2012). Ripe banana peel has a 15-45% phenolic compound compared that in green peel (Fatemah *et al.*, 2012). Thermal processing could also change antioxidant partitioning as the lipid melts and re-solidifies as the crystallization of lipids can often cause the expulsion of minor lipid components (Berton-Carabin *et al.*, 2013).

Table 3. TBARS values of fish patties with different levels of banana peels powder during storage at 4°C

Day	TBARS (mg MDA /kg)			
	BPP0	BPP2	BPP4	BPP6
0	0.048±0.004 ^{dD}	0.069±0.013 ^{cC}	0.092±0.012 ^{eB}	0.140±0.002 ^{dA}
7	0.130±0.051 ^{cB}	0.114±0.019 ^{cB}	0.234±0.048 ^{dA}	0.247±0.068 ^{cA}
14	0.150±0.032 ^{cC}	0.229±0.084 ^{bB}	0.322±0.012 ^{cA}	0.300±0.026 ^{bcA}
21	0.259±0.003 ^{bcC}	0.294±0.066 ^{abBC}	0.386±0.001 ^{bA}	0.334±0.041 ^{abAB}
30	0.429±0.024 ^{aA}	0.320±0.028 ^{aC}	0.446±0.002 ^{aA}	0.386±0.044 ^{aB}

¹Samples: BPP0, control (without banana peels powder); BPP2, added 2% banana peels powder; BPP4, added 4% banana peels powder; BPP6, added 6% banana peels powder

²Values followed by the same small letter within the same column are not significantly different ($p>0.05$) whereas values followed by the same capital letter within the same row are not significantly different ($p>0.05$)

Conclusion

The results suggested that the incorporation of BPP in fish patty does provide the dietary fiber to the consumer. In the meantime, it can be the inexpensive dietary fiber source to be incorporated in meat products. However, further research is crucial to understand their physiological effects during storage and processing as the addition of BPP increase the lipid oxidation in a fish patty. Furthermore, more research needed to be done on how to optimize the formulation by increasing the dietary value of the meat without decreasing its protein content.

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Speciation of copper during vermicomposting process using the swine manure and cassava peel mixture with rice husk ash by *Eisenia foetida*

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Abstract

This study was conducted to evaluate the speciation of copper in swine manure when mixed with rice husk ash, cassava peel and Korat soil series under the vermicomposting process by using Tessier sequential extraction method. The result showed that the pH was reduced while the electric conductivity increased after 60 days vermicomposting. Moreover, the addition of earthworm could accelerate organic stabilization in vermicomposting. The copper in final vermicomposting were higher than the initial values and control without earthworm. Sequential extraction indicated that vermicomposting help decrease and migrate the availability of copper and the earthworm could reduce the mobile fraction, while increase the stable fraction of copper. Speciation of copper showed that copper in form organic matter bond was found highest followed by Fe/Mn Oxide bound, Residual, Carbonate bound and Exchangeable form respectively. Vermicomposting process could reduce toxicity of copper in swine manure. Copper in toxic form can change into nontoxic form. Therefore, vermicomposting process from swine manure, cassava peel, rice husk ash and Korat soil series could reduce the distribution of copper into the environment and food chain.

Keywords: swine manure, vermicomposting, earthworms

Introduction

The intensive swine farming has developed rapidly in recent two decades. As a consequence, the amount of swine manure increases significantly. Swine manure has traditionally been used as land fertilizer. However, swine manure is moderately to heavily polluted with heavy metals, due to feed additives overuse (Li *et al.*, 2010). Continuous heavy manuring may pose environmental problem, such as odor, pollution of ground and surface waters due to leaching and run-off of organics and nutrients, and soil accumulation of heavy metals (Lu *et al.*, 2014). Vermicomposting is a novel technique that utilizing the joint action of earthworm and microorganism under aerobic condition. In this method, most of the organic components can be degraded and the residuals are transformed into stabilized vermicompost which is rich of nitrogen, phosphorus, potassium and humic substance (Song *et al.*, 2014; Lv *et al.*, 2016). During this process, complex interaction among the organic waste, microorganism and earthworm could result in the stabilization some element in wastes (Song *et al.*, 2014). Total

metal concentration is an important indicator of pollution risks, but it provides little or no indication of their speciation bioavailability or mobility. The knowledge of metal speciation may be useful to assess the potential environmental effects as metal associated with different fraction have different impacts on the environment (Lu *et al.*, 2014). The sequential extraction procedure could provide the understanding of the natures of metal fraction and allow the prediction of metal mobility or bioavailability. The present study aimed the variation speciation of Copper in raw substrates and the final vermicompost during vermicomposting process using the swine manure and cassava peel mixture with rice husk ash by *Eisenia foetida* and evaluate the effect of vermicomposting on mobility and bioavailability of Cu.

Materials and Method

Substrates and vermicomposting

Materials used in the experiment included swine manure, cassava peel, rice husk ash and Korat soil series. Swine manure was procured from a livestock farm in Khon Kaen province, northeast of Thailand. The raw manure was dried in ambient temperature around 15 days with periodic stirring before the experiment. The basic physico-chemical properties of all the three raw materials were analyzed (Table 1).

Table 1. Chemical properties of raw materials used for experiment.

Parameter	Swine manure	Cassava peel	Rice husk ash
1. pH (1:10)	7.4	6.7	8.5
2. EC (1:10; ds/m)	2.2	0.5	0.2
3. Total Nitrogen (%)	2.3	0.3	0.2
4. Total Phosphate (%)	9.4	0.04	1.4
5. Total Potash (%)	1.3	0.4	0.6
6. Organic Carbon (%)	30.6	17.8	3.5
7. Organic Matter (%)	52.7	29.8	5.9
8. C/N	13/1	59/1	17.5/1
9. Copper (Cu; mg/kg)	731.7	5.0	24.5
10. Ferrous (Fe; mg/kg)	2,594.1	2,939.4	956.2
11. Manganese (Mn; mg/kg)	1,162.7	133.8	954.9

The earthworm species *Eisenia foetida* was used for the vermicomposting. Vermicomposting was carried out in plastic container with each 200 g mixtures of swine manure, cassava peel, Korat soil series and rice husk ash ratio 2:6:1:0 and 2:6:1:1 respectively, an optimal of 1.60 kg worm/m² was adopted as worm stocking density (Ndegwa *et al.*, 2000). Three replicates were established for each reactor and the container without earthworm and rice husk ash were conducted as control. The vermicomposting container were kept in room temperature and moisture content of mixtures in each container was kept at 70-90 °C of water holding capacity by periodic sprinkling of distilled water. The sample collected at initial (0 day) and the end of experiment (60 days) from vermicomposting and control were air-dried, ground and sieved for further analysis.

Physico-chemical properties analysis

All the sample of pre- compost mixture, compost and vermicompost were homogenously mixed for analyses of pH, electric conductivity (EC), organic matter (OM), total nitrogen

(TN), total phosphorus (TP), total potassium (TK) and total copper. The pH and EC was determine with 1:10 dry soil-water suspension. The organic matter was measured by Walkley and Black (1934). TN was measured by micro-Kjeldhal method. TP, TK and Cu were determined by digested with 10 ml of mixed HNO₃:HClO₄ (1:1), the digests and extracts were detected, TP by Spectrophotometric molybdovanadophosphate method, TK by absorption spectrophotometer (AAS) and Cu by Inductively coupled plasma emission spectrometer (ICP-OES).

Sequential extraction

Sequential extraction recommended by Tessier *et al.* (1979) was used to determine the speciation of Cu in samples. Cu was divided into five fractions include (F1) exchangeable, (F2) bound to carbonate, (F3) bound to Iron and Manganese oxide, (F4) bound to organic matter and (F5) Residual. The extractant, experimental condition and nature of each Cu fractions were show in Table 2. After centrifugation and filtration, the supernatant from each extraction was analyzed by Inductively coupled plasma emission spectrometer (ICP-OES).

Statistical analysis

All results were presented as the average of three replicates. The Cu contents were expressed on dry weight basis. One-way ANOVA following Statistic 10 was used to identify the significant differences between final product with or without earthworm by least significance difference (LSD). The probability level used for statistical significance was at $p < 0.05$ for all test.

Table 2. The extractants, experimental condition and characteristics of various extractable Cu forms with sequential extraction.

Speciation	Extractant	Environmental Condition	Characteristics
F1: exchangeable	1.0 M NaOAc (pH = 8.2)	With agitation at 3,500 rpm for 1 h at 25 °C	Readily influenced by change of ionic composition in the liquid.
F2: bound to carbonate	1.0 M NaOAc (pH = 5.0)	With agitation at 3,500 rpm for 5 h at 25 °C	Susceptible to pH variations.
F3: bound to Fe/Mn oxide	0.04 M NH ₂ OH.HCl in 25% HOAc (v/v)	6 h at 96±3°C in water bath with occasional agitation	Unstable in reductive conditions.
F4: bound to organic matter	- 0.02 M HNO ₃ , 30% H ₂ O ₂ . - After 2 h, 30% H ₂ O ₂ was added. -After cooling, 3.2 MNH ₄ Oac in 20% HNO ₃ (v/v) was added.	-2 h at 85±2°C -3 h at 85±2°C with occasional agitation -agitation for 30 min at 25 °C	Decomposes and change under the oxidizing condition.
F5: Residual	HNO ₃ : HClO ₄ (1:1) digestion procedure		Fixed in crystal lattice and not enter the food chain.

Results and Discussion

Physico-chemical properties of pre-compost mixture, compost and vermicompost

The compost (i.e without earthworm) and vermicompost (i.e. with earthworm) at final sampling significantly differed from the initial pre- compost mixture in term of pH and EC. There was increase in pH value in both compost and vermicompost as compared to that of initial pre- compost mixture (Table 3). Possible contribution to the changes in pH could be due to the production of CO₂, NO₃⁻N and organic acids during waste mineralization in composting and vermicomposting process (Nuhaa *et al.*, 2015; Song *et al.*, 2014; Suthar, 2010). Both compost and vermicompost significant decrease the EC compared to the initial pre- compost mixture, the change in EC of compost and vermicompost might have been due to loss of organic matter and release of different mineral salts in available forms such as phosphate, ammonium, potassium, etc. (Gupta and Garg, 2008).

Table 3. Physico-chemical properties of pre-compost mixture, compost and vermicompost.

Properties	Mixture 1			Mixture 2		
	Pre-compost	Compost	Vermicompost	Pre-compost	Compost	Vermicompost
pH(1:10)	7.56f	8.21a	8.09b	7.62e	7.94c	7.71d
EC(1:10; ds/m)	1.056c	0.938e	0.931e	1.12a	1.013d	1.102b
OM (%)	29.39a	20.72b	17.29b	26.86a	19.78b	16.85b
TN (%)	0.59a	0.64a	0.57a	0.60a	0.63a	0.54a
TP (%)	0.92cd	1.03bc	1.18a	0.89d	1.10ab	1.20a
TK (%)	0.64c	0.75b	1.08a	0.56c	0.78b	0.80b

Different letters in in the same row are statistically different (LSD test, p< 0.05)

Mixture 1: Swine manure: Cassava peel: Soil: Rice husk ash (2:6:1:0)

Mixture 2: Swine manure: Cassava peel: Soil: Rice husk ash (2:6:1:1)

Total contents of Copper

Total contents of Copper increased after the treatment with and without earthworm in comparison to initial values (Figure 1). Two pathways could affect the Cu content in vermicomposting process, the bioaccumulation of earthworm, volume reduction caused by organic decomposition. The weight and volume reduction resulted by mineralization and decomposition of organic matter during vermicompost could concentrate and increase the heavy metal (Lv *et al.*, 2016).

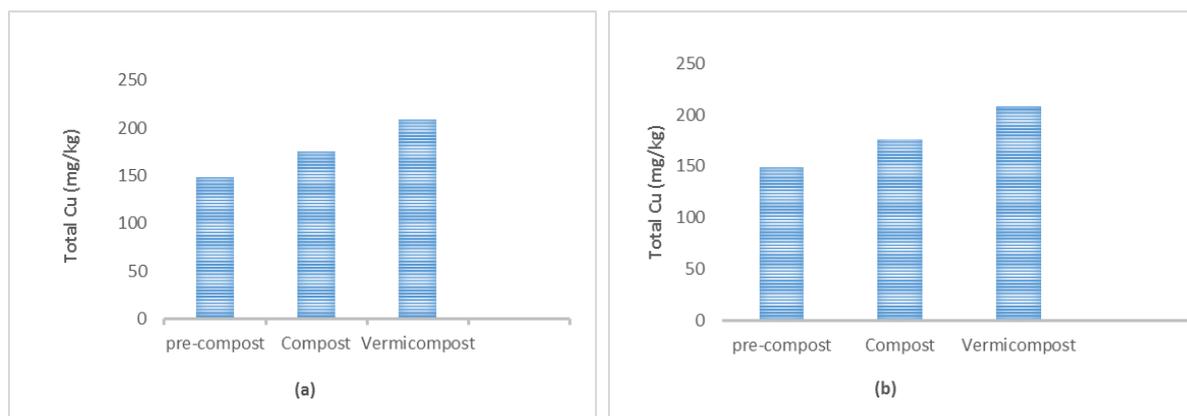


Figure 1. Total concentration of copper in initial mixture, compost and vermicompost;
 (a) Mixture 1: Swine manure: cassava peel: soil: rice husk ash (2:6:1:0)
 (b) Mixture 2: Swine manure: cassava peel: soil: rice husk ash (2:6:1:1)

Chemical speciation of Copper

Chemical speciation of Cu in initial mixture, compost and vermicompost as show in Figure 2. The treatment with and without earthworm in Mixture 1 (Swine manure: cassava peel: soil: rice husk ash, 2:6:1:0) and Mixture 2 (Swine manure: cassava peel: soil: rice husk ash, 2:6:1:1) had similar trend for decreased the F1 and F2 fraction, while increased the F4 and F5 fraction. Vermicomposting process in Mixture 1, earthworm cloud reduced F1 fraction from 20.96 to 9.13 mg/kg, F2 fraction from 16.5 to 14.83 mg/kg and F3 fraction from 52.55 to 49.29 mg/kg but increased F4 fraction from 46.54 to 102.71 mg/kg and F5 fraction from 6.35 to 8.50 mg/kg respectively. In Mixture 2, earthworm cloud reduced F1 fraction from 20.99 to 8.15 mg/kg, F2 fraction from 14.50 to 9.83 mg/kg but increased and F3 fraction from 52.35 to 61.59 mg/kg, F4 fraction from 54.91 to 115.39 mg/kg and F5 fraction from 5.86 to 13.30 mg/kg respectively (Table 4).

Table 4. Speciation of Cu in pre-compost mixture, compost and vermicompost.

Speciation of Cu	Cu (mg/kg)					
	Mixture 1			Mixture 2		
	Pre-compost	Compost	Vermicompost	Pre-compost	Compost	Vermicompost
F1: exchangeable	20.96a	9.74b	9.13b	20.99a	8.44b	8.15b
F2: bound to carbonate	16.50a	17.09a	14.83b	14.50b	10.22c	9.83c
F3: bound to Fe/Mn oxide	52.55b	66.19a	49.29a	52.35b	50.58b	61.59a
F4: bound to organic matter	46.54d	88.15c	102.71b	54.91d	97.2bc	115.39a
F5: Residual	6.35c	8.13b	8.50b	5.86c	9.03b	13.30a

Different letters in in the same row are statistically different (LSD test, $p < 0.05$)

Mixture 1: Swine manure: Cassava peel: Soil: Rice husk ash (2:6:1:0)

Mixture 2: Swine manure: Cassava peel: Soil: Rice husk ash (2:6:1:1)

Vermicomposting significantly reduced the exchangeable fraction of Cu in both of the two mixture (Figure 2). The uptake into earthworm body might be one reason for this reduction. *E. foetida* did absorb Cu and this bioaccumulation had strong positive correlation with the concentration of exchangeable fraction (Li *et al.*, 2010; Lv *et al.*, 2016). Besides, vermicomposting result in slightly lower F1 fraction and highest among of F4 and F5 fraction compared to the control. It is the report that Cu could induce the synthesis of metallothionein isoform in earthworm intestines, then bind metal ions by forming organo-metallic ligands and thus reduce the exchangeable fraction of Cu. Earthworm enhanced F4 fraction as relative to the no-worm control. An increase of cu bound into organic matter was also reported for sewage sludge and water hyacinth (Lv *et al.*, 2016)

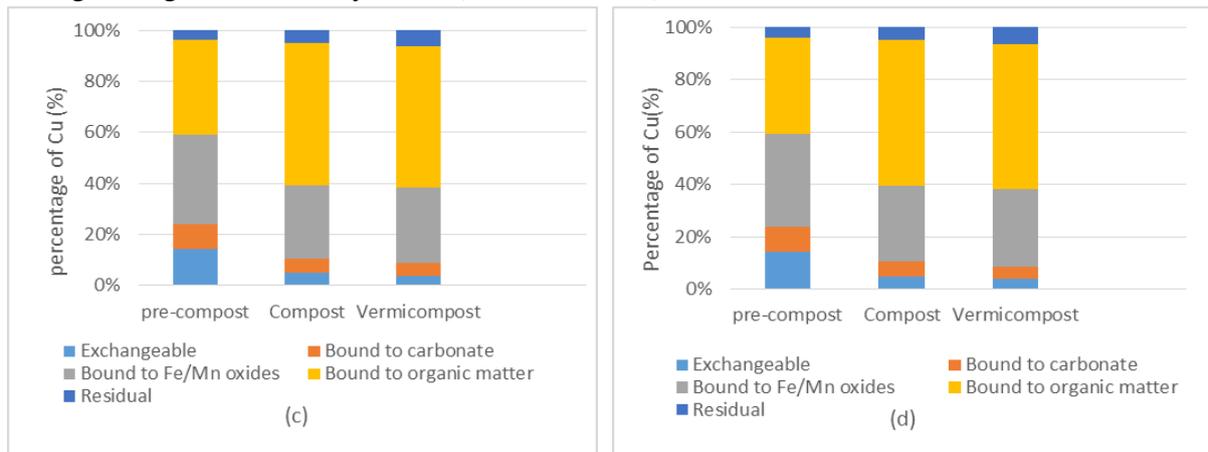


Figure 2. Speciation of Cu in in initial mixture, compost and vermicompost
(c) Mixture 1: Swine manure: cassava peel: soil: rice husk ash (2:6:1:0)
(d) Mixture 2: Swine manure: cassava peel: soil: rice husk ash (2:6:1:1)

Conclusion

In all, we concluded from this studied that the accumulation by earthworm and changes on physico-chemical properties in substrate could affect the transformation of Cu during vermicomposting and alleviate the mobility and availability of Cu finally. Vermicomposting process could reduce toxicity of copper in swine manure. Copper in toxic form can change into nontoxic form. Therefore, vermicomposting process from swine manure, cassava peel, rice husk ash and Korat soil series could reduce the distribution of copper into the environment and food chain.

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Phenolic, flavonoid content and DPPH scavenging activity of fruits, leaves and twig of *Garcinia forbesii* King (Assam aroi-aroi), the red mangosteen.

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Abstract

Garcinia forbesii King or locally known as “Assam aroi-aroi” or Brunei cherry is a small tree which is scattered throughout the lowlands of Malaysia. The skin of the aroi-aroi fruit were sliced and dried and being used in cooking to give a sour taste in local cuisines. The dried aroi-aroi were also used as medicine to treat women after childbirth, stomach ache and a cough relief, a traditional practice to cure many illnesses and diseases and to maintain general health. In South Kalimantan, it was called red mangosteen but the fruit is rarely eaten. The fruit has white flesh with a sweet flavour and fresh red rind with strong sour taste. The present research showed that the plant is not popular compared to the other *Garcinia* species. The objectives of this research are to study the phenolic and flavonoid content and DPPH scavenging activity of different plant parts (fruits; whole fruits, unripe rind, unripe flesh, ripe rind, ripe flesh, young leaves, mature leaves and twig) extracts of *Garcinia forbesii*. The highest antioxidant capacity and highest phenolic and flavonoid contents were found in twig extracts (87.73 – 91.05 %) scavenging activity using standard ascorbic acid at the concentration of 0.015 to 1.0 mg/ml in methanol), followed by mature leaves, young leaves and non-polar extracts of the fruits. Lower antioxidant capacity in young leaves compared to mature leaves and higher antioxidant capacity in ripe fruits when compared to unripe condition. The higher content of phenolic compounds and total flavonoids of some parts of plants sample indicated that these compounds contribute to the antioxidant activity which can be further studied in the identification of bioactive compounds.

Keywords: *Garcinia forbesii* King, Assam aroi-aroi, Antioxidant, red mangosteen.

Introduction

Garcinia forbesii King are belonging to the genus *Garcinia*, a member of the Guttiferae which consist about 400 species concentrated mainly in south-east Asia, India and West Africa. Some occur as shrubs and usually produce hard timber (Jabit *et al.*, 2009; Corner, 1988). Most of the members are found to be rich in source of xanthenes which had chemotaxonomic interest (Harrison *et al.*, 1993). Phytochemical studies of this genus are commonly reported also to contain benzophenones, triterpenes, biflavonoids and benzoquinone (Waterman and Hussain, 1983). Previous studies also found that this genus contains bioactive constituents which showed activity towards antimicrobes, anticancer, antioxidant and anti-diabetic as well (Alen *et al.*, 2008). The most famous plant of this genus

and widely used in Malaysia, *Garcinia atroviridis* or known as asam gelugur exhibited strong antioxidant properties and help in lowering the progression of atherosclerosis by reducing the oxidative stress (Amran *et al.*, 2010) and CETP inhibition (Abdul Sani *et al.*, 2016).

In Malaysia, *Garcinia forbesii* King are scattered throughout the lowlands region and tropical region of Thailand. In Indonesia, the plants are scattered in region of Sumatera, Java, Kalimantan, Sulawesi, Maluku and Papua. The plants are known as entelang, ete, gendis, kandis, kumanjing, mundar/bundar, manggis merah or manggis hutan by the people in Kalimantan, while known as kundong or walung by the Javanese and kiceuri by Sundanese. Other names are asam aur-aur (Brunei); asam aroi-aroi, kundung, yellow kandis (Sabah, Malaysia) and Brunei cherry (America).

The phytochemistry among the popular *Garcinia* species, such as *Garcinia mangostana* Linn. and *Garcinia kola* Heckel has been extensively investigated, but only a few studies involving other less popular species including *Garcinia forbesii* King (Jabit *et al.*, 2009).

Materials and Methods

Plant Materials

The plant samples, *Garcinia forbesii* King (fruits, leaves and twig) were collected from Jalan Masjid Kampung Tengah, Bongawan. The location coordinates are 5°32'04.0"N 115°51'29.7"E. The sample was identified by botanist, Mr. Jonhhny Gisil from Institute for Tropical Biology and Conservation (ITBC) and the voucher specimen S.AHMAD 00001 was deposited in herbarium BORNEENSIS, Universiti Malaysia Sabah in December 2017. Different parts of *Garcinia forbesii* (fruits, leaves and twig) were cut into small pieces and air dried at room temperature (37- 40 °C). The samples then ground into powdered form.

Extraction

The extractions were conducted through cold maceration using organic solvent with different types of polarity (hexane, chloroform, ethyl acetate and methanol). Dried samples were soaked in different polarities of solvents including hexane, chloroform, ethyl acetate and methanol to obtain different polarities of crude extracts. The ratio of extractions was 1:10 which means 10 g of samples were soaked in 100 ml of solvent. The maceration processes were done for 3 times and each time took 3 days or more. Then the solvent extracts were filtered and the filtrates were placed in the rotary evaporator (50 – 60 °C) to remove residual solvents and concentrate the crude samples.

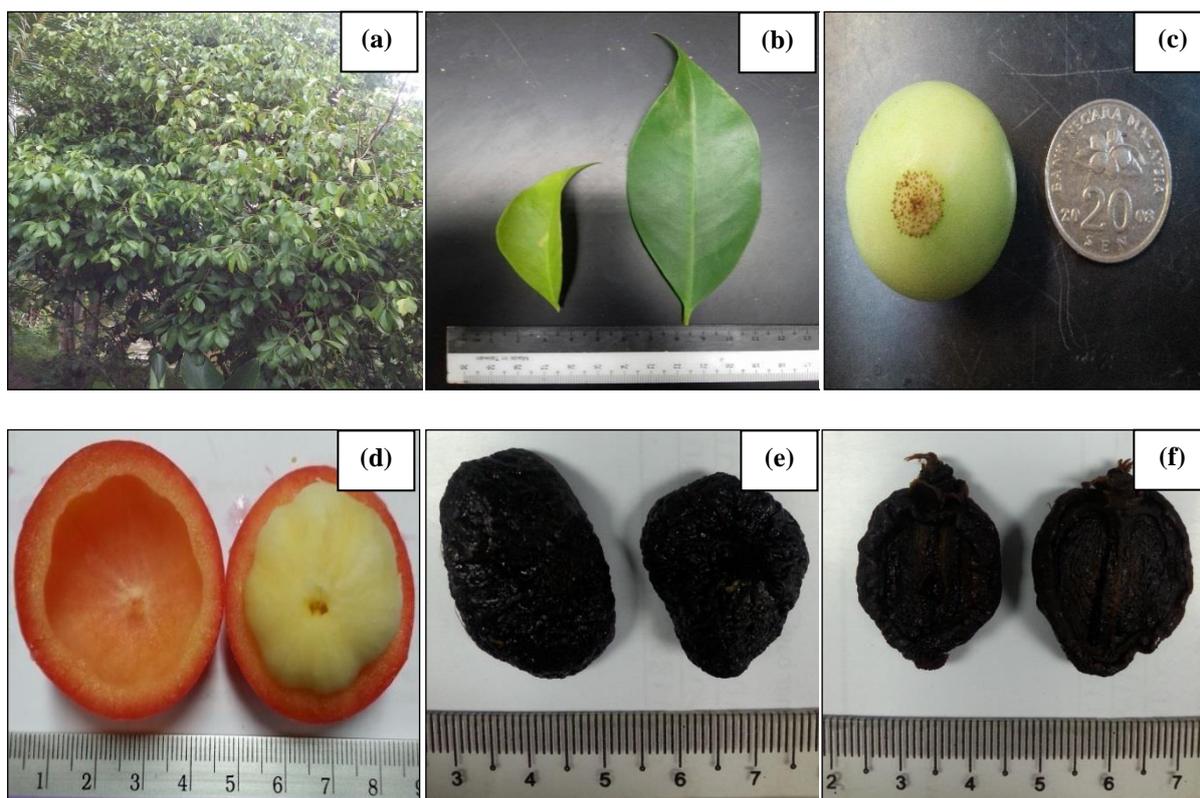


Figure 1. *Garcinia forbesii* tree (a), leaves (young and mature) (b), unripe fruits (c), ripe fruits (d), dried rind (e) and dried fruits (f).

Total Phenolic Content (TPC)

The total phenolic content (TPC) was determined by using colorimetric Folin-Ciocalteu method of Velioglu *et al.* 1998. 100 μ l (1000 μ g/ml) of extract was mixed with 0.75 ml of Folin-Ciocalteu reagent from Merck. It was vortex and allowed to stand at 22°C for 5 minutes. Then, 0.75 ml of Na₂CO₃ (60 g/L) solution was added to the mixture. It was then left undisturbed for 90 minutes at 22°C. The absorbance was measured at 760 nm using UV spectrophotometer. Standard graph was prepared by using different concentration of gallic acid (125, 250 and 1000 μ g/ml).

Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was measured by aluminium chloride colorimetric assay as stated by Zhishen *et al.*, 1999. Then, 500 μ l of extract was prepared with concentration of 100 mg/ml. 2.25 ml distilled water was added into the extract and followed by the addition of 150 μ l of NaNO₂. At 5th minute, 300 μ l of 10% AlCl₃ was added. After 6 minutes later, 300 μ l of 1 M NaOH was mixed with the solution. The absorbance was measured immediately against prepared reagent blank at 510 nm by using UV spectrophotometer (Kamtekar *et al.*, 2014). Standard graph was prepared by using different concentration of quercetin (125, 250 and 1000 μ g/ml).

DPPH assays

The scavenging activities of the crude extracts were determined by using 1, 1-diphenyl-2- pycrylhydrazyl (DPPH) for all samples (fruits; whole fruits, unripe rind, unripe flesh, ripe rind, ripe flesh, young leaves, mature leaves and twig). Stock solutions of fruits; whole fruits, unripe rind, unripe flesh, ripe rind, ripe flesh, young leaves, mature leaves and twig extracts were prepared using methanol with concentration of 10 mg/mL, and 1 mg/mL for pure compounds. A series of tested solutions with different concentrations were prepared by two fold dilution in 96-well plates (1000, 500, 250, 125, 62.5, 31.25 and 15.63 $\mu\text{g/mL}$) using methanol as solvent. Each well was added with 100 μL DPPH reagent which was freshly prepared. Then the plate was shaken gently before left in the dark for 45 minutes at room temperature. The absorbance of mixture was determined at 517 nm using UV spectrophotometer (Ekaprasada *et al.*, 2009). The IC_{50} was determined by plotting percentage of inhibition against concentration range from 500 to 15.63 $\mu\text{g/mL}$ of the sample. The IC_{50} value was defined as the inhibition concentration of sample to reduce 50% the absorbance of DPPH. All of the assays were conducted in triplicates and the average readings were calculated. The absorbance value was converted to percentage of inhibition by applying the formula:

$$\% \text{ scavenging activity} = \frac{[(\text{Abs}_{\text{sample+DPPH}}) - (\text{Abs}_{\text{sample blank}})]}{[(\text{Abs}_{\text{DPPH}}) - (\text{Abs}_{\text{Solvent}})]} \times 100 \quad \text{-----Expression (1)}$$

Results and Discussion

Determination of total phenolic content

The total phenolic content for different plant parts (fruits; whole fruits, unripe rind, unripe flesh, ripe rind, ripe flesh, young leaves, mature leaves and twig) extracts of *Garcinia forbesii* were estimated by Folin Cioaltea's method using gallic acid as standard. The reagent is formed from a mixture of phosphotungstic acid and phosphomolybdic acid which after oxidation of the phenols are reduced to a mixture of blue oxides of tungsten and molybdenum. The blue coloration produced has a maximum absorption in the region of 760 nm and proportional to the total quantity of phenolic compounds present in the tested samples (Kamtekar *et al.*, 2014). The gallic acid with concentrations (125 -1000 ppm) conformed to the Beer's Law with a regression co-efficient (R^2)=0.999. The plot of graph has a slope $m=0.003$ and intercept 0.045 as the equation of standard curve is $y= 0.003x + 0.045$ (Figure 2). The summary of total phenolic content were tabulated in Table 1. From the data the highest content of phenolic compounds present in the sample of ethyl acetate extracts of the twig ($328.44 \pm 0.73 \mu\text{g GAE/mg}$), followed by ethyl acetate extract of mature leaves ($211.78 \pm 1.62 \mu\text{g GAE/mg}$) and the young leaves ($156.11 \pm 0.96 \mu\text{g GAE/mg}$).

The total phenolic content for fruits part (Figure 3) can be concluded that ripe fruits contained more phenolic compounds rather than unripe fruits. Flesh parts are higher in phenolic content than rind part. Non polar parts (hexane and chloroform) of extracts more contained phenolic compounds rather than polar extracts (ethyl acetate and methanol). Meanwhile, less significance number of phenolic content showed by ethyl acetate extracts for all fruit parts.

The total phenolic content for leaves part (Figure 4: young and mature leaves) can be concluded that ethyl acetate extract of mature leaves showed highest phenolic content ($211.78 \pm 1.62 \mu\text{g GAE/mg}$) compared to ethyl acetate extract of the young leaves ($156.11 \pm 0.96 \mu\text{g GAE/mg}$). Meanwhile, less significance number of phenolic content showed by the hexane and chloroform extracts (Table 1). This can be concluded that mature leaves contained higher phenolic compound than the young leaves.

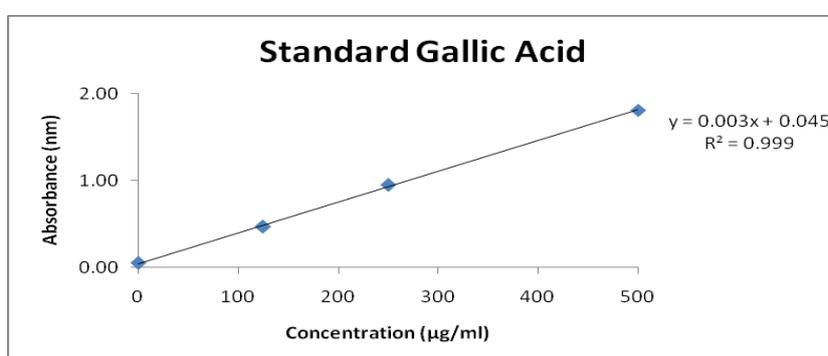


Figure 2. Total phenolic content for standard gallic acid

Table 1. The summary of DPPH activity, TPC and TFC content of the samples.

Samples/Extracts		DPPH IC ₅₀ (µg/mL)	TPC ± SD (µg GAE/mg)	TFC ± SD (µg QE/mg)
Fruits				
Ripe rind (RR)	Hexane	251.96 ± 0.91	48.67 ± 0.09	51.33 ± 0.53
	Chloroform	211.84 ± 1.93	34.09 ± 0.87	36.67 ± 0.76
	Ethyl Acetate	>300	3.33 ± 0.69	4.00 ± 1.21
	Methanol	>300	10.56 ± 0.91	3.22 ± 2.01
	Hexane	274.85 ± 0.96	27.00 ± 0.83	29.67 ± 0.91
Unripe rind (UR)	Chloroform	267.93 ± 0.84	25.44 ± 0.76	28.11 ± 0.63
	Ethyl Acetate	>300	2.67 ± 0.99	4.33 ± 1.34
	Methanol	>300	1.56 ± 1.06	3.00 ± 0.97
	Hexane	248.04 ± 0.66	89.56 ± 0.95	92.22 ± 0.89
	Chloroform	219.37 ± 0.12	45.01 ± 0.98	47.67 ± 0.67
Ripe flesh (RF)	Ethyl Acetate	>300	11.00 ± 1.07	13.67 ± 1.13
	Methanol	>300	23.89 ± 1.23	26.56 ± 0.92
	Hexane	269.57 ± 0.72	77.67 ± 1.50	83.11 ± 0.73
	Chloroform	255.84 ± 0.92	24.56 ± 0.94	34.44 ± 0.85

Unripe flesh (UF)	Ethyl Acetate	>300	5.22 ± 0.99	8.11 ± 1.02
	Methanol	>300	16.56 ± 1.01	13.22 ± 1.11
Samples/Extracts		DPPH IC ₅₀ (µg/mL)	TPC ± SD (µg GAE/mg)	TFC ± SD (µg QE/mg)
Leaves				
Shoots (YL-young leaves)	Hexane	>300	50.56 ± 1.31	311.30 ± 1.24
	Chloroform	>300	56.89 ± 1.24	464.93 ± 1.09
	Ethyl Acetate	257.67 ± 0.58	156.11 ± 0.96	482.32 ± 1.33
	Methanol	>300	99.89 ± 0.92	270.73 ± 0.95
Mature leaves (ML)	Hexane	>300	28.67 ± 0.86	380.87 ± 1.71
	Chloroform	>300	42.56 ± 0.88	506.96 ± 0.98
	Ethyl Acetate	154.47 ± 0.75	211.78 ± 1.62	812.75 ± 0.77
	Methanol	>300	89.67 ± 1.72	324.35 ± 0.94
Twig				
	Hexane	50.63 ± 0.97	147.78 ± 0.47	763.48 ± 0.84
	Chloroform	49.34 ± 1.13	142.33 ± 0.56	712.75 ± 0.95
	Ethyl Acetate	30.76 ± 0.98	328.44 ± 0.73	846.09 ± 0.99
	Methanol	>300	116.00 ± 0.95	215.65 ± 1.03

**DPPH IC₅₀: < 10 µg/mL: Highly active, 10-50 µg/mL: Active, 50 to 150 µg/mL: Moderately active, 150 to 300 µg/mL: Weakly active, > 300 µg/mL: Inactive. **TPC & TFC content compared to standard gallic acid and quercetin at 1000 µg/mg.

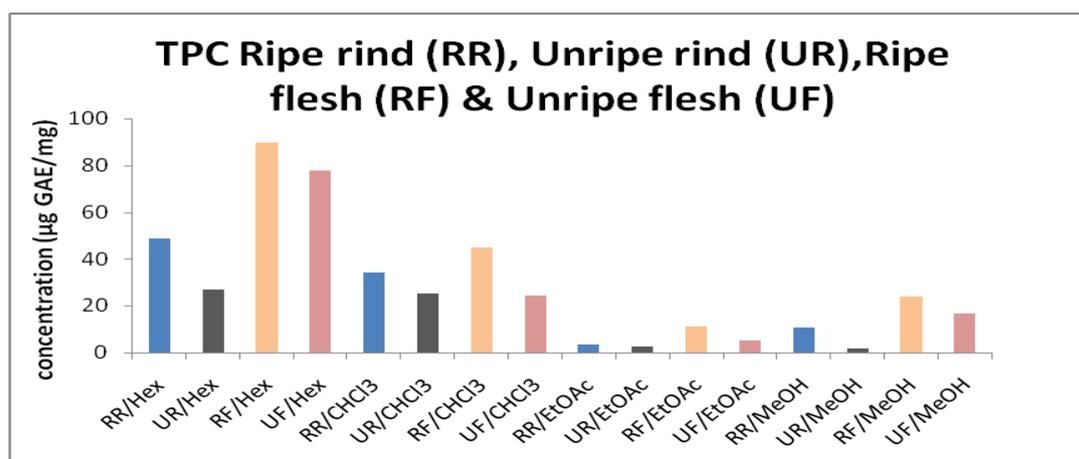


Figure 3. Total phenolic content for part of fruits (RR) Ripe rind, (UR) Unripe rind, (RF) Ripe flesh and (UF) Unripe flesh.

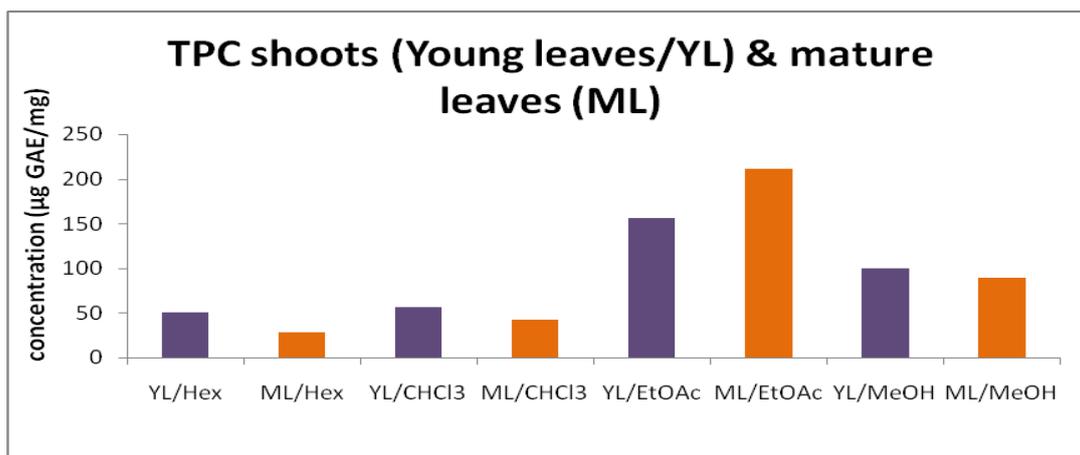


Figure 4. Total phenolic content for part of leaves (YL) young leaves and (ML) mature leaves.

For twig extracts (Figure 5), ethyl acetate showed the highest content of phenolic compound ($328.44 \pm 0.73 \mu\text{g GAE/mg}$), followed by hexane extract ($147.78 \pm 0.47 \mu\text{g GAE/mg}$), chloroform extract ($142.33 \pm 0.56 \mu\text{g GAE/mg}$) and methanol extracts ($116.00 \pm 0.95 \mu\text{g GAE/mg}$). Twig extracts showed the highest phenolic content compared to leaves and fruits part.

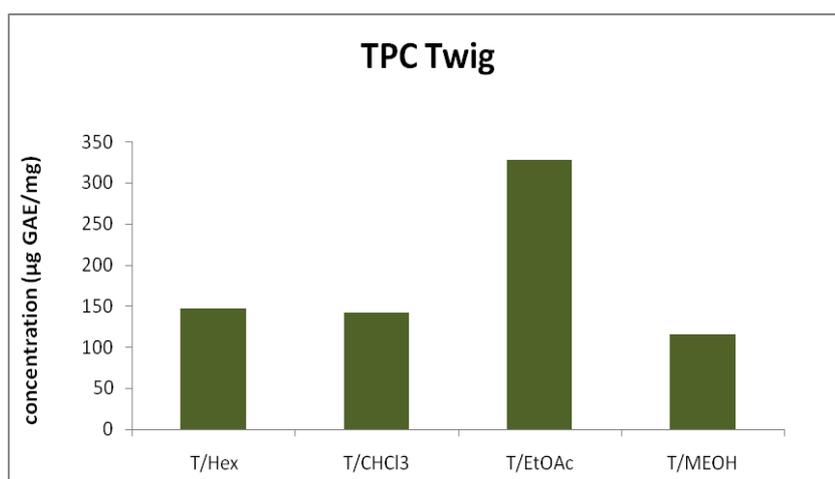


Figure 5. Total phenolic content for part of Twig (T).

Determination of total flavonoid content

The total flavonoid content for different plant parts (fruits; whole fruits, unripe rind, unripe flesh, ripe rind, ripe flesh, young leaves, mature leaves and twig) extracts of *Garcinia forbesii* were estimated by aluminium chloride colorimetric assay using quercetin as standard (Figure 6). Aluminium chloride forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxide group of flavones and flavonols. It also forms liable complexes with ortho dihydroxide groups in A/B rings of flavonoids. The quercetin concentration of 125 - 1000 ppm conformed the Beer's Law at 510 nm with a regression co-efficient (R^2) = 0.98812. The plot has slope (m) = 0.00023 and intercept at 0.0074. The equation of standard curve is $y=0.00023x + 0.0074$ (Figure 5). The summary of total flavonoid content were tabulated in Table 1. From the data the highest content of flavonoid compounds present in the sample of

ethyl acetate extracts of the twig ($846.09 \pm 0.99 \mu\text{g QE/mg}$), followed by ethyl acetate extract of mature leaves ($812.75 \pm 0.77 \mu\text{g QE/mg}$) and the young leaves ($482.32 \pm 1.33 \mu\text{g QE/mg}$).

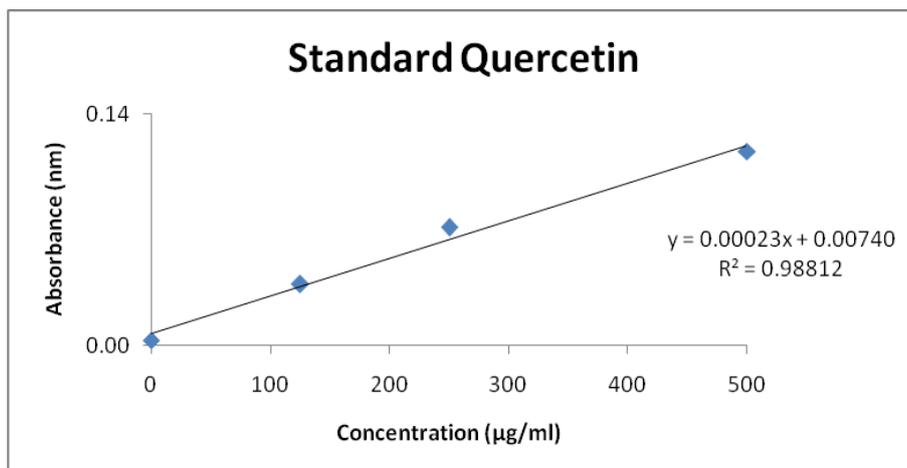


Figure 6. Standard calibration curve for standard quercetin.

The total flavonoid content for fruits part (Figure 7) can be concluded that ripe fruits contained more flavonoid compounds rather than unripe fruits. Flesh parts are higher in flavonoid content compared to rind part. Hexane and chloroform extracts are higher in flavonoids content compared to extracts of ethyl acetate and methanol.

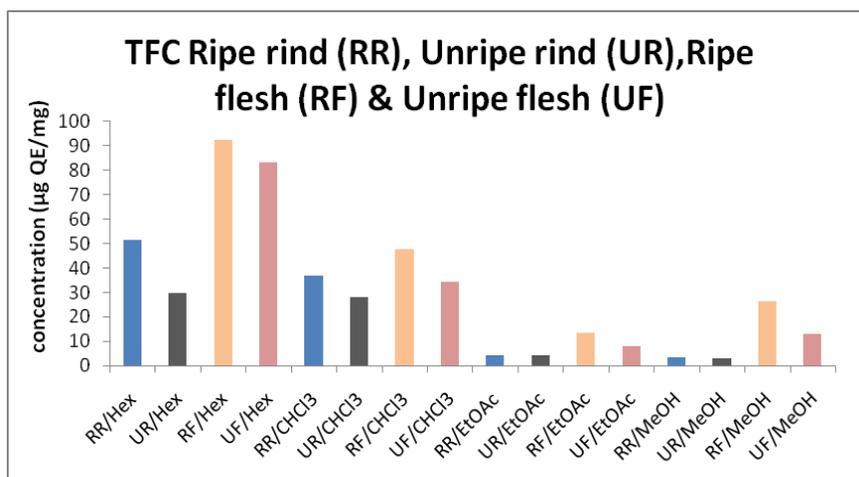


Figure 7. Total flavonoid content for part of fruits (RR) Ripe rind, (UR) Unripe rind, (RF) Ripe flesh and (UF) Unripe flesh.

The total flavonoid content for leaves part (**Figure 8**: shoots and mature leaves) can be concluded that ethyl acetate extract of mature leaves showed highest flavonoid content ($812.75 \pm 0.77 \mu\text{g QE/mg}$) compared to ethyl acetate extract of the shoots ($482.32 \pm 1.33 \mu\text{g QE/mg}$). Chloroform extracts also showed high flavonoid content, followed by hexane extracts and methanol.

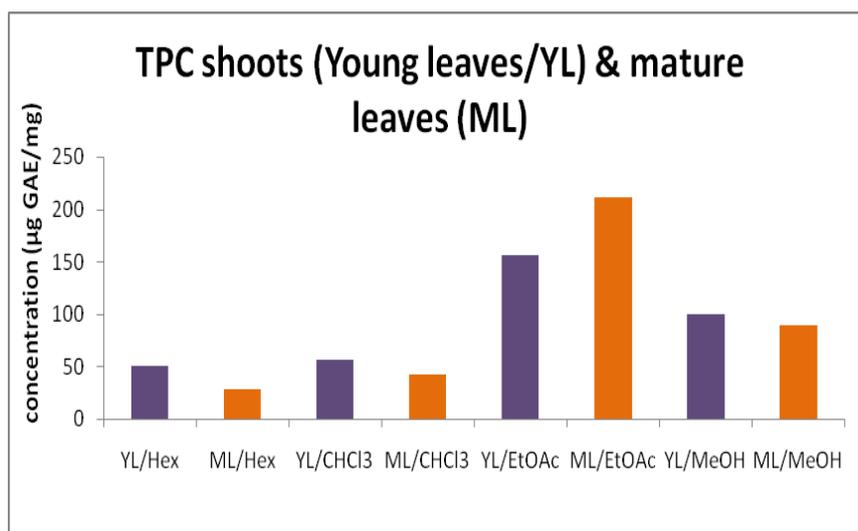


Figure 8. Total flavonoid content for part of leaves (YL) young leaves/shoots and (ML) mature leaves.

For twig extracts (**Figure 9**), ethyl acetate showed the highest content of flavonoid compound ($846.09 \pm 0.99 \mu\text{g QE/mg}$). Hexane extract showed high content of flavonoid ($763.48 \pm 0.84 \mu\text{g QE/mg}$) followed by chloroform extract ($712.75 \pm 0.95 \mu\text{g QE/mg}$). Methanol extract showed the lowest flavonoid content for the twig extracts ($215.65 \pm 1.03 \mu\text{g QE/mg}$). Twig extracts showed the highest flavonoid content compared to leaves and fruits part.

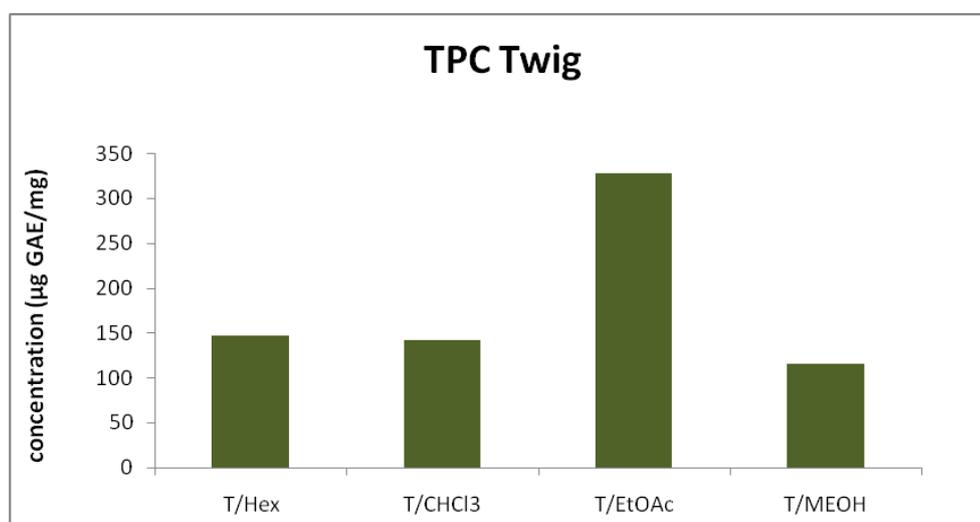


Figure 9. Total flavonoid content for part of Twig (T).

Determination of DPPH scavenging activity

The scavenging activity content for different plant parts (fruits; whole fruits, unripe rind, unripe flesh, ripe rind, ripe flesh, shoots, mature leaves and twig) extracts of *Garcinia forbesii* were tested using DPPH assay. The scavenging activity of the samples measured as decreasing in absorbance at 517 nm of the stable free radical DPPH. In this assay, the scavenging activity against free radicals. The purple-coloured free radical reacts with scavenger to yield the yellowish product, 1, 1-diphenyl-2-picrylhydrazine. The percentage of radical scavenging calculated using **expression (1)**.

The activity was expressed in the term of IC₅₀ (concentration of sample scavenging 50% of the initial DPPH radical). It were determined by plotting the graph of percent scavenging activity against different concentrations. From the twig extracts (ethyl acetate, chloroform and hexane) exhibited active activity with IC₅₀ value below 50 µg/ml. Extract ethyl acetate of mature leaves exhibited moderate activity with IC₅₀ value of 154.47 µg/ml. Some of extracts exhibited weak activity which summarized in Table 1. The percentage of scavenging activity are as presented in Figure 10 which the scavenging activity at concentration of 1000 µg/ml was calculated using ascorbic acid as standard.

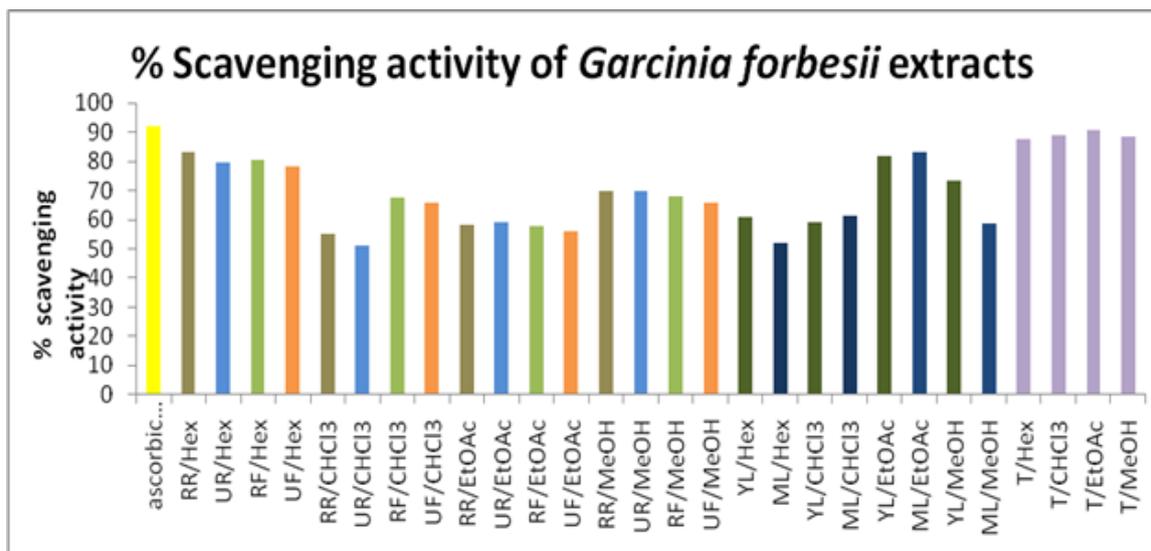


Figure 10. % of scavenging activity of *Garcinia forbesii* samples [ripe rind (RR), unripe rind (UR), ripe flesh (RF), unripe flesh (UF), young leaves (YL), mature leaves (ML) and twig (T)]

The antioxidant activity of fruits part can be concluded that ripe fruits contained more antioxidant content compared to unripe fruits. Ripe rind and ripe flesh showed the lowest IC₅₀ among fruits extracts (211.84 -251.96 µg/ml). Hexane and chloroform extracts showed high antioxidant activity compared to ethyl acetate and methanol extracts. For leaves extract, mature leaves showed the highest antioxidant activity with IC₅₀ of 154.47 ± 0.75 µg/ml. The other extracts of twig showed IC₅₀ more than 300 µg/ml which considered not active. Twig extracts exhibited antioxidant activity except methanol extracts which the highest IC₅₀ belongs to ethyl acetate extracts (30.76 ± 0.98 µg/ml). From the IC₅₀ value (Table 1) and scavenging activity (Figure 10), the antioxidant activity can be concluded that twig extracts (ethyl acetate) showed highest antioxidant activity, followed by mature leaves (ethyl acetate) and ripe rind (hexane).

Conclusion

The different plant parts (fruits; whole fruits, unripe rind, unripe flesh, ripe rind, ripe flesh, young leaves, mature leaves and twig) extracts of *Garcinia forbesii* showed the presence of phenolic and flavonoid compounds and antioxidant activity. Twig is the most potential antioxidant sample, followed by mature leaves, young leaves, ripe fruits and unripe fruits. Thus further studied should be conducted to identify the chemical constituents which responsible to the activity.

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Species variation in home garden practices at the residential area of Penampang, Sabah and the residents' perception towards the land use of limited land space

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Abstract

A homegarden is one of the agroforestry practices that usually implemented for its benefits towards communities in rural areas. However, urban residential areas also have implemented the homegarden practices but on a small scale due to the limited space of their residential area. This research has been conducted at the residential area of Penampang, Sabah to identify species variation planted and residents' perception towards limited land space at 10 residential areas in Penampang, Sabah namely Taman Kohizan, Taman Muntahan, Taman Dixon, Taman Palas, Taman Oriental, Taman Dongonggon, Taman Gunung Ledang, Taman Ridgeview, Taman Dabak and Taman Millenium. A total of 197 questionnaires have been randomly distributed to the residents of the selected residential area. The findings of this study indicated that there were 48 species of crops planted as a food source and ornamental plants for aesthetic value such as *Aloe vera*, *Curcuma longa*, *Nephelium lappaceum* and *Bougainvillea glabra*. The results also showed that a majority of residents strongly agreed that limited land space of their house could contribute in promoting green world campaign in residential areas. Meanwhile, 44.2% of the residents strongly agreed that home garden activities in limited land space of their house could improve the health for well being of communities. In conclusion, the communities are optimizing the limited space of land use in residential areas for sustainable development in the future.

Keywords: Homegarden, land use, residential area

Introduction

Home garden practice is an agroforestry system that has been implemented a long time ago and normally carried out at home or surrounds the house. According to Nair (1993), a homegarden can be found in both rural and urban areas in a small-scale, mainly as a food source to the households. Besides, species that cultivated in homegarden practices also can be used as a medicine and as an ornamental plant. In the urban area, a home garden can be known as urban farming (Bailkey and Nasr, 2000). Although land areas in an urban area were limited, innovation and technologies sometimes were applied to implement home garden activities (Nair, 1993). This home garden is practiced as a hobby and food source for the households and in some instances as an extra income source for household members and sometimes the harvest shared with other community and residents as a part of typical

Malaysian cultural trait (Rasmuna and Mohd, 2015). According to Malaysian International Trade and Industry (2015), world population will increase to 10 billion people and 80% of the population projected to live in the cities. Due to the result, the demand for food supply and the cost of living will increase. As a solution to the problem, a home garden can be implemented to supply food source in urban residential areas in the urban area along to reduce the cost of living (Milah and Siti, 2017). In this study, species variation planted was documented and the residents' perception towards limited land space at 10 residential areas in Penampang, Sabah was determined.

Materials and methods

The study area was located in Penampang District of Sabah with the total area of Penampang is 178,948 m² with 137,002 of peoples (Bancian Penduduk dan Perumahan Malaysia Tahun 2000). The data of this study was collected with a total of 197 respondent will be randomly selected by using Yamane (1967) formula. There are 10 residential areas have been selected randomly, with three residential areas, Taman Kohizan, Taman Muntahan and Taman Dixon represented by 19 respondents whereas the Taman Palas, Taman Oriental, Taman Dongonggon, Taman Mount Ledang, Taman Ridgeview, Taman Dabak and Taman Millennium represented by 20 respondents. The questionnaire form was divided into four sections; Section A (Demographic information), Section B (Types of Land Use), Section C (Community Perception towards Land Use on Limited Space), and Section D (Land Use Benefits). Respondents were asked to state the type of land use their implement, species planted, their perception and the benefits of maximizing the land use in the limited space of their residential area. Their perception and the benefits of maximizing the land use in the limited space were valued by using Likert Scale: (1= Strongly disagree, 2= Disagree, 3= Not sure, 4= Agree, 5=Strongly agree).

Result and discussion

Socio-demographic Profile

The total number of respondents interviewed was 197, comprising 86 (43.7%) males and 111 (56.3%) females and the majority were already married (76.2%). The largest group was 31-40 years old (41.6%), followed by 41-50 years old (20%), 21-30 years old (17%), 51-60 (12.2), above 61 years old (4.6%) and less than 21 years old (4.1%). About 60% of the respondents have finished their education until SPM and mostly were employed in the private sector (29.9%) as showed in Figure 1.

The average monthly income of the respondents is mostly below RM1000 with 25.8% of the respondents as showed in Figure 2 since most of the residents finished their education level up to SPM (*Sijil Pelajaran Malaysia*) followed by RM2001-3000 monthly income with 25.4% of the respondents because most of the respondents were employed in private sector that usually can offer high salary to the respondents.

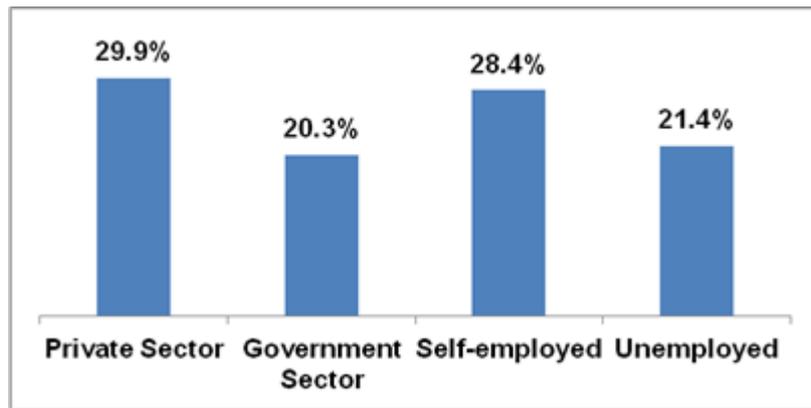


Figure 1. Types of employment of the respondents

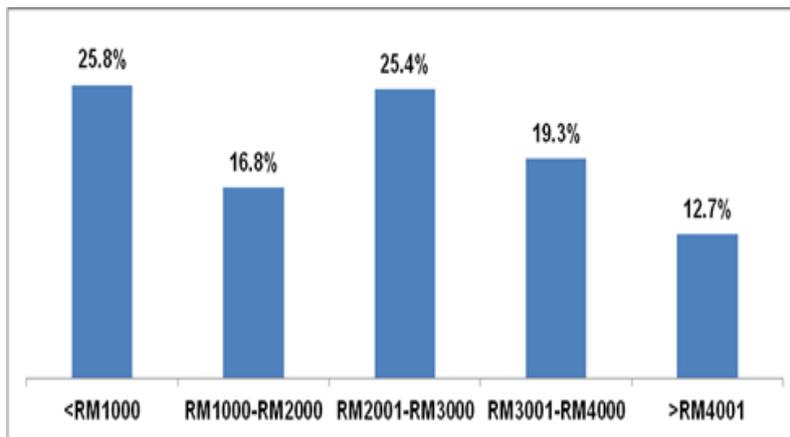


Figure 2: Average monthly income (RM) of the respondents

Land Use in The Residential Area

Total free land area of their housing is mostly 15-20m² (Figure 3) and due to the limited land space, the residents tend to optimize the land use of the limited space. According to Oliver *et. al.* (2016), most of urban residents prefer landed housing types as the landed area can offer free land space to the residents.

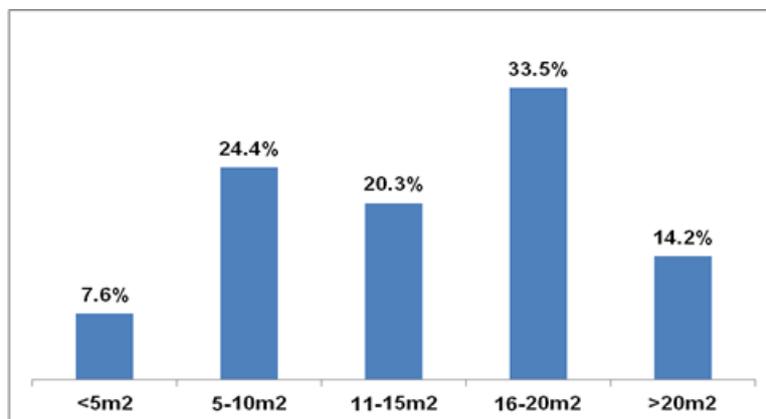


Figure 3. Free space of land area (m²) at the residential areas

Most of the respondent done homegarden activity at the free space of their housing area with 34% of the respondent used the land to cultivate ornamental plant and 32.5% cultivate vegetables/herbs/fruits (Figure 4). The result supported by Galhena *et. al*, that homegarden activity gaining interest nowadays because its promising a healthy food supply and reduce cost of living .

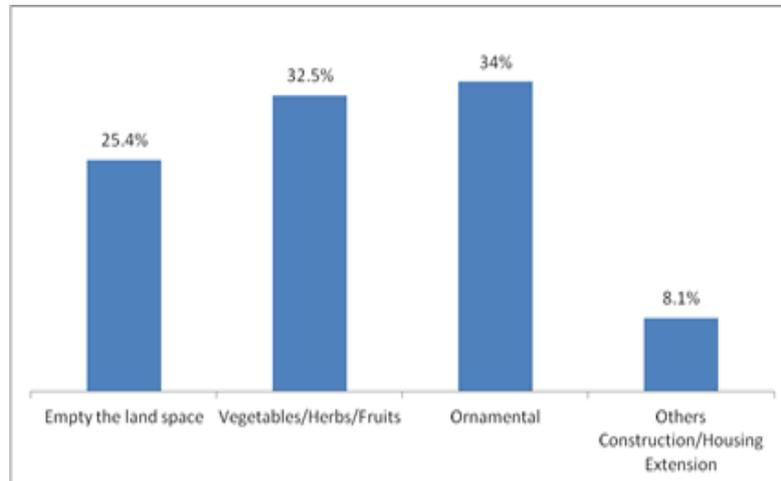


Figure 4. Types of land use in the residential areas

Species Variation Planted in the Residential Areas

There were 48 species of vegetables, herbs, fruits and ornamental plants documented in this study as shown in Table 1. Most of the plants that cultivated by the residents are vegetables because it easily planted and grown. Besides, the residents believed that they can reduce their cost of living and get healthy food resources if their plant the vegetables on their own.

Table 1. Species cultivated in the residential land area

No.	Types of plant	Local Name	Scientific Name
1	Vegetables	Bitter gourd	<i>Momordica charantia</i>
2		Chili	<i>Capsicum frutescens</i>
3		Curry tree	<i>Murraya koeniggi</i>
4		Eggplant	<i>Solanum melongena</i>
5		Long bean	<i>Vigna unguiculata</i>
6		Mustard	<i>Brassica juncea</i>
7		Onion	<i>Allium cepa</i>
8		Peanut	<i>Arachis hypogaea</i>
9		Butternut Pumpkin	<i>Cucurbita moschata</i>
10		Spinach	<i>Amaranthus sp.</i>
11		Sweet leaf	<i>Sauropus androgynus</i>
12		Sweet potato	<i>Ipomoea batatas</i>
13		Taro	<i>Colocasia esculenta</i>
14		Water spinach	<i>Ipomoea aquatica</i>
15		Wax gourd	<i>Benincasa hispida</i>
16		White chili	<i>Capsicum frustecens Linn</i>
17		Herbs	Cheese fruit
18	Ginger		<i>Zingiber officinale</i>
19	Lemongrass		<i>Cymbopogon citratus</i>
20	<i>Lidah buaya</i>		<i>Aloe vera</i>
21	Screwpine		<i>Pandanus amaryllifolius</i>
22	Sneak grass		<i>Clinacanthus nutans</i>
23	Turmeric		<i>Curcuma longa</i>
24	Fruits	Banana	<i>Musa spp</i>
25		Black Mulberry	<i>Morus nigra. L</i>
26		Coconut	<i>Cocos nucifera L.</i>
27		Corn	<i>Zea mays</i>
28		Dragon fruits	<i>Hylocereus undatus</i>
29		Guava	<i>Psidium guajava</i>
30		Limau kapas	<i>Citrus aurantifolia</i>
31		Limau kasturi	<i>Fortunella japonica</i>
32		Manggo	<i>Mangifera indica</i>
33		Papaya	<i>Carica papaya L.</i>
34		Pomegranate	<i>Punica granatum</i>
35		Rambutan	<i>Nephelium lappaceum</i>
36	Ornamentals	Betel nut	<i>Areca catechu</i>
37		Cactus	<i>Cactaceae spp.</i>
38		Cat's whiskers	<i>Orthosiphon stamineus</i>
39		Chinese hibiscus	<i>Hibiscus rosa sinensis</i>
		Common	
40		Sunflower	<i>Helianthus annuus</i>
41		Jungle geranium	<i>Ixora coccinea.</i>
		Madagascar	
42		periwinkle	<i>Catharanthus roseus</i>
43		Money Plant	<i>Epipremnum aureum</i>
44		Morning glory	<i>Ipomoea nil</i>
45		Orchid	<i>Orchidaceae sp.</i>
46	Paper flower	<i>Bougainvillea glabra</i>	
47	Pine	<i>Pinus mugo</i>	
48	Rose	<i>Rosa spp.</i>	

Residents' Perception towards Limited Land Space

There were 44.2% respondents strongly agreed that home garden activities in limited land space of their house could improve the health for well being of communities as shown in Table 2. Most households implement homegarden because the activity can improve their health as an exercise activity, reduce food insecurity and improve dietary intake among the residents (Carney *et. al.*, 2012, Eichemberg *et. al.*, 2013 and Galhena *et. al.*, 2013)

Perception	Strongly disagree (1)	Disagree (2)	Not sure (3)	Agree (4)	Strongly Agree (5)	Mean
A1	1 (0.5%)	4 (2%)	27 (13.7%)	95 (48.2%)	70 (35.5%)	4.16
A2	1 (0.5%)	5 (2.5%)	19 (9.6%)	100 (50.8%)	72 (36.5%)	4.20
A3	2 (1%)	16 (8.1%)	41 (20.8%)	75 (38.1%)	63 (32%)	3.92
A4	1 (0.5%)	3 (1.5%)	20 (10.2%)	86 (43.7%)	87 (44.2%)	4.30

Where:

A1: Limited land space can contribute to increasing residents economy

A2: Crops that planted in limited land space contribute to oxygen supply

A3: Variety types of crops cultivated will increase soil nutrients

A4: Agriculture activities will increase the health level of the residents

Conclusion

In conclusion, this study has documented 48 species planted as a food source and ornamental plants for aesthetic value and the communities believe that optimizing the limited space of land use in residential areas is crucial for sustainable development in the future.

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Pennisetum setaceum and Digitaria setivalva treated with Palm Oil Mill Effluent Sludge as an organic fertilizer for shear strength enhancement

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Abstract

Using specific vegetation for slope stability is crucial for erosion control on the cut slope. In this study, the effects of treated palm oil mill effluent sludge and chicken dung as soil amendments was determined by growing *Pennisetum setaceum* and *Digitaria setivalva*. On the cut slope, the vegetation effect towards the interaction of root-shear strength relationships was observed. The objectives of the study (1) to investigate the effects of palm oil mill effluent sludge on soil physical properties and root length density, (2) to determine the root-shear strength relationships. The Bungor series soil slope was acidic (pH 4.43), high exchangeable aluminium (32.2 mg kg⁻¹), and low soil organic carbon (0.43%), total N (0.02%), available P (0.17 mg kg⁻¹) and trace elements (Fe, Mn, Cu and Zn) (<0.5 mg kg⁻¹), indicating low fertility status. Therefore, the soil porosity and hydraulic conductivity in the dumping sludge > chicken dung > mixing sludge was significantly difference compared to the control in *Pennisetum setaceum* and *Digitaria setivalva*. Different trend was observed in bulk density; control > mixing sludge > chicken dung > dumping sludge and root length density (g m⁻³) in *Pennisetum setaceum* and *Digitaria setivalva*; dumping sludge (389.69) (334.28) > chicken dung (271.80) (273.15) > mixing sludge (156.26) (138.43) > control (83.64) (76.69). The results showed that the dumping sludge application effectively enhanced vegetation growth of *Pennisetum setaceum* and *Digitaria setivalva* and increased soil shear strength. The increased of shear strength was correlated to the increased of root length density and rapid vegetation growth. There was significant correlation identified in an organic amendments for all the treatments ($R^2 = 0.92$). These grass vegetation-soil interactions were significant in controlling erosion and slope stability.

Introduction

In Malaysia, annual rainfall could reach as high as 4500 mm. This combined with yearlong high temperatures causing intense chemical weathering and formation of thick residual soil profiles which in certain locations could reach 100 m in depth. With these set of climate and geological conditions, combined with other causative factors, landslides are thus one of the most destructive natural disasters in Malaysia. The most common types of landslides in Malaysia were shallow slides where the slide surface was usually less than 4 m deep and occurred during or immediately after intense rainfall (Pradhan and Lee, 2010). The inability

of vegetation to grow on this cut slopes contributed to soil erosion and in extreme cases slopes failures may occur. The cut slope was difficult for plants to grow without application any soil amendments. Appropriate soil amendments and suitable vegetation species to be grown on this cut slopes become vital and needs immediate action.

The Organic amendments was becoming vital in improving soil fertility status. Organic amendments based on oil palm waste was potential to be used as fertilizer including palm oil effluent. According to Khairuddin et al. 2017 stated that the sludge from palm oil mill effluent can be utilized as nutrients in soil. Nowadays, mostly chicken dung was applied as main organic amendments. The roots system of plant species plays an important role on soil erosion control by binding soil particles physically into stable aggregates. The influence of the physical condition of soils on plant growth and associated with agricultural practices has been emphasized, but the soil physical properties has equal effects on the use of soils and soils material for non-agricultural purposes.

The most important property of a soil for engineering uses is soil strength (Coppin and Richards, 1990). The method to determine the strength of a soil which was obtained using shear strength analysis. Shear strength of a soil mass is the internal resistance per unit area that the soil mass can offer to resist failure and sliding along any plane inside it (Bell, 2013). The presence of vegetation roots would result in an overall increase in the soil strength. This arises from combined effects of soil reinforcement by a mass of roots and soil moisture depletion by evapotranspiration (Abernethy and Rutherford, 2001). Other factors, which affect the strength of a soil, were soil moisture content, particle size distribution (soil texture), and the mineralogy of different soil particles. The objectives of the study were to investigate the effects of palm oil mill effluent sludge on soil physical properties and root length density, and to determine the root-shear strength relationships.

Materials and Methods

Soil samples site:

The site was located along the Persiaran UPM-MARDI where the horizon A, B and C were exposed. Vegetation was able to establish on Horizon A and B but no vegetation was observed at Horizon C. Horizon C was identified as rock and infertile for successful root development. It was also acidic and hardened under dry condition which prevents roots penetration for anchorage and water absorption (Hodge, 2010). The site was prone to erosion due to the high rainfall events (2000-3000 mm/year) and temperatures ranging from 25.6 to 27.8°C throughout the year (Pradhan et al., 2010). Most roads in Malaysia were constructed from rugged hills and mountain terrains and cutting the slopes was the common practices for a new road development. Poor growth of vegetation on the slope probably causes a major soil erosion and in extreme cases the occurrence of landslide.

Soil sampling and physicochemical analysis:

Samples of Bungor soil slope were obtained from a cut slope along Persiaran UPM-MARDI at Universiti Putra Malaysia in Serdang, Malaysia (N 2.98343, E 101. 73806). The shale was air-dried, ground and sieved through a 2.0-mm sieve. The samples were analyzed for pH, total nitrogen (TN), soil organic carbon (SOC), available phosphorus, using standard analytical methods (Johnson et al, 2007). The pH was determined in 1: 1 soil: water suspension using a pH electrode. Soil shear strength or shear stress at failure was determined by the direct shear box test (Gan, et al., 1988). Briefly, 25-cm thick intact soil samples (20 cm x 20 cm x 100 cm) were cut at 25-cm intervals in each wooden box and the edges carefully trimmed. The sample was packed in a metal shear box following the procedure described in literature (Abou-Chakra and Tüzün, 1999). The angle of internal friction was determined by placing the soil in a rigid metal box, square in plan, consisting of two horizontal halves.

Experimental layout

The experiment was set up in a complete randomized design with five replications for each treatment. In each box, a mixture of perennial bunch grass (*Pennisetum setaceum*) and fast growing grass (*Digitaria setivalva*) was planted using transplanting in the ratio of 1:1. The selection of the two species was based on preliminary experimentation that showed rapid growth and high biomass production. The box was grown with mixed plants species (*Pennisetum setaceum* and *Digitaria setivalva*). The soil amendments was applied every 2 weeks. The data was collected after 5 month of planting.

Statistical analysis

Statistical analysis: Data were tested for analysis of variance (ANOVA), respectively. One-way ANOVA was done to test the effects of treatments on soil physical properties, root length densities and shear strength parameters (shear strength, cohesion, and angle of internal friction). All statistical tests were done at probability level, $p = 0.05$ using SAS Statistical Analysis System (SAS, 2007).

Results and Discussion

Chemical properties of Bungor series soil

Table 1 presents a summary of chemical properties of the Bungor series soil. The Bungor series soil was inherently infertile, characterized by high acidity (pH = 3.32), CEC (2.23 meq/100 g soil) and organic carbon (0.94%). Available P (6.04%), Ca (0.06 mg kg⁻¹), K (0.05 mg kg⁻¹), Mg (0.08 mg kg⁻¹) and available P (6.04 mg kg⁻¹) were very high (Table 1).

Table 1. Chemical characteristics of Bungor soil series a cut-slope at Persiaran UPM-MARDI, Universiti Putra Malaysia, Serdang, Malaysia.

Chemical properties	Value
pH	3.32
CEC (meq/100g soil)	2.23
Organic carbon (%)	0.94
Available P mg kg ⁻¹	6.04
Ca mg kg ⁻¹	0.06
K mg kg ⁻¹	0.05
Mg mg kg ⁻¹	0.08

3.1 Physical properties of Bungor soil series

Figure 1 shows that treatment with the dumping sludge (DP) gave the lowest bulk density in *Pennisetum setaceum* (PS) and *Digitaria setivalva* (DS) which was significantly different compared to the chicken dung, mixing pond (MP) sludge and control. Soil bulk density is a basic soil property to indicate soil compaction, which influenced by some soil physical and chemical characteristics such as soil texture, amount of organic matter in soils, constituent of minerals and porosity (Ghestem et al., 2013).

The porosity status after treatments application, showed that the control and DP was significantly different compared to MP sludge and chicken dung (Figure 1). Bulk density is closely related to soil porosity. Soil porosity in the control, chicken dung, MP sludge and DP sludge increased and the bulk density decreased. Soil porosity is important for water infiltration, water and nutrient movement within soil, and ability of the soil to hold water (Bronick and Lal, 2005)

The soil hydraulic conductivity (Figure 1) of *Pennisetum setaceum*(PS), the DP sludge (0.62 cm min⁻¹) and chicken dung (0.60 cm min⁻¹) was significantly different compared to the control (0.32 cm min⁻¹) and MP sludge (0.41 cm min⁻¹). Meanwhile, *Digitaria setivalva* (DS) showed that the DP sludge (0.59 cm min⁻¹) and chicken dung (0.57 cm min⁻¹) was no significant difference. But, there was significantly different between the MP sludge (0.40 cm min⁻¹) and control (0.30 cm min⁻¹). There were many factors that could affect the soil hydraulic conductivity. Soil with higher percentage of clay indicates slow infiltration rate than sandy soil. High bulk density and less soil pores also may cause low soil hydraulic conductivity (Osunbitan et al., 2005). The addition of amendments in soil was associated with the increased of soil aggregation and macro-porosity, which lead in increasing soil hydraulic conductivity (Gwenzi et al., 2011).

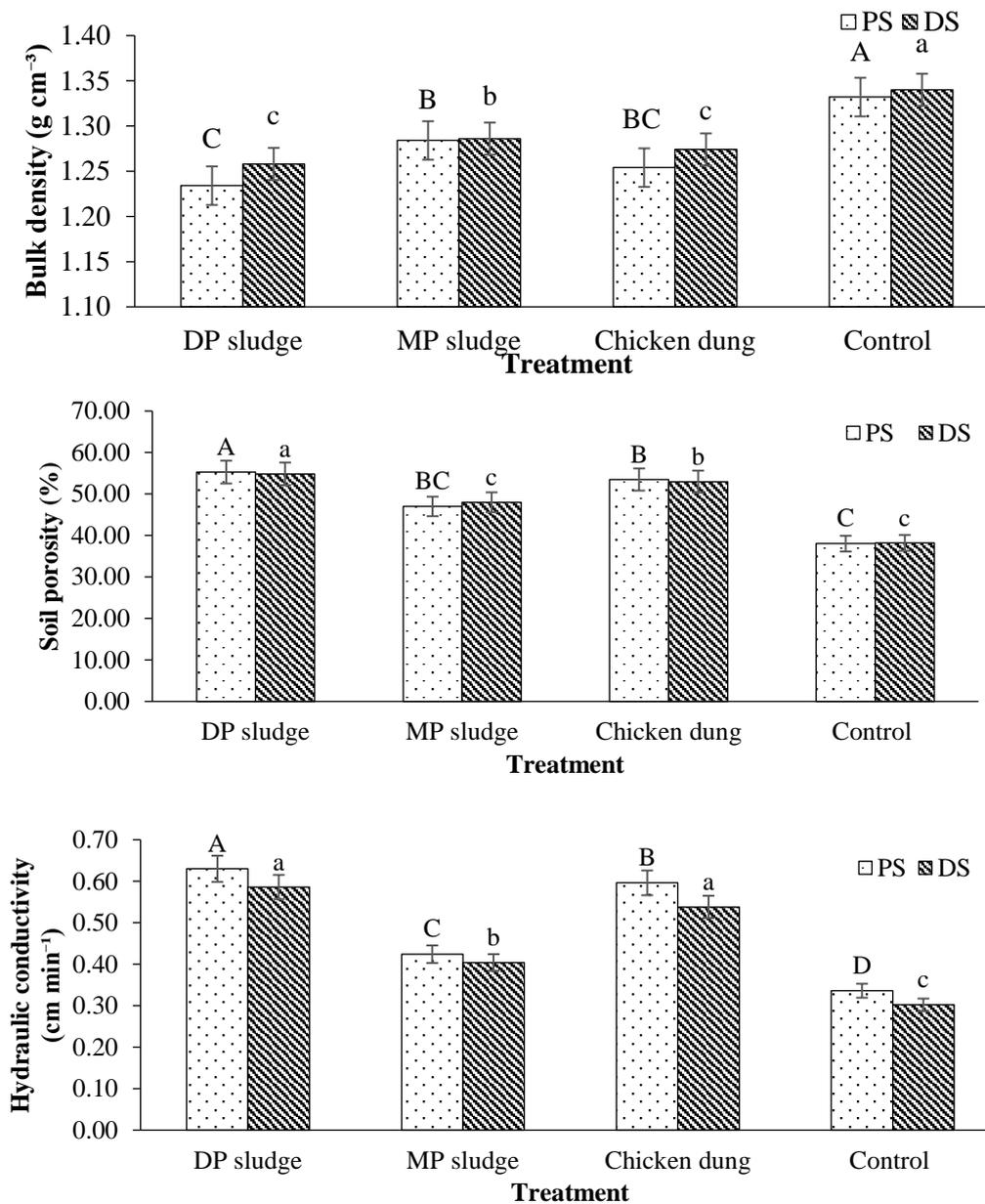


Figure 1. Bulk density, porosity and hydraulic conductivity measurements after application of treatments. (Means with the same letter are not significantly different at $p < 0.05$)

Root length density

Root length density (g m^{-3}) varied significantly ($p < 0.05$) among all the treatments in *Pennisetum setaceum* (PS) and *Digitaria setivalva* (DS), which showed the increasing trend following the control (83.64 g m^{-3}), (76.69 g m^{-3}) < MP sludge (156.26 g m^{-3}), (139.43 g m^{-3}) < chicken dung (271.80 g m^{-3}), (273.15 g m^{-3}) < DP sludge (389.69), (224.28). From Figure 2, the DP sludge application increased root length density of Bungor series soil approximately between 60 to 70% compared to the control treatment. As expected, root biomass showed a general increased with the decreased of soil depth in all the treatments. The maximum decline in root density occurred at 25-50 cm depth in all cases, although more gradual than the sharp exponential decline often observed in field studies (Gwenzi et al., 2011). The observed root profiles have corresponding implications on depth distribution of shear strength parameters.

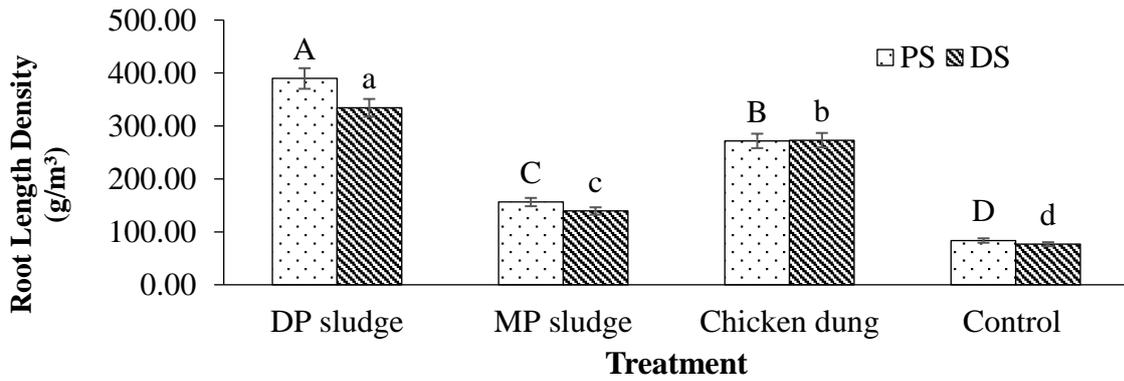


Figure 2. Effects of DP sludge, MP sludge, chicken dung and control application of root length density on slope planting with *Pennisetum setaceum*(PS) and *Digitaria setivalva* (DS). (Means with the same letter are not significantly different at $p < 0.05$)

Shear Strength characteristic

Figure 3 shows the shear strength results after treatments application, indicating that there was significant different in all the treatments in *Pennisetum setaceum*(PS) and *Digitaria setivalva* (DS). Furthermore, the pattern had shown the DP sludge was high in shear strength compared to the chicken dung, MP sludge and control. Khairuddin et al. 2017 also mentioned that the root density increased shear strength of the soil.

The roots growth applied with DP sludge and chicken dung amendments in *Pennisetum setaceum* (PS) and *Digitaria setivalva*(DS) had significantly affect the cohesion and angle of internal friction (Figure 3). At the normal stress of 153 kN m^{-2} , the DP sludge amended the Bungor series soil with the cohesion (c) and angle of internal friction (ϕ) of (75 kN m^{-2}) (80 kN m^{-2}) and (21°) (22°), respectively and a shear strength of 396.18 kN m^{-2} and 390.80 kN m^{-2} (Table 2). On the other hand, the chicken dung possessed the normal stress of 157 kN m^{-2} indicated a cohesion (c) and angle of internal friction (ϕ) of (95 kN m^{-2}) (90 kN m^{-2}) and (23°) (24°) with a shear strength of 354.50 kN m^{-2} and 354.31 kN m^{-2} (Table 2). This shear strength was lower than the corresponding values in the MP sludge and control. The present of roots would result in increasing of soil shear strength (Khairuddin et al., 2017).

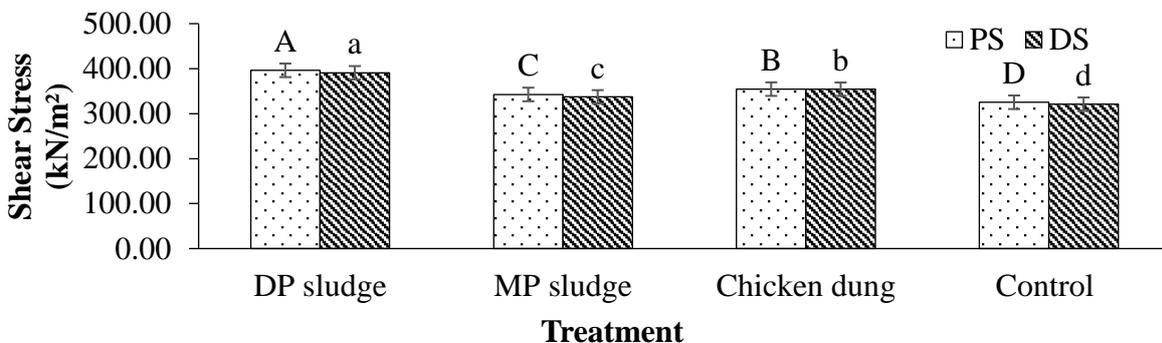


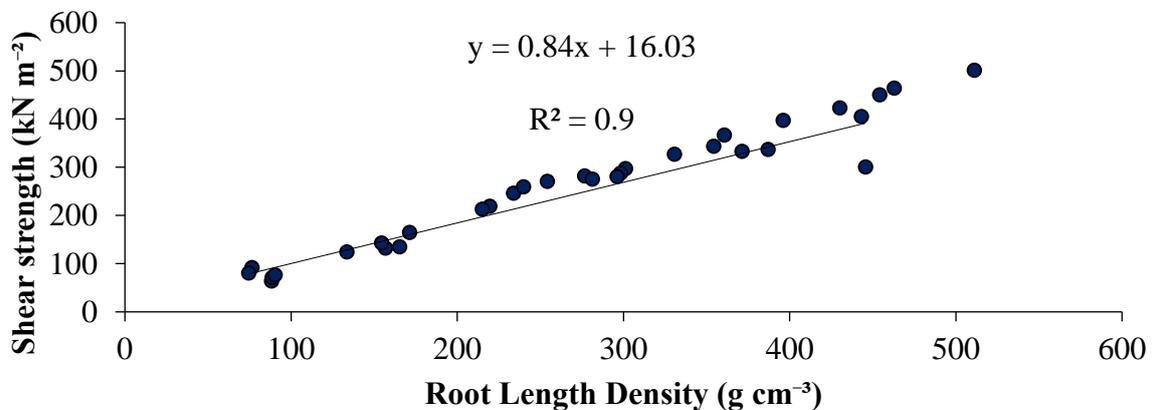
Figure 3. Shear strength measurement after application of treatments. (Means with the same letter are not significantly different at $p < 0.05$).

Table 2. Treatments indicated shear strength parameters

	Angle of Internal Friction (ϕ)		Cohesion (c)	
	PS	DS	PS	DS
DP	21	22	75	80
MP	24	26	100	110
Chicken dung	23	24	95	90
Control	32	32	130	130

Relationship between Shear Strength and Root length density

Data in Figure 4 indicated that the results of four different treatments on the effect of roots reinforcement towards the shear strength. The *Pennisetum setaceum* (PS) and *Digitaria setivalva*(DS) treated with the DP sludge was significantly difference in shear strength analysis and root length density (RLD). The high densities of plant roots in this treatment enhanced better anchorage and provide better aggregates stability to the Ultisols. POME sludge could increase the soil water holding capacity (SWHC) with significant improvement on root growth (Isa et al., 2017). Generally, the graph showed that the shear strength was obtained after the application of normal stress at 100 kN m^{-2} , 200 kN m^{-2} and 300 kN m^{-2} in all the samples.

**Figure 4.** Correlation between shear strength (kN m^{-2}) and root length density (cm cm^{-3}).

Conclusion

Overall, the current study provided insights of organic fertilizer application, practical amelioration strategies in achieving rapid and successful vegetation growth and stabilization of fresh geological substrates associated with artificial landforms (cut slope). Subsequent dumping pond (DP) sludge applications effectively ameliorated the vegetation growth on root parameters and distribution, hydrological behaviour, soil erosion and slope stability on the artificial landforms. Enhanced root growth stimulated by the dumping pond sludge (DP) nutrients application had a profound effect on the shear strength by decreasing the angle of internal friction and cohesion compared to the other treatments. The findings had confirmed the original hypothesis that nutrients from the dumping pond sludge (DP) applications enhanced vegetation growth, which in turn increased shear strength of the substrate.

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The potential of agro-tourism attraction at Kaiduan Village, Papar, Sabah: visitors and tourist agencies perceptions

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Abstract

Agro-tourism is an integrated concept of agriculture and tourism that has received positive reception from among locals and foreign tourists. Agro-tourism based activities has the proven potential to help increase revenue by strengthening and diversification of the economic activities of local residents. The objectives of this study was to determine the visitors and tourist agencies perceptions towards agro-tourism activities development and contributions to the local resident at Kaiduan village. A total of 100 respondents comprising of 50 visitors and 50 tourist agencies were interviewed using semi-structured questionnaire. Based on the survey results, there were six potential agro-tourism activities to can be developed, namely, livestock feeding, fruit picking, fishing, homestay, kayaking, rubber tapping and park recreation. On the agro-tourism contributions, there were six potential contributions to the socio-economic development of the local resident at Kaiduan village, namely, to increase their income, to increase their knowledge related to agro-tourism, to introduce the cultures and customs of their village, to create a spirit of entrepreneurship among them, make use of idle land and improving human resource development.

Keywords: agro-tourism activities, agro-tourism contributions, Kaiduan village

Introduction

One of the alternative forms of tourism is agro-tourism (Lopez and Garcia, 2006). Agro-tourism basically refers to tourism activities conducted in rural areas by groups of individuals who are involved in agriculture, and promotes recreational activities amongst tourists for firsthand experience of the living environment within the farming community, either as resident guests or day trippers (Busby and Rendle, 2000). Agro-tourism activities are not only beneficial in term of economics to the farming community, but also assist in addressing the problem of unemployment and rural migration, since the younger generation has the opportunity to generate income without the need to migrate to a bigger city (Dernoi, 1983). This type of alternative tourism plays an important role in ensuring rural development due to the tremendous positive impact on farmers. Agro-tourism can be classified as an effective development strategy within the context of rural communities due to its substantial positive impact on the livelihoods of the farmers (Hjalager, 1997). Community involvement is a very important aspect in agro-tourism activities, and may be defined as “a form of voluntary action through which individuals take the opportunity to uphold social responsibility” (Tosun, 2000). Similar to any other development projects, there will be those who support and those who oppose agro-tourism activities, based on their very own distinctive views. Various

reasons are put forward by those who are in opposition. In accordance with Tosun's (2006) findings, the majority (more than 80%) of communities in local destinations are eager to take a leading role, either directly or indirectly, in agro-tourism activities. However, it is up to the authorities to improve the participants' confidence levels and ensure the success of agro-tourism projects, which will improve community involvement in this industry.

Malaysia is one of several other countries that have transformed their economies by developing their tourism potential (Norudin *et al.*, 2015). Under the Ninth Malaysian Plan (2006–2010), agro-tourism was one of the new developing product for tourism strategy development in Malaysia that has opened opportunities for farmers to expand and diversify agricultural products and related industries (Kunasekaran *et al.*, 2011). Agro-tourism is a type of rural tourism that allows the tourist to visit farms to get close awareness and experience a farmer's daily life within the agricultural areas (Eduardo & Javier, 2006); as an important tool in the development of the rural community, due to the significant positive impacts to farmers (Hjalager, 1997); allows farmers to enjoy greater economic benefits, helps maintain the next generation of the farming community in the rural area, instead of migrating to towns (Dernoi, 1983). In the Malaysian context, agro-tourism is defined as an activity, which maximizes the use of farm settings and the environment, with hospitality (Ministry of Agriculture and Agro based Industry, 2005). Agro-tourism is also associated with cultural and heritage tourism in order to promote the uniqueness of the rural community (Kunasekaran *et al.*, 2011). In Sabah, the local communities played a vital role in tourism development as well as in agro-tourism development as they provide essential services to the visitors. The local residents' participation in agro-tourism development has been widely discussed. However the potential of agro-tourism attraction in rural areas based on visitors and tourist agencies perceptions are quite limited. The development of an agro-tourism industry in the village or rural areas has been slow due to numerous obstacles such as a lack of entrepreneurial skills and the know-how to develop and promote agro-tourism products. Kaiduan village was selected in this study because of their residents' interest in developing Kaiduan village as an agro-tourism destination. Thus, this study will focus on the agro-tourism activities as the potential agro-tourism attraction as well its contributions to the socio-economic development of the local resident at Kaiduan village.

Materials and Methods

This study was carried out at Kaiduan village in Papar district of Sabah. The objectives of this study were to identify the potential agro-tourism activities as a potential agro-tourism attraction for the agro-tourism development as well its contributions to the socio-economic development of the local resident at Kaiduan village. Target respondent for this study refers to the two groups which are visitors and tourist agencies. A total of 100 respondents comprising of 50 visitors and 50 tourist agencies were interviewed using semi-structured questionnaire. The visitors were randomly selected during their visit, while the tourist agencies representative were selected amongst around the district of Papar and Kota Kinabalu. The questionnaire form were divided into three sections; Section A (respondent demographic data), Section B (respondent perception on the agro-tourism activities), and

Section C (respondent perception on the contribution of agro-tourism). Respondents were asked to state the potential activities in the agro-tourism development and the contribution of agro-tourism to the socio-economic development of the villagers. The activities and contributions of agro-tourism encountered were valued by using four-point Likert Scale with 1 representing “strongly disagree”, 2 representing “disagree”, 3 representing “agree” and 4 representing “strongly agree”. The scoring of perception were determined as the maximum number of mean score (4.0) was divided equally into three categories namely low (1.00-1.99), moderate (2.00 to 2.99) and high (3.00 to 4.00). Factor analysis was used to determine the significant value. The data obtained were statistically analyzed using Statistical Package for Social Sciences (SPSS) to obtain descriptive statistics; and factor analysis was used to determine the significant value of the potential agro-tourism activities and the contributions of the agro-tourism development in addressing the socio-economic development of the villagers.

Results and Discussion

Respondents Demographic Data

The total number of respondents interviewed were 100, comprised of 50 visitors and 50 tourist agencies representative. The respondent population comprised 38% males and 62% females. A total of 46% of the respondents come from the age group 18 to 28; 31% were aged between 29 to 39; 16% were aged between 40 to 49; while the remaining 7% were aged more than 50. In terms of highest level of education, the majority (45%) had obtained secondary school education, followed by 31% who had been educated to university level or held a degree, 19% who had completed primary school education, and 5% who did not have any formal education (Table 1).

Table 1. Demographic Profile of Respondents

Gender		N		%
		Visitor	Tourist Agency	
Male		21	17	38
	Female	29	33	62
Age	18-28	15	31	46
	29-39	18	13	31
	40-49	12	4	16
	>50	5	2	7
Academic qualification	No education	5	0	5
	Primary school	18	1	19
	Secondary school	24	21	45
	Diploma/Degree	3	28	31
Main occupation	Government servant	1	0	1
	Private	1	50	51
	Have own business	30	0	30
	Currently not employed	18	0	18

Perception on the agro-tourism activities

Table 2 shows the ranking for the potential of agro-tourism activities. The mean score of these items from visitor perception range from 2.22 to 3.50 with standard deviation of 0.587 to 1.200. While, the mean score of these items from tourist agency perception range from 2.52 to 3.38 with standard deviation of 0.565 to 0.886. The respondents in this study indicated that most of them agreed regarding the activities that influenced agro-tourism potential attraction.

Table 2. Perception of visitors and tourist agencies on the agro-tourism activities

The list of activities	Visitor		Tourist agency		Sig. Value
	Mean score	Std. Deviation	Mean score	Std. Deviation	
Farming(example: vegetables and fruits)	2.94	0.843	3.10	0.789	0.330 ^{ns}
Feeding livestock such as goat and fish	2.64	0.749	3.04	0.699	0.007*
Picking fruits	2.74	0.777	3.26	0.565	0.000*
Fishing	2.74	0.600	3.04	0.755	0.030*
Visiting recreational garden and traditional house	2.86	0.756	3.26	0.694	0.007*
Jungle Tracking	3.10	0.789	3.36	0.776	0.100 ^{ns}
Camping	3.24	0.687	3.38	0.635	0.293 ^{ns}
Rubber taping	3.32	0.587	2.90	0.789	0.003*
Paddy planting	3.24	0.744	3.00	0.728	0.106 ^{ns}
Kayaking	2.62	0.945	3.00	0.857	0.038*
Riding buffalo	2.22	1.200	2.52	0.886	0.158 ^{ns}
Making handcraft	2.94	0.767	3.16	0.817	0.168 ^{ns}
Local festival	3.50	0.614	3.26	0.777	0.090 ^{ns}

(Keiser-Meyer-Olkin (KMO) = 0.80; Mean score values were significantly different at $p < 0.05$); ^{ns} Indicates no significant value at $p < 0.05$; * Indicates significant value at $p < 0.05$

There were 13 potential of agro-tourism activities listed by the respondents. Based on the perception of visitors, there were five activities with high mean score (3.00-4.00), namely, jungle tracking (3.10), camping (3.24), rubber taping (3.32), paddy planting (3.24) and local festival (3.50). Based on tourist agencies perception there were eight activities with high mean score (3.00-4.00), namely, farming (3.10), feeding livestock (3.04), picking fruits (3.26), fishing (3.04), visiting recreational garden and traditional house (3.26), jungle tracking (3.36), camping (3.38), paddy planting (3.00), kayaking (3.00), making handcraft (3.16) and local festival (3.26). But, based on factor analysis there were six agro-tourism activities that showed significant value of perception between visitors and tourist agencies on the agro-tourism activities. The significant activities were feeding livestock ($p=0.007$), picking fruits ($p=0.000$), fishing ($p=0.030$), visiting recreational garden and traditional house ($p=0.007$), rubber taping ($p=0.003$) and kayaking ($p=0.038$). The significant activities encourages visitors to experience the limelight of agricultural life at first hand, with cultural and heritage values, and the uniqueness of the rural community (Norudin *et al.*, 2015), activities conducted on a working farm for the enjoyment and education of visitors (George, 2008). This includes activities such as picking fruit and feeding animals (Dennis & Richard,

2004), harvesting and processing locally grown crops (Nilsson, 2002; Weaver and Fennel, 1997).

Perception on the potential contributions of agro-tourism to the socio-economic development

Table 3 shows the ranking for the agro-tourism contribution to the socio-economic development. The mean score of these items from visitor perception vary between 2.74 to 3.28 with standard deviation of 0.544 to 0.756. While, the mean score of these items from tourist agency perception vary between 3.00 to 3.54 with standard deviation of 0.542 to 0.708. The respondents in this study indicated that majority of them agreed regarding the agro-tourism contribution to the socio-economic development of Kaiduan villagers.

Table 3. Perception of visitors and tourist agencies on the potential contributions of agro-tourism to the socio-economic development

List of contribution	Visitor		Tourist agency		Sig. value
	Mean score	Std. Deviation	Mean score	Std. Deviation	
Giving job oppurtunity	3.20	0.728	3.46	0.676	0.067 ^{ns}
Increase the income of villagers	3.16	0.710	3.48	0.677	0.023*
Improve the infrastructure	3.14	0.670	3.34	0.658	0.135 ^{ns}
Increase the villagers standard of living	3.10	0.544	3.22	0.708	0.344 ^{ns}
Increase the knowledge about agrotourism	3.00	0.606	3.36	0.598	0.004*
Increase the work effeciency	2.74	0.723	3.00	0.700	0.071 ^{ns}
Introduce the culture and custom the village to the tourist	3.28	0.701	3.54	0.542	0.041*
Create a spirit of entrepreneurship	2.76	0.687	3.18	0.691	0.003*
Make use of idle land	3.00	0.756	3.38	0.602	0.007*
Improve Human Resource	2.86	0.729	3.22	0.648	0.010*

(Keiser-Meyer-Olkin (KMO) = 0.64; Mean score values were significantly different at p<0.05); ^{ns} Indicates no significant value at p<0.05; * Indicates significant value at p<0.05

There were ten potential contributions of agro-tourism to the socio-economic development of the villagers. Based on the perception of visitors, there were seven activities with high mean score (3.00-4.00), namely, giving job oppurtunity (3.20), increase the income of villagers (3.16), improve the infrastructure (3.14), increase the villagers standard of living (3.00), introduce the culture and custom the village to the tourist (3.28) and make use of idle land (3.00). On the other perception by the tourist agencies, all the ten potential contributions

of agro-tourism to the socio-economic development of the villagers with high mean score (3.00-4.00), namely, giving job opportunity (3.46), increase the income of villagers (3.48), improve the infrastructure (3.34), increase the villagers standard of living (3.22), increase the knowledge about agrotourism (3.36), increase the work efficiency (3.00), introduce the culture and custom the village to the tourist (3.54), create a spirit of entrepreneurship (3.18), make use of idle land (3.38) and improve human resource (3.22). But, based on factor analysis results there were only six showed significant value of perception between visitors and tourist agencies on the potential contributions of agro-tourism to the socio-economic development of the villagers. The significant contributions were to increase the income of villagers ($p=0.023$), increase the knowledge about agrotourism ($p=0.004$), introduce the culture and custom the village to the tourist ($p=0.041$), inspire the spirit of entrepreneurship ($p=0.003$), make use of idle land ($p=0.007$) and improve human resource ($p=0.041$). According to Brooke *et al.* (2016), agro-tourism can increase the local benefits of tourism in rural areas, with the potential opportunities that can be captured through agro-tourism activities (Dorra, 2006), as a process of attracting visitors for educational and recreational purposes (Veeck *et al.*, 2006; Nilsson, 2002; Weaver and Fennel, 1997).

Recommendations

Improving facilities and infrastructure is vital to transform Kaiduan village into a viable agro-tourism attraction. Support and assistance from both the public and private sectors are essential, as the community alone is not yet well placed to initiate and maintain the agro-tourism activities. In order to promote the success of the agro-tourism activities, it is important to build the capacity building by training or recruitment of key personnel to strengthen the management. The community should also establish and expand their network to foster the exchange of knowledge, best practices and experiences in agro-tourism development. As the Internet becomes more accessible to the general public, creating a website on Kaiduan village could be very useful to promote the range of products and services that the community can offer to potential clients.

Conclusion

Based on the survey results, six significant activities were identified to be the potential agro-tourism attractions, namely, feeding livestock, picking fruits, fishing, visiting recreational garden and traditional house, rubber tapping and kayaking. The survey results also shows six contributions of agro-tourism to socio-economic of communities at Kaiduan village, namely, to increase the income of villagers, to increase the knowledge about agrotourism, to introduce the culture and custom of the village to the tourist, to create a spirit of entrepreneurship among the communities, to make use of idle land and to improve human resource. Thus, Kaiduan village has a potential to be develop as an agro-tourism attraction in Sabah.

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Tank background color preference by Elvers of Tropical Eel *Anguilla* sp.

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Abstract

Tank coloration is important for fish welfare in captivity and particularly those species have colour vision, thus tank color preference was determined to further enhance growth performance of tropical eel *Anguilla* sp. The test aquaria was covered with a pair of two-colour papers contained with a group of 10 elvers, and preference of elvers on each colour background was recorded. The background colours tested were white, black, yellow, red, green and blue. Preference was analyzed by the Thurstone's law of comparative judgment for paired-preference test. The elvers' performance is not affected by background colours of tanks. Better growth ($40.6 \pm 9.96\%$) and feeding intake (1.95 ± 0.03 g/day) were observed in black background colour tank, with no significant difference in both treatments. In conclusion, tropical long-finned eel with the presence of light adapted retina are behaviourally preferred white background colour but growth were not affected either black or white tank. Confinement in white background colour tank is recommended for better welfare of the species.

Keywords: tropical long-finned eel; elver; background colour preference; colour vision

Introduction

The recent statistic shows the world supply of eel for human consumption is completely depending on wild catch (Dekker et al., 2003). The current eel population has been claimed unsustainable and outside the safe biological limits (Dekker, 2003; Dekker et al., 2003; Arai, 2014b) owing to the overexploitation of wild stock, loss of habitat, climate changes, diseases and environmental pollution (Marcogliese and Casselman, 2009).

Since 2009, several eel species already been listed under Appendix II by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and put under protection in March 2009 (Crook and Nakamura, 2013). The issue has completely altered the international trade for eels worldwide. Hence, eel has now becoming new emergence of aquaculture species as substitute for the decline wild stock (Arai, 2014a).

However, the artificial breeding techniques for eel is not yet firmly established anywhere. Hence, the present study was highlighting the elver stage of tropical long-finned eel as to impose proper rearing technique for better animal welfare. One environmental characteristic

that affects fish physiology is the background color, in which particularly important for eel, a visual feeder.

However, in some species the visual system is not yet well-developed at the early larval stage. Practically, the selection of tank with suitable colour may help the fish to increase their chances to detect the feed or prey. Though studies on background and tank wall colour have been conducted on other fish species little is known regarding the effects of tank background colour on the survival and growth performance of tropical long-finned eel. Thus, it is crucial to study the effects of tank background color preference by elvers of tropical eel *Anguilla* Sp.

Materials and methods

The present study examined tank color preference by elvers of tropical eel 1) behaviorally and 2) on site rearing trial.

Exp 1: Background colour preference of tropical long-finned eel as determined behaviourally

The experiment methodologies were modified based on preference index study of Nile tilapia towards different background colour (Maia and Volpato, 2017). This study was conducted by using five background colours: black, red, blue, green and yellow colour papers. White background colour was later added in the background colour preference test as white background colour tank is very common in aquaculture practice. The light reflectance spectra for each colour paper were first measured under a spectroradiometer (HSR-8100; MAKI Manufacturing, Co. Ltd., Hamamatsu, Japan) in the wavelength range 350 – 750 nm to ensure each colour paper is in the correct range of wavelength (Figure 1.0). Fifteen paired colour papers were prepared for the experiment.

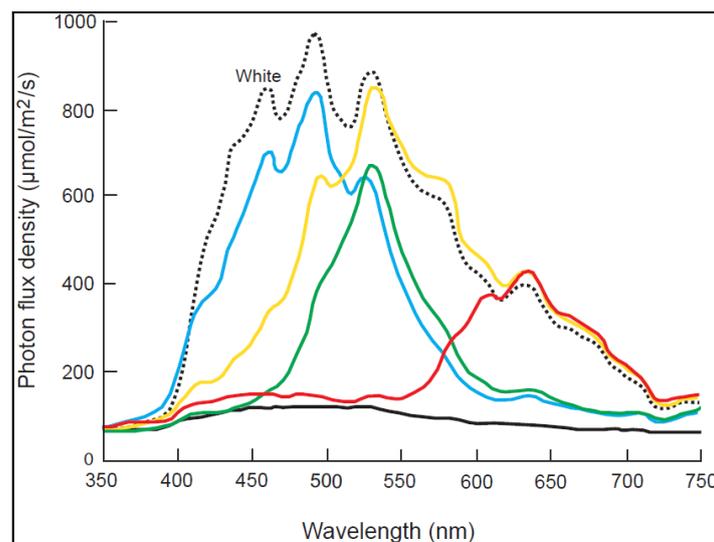


Figure 1. The light reflectance spectra of six colour papers for background colour preference test of tropical long-finned eel.

Next, three round transparent glass aquaria with 10 cm depth and 23 cm diameter width were wrapped with the paired of different colour papers. There were ten elvers were introduced into each glass aquarium with stocking density of 1 elver/1.5 L. Five observations were made in five minutes interval (total 25 min) and the elvers were photographed by using a digital camera (IXUS 160, Canon, Tokyo, Japan) at the end of each observation. The positions of the aquaria were rearranged by using random number table at the end of each observation to expose the aquaria to all possible light ambiences and avoid nuisance factors. The number of head position of elvers on each colour was recorded and counted (one session). The above steps were repeated using different pairs of colour papers for all combinations.

After first and second session, the elvers were changed with ten naive new specimens. The test was repeated for three times. The data of the background colour preference by the elvers were analysed by using Thurstone's law of comparative judgement (Thurstone, 1927) and evaluated by 95% confidence intervals.

Exp 2: Effects of different tank background colours on survival and growth performance of tropical long-finned eel

White background colour was significantly preferred by the elvers as determined behaviourally. Therefore, black and white tank background colours were chosen for the treatments. A recirculating aquaculture system was used in this experiment. Elvers of body weight 12.18 ± 0.12 g and total length of 20.08 ± 0.28 cm were used. A polypropylene cylindrical-shaped shelter (length=30 cm; diameter=8 cm) was installed in each aquarium. The elvers were fed twice daily, 8:00 AM and 4:00 PM using marine sinking pellets (1 mm) to satiation. The total feed intake was recorded daily. The temperature ($^{\circ}\text{C}$), DO (mg/L) and pH were maintained as follows throughout the experiment; temperature ($27.2 \pm 0.38^{\circ}\text{C}$), DO (7.87 ± 0.2 mg/L) and pH (8.15 ± 0.18). The rearing experiment was conducted for 28 days.

Results and discussion

Exp 1: Background colour preference of tropical long-finned eel as determined behaviourally

The elvers of tropical long-finned eel showed active swimming behaviours in random directions inside the aquaria. The mean z-score of the background colour preference test showed that the elvers has significantly highest preference towards white background colour and lowest for yellow (Figure 2). However, there is no significant difference ($P > 0.05$) among red, black, blue, green and yellow background colours. Therefore, it concludes that the tropical long-finned eel has significant biased preference towards white compared to other background colours.

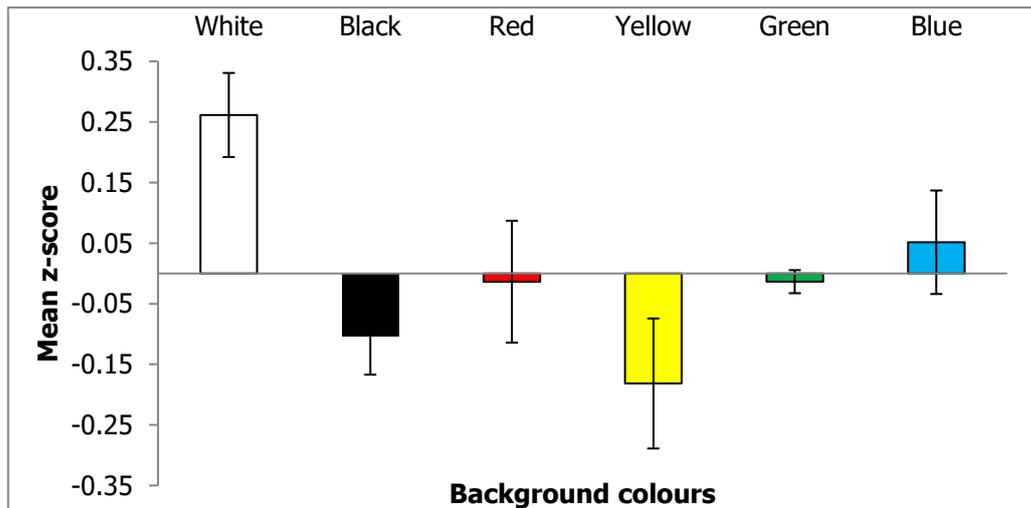


Figure 2. Mean z-score of background colour preference by tropical long-finned eel at 95% confidence interval. Different letters denote significant differences at $\alpha = 0.05$.

As the eels are nocturnal species, it is expected that they favour black substrate and low light intensity. Gong et al. (2015) reported significant preference for black substrate and dim light (5-10 lx) in glass eel of the Japanese eel. On the contrary, the elver stage of the tropical long-finned eel in the present study preferred white background colour as determined behaviourally.

The result might raise a debate on whether the species is actually attracted to the brightness of the background colour, instead of actual preference towards the colour itself. The light reflectance spectra for each colour paper has been measured and it shows that the white colour is indeed the brightest, but the least preferred colour in both experiments with and without white background colour is yellow which is among the bright colour as well. Hence, the colour preference in tropical long-finned eel is achieved by chromatic mechanism and cannot be based on the brightness.

The elvers reared in black background colour tank showed active swimming behaviour across the tank, with no distinct difference in the swimming pattern observed during the day (high light intensity) and late evening (low light intensity). The percentage of survival (%) (96.67% for both) of tropical long-finned eel reared in black and white background colour tank is shown in Figure 3A.

Tropical long-finned eel reared in black and white background colour tank showed mean increment of body weight (Figure 3B) of $40.60 \pm 9.96\%$ and $31.52 \pm 9.64\%$ respectively, but there is no significant difference (Mann-Whitney U test, $z = -0.655$, $P = 0.700$) between the treatments. The species showed higher increment in total length (Figure 3C) in black and white background colour tank with the value of $11.6 \pm 1.17\%$ and $10.12 \pm 3.29\%$ respectively with no significant difference (Mann-Whitney U test, $z = -0.218$, $P = 1.000$) shown in both treatments.

The mean specific growth rate (Figure 3D) of tropical long-finned eel reared in black background colour tank was 1.21 ± 0.25 %/day, which was 0.24% higher than white background colour tank (0.97 ± 0.27 %/day). However, there was no significant difference (Mann-Whitney U test, $z = -0.655$, $P = 0.700$) in both treatments.

The tropical long-finned eel showed slightly better feeding performance in black background colour tank with mean feed intake rate (Figure 4) of 1.95 ± 0.03 g/day. however, there is no significant difference (Mann-Whitney U test, $z = -0.918$, $P = 0.365$) in both treatments.

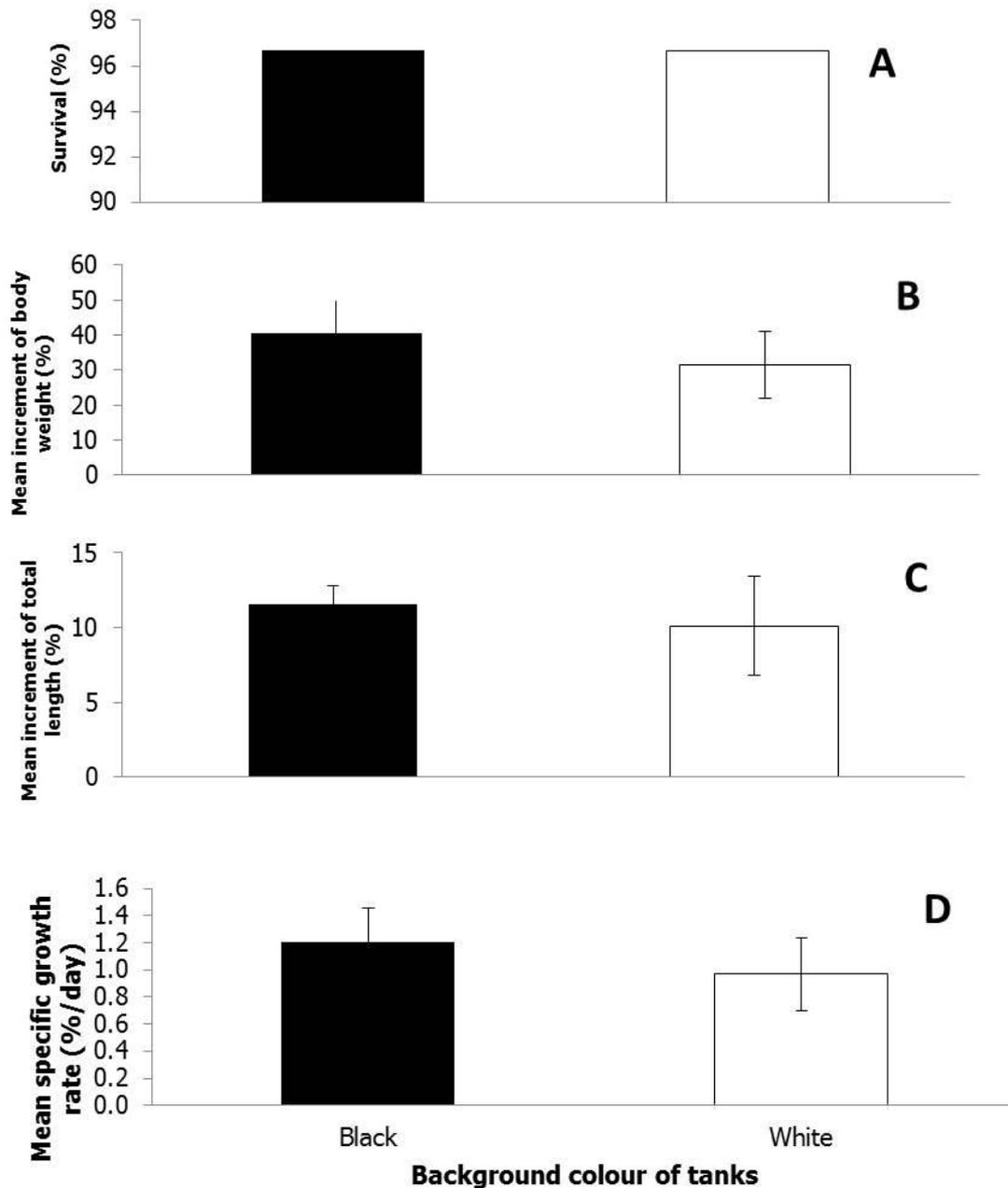


Figure 3. Growth Performance of tropical long-finned eel reared in different background colour tanks.

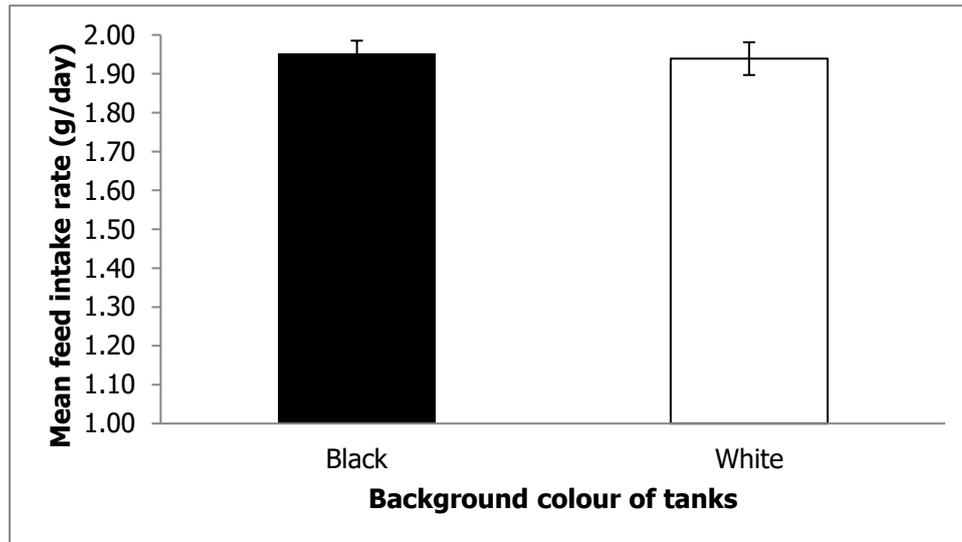


Figure 4. Mean feed intake rate of tropical long-finned eel reared in different background colour tanks. The vertical bar, standard deviation.

Previous studies on tank colour effects on fish performance revealed that the results were species specific. The rearing experiment revealed that there are no significant differences in overall growth performance of the tropical long-finned eel cultured in black and white rearing tanks. The European perch (*Perca fluviatilis*) larvae were clearly affected by the brightness of tanks (black, white grey, light grey and white); survival and growth were best in light grey and white tanks but lowest in black tank (Tamazout et al., 2000).

Tank background colour promotes better feeding performance and hence, contributes to high survival and growth of cultured species (McLean et al., 2008).. White background colour tank may have given the best contrast for elvers to detect food item, but high vision acuity in dark environment may also aid in the elvers feeding performance. The background colour preference does not necessarily coincide with the results of rearing experiment.

Conclusion

There is no effect of the tank colour was detected in the rearing experiment, the tropical long-finned eel exhibited significant background colour preference for white behaviorally but growth was not affected either black or white tank.

Acknowledgments

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Effects of fertilizers on growth of oil palm (*Elaies guineensis*) clonal ramets in main nursery

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Abstract

Clonal oil palm offers the potential for greater productivity because it is possible to establish uniform tree stands comprising identical copies (clones) of a limited number of highly productive oil palms. There is limited information on the most suitable fertilizer type, method and rates of application for these clonal ramets. The same fertilizer type, method and rates of application for DXP seedlings is being used by Sawit Kinabalu Sdn Bhd for clonal ramets at nursery stage. This research was therefore conducted to investigate the effects of various types, methods and rates of fertilizers on the growth of clonal ramets. Factorial completely randomized designed polybag experiments were conducted at Gomantong Estate, Kinabatangan. Experiment showed no significant differences between all fertilizer application methods and rates on the vegetative growth of the clonal ramets. Result suggest that conventional fertilizer application that used for oil palm seedlings is adequate to recommended to the clonal ramets in order to achieve standard vegetative growth before field planting. Study also shown that, there is no response of higher dosage of fertilizer applied. Whilst, no significant amongst the treatments, result suggested that the selection of fertilizers have to look into size of the nursery, watering system used and most important is the availability of workers. Besides selection of fertilizers, the source of fertile soil medium for clonal ramets play important role to accommodate vegetative growth of the clonal ramets.

Keywords: Clonal ramets, Fertilizer inputs, vegetative growth

Introduction

Clonal oil palm offers potential for greater productivity because it is possible to establish uniform tree stands comprising identical copies (clones) of a limited number of highly productive oil palms. In addition, improved standards of field agronomy have a greater effect on productivity. The specific clonal ramets growth characteristic is highly dependant on the right functioning of the root system. The difference in physical and chemical properties of planting media which reflects to the variation in physical and biomass allocation of the clonal ramets can be used to classify the suitability of the planting media in relation to the needs of the vegetative growth. Greater amounts of fertilizer nutrient inputs are required to sustain higher yields in clonal oil palm, but clonal oil palms also use fertilizer nutrients more efficiently than DXP seedlings (Woo et al., 1994). Effort to increase the fertilizer dosage is accompanied by high cost and probable degradation of soil by continuous use (Folorunso, 1999). Thus, the purpose of this study were to determine the most suitable fertilizer methods and rates on the clonal ramets vegetative growth at nursery stage in order with standard nursery management practices. By knowing the appropriate method and ratio of fertilizer application, the potential

yield of clonal palms which is more 10%-15% better than conventional DXP material can be archived.

Current Fertilizer Application Scenario

Based on conventional fertilizer application for nursery practise, the first four months after transplanting from the pre-nursery, a N P K Mg fertiliser of 15:15:16:4 composition is normally used, and from the fifth month onwards the potassium content is increased using a 12:12:17:2 formulation. A trace element addition is sometimes included in the compound but its benefit is uncertain. The first fertiliser application at one month is made in solution from since the amount given per seedling is very small. Care is required when solid fertilizer of the granular type is applied since some granular fertilizers frequently become lodged against the young unopened leaves, causing serious scorching there.

It is sometimes recommended that liquid fertilizer be washed off after application. This might be needed for compounds in solution but is not necessary for proper foliar fertilizers applied at the correct dilution. Too high concentration can lead to scorch, particularly of the spear leaf at its point of emergence, usually seen as a line of spots across the leaf as it expands.

The SRF rates should be compatible to the growth rate of the seedlings. SRF fertilizer is very expensive, too low dosage to save cost may result in poor growth and overdoses can result in toxicity and increase the cost.

Materials and Methods

Location, source of ramets and growth medium

Two experiments were conducted in Ladang Gomantong, Kinabatangan, Sabah. The location of the studies was at latitude 5° 55'N and longitude 118° 02'E at the nursery site called Annex nursery owned by Sawit Kinabalu Sdn Bhd. Germinated ramets of clonal palm were obtained from Sawit Kinabalu's tissue culture lab. The age of the ramets received was 4 months after hardening. There was no pre-nursery process for clonal palm seedlings. The source of soil for the experiments was from Gomantong estate area which was used as the Sawit Kinabalu nursery growth medium. The soil was classified as *Kuah series*. Shading of clonal palms was also provided at the site to avoid ramets from exposer to dry weather and heavy rain.

Duration of the study

The study was conducted over a period of about 32 months. It started on 21st August 2014 with preparation of research proposal, selection of experiment, site preparation, literature reviews, includes the 12 months of field data collection in 2015 to 2016 until receipt of soil and foliar analysis from the Sawit Kinabalu central laboratory in March 2017.

Experimental design and treatments

This study was conducted using a 3X4 factorial CRD experimental design. There were two factors (fertilizer application methods and fertilizer rates). There were 3 fertilizer application methods each at 4 application rates. Each treatment combination was replicated 3 times. Therefore the total number of experimental plants were 36. The treatments are shown in Table 1. The types of fertilizers used were foliar fertilizer (F1), slow release fertilizer (F2) and NPKBlue (F3). The nutrient composition of each fertilizer is shown in Table 2.

Table 1. Application rates for each fertilizer application methods

Fertilizer	Rates	T1 (Control)	T2	T3	T4
Foliar (F1)		+0%	+5%	+10%	+15%
SRF (F2)		+0%	+5%	+10%	+15%
NPKBlue (F3-Control)		+0%	+5%	+10%	+15%

T=rates (The percentage value is that above the conventional application rates)

Table 2. Percentage of nutrient composition of each fertilizer

Nutrients	Foliar	SRF	NPKBlue
%N	12	17	12
%P	6	8	12
%K	8	9	17
%Mg	0	3	2

Results

Leaf area (cm²) and Leaf dry weight (gram)

The principal vegetative components of clonal palm are root, trunks or stem, foliage and height. Measurement of growth parameters were the petiole cross section, frond length, total frond number, pinna number and length to calculate leaf area (LA) and leaf dry weight (LWD). The results presented in the Table 3 showed the effect of fertilizer application methods at different LA and LDW of clonal ramets. There is no significant interaction effect of the all treatments.

Table 3 showed that the fertilizer application methods had no significant effect ($p>0.05$) on LA of clonal ramets. The application of the different rates also had no significant effect ($p>0.05$) on LA measured.

Table 3 also showed the fertilizer application methods had no significant effect ($p>0.05$) on LDW of clonal seedlings. Similarly, application at different rates also shown no significant effect ($p>0.05$) on the LDW of clonal ramets.

Table 3. Effect of fertilizer application methods at different application rates on mean of leaf area and leaf dry weight

	Leaf Area (cm ²)	Leaf Dry Weight (g)
Methods		
F1	172.73	332.75
F2	192.85	334.15
F3 (control)	193.49	340.39
Lsd (0.05)	41.04	19.76
Pr	0.50	0.70
Rates		
T1 (control)	171.58	345.09
T2	206.83	343.93
T3	194.99	329.47
T4	172.03	324.56
Lsd (0.05)	47.39	22.82
Pr	0.35	0.19
Pr F fert* Rates	0.57	0.57
CV (%)	26.13	6.99

Discussions

By comparing the treatments, result showed that F3 (control) and T2 (increased 5% from recommended dosage) respectively showed the highest mean of leaf area as compare to the others. The highest mean leaf dry weight were showed in F3 (control) on application methods and T1 (Control) on the different rates. The development of each parameters is both interrelated with, and determined by, the environment of the area in which the clonal ramets is growing. Growth measurements give a good guide development when comparing growth of similar material in different environments. The study was conducted in Sandakan region which is receiving more than 2000mm per year. It is also suggested to have further study on this planting material to the other regions.

From the MPOB statistic data in 2017, it was stated that industry will facing 27% of declining of foreign workers in Malaysia. Other aspects that need to be considered are the minimum wages, MSPO compliances, the increasing of workers levy and these will leads to increase the overhead cost. Foliar fertilizer is the cheapest fertilizer amongst the other fertilizers but need frequent applications. Distribution of the fertilizer in a broad band around the seeding is important, with care also being taken to keep materials away from the stem base. Effort to use slow release fertilizer are accompanied by high cost, strengthen security and supervision during application is very crucial nevertheless needs very less dosage and workers compared to the other fertilization methods.

Conclusion

Based on the experiments, a few recommendations can be suggested to planters or individuals who are involved in the new planting material of clonal palm to improve vegetative growth and achieve optimum nutrient uptake for better field planting.

Based on the result of the vegetative growth, the conventional fertilizer recommendation is applicable for clonal ramets at nursery stage. However, there is an option by the nursery operator to decide the methods of application and types depends on the size of the nursery, number of workers availability and environment factors

There is need to evaluate the optimal rates and behavior of fertilizer especially slow release available and marketed to the oil palm industry. All slow release fertilizers may not necessarily give the desired result in responding to growth. Nevertheless it is obvious that not all the slow release fertilizer behave equally in responding to seedlings growth due to their way of releasing nutrients, the type and thickness in coating and behavior to the external factor likes temperature, moisture *etc* (Mathew, 2010). It is important to check the soil media before planting the clonal ramets. Sending soil sample to laboratory is highly recommended.

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Investigation on vegetable growth using aero farm technique for indoor farming

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Abstract

Indoor planting or better known as indoor farming has become more widely used method as it is able to provide higher yields throughout the year. Currently most of the developed countries are shortage of land for agriculture and the time is a major factor effecting agriculture sector, it is a global trend. In this technique, one of the most important things is the use of artificial light sources to replace the sun in indoor environment. Besides the artificial lights, medium of the soil is replaced by a plain towel as a trial run for the growth rate of vegetables. In this study, LED light with three different LED color lights, 5W and a piece of plain white towel were used as a replacement of soil medium. Monitoring process by watering and adding some fertilizers to the plant were done. The height or growth of the plant was taken and recoreded for 4 weeks period. At the end of the study, it was found that there is growth of vegetable, mustard and spinach in the towel. The four weeks mustard growth was by an average around 1.5 cm and average spinach growth is around 4.5 cm. The study shows LED color lights create an impact on the growth of a vegetable. This study is useful for researchers who focus on indoor farming using artificial light as a source of sunlight and plain cloth as a growing media of soil in future.

Keywords: In door farming, Aero Farm, LED lamp, Grow Light, Growth Height

Introduction

Agriculture is becoming a popular industry in the current world. Many researchers published their work about this industries in various conferences and journals (Anem, M., 1970), (Maisurimianti, 2011), (Shahimi, O. I. 2015). One of the current trends in this field is Aero Farm. Aero Farm is one of the high-tech methods of cultivation that does not use elements such as soil or sunlight and it reduces the use of (Team, A., 2015). This is because this technology only applies spraying method in the watering process. Furthermore, this method uses cloth as a substitute for the growing medium and requires Light-Emitting Diode (LED) lighting as a substitute for sunlight to complement the plant photosynthesis process. By applying this technology, special nutrients or fertilizers are used directly through plant root system to reduce the use of excessive fertilizers to plants. Hence, the application of Aero Farm can help farmers or housewives in growing crops in a small scale both either at home or in any parts of building.

The Aero Farm method can only be used in planting small plants such as salad, mustard or spinach due to humidity condition that can accommodate crop growth. Therefore, nowadays the Aero Farm method is applicable because it does not require large space and can overcome the decreased area of agricultural land problem due to development and urbanization (Trizelia,

T, 2015), (Wirayanti, A, 2013). As an example, in New Jersey, people suffered from food shortage problem as the food source decreases from time to time. Thus, in order to overcome the problem of food supply, New Jersey built the Vertical Agricultural Industry in New York to tackle food supply problem. As a result, the cultivation process that uses this method was a success and it can produce over 900 tonnes of crops per year by using this technology. Some studies have shown that the use of this technology can encourage agricultural activities in urban area which in turn can develop awareness among the public about the benefit of applying Aero Farm method for future generation. In addition, the Aero Farm method helps the public to understand high-tech agricultural techniques and also in reducing the land use in the future (Ken Utami, 2016)

Problem Statement

Some people are unable to carry out agricultural activities for certain purposes. Among the problems faced are as follows:

Limited cultivation area for agriculture due to the increased activities in industrial and housing development.

The country's agricultural sector is still too dependent on imported products from other countries.

Objective

The goal of this project was to design and build Aero Farm system so that it could be integrated into agriculture as well as to introduce it to the community, especially farmers. However, this project focuses on small scale. The following are the objectives of this project:

To utilize undeveloped area and old buildings in urban area.

To reduce the dependence on imported goods, especially agricultural products mainly vegetables.

Scope

Vegetable Crops

The Aero Farm system is one of the innovations made for the purpose of cultivating high quality vegetable without the use of large space in producing high quality vegetable.

The technology of fertigation

Fertigation system is one of the cultivation technology systems that uses liquid chemical fertilizer. Nowadays, consumers are very concerned when selecting which products to use by considering many factors such as issues related to health, food and environmental pollution.

Modern Technology

The use of the Aero Farm system can help farmers who are interested in expanding their agriculture by taking steps to overcome future problems especially limited space for agricultural activities.

Methodology

This project was implemented by following the sequence of steps which is shown in Figure 1 to ensure it was done perfectly according to the objectives of the study. This project was started by conducting a literature review as stated in the diagram whereby all information related to this project was reviewed and collected to provide background information about this project. After that, project materials were gathered and the project was done by stages. Once the project was completed, it was tested from its ability of producing good quality products without the application of soil.

Findings and Data Analysis

Throughout the entire implementation of the project, various data results of the project were collected and analyzed accordingly. The plants in the Aero Farm system were similar to the plants which were planted on the ground and each of them required 16 elements to grow well. In the Aero Farm system, these elements were dissolved in water to enable them to be absorbed by the roots and this solution is called as nutrient solution or fertilizer solution. Normally, the materials are used in the form of mineral salt or liquid. The ratio of the nutrient portion dissolved into the water was referred to determine the exact measurement. If 1 milligram of A + B fertilizer is dissolved in a 1 liter of clean water, the concentration of the solution is read as 1 ppm while the clean water that has not been mixed with fertilizer has 0 ppm. To ensure excellent growth of crop, ppm value need to be measured frequently by referring to the standard work concentration value which is 1280 ppm. Besides that, the experimental test on the durability of the system was also conducted through 2 stages. In the early stage, the cloth used was viewed as too thin which resulted the inability of the plant to survive and the growth of the plant was affected because the watering process was done once in a day. Meanwhile, in the second stage of the experiment, the problem was fixed by changing the previous cloth to a thick and furry cloth to enable good crops production. In addition, the watering process was done twice daily to increase crop growth as proactive measures and taking necessary steps are imperative to maintain the project running smoothly. Figures 2 and 3 show the results of the study on the growth of vegetables such as spinach and mustard by using a cloth. The reliability and validity of all the data taken and measured is around 90% for this experiment.

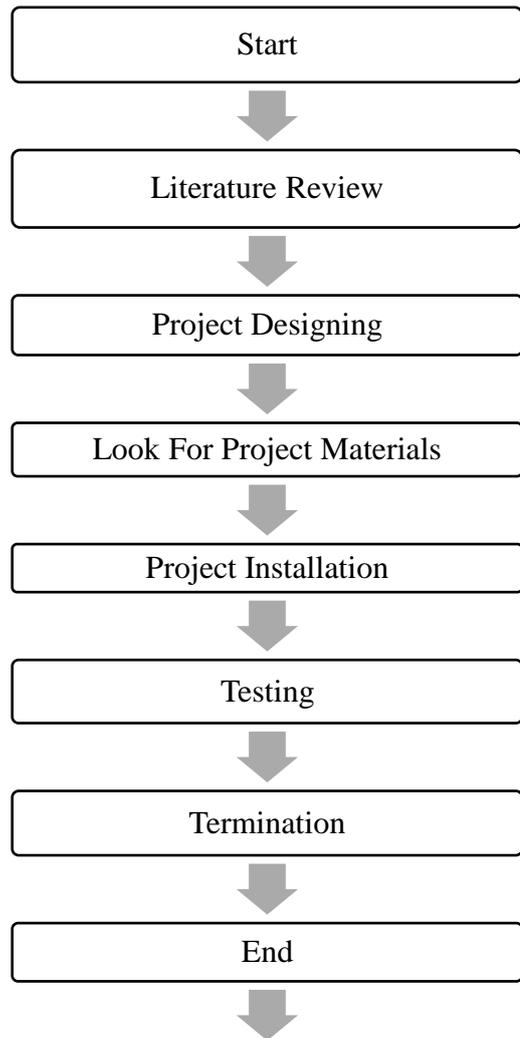


Figure 1: Project Flow Chart



Figure 2. The structure and growth of vegetables on a cloth.



Figure 3. The growth of spinach and mustard on a cloth

Data of Vegetables Growth

The measured data in this experiment has been captured and presented in table 1 and figure 4. It was observed the spinach growth rate was much better than he mustard.

Table 1. Vegetable height by week

Week	Temperature	Crop	
		Mustard	Spinach
1	23 ° C	3.5 cm	5.5 cm
2	24 ⁰ C	3.7 cm	5.9 cm
3	23 ⁰ C	4.0 cm	8.0 cm
4	24 ⁰ C	5.0 cm	10.0 cm

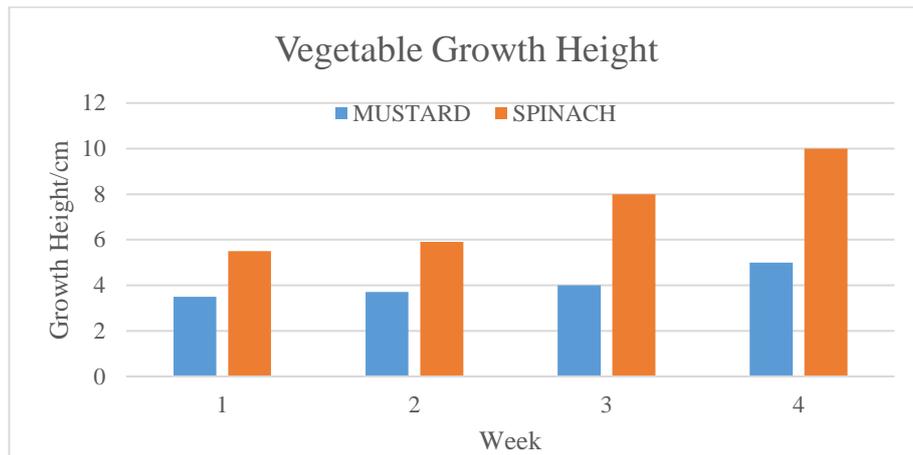


Figure 4. Comparison of vegetable growth height

Conclusion

First and foremost, the preparation process of a project need to be planned carefully and throughout the entire project, there were various obstacles and problems which led to unsatisfactorily results. Even though this is a trial run project with limited time period, the project is still considered a success by growing a vegetable in a plain cloth without using soil as a medium of farming, though the outcome might be slightly beyond expectation. The Aero Farm System project has materialized through the observation from large industries which has potential and can be beneficial to the community because it does not require large space to implement this project. The objective of the Aero Farm System project is to utilize the undeveloped area and the old buildings in urban area. Besides that, this project can be considered as a solution to mitigate the problem of groundwater contamination and to improve nutrient amount in soil. In conclusion, the project is able to assist the agricultural industry in providing more space for agriculture sector.

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Diversity of insect within the core zone of Crocker range biosphere reserve at Ulu Membakut, Sabah

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Abstract

Crocker Range Biosphere Reserve (CRBR) is one of the hundreds of UNESCO'S Man and Biosphere (MAB) programme, which protects the areas from biodiversity losses. Biosphere reserve consists of three zones that are the core zone (known as the protected area), the buffer zone (a region that surrounds the core zone) and the transition zone (an outer region that involves activities such as agriculture). These areas are the habitat for insects, as their role is vital for the foundation of biodiversity. The purpose of this study is to assess the insect diversity within the core zone of biosphere reserve. Ulu Membakut area of CRBR has no designated buffer zone; instead, a river acts as a protective outer layer of the core area from any human disturbance and land use. Insect collection carried out within the core zone of CRBR Ulu Membakut Sabah using yellow pan trap, pitfall trap, bait trap and active collection (light-trapping cloth, hand pick and sweeping net). 427 individuals were collected which consisted of ten orders of insects that are Blattodea, Coleoptera, Ephemeroptera, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Mantodea, Orthoptera and Odonata. The predominant insect order presence belongs to Hymenoptera with 48.5% of overall samples and the least was Blattodea and Mantodea with 0.5% occurrences. However, Coleoptera showed to be prominent in terms of the highest insect diversity and richness ($H' = 1.483$, $S' = 1.169$). Meanwhile, the highest insect evenness was Odonata ($E' = 0.971$). Using Shannon-Wiener Diversity, Margalef Richness and Pielou Evenness indices, the mean shows $H' = 0.4906$, $S' = 0.4734$ and $E' = 0.6321$, respectively. Increase in value of those indices indicates the healthiness of insect diversities in the area. The mean of Shannon diversity index are low compared to other part of the CRBR, with value of $H' = 4.77$ due to some dominant insect presence and low insects occurrences for some families. Crocker Range, Sabah area consists of various endemic and hyper-endemic insects that only existed within the CRBR. Unfortunately, there is no endemic and hyper-endemic insect recorded in this study. Past and future studies are vital to monitor the presence of insect in CRBR to evaluate the differences or changes in diversity within the core, buffer and transition zone. The absence of buffer zone as an area can lead to low insect diversity as it can easily interferes with the idea of CRBR to minimize the loss of biodiversity as the core area is exposed to land use and human disturbance that active within the outer layer that is transition zone.

Keywords: Insect, Crocker Range Biosphere Reserve, Diversity

Introduction

For centuries, insect outnumbered other animals regardless of their species. Insect has complex and diverse species which are not only different in sizes, habitat, and requirement for survival, but, it also exceeds more than 75% species diversity globally (Adjaloo, 2012; Chapman, 2016). To put it into perspective, insect colonization reaches millions for an acre of area (Triplehorn et al., 2005). The arthropod plays an important role in assisting the production of a produce as source of food in food web and biological control (Bartomeus et al., 2014). Located in Malaysia as one of the largest palm oil producers, Sabah is the leading state in production whose land use increases from 0.92 million hectares in year 2000 to 1.55 million hectares land in 2016 for cultivating oil palm, and most of the area was forest (Teoh, 2000; Malaysian Palm Oil Boards, 2016). With the forest degradation in an alarming stage, there is a possibility that we might lose a huge number of insect species without even identifying them due to poor documentation record of insect diversity in tropical forest in comparison to temperate ecosystem (Lewis & Basset, 2007). Apart from the Heart of Borneo (HoB) Initiative, MAB or Human and Biosphere Reserve aim to protect biological diversity as well as preserve and maintain an area against any human disturbance and forest conversion. MAB also works as research centres, trainings, a significant area for education and models for any development and sustainable conservation (Ahmad et al., 2013). With an area of 350,584 Ha, the Crocker Range Biosphere Reserve is one out of hundreds of UNESCO'S Biosphere Reserve program declared in 2014 under the protection of Sabah Parks. It holds more than 60 species of endemic insect that only found in this area nowhere else in the world (Chung et al, 2016). In order to achieve the goal, Biosphere Reserve consists of three designated areas (Hamilton, 2013). First is the core zone, which is the area that is being protect against any harmful activity. The second area known as the buffer zone, which surrounds the core zone. Hence, any activity and land use has to be align with the idea of protecting and sustaining the core area. In the case of Ulu Membakut Biosphere Reserve, there is no area classified as buffer zone as it has river, which acts as a protective layer to core area. The outer layer or third zone known as transition zone, which involves the activity of forestry, agriculture, recreation and other purposes. Plantation sector utilizes this area for planting major crops including oil palm and rubber within transition in Ulu Membakut.

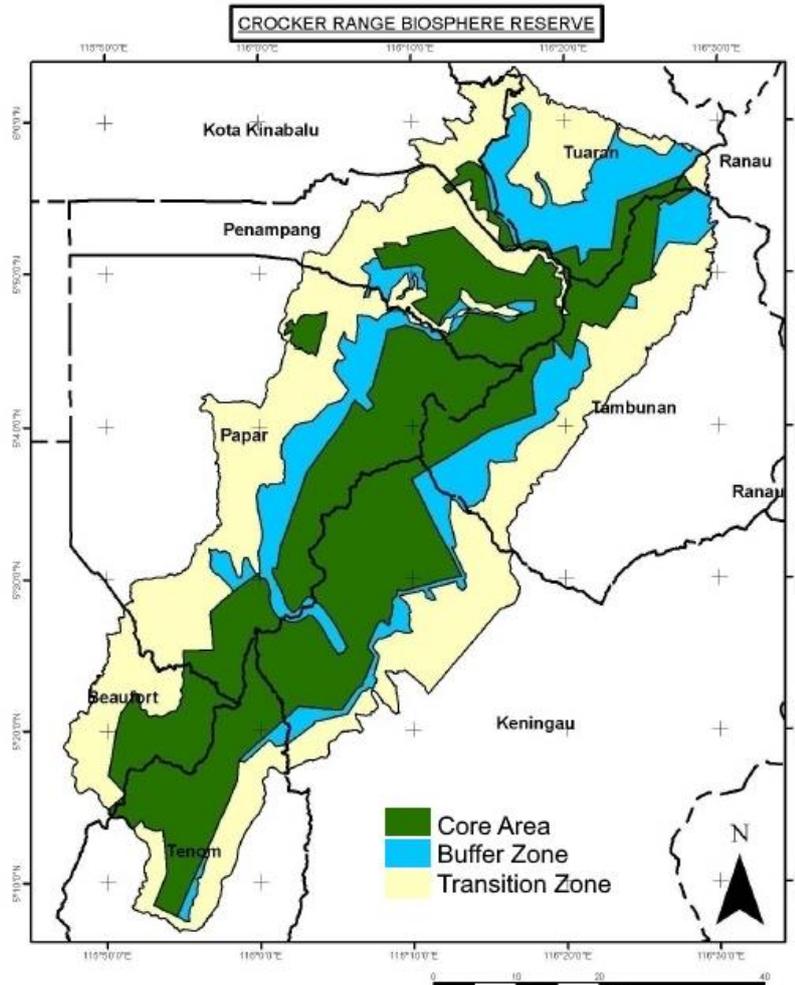


Plate 1. Three zoning of Crocker Range Biosphere Reserve
 (Source: Sustainable Development for Biodiversity and Ecosystems Conservation, 2015)

Crocker Range covers a vast area, with an estimation of 5 to 10 million species from different families of insects in tropical forest (Lewis & Basset, 2007). Previous study provides list and references of endemic insect of Borneo and it helps in identification of samples afterwards.

Materials and Methods

The study conducted at Ulu Membakut, Sabah from 25 to 27 of April 2017 as one of the expeditions in Man and Biosphere Program 2017. It is one of the areas of Crocker Range Biosphere Reserve with a distance of 98 km from Kota Kinabalu. It is where Ulu Membakut Substation is located. The area has an annual average temperature of 25.8°C, relative humidity of 78.9%, surface wind speed of 0.94 Meter and per second and a mean of monthly rainfall of 131.8 mm with April being the peak period of rainfall.

Ulu Membakut,
Biosphere Reserve

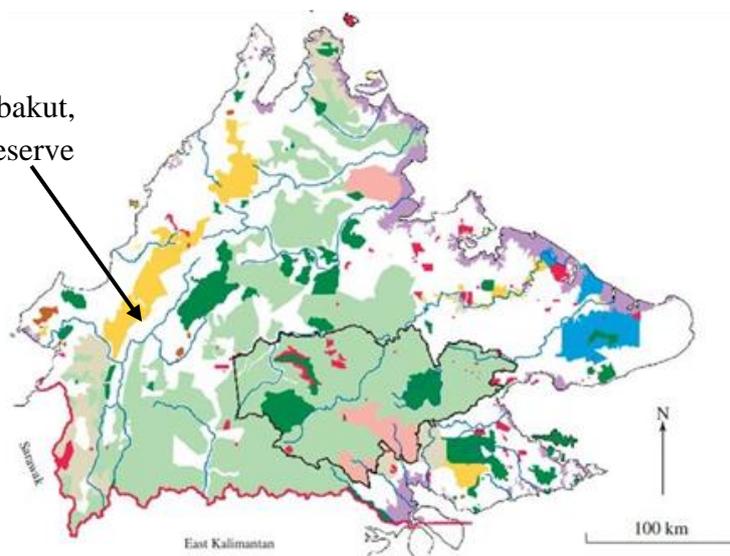


Plate 2. Location of Sampling Site
(Source: Reynold et al., 2011)

Yellow Pan and Pitfall Trap

There are four sampling points randomly selected within 50 hectares area. Both yellow pan and pitfall trap were set up in each of the sampling point. Yellow pan trap attracts flying and jumping insect, which attracted by the colours, such as the Coleoptera, Diptera and Orthoptera. The idea of a pitfall trap is to collect any crawling or jumping insects on the ground mostly Coleoptera, Orthoptera and Hemiptera. Effective pitfall traps constructed by burying a cup filled with killing agent at a ground level.



Plate 3 and 4. Yellow Pan Trap and Pitfall Trap

Bait Trap

Lepidoptera escape response is to fly upward as their natural act of survival and flight direction upon take off. The key to collect this order of insect is to attract and lure them into the bait by using two over ripe banana and mash with two tablespoon of sugar. The placement of bait trap is along the open area and river.



Plate 5. Bait Trap

Light Trap

Light trap was set up facing the forest using a vertical sheath with 2 x 2 meter size to attract nocturnal insect and are collected using forceps and aspirator. The rapid biodiversity assessment held within the 1 X 1 m square carried out from 8:30 pm to 10:00 pm, to evaluate diversity of the sampling area.

Preserving and Mounting of Insect

Dead, pinned insects are very brittle and easy to break. Besides that, damaged specimens considered worthless and identifying insect order and family is impossible with damaged sample. It needs precautions in handling collected samples. Specimen collected in the field is either directly mounted and pinned properly or brought back to lab. Small size of papers cut about ¼ an inch wide and 8 to 10 inches long folded to preserve the Lepidoptera. Small insects collected through aspirator are stored in a liquid of 70% ethyl alcohol.

Identification and Indices

The process of mounting, preserving and identifying samples to family level took place in Science and Agrotechnology Laboratory Complex, UiTM Kota Kinabalu. ‘Borror and DeLong, Introduction of the Study of Insects’, a book authored by Triplehorn and Johnson is used as reference for insect identification. There are few indices proposed in quantifying biodiversity as system of natural properties. Furthermore, those indices give us the capability to see clearly and compare the differences in terms of taxa, regions and level of trophic. Any data collected involving several taxa can be easily interpret by using those indices in a mathematical form. Thus, this helps to increase the understanding of the role, relationship of biodiversity for any kind of usage for plants, arthropods, mycorrhizal fungi, and so on. Previous studies show a comparison between Shannon’s (H’) diversity index and Simpson’s (D1’) diversity index, whereby both have the component in determininh data’s richness and evenness. Between those two indices, it was stated that Shannon’s diversity index is more significant than Simpson’s diversity index. For instance, H’ is sensitive to both abundant and rare species whereas D1’ is only sensitive to abundant species. The number of relationship

was much clearer when using Shannon's diversity as well. In this study, the diversity of insect is analysed by using Species Diversity (Shanon-Wiener Diversity Index), Species Richness (Margalef's Index) and Species Evenness (Pielou's Index).

Shanon-Wiener Diversity Index

One of the important components in biodiversity is species diversity, which represents the number of species or family from different taxa (Oshim, 2015). Aslam (2009) has stated that diversity is finding the number of different species or family. The value calculated in this index normally ranges from 1.5 to 3.5, but in a large sample that exceeds five families, the index ranges up to 4.6 using natural log and it means the individuals are distributed equally (Mohan & Padmanaban, 2013). Meanwhile, value near to zero means every family in the sample are no different and practically the same.

$$H' = - \sum p_i \ln (p_i)$$

$$p_i = S/N$$

S = Number of individual of one family

N = Total number of all individual in the order

In = Logarithm to base

Margalef's Richness Index

Often there is a misconception between diversity and richness. Diversity focuses on determining the distribution amongst species while species richness related to number of species that exists in particular zone. Species richness index can be successfully calculate when there are clear distinctions in the sample. Margalef's Richness Index is much simpler. Higher value in this index therefore, prove in greater diversity.

$$R = (S-1) / \ln N$$

S = Total number of family

N = Total number of individuals in the order

In = Logarithm to base e

Pielou's Evenness Index

Evenness brings a meaning of the individual allocation over species or family. In calculating the evenness index, best-used method is the Pielou's Evenness Index. Evenness is a calculation, which determines how well and equal is the individual allocated or distributed among the population. The result is focus on low and high values, which shows low evenness due to domination of one or few species and how well distributed and equal the species are without domination, respectively.

$$E = H' / \ln S$$

H' = Shannon wiener diversity index

S = Total number of families in the sample

In = Logarithm to base e

Results and discussions

From this study, the method of trapping used collected various families of insects within a short sampling period that consists of rainy days. Within the sampling period, 427 individuals of insects from 10 order are recorded.

Table 2. Total Insect collected and Methods of Sampling

Order	Family	Active		Yellow Pan	Pitfall	Bait Trap	Total
		Diurnal	Nocturnal				
BLATTODEA	Blabeidae		2				2
	Cerambycidae		4				4
COLEOPTERA	Scarabaeidae		24	4			28
	Carabidae		3	2			5
	Anobiidae		19				19
	Rhynchitinae		2				2
	Staphylinidae		1	2	11		14
	Calliphoridae		15				15
DIPTERA	Mycetophilidae	1	1				2
	Tabanidae		1				1
	Stratiomyidae			1			1
	Cicadidae		7				7
HEMIPTERA	Reduviidae	1		3			4
	Cicadellidae		1	21	1		23
	Formicidae		53	15	134		202
HYMENOPTERA	Vespidae	1	1				2
	Apidae	2					2
	Sphecidae	1					1
	Termitidae		2				2
ISOPTERA	Nymphalidae	8					8
	Noctuidae		19			7	26
LEPIDOPTERA	Mantidae		2				2
	Acrididae		2				2
MANTODEA	Gryllidae			24	19		43
	Tettigonidae		5				5
	Calopterygidae	2					2
ORTHOPTERA	Libellulidae	3					3
	Total	19	164	72	165	7	427

Table 3. Insect order and their total

Order	Total of Individual	Total of Family	Shannon-Wiener Diversity Index	Margalef Richness Index	Pielou Evenness Index
Blattodea	2	1	0	0	-
Coleoptera	72	6	1.483	1.169	0.827
Diptera	19	4	0.734	1.019	0.529
Hemiptera	34	3	0.842	0.567	0.766
Hymenoptera	207	4	0.139	0.563	0.100
Isoptera	2	1	0	0	-
Lepidoptera	34	2	0.546	0.284	0.787
Mantodea	2	1	0	0	-
Orthoptera	50	3	0.489	0.511	0.445
Odonata	5	2	0.673	0.621	0.971
Total	427	27			
Mean			0.4906	0.4734	0.632142857

The highest diversity index belongs to Coleoptera with index of $H' = 1.483$ with six families in total. The second highest is Hemiptera that has a value of $H = 0.842$ followed by Diptera with value of $H = 0.734$. This is followed by Odonata ($H' = 0.673$), Lepidoptera ($H' = 0.546$), Orthoptera ($H' = 0.489$) and Hymenoptera ($H' = 0.139$). For Blattodea, Isoptera and Mantodea, they have the same value that is 0 due to a single family in those order. For Margalef's Richness Index, it shows that the highest index belongs to Coleoptera value of $R = 1.169$, followed by Diptera ($R = 1.019$), Odonata ($R = 0.621$), Hemiptera ($R = 0.567$), Hymenoptera ($R = 0.563$), Orthoptera ($R = 0.511$) and Lepidoptera ($R = 0.284$) with the remaining order having a value of zero. Different from the others indices, Evenness focuses on the number of insect family with the number of total individuals. The nearer the value is to 1, the greater is the Evenness. It shows that Odonata has the highest Evenness Index that is $E = 0.971$ followed by Coleoptera with value of $E = 0.827$, which means that the total number of families are almost the same with the total number of individual presence. The Evenness Index used to determine the evenness of families within an order. It requires more than one family within an order to be calculated. Hence, Blattodea, Isoptera and Mantodea do not have Evenness Index since there is no way it can be calculated. The even number should be determined with 1:1 ratio and it shows how well distributed the order is.

Mean of indices shows that the diversity index in Ulu Membakut is lower compared to other part of CRBR in Penampang with a value of $H' = 4.77$ (Chung et al., 2016). Despite the high number of insect collected, number of individuals distributed among the families are significantly different, hence producing low index value. For example, Hymenoptera consists of Formicidae, Vespidae, Apidae and Sphecidae with number of individuals 202, 2, 2, 1, respectively. Apart from that, Blattodea, Isoptera and Mantodea order comes with $H' = 0$ value with only one type of family collected. It is self-explanatory when the mean of the three indices are quite low due to how some of the families are dominant and it cannot be tolerate with the indices.

The insect sampling are not evenly distributed and cause low diversity index due to the less insect presence for some families. Insect distribution and abundance driven by biotic or abiotic factors (Savopoulou-Soultani, 2012). Low diversity index in this study related to a decent amount of rain in the month of April as well as rain along the sampling period with April having the highest rainfall in a year. Insects are sensitive to temperature for being ectothermic and it influences their rate of metabolism, development, consumption and life cycle (Hushaw, 2015). It effects the availability of food, temperature, humidity, predators or contender (Gullan & Cranston, 2014). April has the highest temperature up to 28°C increase in temperature. It gives varying responses to insect in general (Jaworski & Hilszczanski, 2013). For example, increase in temperature causes higher mortality for Lepidoptera *Lymantria monacha* but increases the number of generation for other insect species. As one of the driving forces for insect development, climate plays important role in determininh insect population (Palumbo, 2011; Damos and Savopoulou-Soultani, 2012).

Ulu Membakut area of CRBR has no designated buffer area, which covers the core area. Instead, the core and transition zones separated by only a river as it acts as a boundary or protective outer layer of core area before transition zone (Hamilton, 2013). Most of the locals take advantage of the transition zone area to cultivate monocrop such as oil palm and rubber and other local produce, which is fruits. This study only covered the core area of CRBR and leads to no certainties without the data from the transition zone, it is possible that the cause of low diversity insect is insect migrate to the plantation area that is adjoining with the local ecosystem since oil palm plantation provides low temperatures and high humidity (Kalidas, 2012). In that case, there is a risk of insect colonization and infestation in oil palm plantation as much as their natural habitat, which is forest even though it is a monoculture. Human activities and land disturbance decrease the insect composition and diversity for the local ecosystem (Perry, 2016). The transition zone provide vast and various suitable habitat for them.

Crocker Range Biosphere Reserve governs a number of endemic and hyper-endemic insects that only existed in Borneo and a small part of Crocker Range, respectively (Chung et al., 2016). Unfortunately, with the same sampling methods used with previous study, there is no single endemic or hyper-endemic insects recorded, for example the Bornean endemic Kinabalu Tiger butterfly, *Parantica crowleyi* and hyper-endemic stag beetle *Cyclommatus chewi*.

This study is one of the many steps and provides knowledge in assessing the diversity of insect within CRBR zones. Indices may seem unimportant and incapable to provide sufficient data for interpreting the relationship with the change in insect occurrences for time being, the insect sampling only took place within the core zone of CRBR. However, with enough data of diversity index within the zones in every part of CRBR, it could provide us with the impact of the zones introduced by UNESCO in order to properly manage, protect and minimize the loss of biodiversity especially insects.

Conclusion

A continuous study to monitor the insect diversity within the three designated zones of Core, Buffer and Transition in CRBR is important to understand the occurrence, activities, mobility and type of insects in a particular area and time. Monoculture, fragmentation and other human activities are a threat to decrease diversity (Sutrisno, 2010). With a vast area of the CRBR and no data on the transition zone adjacent to the core, there are possibilities it will affect that the insect diversity due to land use for agricultural purposes and other human disturbance adjacent to the core area. Ulu Membakut does not have a designated buffer zone to protect the core zone as land disturbance affect the insect composition and their diversity. It is important to revise the absence of buffer zone in terms of insect diversity. Without a proper buffer zone, a river might protect the core area from any land disturbance. However, does it protect insect, especially any endemic species that lives within?

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Effect of clone and fermentation duration on cocoa flavor of three Malaysian cocoa clonal varieties

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Abstract

Cocoa flavor is an important aspect of cocoa quality. It is contributed by several factors such as genetics and fermentation processes. A good flavor is usually determined by high cocoa flavor with a low and balanced bitterness, acidity, and astringency. This study aimed to determine the effect of clone and fermentation duration on cocoa flavor and other sensory profiles. Three Malaysian cocoa clones (MCBC 5, 4, and 2) were fermented for six days in a shallow box. Beans from each clone were randomly taken everyday and subsequently dried. A part of the dried beans was subjected to cut test, pH, and fermentation index (FI) analysis to determine fermentation quality while the other part was roasted and ground into cocoa liquors for sensory evaluation. Cut test analysis showed that MCBC 2 beans were fully fermented on day four (65% fully brown beans), one day earlier compared to other clones. Based on FI analysis, however, beans from all tested clones were fully fermented as early as day two. pH was gradually decreased from 6.13 to 5.32 as fermentation progressed but increased later at to 7.01 at the end of fermentation. Although pH values of the three clones were slightly different during fermentation, they showed similar pattern. Sensory evaluation for cocoa flavor, bitterness, acidity, and astringency were conducted by trained panelists using Ghanaian cocoa beans as standard (4.89 ± 1.64), (3.06 ± 1.42), (0.83 ± 0.75), (3.33 ± 1.35), respectively. Results from two-way ANOVA showed that there was no significant differences for the sensory evaluation between cocoa clones and fermentation. The most intense cocoa flavor was MCBC 2 fermented for five days (4.06 ± 0.95) which was close to the standard value. The higher intensity of the cocoa flavor in this sample could be due to the low bitterness (2.83 ± 0.87) and astringency (3.56 ± 0.85) with average acidity level (1.56 ± 0.68) compared to other two clones.

Keywords: *Theobroma cacao*; cocoa flavor; fermentation; sensory evaluation.

Introduction

As known around the world, the major and main use of cocoa is to produce a popular consumer goods which is chocolate. There are several factors that determine the quality of cocoa beans such as bean count and size, colour, and acidity. However, the most crucial parameter of the cocoa is the flavour characteristic (Kongor *et. al.*, 2016). The unique flavour of cocoa is produced

during the fermentation of the beans. Without fermentation, the cocoa flavour will not be present (Aprotosoai et. al., 2015).

During fermentation process, the exposure of the sweet pulp of cocoa to the surrounding environment attracts the colonization of yeasts as well as acetic acid and lactic acid bacteria (Ho et. al., 2014). This reaction produces heat, lactic acid and acetic acid which will penetrate the bean cotyledon and causing its death (Hernández et. al., 2015). Biochemical reactions in the cotyledon of the dead bean leads to the production of the cocoa flavour and aroma precursors which will be further expressed during roasting (Aprotosoai et. al., 2015).

Fermentation process should be conducted for the right time in order to produce a good quality of beans with best cocoa flavour. Under-fermented and over-fermented beans will produce beans with off-flavour and low cocoa intensity (Aprotosoai et. al., 2015). In Malaysia, mixture of cocoa beans from different clones are usually fermented for five days in fermentation box (Khairul Bariah, 2014). However, a pulp-preconditioning technique prior to the fermentation can increase the percentage of well-fermented beans in shorter time (Afoakwa et. al., 2013; Kongor et. al., 2016). In regard to this, a study by Khairul Bariah (2014) also showed that good quality of Malaysian cocoa beans can be attained as early as on the third day of fermentation.

Flavour of cocoa is also affected by the genotype of the cocoa tree (Kongor et. al., 2016). Different cocoa clones have different amount of certain components such as theobromine. Therefore, they produce different intensity of cocoa flavour (Suzannah, 2006; Othman et. al., 2008). Despite this, there are no research done on the sensory evaluation of different clones during fermentation. Therefore, this study aimed to determine the effect of Malaysian cocoa clones and fermentation duration on the cocoa flavour and other sensory profiles.

Materials and Methods

Sample preparation

Ripe cocoa pods from three Malaysian cocoa clones were harvested from Cocoa Research and Development Centre (CRDC), Hilir Perak. The selection of clones was based on the MCB's recommendation on the intensity of cocoa flavour. MCBC5 was used for a representative of good cocoa flavour, MCBC4 for medium cocoa flavour, and MCBC2f for low cocoa flavour). The pods were harvested and fresh beans were fermented in the same day using shallow fermentation box (1 x 1 x 1 ft) for six (6) days. Temperature of the surrounding environment and fermenting mass were measured with thermometer for every 24 hours. About 1 kg of samples were taken from each clone for every 24 hours. The samples taken were subjected to oven-drying at 45⁰C for 8 hours per day until the moisture content reduced to 7 – 7.5 percent. Dried beans were kept in sealed plastic bags for further analysis.

Cut test

A hundred (100) dried beans were taken randomly from each sample. The beans were cut lengthwise into half to expose the inner surface of the beans. The surface of both sides of the cut beans was inspected carefully under artificial light and were divided into four groups based

on the colour (fully brown, partly purple, partly brown, fully purple, and slaty). The samples with more than 60% of fully brown beans are considered good quality of fermented beans. While, samples with 45 – 60% of fully brown beans are of medium quality. Lastly, samples that contained less than 45% of fully brown beans are considered as low quality of beans. The score for the Equivalent Percent Fully Brown (EB) was counted by using the equation below according to Khairul Bariah (2014).

$$EB = [(1 \times \%FB) + (0.7 \times (\%PB + \%PP)) + (0.5 \times (\%FP)) + (0.3 \times (\%slaty))]$$

Fermentation Index (FI)

Dried cocoa beans were de-shelled manually and the nibs was grinded in conventional grinder into powder. 0.5 g of the grinded cocoa nibs was mixed with 50 ml of methanol:HCl (97:3) solution and was incubated overnight at 4°C. After incubation, the solution was filtered with filter paper and the solution was made up to 50 ml with methanol:HCl solution. By using spectrophotometer, the absorbance of the solution was measured at 460 and 530 nm. The absorbance value of 1.000 and below is considered under-fermented, those with absorbance value of 1.000-1.599 are deemed as completely fermented and cocoa beans with absorbance value of 1.600 and above is considered as over-fermented.

pH nibs of dried beans

5 g of de-shelled beans was grinded and mixed with 50 mL of hot distilled water. The mixture was then stirred and filtered with filter paper. The filtered mixture was let cool until 20-25°C and the pH level will be measured by using calibrated pH meter.

Sensory Analysis

Dried cocoa beans were roasted by according to Farah (2012). The shells were removed and the cotyledon were cut into 10-5mm pieces. The roasting was done for 25 minutes at 127°C with conventional oven. The roasted cocoa nibs were then grinded into smooth cocoa liquor with mortar and pestle. The cocoa liquor made for each samples were then sent to the Cocoa Innovation and Technology Centre (CITC), Nilai for sensory evaluation by 9 trained panellists. The intensity of the cocoa flavour along with the acidity, bitterness, and astringency of the beans were scored from 0 to 10. Score of 0 is considered as the lowest intensity and 10 as the highest intensity. Ghanaian cocoa liquor was made with the same technique and was made as the standard.

Statistical Analysis

By using Statistical Package for the Social Sciences (SPSS), in completely random design (CRD), the effect of clones and fermentation level to the intensity of cocoa flavour was investigated with Two-way ANOVA.

Results and discussions

pH of dried beans

Dried beans fermented for 0, 24, 48, 72, 96, 120, 144 hours were analysed for their acidity level (Fig. 1). All the unfermented cocoa clones started within close range of pH level, (5.56 to 6.13) and started to decrease during the first 24h of fermentation. This may signify the start of the microbial reaction with the pulp which emanates heat, lactic acid, and acetic acids (Hollywn, 2008). This pattern was observed as well by Nazaruddin et. al., (2006) and Carmo Brito et. al., (2017). However, the pH value for the MCBC 5 increased from 5.56 to 5.77 opposed to the other two clones. Nevertheless, all three clones exhibited decreasing pH from day 1 to day 2. This is expected from the release of organic acids during fermentation by the reaction of yeast, acetic acid bacteria and lactic acid bacteria with the pulp into the beans cotyledon (Guehi et. al., 2010).

The acidity level started to increase again from day 2 to day 3 for MCBC 4. While, the other clones were still slightly decreasing on their pH. From day 5 to day 6, all three clones presented the same pattern of increasing acidity level. MCBC 5 showed lower pH at day 6 compared to other two clones probably because of its lower pH from the beginning of the fermentation. Even though the concentration of acidic compounds increased in the beginning of the fermentation, well fermented beans (day 6) has higher pH value. The increasing pH may indicate the depletion of acetic acids in the beans as the fermentation went on (Guehi et. al., 2010).

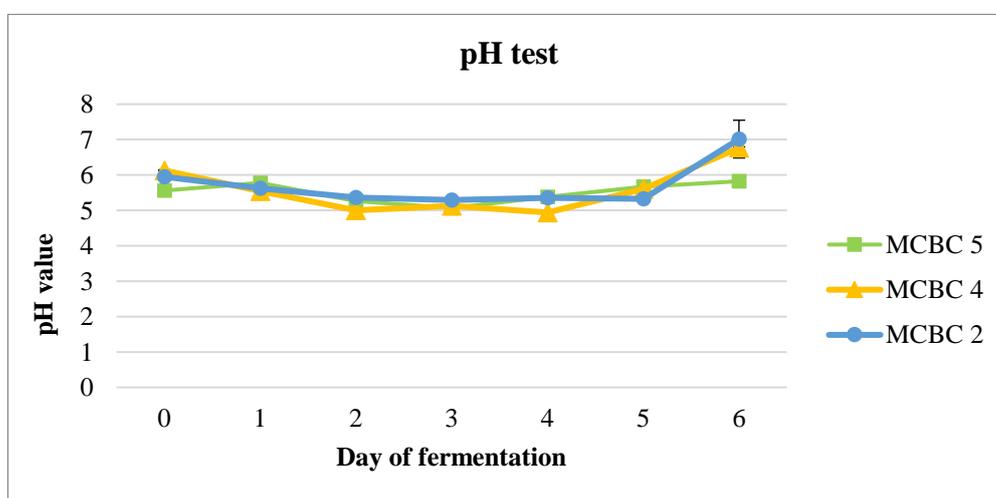


Figure 1. The pH of dried cocoa beans during fermentation process.

Cut test and Fermentation Index (FI)

The level of fermentation beans was determined from two tests. The first one was the cut test which is the observation on the colour changes of the bean cotyledons under artificial light (Table 1). Fresh cocoa beans are rich with polyphenols which are stored in the storage cells of the bean cotyledons. The purple colour of the cotyledon is due to the presence of anthocyanin which is one of the polyphenols. During fermentation, the anthocyanin was hydrolysed rapidly by glycosidase into anthocyanidins and sugars causing the bleaching of the purple pigments into brown colour (Camu *et. al.*, 2008; Khairul Bariah and Tajul Aris Yang, 2015). Therefore, anthocyanin is used by cocoa producers and buyers as a marker to indicate the degree of cocoa bean fermentation.

MCBC 2 showed well-fermented beans on day 4 with 65% of fully brown beans, a day earlier to other clones. As mentioned by Misnawi *et. al.*, (2003), about 93% of anthocyanin will be lost after 4 days of fermentation. The percentage of fully brown beans for each clone increased as the fermentation duration increased except MCBC 2. The percentage of fully brown beans of MCBC 2 decreased from day 5 (85%) to day 6 (81%) which is in accordance with the FI value (Table 1).

The second test was the fermentation index (FI) which is more accurate compared to the cut test by using spectrometer. The data revealed that the cocoa beans from all clones were well-fermented as early as day 2 (Table 1). This is also in line with the study by Khairul Bariah (2014) which showed well-fermented beans through fermentation index on day 3.

As duration of fermentation increased, the fermentation index should increase due to the increasing formation of anthocyanidins from anthocyanins (Kongor, 2013). Yet, the level of the fermentation index for MCBC 5 and MCBC 2 started to decrease from day 5 to day 6. MCBC 4 showed the same pattern but it decreased from day 4 to day 5 and increased again until day 6. Results from cut test and FI showed different days required for well fermented beans may be due to the difference in the analytical method as the FI is more complicated and detailed compared to cut test.

Table 1. Fermentation level of cocoa bean sample from cut test and Fermentation Index (FI).

Clones	Day of fermentation	% of fully brown beans	EB Score	Fermentation Index (FI)
MCBC 5	0	23	75	0.850 ± 0.026
	1	38	80	0.856 ± 0.062
	2	38	80	1.005 ± 0.103
	3	42	81	1.477 ± 0.271
	4	57	85	1.308 ± 0.235
	5	65	88	1.500 ± 0.097
	6	67	89	1.485 ± 0.197
MCBC 4	0	6	68	0.401 ± 0.047
	1	35	80	0.896 ± 0.292
	2	41	81	1.330 ± 0.564
	3	40	81	1.643 ± 0.305
	4	52	86	1.502 ± 0.022
	5	70	91	1.404 ± 0.040
	6	75	93	1.424 ± 0.222
MCBC 2	0	20	71	0.564 ± 0.029
	1	40	82	0.699 ± 0.009
	2	54	86	1.416 ± 0.140
	3	50	85	1.586 ± 0.212
	4	65	89	1.521 ± 0.448
	5	85	95	1.737 ± 0.382
	6	81	94	1.589 ± 0.007

Sensory evaluation profiles and statistical analysis

By using Ghanaian cocoa liquor as the standard, the cocoa liquor of each sample was evaluated by the trained panellists (Table 2). The highest cocoa flavour was from MCBC 2 at fifth day of fermentation (4.06) with the nearest score to the standard so far. The lowest cocoa flavour was identified as the unfermented MCBC 4 (0.72). MCBC 5 showed a moderate and quite high score of cocoa flavour throughout the fermentation, especially for unfermented beans (3.17).

Even so, unfermented MCBC 5 has the second most bitter taste compared to other samples while the lowest score for bitterness is again the MCBC 2 fermented for 5 days. However, unfermented MCBC 2 displayed the highest score on the bitterness (6.00), doubled from the standard score. All samples showed no steady increasing or decreasing pattern of score on the astringency flavour. The lowest astringency score is once again won by the MCBC 2 fermented for 5 days (3.56) which is a quite close score to the standard (3.33). Yet, unfermented MCBC 2 had the highest astringency score (6.78) compared to unfermented MCBC 5 (5.22) and MCBC 4 (5.06).

Contrast to the other attributes, unfermented MCBC 4 revealed a good score on the acidity (0.56) which is even lower than the standard (0.83). The higher acidity of MCBC 4 beans at day 2 to day 4 is in accordance with the pH level (Fig. 2). Meanwhile, MCBC 5 fermented for three days was the most acidic compared to the other clones. Interestingly, for all four attributes in every clone, the score is about the same range at the end of fermentation (day 6).

This pattern may show that the Malaysian cocoa beans fermented for 6 days are considered as over-fermented as recommended by Malaysian Cocoa Board.

As opposed to the first criteria of the cocoa clone selection, the MCBC 5 which was selected as one of the Malaysian cocoa clone with good cocoa flavour turned out to give moderate score. This may be due to the high bitterness, astringency and acidic taste that masked the cocoa flavour, causing the detection of cocoa flavour to be difficult (Guehi et. al., 2010). This can be seen from the high intensity of cocoa flavour evaluated from MCBC 2 fermented for day 5 which may be due to the low bitterness and astringency enabling the cocoa flavour to be detected easily. However, there is no correlation proved between different clones and fermentation duration on impacts to the cocoa flavour. Further study should be done in future to determine the factors that correlated to give the unique cocoa flavour.

Table 2. Average sensory evaluation of cocoa liquor from different clones at different day of fermentation with Ghanaian as the standard. The **bold** value indicates best score for the respective attributes.

Clones	Day of fermentation	Cocoa	Bitter	Astringent	Acid/sour
Ghana		4.89 ± 1.64	3.06 ± 1.42	3.33 ± 1.35	0.83 ± 0.75
	0	3.17 ± 1.12	5.11 ± 1.45	5.22 ± 1.09	0.73 ± 0.65
	1	2.83 ± 2.47	4.78 ± 2.40	5.06 ± 2.26	1.06 ± 0.92
	2	3.33 ± 2.65	4.44 ± 2.74	5.39 ± 3.10	1.67 ± 0.97
	3	2.44 ± 1.13	4.78 ± 1.30	5.78 ± 1.48	3.00 ± 0.66
	4	3.17 ± 1.22	4.94 ± 1.38	4.94 ± 0.53	2.06 ± 0.73
	5	3.33 ± 1.03	4.00 ± 1.12	4.61 ± 1.32	1.94 ± 0.85
MCBC 5	6	3.11 ± 1.08	3.50 ± 0.87	4.56 ± 1.01	1.22 ± 0.97
	0	0.72 ± 0.83	4.61 ± 1.05	5.06 ± 0.95	0.56 ± 0.68
	1	3.22 ± 1.09	4.56 ± 1.33	4.72 ± 1.09	1.39 ± 1.27
	2	2.56 ± 1.51	4.28 ± 1.09	4.89 ± 1.27	2.94 ± 0.81
	3	2.78 ± 1.72	4.22 ± 2.45	5.17 ± 1.94	2.22 ± 1.23
	4	2.33 ± 0.87	4.00 ± 1.41	4.67 ± 1.35	2.94 ± 0.77
	5	2.56 ± 1.57	4.83 ± 0.87	5.22 ± 1.39	1.61 ± 0.78
MCBC 4	6	3.11 ± 1.05	4.17 ± 1.46	4.83 ± 1.22	1.28 ± 0.87
	0	0.89 ± 0.93	6.00 ± 1.71	6.78 ± 1.79	1.22 ± 0.87
	1	1.67 ± 1.22	5.06 ± 1.38	5.67 ± 1.50	0.78 ± 0.79
	2	3.28 ± 1.20	3.94 ± 1.42	4.56 ± 1.33	1.00 ± 0.75
	3	3.44 ± 2.79	4.67 ± 2.49	4.67 ± 2.44	2.11 ± 1.14
	4	3.78 ± 2.79	3.89 ± 2.76	4.83 ± 2.56	1.78 ± 1.06
	5	4.06 ± 0.95	2.83 ± 0.87	3.56 ± 0.85	1.56 ± 0.68
MCBC 2	6	2.78 ± 1.20	3.94 ± 1.24	5.00 ± 0.90	1.17 ± 0.50

Conclusion

The temperature and pH of the cocoa beans throughout fermentation indicated the succession of the process. The cut test of the beans showed increasing percentage of well fermented beans as the fermentation increased. Meanwhile, the FI showed increasing concentration of anthocyanidins presented by the brown pigments of the bean cotyledons. The slight decrease of the FI at the end of fermentation (day 6) may indicate that the fermentation was complete and should be terminated. There was no specific pattern on the sensory evaluation of the different clones at different fermentation duration. However, MCBC 2 fermented for 5 days has the most acceptable score of sensory evaluation compared to other clones.

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Preliminary investigation into chemical characteristics of native organic residues in the Northeast, Thailand

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Abstract

Sandy soils are one of the major soil types in the Northeast, Thailand which are recognized to be infertile and low plant nutrient contents. Organic matter management have been believed to be able to improve the status of plant nutrients in sandy soils. The objective of this research aims at investigating the chemical characteristics of the plant nutrient contents, including boron (B), calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), nickel (Ni), phosphorus (P), and zinc (Zn), in organic residues which are locally available in the Northeast region of Thailand. The organic residues include groundnut stover (GN) (*Arachis hypogaea*), tamarind leaf (TM) (*Tamaridus*), dipterocarp leaf (DP) (*Dipterocarpus tuberculatus*), and rice straw (RS) (*Oryza sativa*). The preliminary results show that the concentrations of plant nutrients are depended on the types of organic residue. Groundnut stover, TM, DP, and RS mostly contain elevated amounts of Fe and K. Groundnut stover dominantly contains P (2025 ± 103 mg/kg) and K (7291 ± 1481 mg/kg). Tamarind mainly consists of B (39 ± 0.36 mg/kg), Ca (19837 ± 1291 mg/kg), Cu (6.3 ± 0.88), and Mg (5723 ± 570 mg/kg). The concentration of Mn (860 ± 79 mg/kg) is highest in DP and RS compared to other organic residues. This research clearly demonstrates that native organic residues in the Northeast Thailand have potential to be used as alternative nutrient supply for plant growing in sandy soils.

Keywords: Sandy soils; Organic residues; Plant nutrients; the Northeast region; Thailand

Introduction

Sandy soils in the Northeast Thailand have been extensively used for agriculture resulting in significant land degradation (Vityakon et al., 2004). Sandy soils in Thailand are recognized to be infertile because they contain high sand content, low water holding capacity, low pH (4.0-5.5), low cation exchange capacity, low soil organic matter, and low plant nutrients (Fujii et al., 2017). In order to enhance the level of nutrients in sandy soils, fertilizers become an essential nutrient source in ensuring sustainability of food production systems. Organic matters (e.g. manures, composts, and plant residues) are believed to be used as alternative sources for supplied nutrients and ameliorated physical and chemical properties of sandy soils (Mackay et

al., 2017). Nowadays, various types of organic residue, including rice straw, some green manures, and tree leaf litters, can be found locally in the Northeast Thailand (Vityakon et al., 2000). In theory, when the organic residues are decomposed, nutrients can be released into soil to supply nutrients for plants. The objective of this research is to preliminarily investigate the plant nutrient concentrations in the organic residues which are locally available in the Northeast, Thailand.

Materials and Methods

Organic residue preparation

Organic residues used in this study are locally available in the Northeast Thailand. The organic residues, including groundnut stover (GN) (*Arachis hypogaea*), tamarind leaf (TM) (*Tamaridus*), dipterocarp leaf (DP) (*Dipterocarpus tuberculatus*), and rice straw (RS) (*Oryza sativa*), were dried in an oven at 70°C until the weight was constant (up to 7 days), then they were cut and grounded to make the samples homogenous.

Organic residue analysis

The pH and electrical conductivity (EC) of the organic residues were determined in a 1:20 deionized water (Milli-Q, 18.2 $\mu\Omega/\text{cm}$) extract. Total organic carbon (TOC) and total nitrogen (TN) were determined by dry combustion on a CN analyzer.

The organic residues were digested in concentrated perchloric acid (HClO_4). Briefly, 0.4 g of ground organic residues was treated with 5 mL of HClO_4 at 190-200°C until the solution is clear. Final extract was diluted with Milli-Q water to a final volume of 20 mL (Ksawery et al., 2010). Total elements in organic residues were determined using inductive coupled plasma-optical emission spectrometry (ICP-OES) (Perkin-Elmer Optima 7300 DV).

Results and Discussion

Organic residue characteristics

The organic residues are locally available in the Northeast Thailand are acidic ranging from 3.7-5.8 (Figure 1). Tamarind leaf has the lowest pH value (3.7 ± 0.04) because TM contains high content of organic acids, particularly tartaric acid (van den Bilcke et al., 2014). The electrical conductivity (EC) in the organic residues are elevated ranging between 2.3 ± 0.03 and 3.0 ± 0.08 mS/cm, except DP ranging between 0.80 and 0.02 mS/cm (Figure 1). This result indicates that GN, TM, and RS contain high amounts of soluble salt.

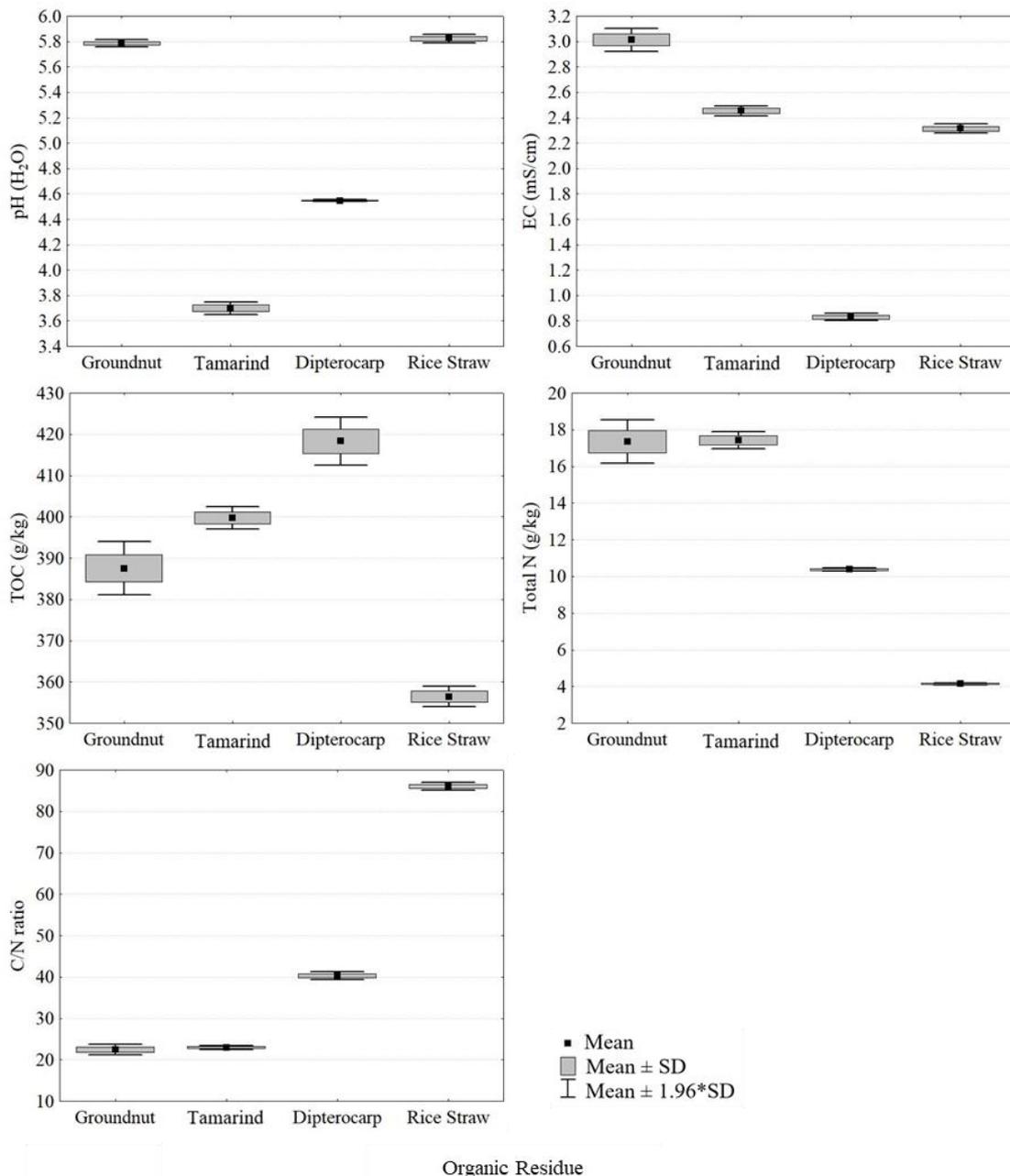


Figure 1. Some chemical properties for native organic residues in the Northeast, Thailand

The average contents of TOC in GN, TM, DP, and RS are 388 ± 5.7 , 400 ± 2.4 , 418 ± 5.1 , and 356 ± 2.2 g/kg, respectively. Groundnut stover and TM contain elevated concentrations of TN (17 ± 1.1 g/kg and 17 ± 0.42 g/kg, respectively) compared to dipterocarp leaf (DP) (10 ± 0.09 g/kg) and rice straw (RS) (4.1 ± 0.06 g/kg). Consequently, the C/N ratio value of GN, TM, DP, and RS are 22 ± 1.1 , 23 ± 0.42 , 40 ± 0.83 , and 86 ± 0.91 , respectively which reflects the rate of organic residue decomposition. On the basis of the C/N ratio result, GN and TM can be classified to be the high quality of organic residue which becomes easily decomposed by soil microorganisms, while RS and DP can be classified as the lower quality of organic residue which is hard to become decomposed (Moritsuka et al., 2004).

Total element concentrations in organic residues

Total element concentrations in locally-available organic residues in the Northeast, Thailand are shown in Table 2.

Table 2. Mean and standard deviation of total element concentration (mg/kg) for native organic residues in the Northeast Thailand

Element	Groundnut	Tamarind	Dipterocarp	Rice Straw
B	15 ± 2.1 b	39 ± 0.36 a	10 ± 0.21 c	2.1 ± 0.23 d
Ca	3490 ± 314 b	19837 ± 1291 a	4130 ± 1105 b	1222 ± 529 c
Cu	2.1 ± 0.67 b	6.3 ± 0.88 a	0.93 ± 0.18 c	0.87 ± 0.07 c
Fe	261 ± 107 a	281 ± 25 a	227 ± 42 a	151 ± 14 b
K	7291 ± 1481 a	6056 ± 1053 a	3334 ± 496 b	5513 ± 1288 a
Mg	1750 ± 148 b	5723 ± 570 a	1343 ± 82 c	597 ± 10 d
Mn	95 ± 18 b	93 ± 13 b	860 ± 79 a	855 ± 32 a
Na	140 ± 7.7 b	61 ± 24 c	48 ± 23 c	253 ± 77 a
Ni	1.7 ± 0.10 a	1.7 ± 0.09 a	1.9 ± 0.21 a	1.1 ± 0.03 c
P	2025 ± 103 a	277 ± 124 bc	163 ± 32	413 ± 16 b
Zn	15 ± 4.5 b	23 ± 7.9 a	4.8 ± 0.13 c	14 ± 2.2 b

The result shows that the concentrations of plant nutrients are depended on the types of organic residue as reported by other studies (Khanna et al, 1994; Yushiharni and Gilkes, 2012). Groundnut stover contains elevated concentration of K (7291 ± 1481 mg/kg), P (2025 ± 103 mg/kg), Fe (261 ± 107 mg/kg), and Ni (1.7 ± 0.10 mg/kg). Tamarind leaf contains high concentrations of Ca (19837 ± 1291 mg/kg), K (6056 ± 1053 mg/kg), Mg (5723 ± 570 mg/kg), Fe (281 ± 25 mg/kg), B (39 ± 0.36 mg/kg), Zn (23 ± 7.9 mg/kg), and Cu (6.3 ± 0.88 mg/kg). Dipterocarp leaf contains high concentration of Mn (860 ± 79 mg/kg), Fe (227 ± 42 mg/kg), and Ni (1.9 ± 0.21 mg/kg). Rice straw contains high concentrations of K (5513 ± 1288 mg/kg) and Mn (855 ± 32 mg/kg).

The finding of this research indicates that GN and TM are suitable to be use as alternative nutrient supplies for plants because GN and TM contains a number of plant nutrients and are easy to be decomposed by soil microbials.

Conclusions

Sandy soils in the Northeast Thailand are infertile, especially low plant nutrients. Organic residues including rice straw, groundnut stover, and tree leaf litters are locally-available in this region. The preliminary study of these native organic residues indicates that these native residues have diverse nutrient concentrations depending on types of residues. Groundnut stover, TM, DP, and RS mostly contain elevated amounts of Fe and K. Groundnut stover dominantly contains P and K. Tamarind mainly consists of B, Ca, Cu, and Mg. The concentration of Mn is highest in DP and RS compared to other organic residues. This research clearly demonstrates that native organic residues in the Northeast Thailand have potential to be used as alternative nutrient supply for plant growing in sandy soils.

Acknowledgements

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The effects of Paclobutrazol on the yield components and beta-carotene content of sweet potato var. VitAto

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Abstract

The effect of paclobutrazol (PBZ) as plant growth regulator on an orange-fleshed sweet potato var. VitAto storage root yield components and β -carotene content was tested. Field experiment laid out using Randomized Complete Block Design (RCBD) replicated four times was conducted by utilising PBZ as the main treatment. The treatments were 0 (control), 10, 20, 40 and 80 ppm PBZ applied as soil drenching per plant. The storage root samples were obtained using destructive sampling on 30, 55, 77 and 99 days after planting (DAP) corresponded to every growth stage of VitAto. The results showed that the 10 ppm PBZ (lowest concentration) increase most of the parameters such as storage root fresh weight, dry mass production and β -carotene content 41.3, 30.9 and 12.6% significantly higher than control treatment at the maturity stage respectively. The storage root number and harvest index also increased by 10 ppm PBZ higher than control although no significant differences were observed. These parameters also showed strong correlation to each other as shown through correlation studies. The application of 10 ppm PBZ was the best option to be used as a supplement to the recommended inorganic fertilization practice for VitAto cultivation on sandy tin-tailing soil.

Keywords: Orange-fleshed sweet potato; VitAto; Paclobutrazol; PBZ; β -carotene; yield components; storage root

Introduction

VitAto is an orange-fleshed sweet potato (OFSP) and that was introduced by Malaysian Agricultural Research and Development Institute (MARDI) in 2007. The terms “VitA” comes from **Vitamin A** and “to” from sweet potato were combined to give a varietal name of VitAto (Tan et al., 2010). There are many beneficial traits of VitAto such as high β -carotene content (precursor of vitamin A) up to 2000 μg per every 100 g fresh storage root, vitamin C, vitamin E and anthocyanin (Tan et al., 2010; Normah, 2010). It can produce yield more than 40 tonnes per ha equal to five times of Telong, Gendut and Jalomas varieties previously introduced by MARDI. With this high yield, farmers can earn high income from the cultivation of this variety up to RM 2000 per ha per month. In Malaysia, the crop can also be cultivated in many soil types including marginal soil such as sandy or Beach Ridges Interspersed with Swales (BRIS) soil. Another unique feature of this crop is that, the storage root of VitAto can be processed into the nutritious flour containing high β -carotene content that has a potential to replace imported wheat flour which cost Malaysia more than RM 800 million per year (Tan et al.,

2010). The dedicated factory for this purpose was recently built in Tembila, Besut in Terengganu, and the factory was supposed to start the operation by early 2015.

Paclobutrazol with chemical name of [(2RS, 3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)-pentan-3-ol] is one of the triazole derivatives (Jaleel et al., 2008a; Kamran et al., 2018). Triazole compounds are a group of fungicides that have plant growth regulating properties (Jaleel et al., 2008a). Currently, many other triazole derivatives are produced including triadimefon, hexaconazole, propiconazole, tebuconazole and bitertanol as reported in many studies (Tuna, 2014; Ijaz et al., 2015; Mourad et al., 2017).

There is limited published information on how PBZ affect growth and yield of sweet potato. However, PBZ has been widely used as treatments in other root crop species for the similar reason. In potato, the application of PBZ decreased the assimilate partitioning to stem, leaves, root and stolon but increased the partitioning of dry mass production to tubers (Tsegaw, 2006). The application of PBZ reduced the number of potato tubers (Tsegaw, 2006; Esmailpour et al., 2011; Mabvongwe et al., 2016), the number and fresh weight of tuber and harvest index of cassava (Medina et al., 2012). The reduction of harvest index of cassava also reported elsewhere (Panyapruet et al., 2016). However, there was an increase in tuber dry mass production and fresh weight of several crops such as elephant foot yam (Gopi et al., 2005), potato (Tsegaw, 2006; Esmailpour et al., 2011; Samy et al., 2014) and carrot (Gopi et al., 2007). The potato plant is generally grown mostly from tubers (CIP, 2015). The small or 'mini-tuber' often used as a mother tuber, the emergence of the new bud sprout become a new plant (Renee's Garden, 2012). The production of potato mini-tuber was decreased at high PBZ concentration (Hughes & Keith, 2003) but increased at low concentration (Bandara & Tanino, 1995).

VitAto has high β -carotene content more than 2000 $\mu\text{g}/100\text{ g}$ fresh weight as described by Tan et al., (2010). However, no study was reported on the approaches or efforts to improve or increase content of this pigment apart from breeding program to produce variety enriched in β -carotene content. The PBZ is a fungicide that can trigger the production of antioxidant such as carotenoid content in plants to fight against oxidative stress (Fletcher et al., 2000; Sivakumar et al., 2010; Tuna, 2014). The PBZ probably be able to increase β -carotene content since it is a major carotenoid content of sweet potato (Woolfe, 1992). The PBZ increase the carotenoid in storage root of carrot (Gopi et al., 2007).

The objective of this experiment was to investigate the effects of different concentrations of PBZ on the yield components and β -carotene content of sweet potato var. VitAto. In addition, the relationships between β -carotene and other yield components as influenced by PBZ were also examined.

Materials and Methods

Experimental Site Description

The area of experiment was located at the Malaysian Agricultural Research and Development Institute (MARDI) Rawang Station, Selangor, (N 3° 16' 17.89", E 101° 30' 52.19") at 66 meter above the mean sea level. The soil type was sandy tin-tailing soil with 90.8, 4.5 and 4.7% of sand, silt and clay, respectively (Vimala, 2005). The average monthly temperature was 23.7 to 28.6 °C, mean relative humidity was 76.2 to 86.5% and rainfall was 3.0 to 8.1 mm (Malaysian Meteorological Department, 2014).

Planting materials

The VitAto stem cuttings used as planting materials were obtained from MARDI's Rawang Station. The length of stem cutting used was 20 to 30 cm with 7 internodes (Tan et al., 2010). These cuttings were planted at 30 ° degrees slanting facing to the east with approximately 5 cm length and consisted of 2 to 3 internodes inserted in the soil. This procedure is important in order to achieve optimum number and reasonable size of storage root (Belehu, 2003).

Treatments and experimental design

Paclobutrazol with 28% w/w of a.i traded as Deltat Cul-tar (Syngenta Ltd, U.K) was the main plant growth regulator used in this study for yield and β -carotene enhancement. The treatments were the various PBZ concentrations namely 0 (control), 10, 20, 40 and 80 ppm per plant. These treatments were diluted in 500 ml water and applied as soil drenching at 20, 40 and 60 days after planting (DAP) modified based on experiment conducted by Gopi et al. (2007) and Jaleel et al. (2009). The five treatments were arranged in randomized complete block design (RCBD) and replicated four times.

Field operation

Chicken manure as basic fertilizer were applied one week before cultivation. The recommended inorganic fertilizer was given to the plant at 28, 35 and 49 DAP. Land preparation, cultivation and other agronomic practices were based on cultivation manual of VitAto on sandy tin tailing soil by MARDI (2008).

Growth analysis procedure

The destructive growth analysis technique based on Hunt (1990) was used for determination of yield and β -carotene content parameters at every growth stages; storage root initiation, early and middle bulking and maturity stages. The plants were harvested in sequence manner to ease in field operation (Jolliffe et al., 1982). In every harvest period, each plant per treatment per replication was harvested. The harvests periods were at 30, 55, 77 and 99 DAP corresponding to every growth stages of VitAto. Plant samples were separated into leaves, stem, root and storage root and placed in the oven for 48 hours at 72° C for dry mass determination.

Yield Component

The yield components can be described as number and fresh weight of storage and pencil size roots, storage root dry mass or harvest index. Storage root and pencil size root were measured at every growth stages starting from the storage root initiation through storage root bulking and finally at the maturity stage. The samplings for biomass primary data were selected to coincide with these growth stages. The root with the diameter length of greater than 15 mm was classified as storage root while those roots with diameter length ranged from 5 to 15 mm was classified as pencil root as described in Borhan et al. (2016). The pencil root was usually thin and elongated with some lignification produced and would not become storage root (Meyers et al., 2014). Pencil root was not generally considered as yield component but the diameter size was used to indicate the smaller storage root known as pencil size root which the diameter length measuring less than 15 mm. The physical characteristics of both roots were examined and identified before the actual measurement. Storage root dry mass and harvest index were measured at the final harvest or at the maturity stage. Storage root dry mass was obtained from the sum of storage root and pencil size root dry mass. Harvest index is the ratio of storage root dry mass to plant total dry mass produced.

Storage root beta-carotene content determination

The β -carotene content of storage root was determined from fresh storage root. The extraction of storage root β -carotene was carried out immediately after harvest since it was crucial to determine the level of β -carotene at that time of harvest to represent the actual content in relation to plant age. The sample used was not selected from any single or selected part of the storage root but instead include all available storage roots per plant to give an average content of β -carotene per plant. This was carried out due to the uneven distribution of this compound, and it was reported that the highest concentration was found in the core (Wu et al., 2008). The β -carotene extraction and determination method was carried out based on Rodriguez-Amaya and Kimura (2004). After extraction, the β -carotene content of the sample was analysed using B13627 Unicam Helios alpha UV-VIS spectrophotometer (Severn Sales Limited, United Kingdom) by taking the absorbance value at 450 nm of wavelength. The absorbance values obtained from the spectrophotometer were used to calculate the total β -carotene content using the following formula (Rodriguez-Amaya & Kimura, 2004):

- Total β -carotene content ($\mu\text{g/g}$) =
$$\frac{A \times \text{Volume (mL)} \times 10^4}{(A_{1\text{cm}}^{1\%}) \times \text{Sample weight (g)}}$$

Where A is absorbance at 450 nm wavelength, volume refers to total volume of extract (50 or 25 ml), $(A_{1\text{cm}}^{1\%})$ is the absorption coefficient of β -carotene in PE (2592). The multiply by 100 to give the β -carotene content in $\mu\text{g}/100$ g of fresh storage root.

Correlation coefficient among growth parameters

Correlation studies were conducted to determine various relationships between the various yield and yield quality measured parameters measured.

Statistical analysis

A statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparisons to evaluate the statistical significance of multiple comparison data of storage root yield components and β -carotene content. The relationship between variables was determined using Pearson correlation. The p values ≤ 0.05 was used to determine the level of significance.

Results

Yield components

The storage root number and fresh weight were generally increased from the initiation to maturity stages (Figure 1a and b). In contrast, pencil size root number and fresh weight were increased from the initiation to middle bulking stages (77 DAP) before declining thereafter (Figure 1c and d). The storage root number for the control treatment was significantly higher than 10 ppm at the early bulking stage (55 DAP) but 10 ppm PBZ showed rapid increased when approaching the maturity stage and tended to be higher than other treatments although no significant difference was observed. The storage root fresh weight for 20 and 10 ppm PBZ were significantly higher than 40 ppm at the middle bulking (77 DAP) and 80 ppm PBZ at maturity stages respectively.

The pencil size root number for 20 ppm was significantly higher than 40 and 80 ppm at the early bulking stage (55 DAP) while 40 ppm was significantly higher than 10 ppm PBZ at the maturity stage. The pencil size root fresh weight for 40 ppm was significantly higher than 10 ppm PBZ at the maturity stage but no significant differences were observed previously. The storage root dry mass production for 10 and 20 ppm were significantly higher than control and 80 ppm treatments while harvest index for control, 10 and 80 ppm were significantly higher than 40 ppm PBZ treatments at the maturity stage (99 DAP) (Figure 2a and b).

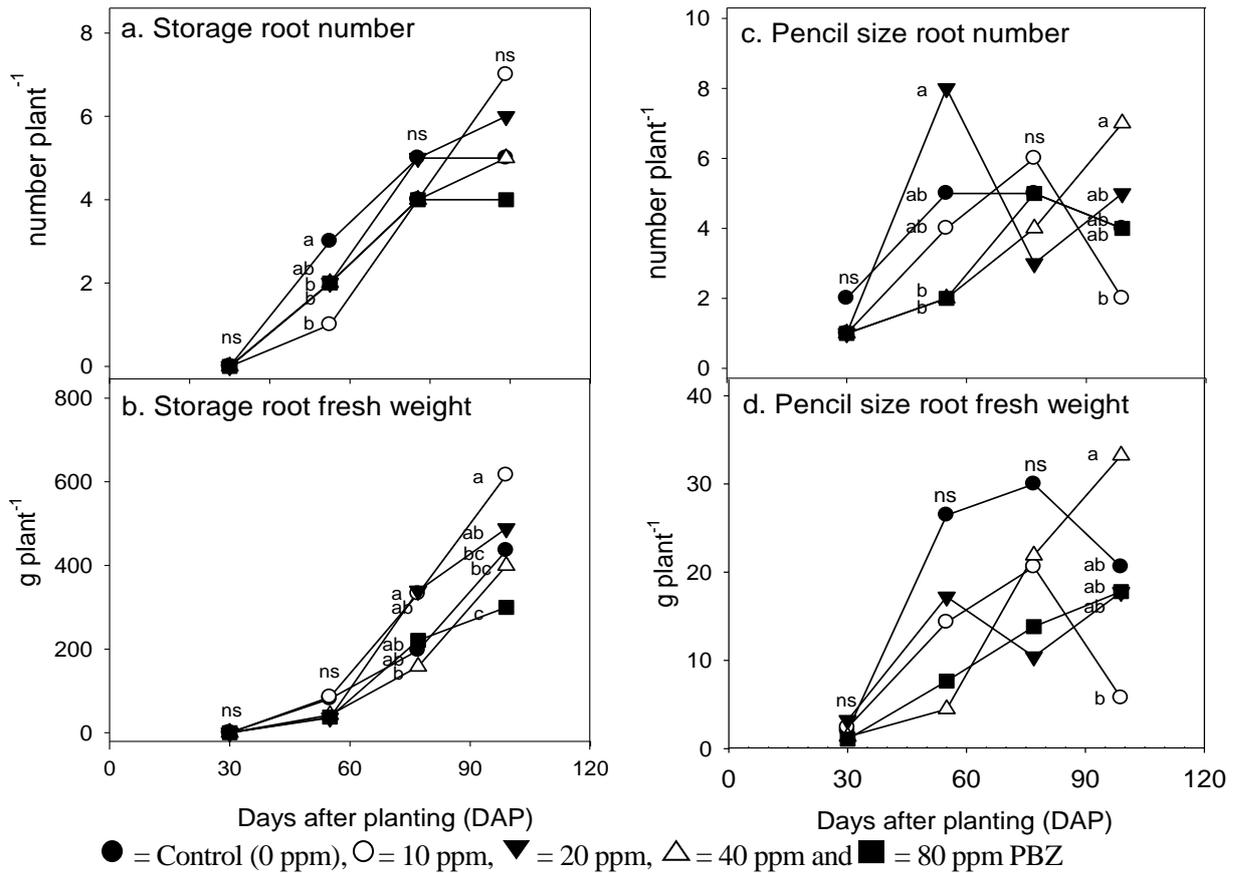


Figure 1. Yield components of VitAto as affected by various concentrations of PBZ treatments throughout the entire cropping period. Different letters follow different treatments at specific harvest time indicate a significant difference at $p \leq 0.05$ while ns indicate no significant. The values are the means of four replicates.

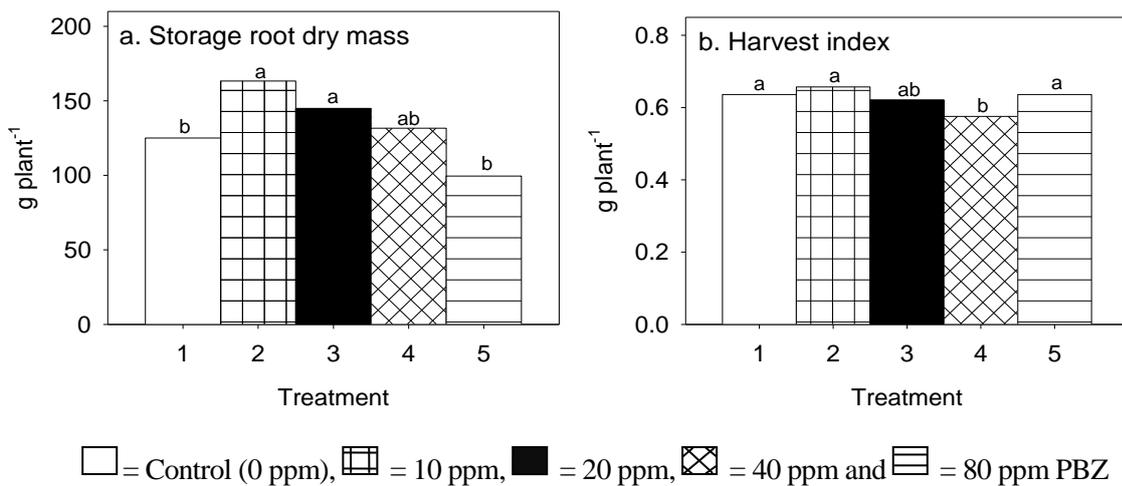
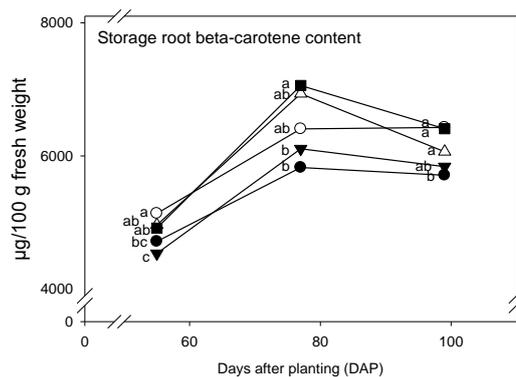


Figure 2. Yield components (storage root dry mass and harvest index) as affected by various concentrations of PBZ treatments at the maturity stage. Different letters follow different treatments at specific harvest time indicate a significant difference at $p \leq 0.05$ while ns indicate no significant. The values are the means of four replicates

Level of storage root beta-carotene content

The storage root β -carotene content for 10 ppm PBZ showed an increment from storage root early bulking (55 DAP) to maturity stages while other treatments were increased only from early bulking to middle bulking stages (55 to 77 DAP respectively) before declined thereafter (Figure 3). The storage root β -carotene content for 10 ppm was significantly higher than control and 20 ppm PBZ treatments at early bulking stage (55 DAP). In contrast, storage root β -carotene content for 80 ppm was significantly higher than control and 20 ppm PBZ at middle bulking stage (77 DAP). The storage root β -carotene content for 10, 40 and 80 ppm PBZ were significantly higher than the control at the maturity stage.



● = Control (0 ppm), ○ = 10 ppm, ▼ = 20 ppm, △ = 40 ppm, ■ = 80 ppm

Figure 3. Storage root β -carotene content as affected by various concentrations of PBZ treatments from the middle storage root bulking to maturity stages. Different letters follow different treatments at specific growth stage indicate a significant difference at $p \leq 0.05$ while ns indicate no significant. The values are the means of four replicates

Correlation studies of various parameters related to yield components and storage root beta-carotene content throughout the entire cropping period

The storage root number had strong positive correlation with storage root fresh weight and storage root dry mass production ($r = 0.70^{***}$ and 0.72^{***} respectively) but was not significantly correlated with pencil size root number, fresh weight and β -carotene content ($r = 0.01, 0.18$ and 0.28 respectively) and at the same time negatively correlated with harvest index ($r = -0.23^*$). The storage root fresh weight was strongly correlated with storage root dry mass ($r = 0.98^{***}$) and β -carotene content ($r = 0.45^{***}$). However, it had no significant correlation with pencil size root fresh weight and harvest index ($r = 0.02$ and 0.01 respectively) and negatively correlated with pencil size root number ($r = -0.11$). The pencil size root number showed strong correlation with pencil size root fresh weight ($r = 0.84^{***}$) and was negatively correlated with harvest index and β -carotene content ($r = -0.60^*$ and -0.04 respectively) but no correlation with storage root dry mass ($r = 0.19$). The pencil size root fresh weight was moderately correlated with storage root dry mass ($r = 0.37^{**}$) but negatively correlated with harvest index ($r = -0.69^*$) and β -carotene content ($r = -0.02$).

Table 1. Correlation coefficient of various parameters related to dry mass production, growth rate components and yield components as affected by various low concentrations of PBZ treatments.

Parameters	SRN	SRTFW	SRDM	PSRN	PSRTFW	HI
SRTFW	0.70** *					
SRDM	0.72** *	0.98***				
PSRN	0.01	-0.11	0.19			
PSRTFW	0.18	0.02	0.37***	0.84***		
HI	-0.23*	0.01	-0.62*	-0.60*	-0.69*	
SRBC	0.28	0.45***	0.38*	-0.04	-0.02	0.26

Note: SRN: Storage root number; SRTFW: Storage root total fresh weight; SRDM: Storage root dry mass production; PSRN: Pencil size root number; PSRTFW: Pencil size root total fresh weight; HI: Harvest index; SRBC: Storage root β -carotene content

*, **, *** indicate significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively while growth parameters without * indicate no significant correlations. The values are the means of four replicates.

Discussions

Yield Components

The response of yield components to various paclobutrazol concentrations

In general, the storage root number and total fresh weight showed positive response to 10 ppm PBZ. The 10 ppm PBZ had increased storage root number as well as storage root fresh weight and dry mass production, indicating the bigger sink size availability to store greater assimilates or inputs. The storage root production for short growing duration cultivars that is source limited would basically depend on sink capacity (strength) rather than source capacity or photosynthetic efficiency (Li & Kao, 1985; Bhagsari, 1990). The low PBZ concentrations were proven to increase tuber fresh weight and dry mass production of potato (Anjanappa et al., 2000), carrot (Gopi et al., 2007) and cassava (Panyapruerk et al., 2016). Bhattarai (2017) showed increment of potato mini-tuber number and fresh weight. However, some other studies that used low PBZ concentrations showed similar effects as high PBZ concentrations used in this study where they increased tuber number but reduced tuber fresh weight of potato (Bandara et al., 1998). There were also several studies shown that the PBZ treatments resulting in the reduction in the number but increased the fresh weight and dry mass production of potato tuber (Tsegaw, 2006) and minituber (Kianmehr et al., 2012). In contrast, the PBZ application in cassava reduced both number and fresh weight of tuber (Medina et al., 2012). These variations of effects were influenced by the different levels of PBZ concentrations used and the degree of plant response.

The relationship among yield components

Storage root number was the first yield component (sink potential) determined if compared to storage root fresh weight and dry mass. The storage root was initiated at the second growth phase from storage root initiation to storage root bulking stages (Somasundaram & Santhosh Mithra, 2008). It is of paramount important to increase the storage root number earlier because it can maximize the marketable yields (Nwankwo & Afuape, 2013; Meyers et al., 2014). The developing storage root would rapidly gain in weight probably at the final growing phase from storage root bulking to maturity stages where most of assimilate was translocated to storage

root (Lebot, 2009; Borhan et al., 2017a). Therefore, the higher storage root numbers the higher storage root fresh weight. The storage root number and total fresh weight were strongly correlated with each other under low PBZ concentrations ($r = 0.70^{***}$). The result was in agreement with the results obtained by Bhagsari (1990) and Borhan et al. (2016). In this study, the storage root number also strongly correlated with the storage root dry mass parallel to finding of (Borhan et al., 2016). The correlation studies from both experiments showed strong correlation between storage root dry mass and fresh weight with corroborate with studies reported (Abdissa & Dechassa, 2012; Borhan et al., 2016).

General trend in storage root beta-carotene content

The storage root β -carotene content responded positively to PBZ treatments especially to 10, 40 and 80 ppm. Since all treatments were significantly higher than the control at the maturity stage. The enhancement of storage root β -carotene started earlier at the storage root middle bulking stage with the value increased from 5826 in the control treatment to a maximum of 7058 $\mu\text{g}/100\text{ g}$ in 80 ppm PBZ. These values were in the similar range as reported in other studies on orange-fleshed storage root of sweet potato with values ranging from 150 to 12390 $\mu\text{g}/100\text{ g}$ (Mbusa et al., 2018), 802 to 1209 $\mu\text{g}/100\text{ g}$ (Williams et al., 2013) and around 5091 to 16456 $\mu\text{g}/100\text{ g}$ (Laurie et al., 2012). Based on the results obtained in this study, it can be concluded that the application of PBZ could be used as a potential additional or optional input to enhance the storage root β -carotene content. The finding of this study corroborate with findings of other studies on the role of Triazole compounds in enhancement of carotenoid content of many crops such as carrot (Gopi et al., 2007), sweet potato (Sivakumar et al., 2009) and tomato (Shanmugapriya et al., 2013; Tuna 2014).

Relationship of beta-carotene content and yield components

The correlation studies shows that the increase in storage root β -carotene was in parallel with an increase in yield component parameters. This was indicated by significant correlation of storage root β -carotene content with storage root fresh weight (0.45^{***}) and storage root dry mass (0.38^*). The 20 ppm PBZ and HEX were shown to enhance tuber carotenoid content, fresh weight and dry mass production of carrot (Gopi et al., 2007).

The increased storage root β -carotene content only up to middle bulking stage (77 DAP) and then the content was decreased gradually approaching maturity. The development pattern of pigment including β -carotene is generally do not exhibit a linear increase but rather in sigmoidal form. The increase in β -carotene is usually parallel with an increase in fresh weight and dry mass of storage root as indicated in the correlation study. However, the content will eventually decrease when the size of storage root exceeding a certain ceiling size. This observation was in agreement with Chattopadhyay et al. (2006), Faber et al. (2013) and Borhan et al. (2017b).

The high versus low concentrations of paclobutrazol

The optimum PBZ concentration for maximum storage root β -carotene content was 80 ppm treatment and took place at during the storage root enlargement stage, however, 10 ppm treatment favored maximum content at the maturity stage. It seemed that low concentration gave better enhancement in term of maximising β -carotene content as well as enhancement of other yield components. Similar results were reported on studies of other root crop species that utilizing low concentrations of triazole compound derivatives as treatments with concentrations ranging from 10 to 30 ppm to increase carotenoid content in the leaves and tuber of white yam (Jaleel et al., 2008b) and in the leaves of *Plectranthus forskholii* (Lakshmanan et al., 2007) and sweet potato (Sivakumar et al., 2009; Borhan et al., 2017b).

The storage root β -carotene content seemed to increase with the increments of storage root dry mass as shown in correlation studies. This finding indicated that, β -carotene content could be preserved in storage even after the storage root undergone processing into flour as suggested by Tan et al. (2010). However, there will be a slight reduction of β -carotene content as the storage root undergone processing processes. The β -carotene content in fresh OFSP is 8.75 mg while 8.04 mg when dried (Emmanuel et al., 2012), while the β -carotene content of VitAto is 2000 μ g/100g on fresh weight basis (Tan et al., 2010) and reduced to 900- 960 μ g/100g (Normah, 2010) after processing to become flour. Ideally, the β -carotene content in the fresh storage root should be high to begin with, and consequently, the content should remain relatively high after processing. Based on this study, the best treatment that can maximize the β -carotene content at harvestable stage (maturity) was 10 ppm PBZ.

Conclusion

Among PBZ treatments that use as supplement to the recommended inorganic fertilizer, the best treatment that can increase VitAto yield components and β -carotene content was 10 ppm PBZ. In general, the effects of PBZ showed that the lower the concentrations used, the higher the yield of VitAto would be obtained. This treatment increase storage root dry mass significantly higher than control at the maturity stage. The higher storagw root dry mass eventually increase the size of storage root. It is evident from this study that low PBZ concentrations were able to increase the sink strength of storage root to attract assimilate at reproductive stage. The 10, 40 and 80 ppm PBZ treatment plants had significantly higher storage root β -carotene content than the control at the maturity stage. However, the 10 ppm PBZ was considered as a better treatment because it showed an enhancement towards overall parameters such as growth, yield as well as storage root β -carotene content at harvestable stage (maturity).

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The effects of Paclobutrazol on the dry mass production and partitioning of VitAto, an orange-fleshed sweet potato

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Abstract

Field experiment was conducted on sandy tin-tailing soil to investigate the effect of paclobutrazol (PBZ) on sweet potato variety VitAto plant dry mass production and partitioning. The treatments were various paclobutrazol concentrations namely 0 (control), 10, 20, 40 and 80 ppm. The layout of experiment was a Randomized Complete Block Design (RCBD) with four replications. Destructive biomass samples were obtained at selected growth stage; storage root initiation, early and middle bulking and maturity corresponded to 30, 55, 77 and 99 days after planting (DAP), respectively. As results, most of the parameters measured responded positively to 10, 20 and 40 ppm paclobutrazol treatments in comparison to control treatment at the maturity stage. In addition, 10 ppm paclobutrazol was the best treatments that can increase most of the parameters measured especially for plant total dry mass and storage root dry mass productions which 28.7 and 30.9% significantly higher than control at the maturity stage. If the total dry mass can be increased, the partitioning of assimilate from above to below ground parts also increased as showed through correlation studies between total dry mass and storage root mass ratio ($r = 0.81^{***}$). The higher total dry mass contributes to the higher storage root dry mass production ($r = 0.98^{***}$). As indicated from this study, paclobutrazol at 10 ppm concentration can be used by farmers as supplement to inorganic fertilizer application in VitAto cultivation on sandy soil.

Keywords: Sweet potato; VitAto; paclobutrazol; dry mass production and partitioning; destructive sampling

Introduction

Paclobutrazol is one of triazole compounds; a group of systemic fungicide that can act as a plant growth regulator (Jaleel et al., 2008; Kamran et al., 2018). Triazole compounds generally are more effective than other plant growth regulators and required only in a relatively low concentration for effective application (Nivedithadevi et al., 2012; Newman, 2014). The effects of triazoles either on stimulation or reduction on the root growth would depends on the concentrations used as well as cultivar response (Davis et al., 1988; Fletcher and Hofstra, 1988). However, this statement is not only applied specifically to root growth but also to other plant parts or the whole plant growth. The PBZ was reported to reduce the total dry mass production of *Lantana camara* (Ruter, 1996), pepper (Grossi et al., 2005) and potato (Tsegaw,

2006). In some species such as strawberry, high PBZ concentrations ranging from 75 to 1200 ppm resulted in the reduction of the above, below ground parts as well as total dry mass production (Deyton et al., 1991). In contrast, low PBZ concentrations such as at 20 ppm increased the total dry mass production of other crop species such as elephant foot yam (Gopi et al., 2005) and carrot (Gopi et al., 2007). The type of crop species may also give differential response to concentrations used. For *Lantana camara*, leaf area, stem length and total dry mass production were reduced at low concentration of 14 ppm but gave stimulatory effects at high concentration of 60 ppm (Ruter, 1996). Most parameters of the above ground parts of root crop species were generally reduced by PBZ application as reported in many studies. These parameters included leaf area of *Catharanthus roseus* (Jaleel et al., 2008), plant height in onion (Ashrafuzzaman et al., 2009), plant height, stem number, leaf and stem dry mass production of potato (Esmailpour et al., 2011), leaf area, total fresh weight and, plant height and stem dry mass production of cassava (Medina et al., 2012; Panyapruet et al., 2016).

VitAto is an orange-fleshed sweet potato (OFSP) variety that was introduced by Malaysian Agricultural Research and Development Institute (MARDI) in 2007 (Tan et al., 2010). The VitAto variety is also listed as one of the recommended crops under East Coast Economic Region (ECER). The crop has been used as a replacement crop for tobacco in Kelantan and Terengganu which widely cultivated on BRIS soil (Tan et al., 2010; Rosly, 2010). Since the introduction of ASEAN Free Trade Agreement (AFTA) in 2010, tobacco as the main crop for BRIS soil was no longer viable and had been gradually replaced with VitAto and kenaf.

The problem arises when the yield obtained by farmers from cultivating VitAto on BRIS soil is usually lower than expected. The low yield of VitAto may be due to several reasons but, it is believed that the amount of fertilizer application is still inadequate for the production of good yield. To overcome this problem, PBZ was used as a supplement to the existing inorganic fertilization practice. If PBZ is effective, under this hormone application, the assimilate as product of photosynthesis can be translocated more to the storage root and not to other plant parts especially during maturity stage. Since, the main action of fungicide under triazole compound is to increase the assimilate translocation from shoot to root (Gomathinayagam et al., 2009; Borhan et al., 2017a; Kamran et al., 2018). Therefore, the objective of this study is to investigate the effect of PBZ on the dry mass production and partitioning of VitAto.

The experiment was conducted on sandy tin-tailing soil although the problem of low yield of VitAto occur on BRIS soil. However, the result from this study probably applicable for BRIS soil because both soil types possessed many similarities in term of their physical, chemical and biological characteristics despite of different localities and origins as shown in Table 1. These soils contain more than 90% of sand and only around 5% of silt and clay, low content of organic matter and nutrients, high soil pH, and poor water holding capacity. Their soil surface temperature is usually high due to their poor or low organic matter content. Both BRIS and sandy tin-tailing soils are either lack or have minimal microbial activities which are important in decomposition and cycling of minerals nutrients in the soil (Bot and Benites, 2005).

Table 1. Comparison of soil properties between BRIS and sandy tin-tailing soils

Parameters	BRIS	Sandy tin-tailing soil
Physical		
Coarse sand (%)	93 – 98.2	90.8 – 92
Fine sand (%)	5	6
Silt (%)	0 – 1.3	0 – 4.5
Clay (%)	2.0 – 7	4.7 – 7
Organic matter	Low	Low
Soil surface temperature	High	High
Structural stability	Poor	Poor
Water holding capacity	Poor	Poor
Moisture content	Low	Low
Chemical		
Nitrogen (%)	0.002 – 0.02	0.01 – 0.07
Organic carbon (%)	0.86	0.042 – 1.79
C:N ratio	43	25.57
Available P ($\mu\text{g/g}$)	0.78	0.48 – 0.76
Ex. K (cmol (+) kg^{-1})	0.01 – 0.04	< 0.01 – 0.03
Ex. Ca (cmol (+) kg^{-1})	0.05 – 0.06	0.02 – 0.07
Ex. Mg (cmol (+) kg^{-1})	0.02 – 0.16	0.003 – 0.059
CEC (cmol (+) kg^{-1})	0.3	0.4 – 1.95
Wet pH	5.0	4.2 – 5.1
Dry pH	6.18	6.15

Note: P = Phosphorus, K = Potassium, Ca = Calcium, Mg = Magnesium, CEC = Cation exchange capacity and Ex. = Exchangeable. (Lim et al., 1981; Ang et al., 2001; Vimala, 2005; Wan Rasidah et al., 2013).

Materials and method

Experimental site description

The experiment was conducted from February to June 2013 at the Malaysian Agricultural Research and Development Institute (MARDI) Rawang Station, Selangor. It was located at N 3° 16'17.89", E 101°30' 52.19" at 66 meter above the sea level. The soil type at the experimental site is sandy tin tailing with 90.8, 4.5 and 4.7% of sand, silt and clay respectively (Vimala, 2005). The selected climatic parameters during the experiment are presented in Table 2 based on Malaysian Meteorological Department (2014).

Table 2. Selected climatic parameters during the experimental period (2013)

Month/ Year	Temperature ($^{\circ}\text{C}$)		Rainfall (mm)	Mean Relative Humidity (%)
	Minimum	Maximum		
February	24.1	28.1	3.3	83.4
March	25.7	28.2	3.3	79.8
April	25.7	28.4	6.3	82.5
May	25.5	28.6	7.1	80.7
June	25.7	28.5	3.0	76.2

Source: Malaysian Meteorological Department (2014)

Experimental details

Field trial was conducted (October 2012 to February 2013) before the actual experiment carried out using various high concentrations of paclobutrazol from 100 to 400 ppm as treatments. The result shows that, most of the parameters measured responded positively to the low concentration of paclobutrazol. Therefore, the actual experiment uses various low PBZ concentrations as treatments.

The stem cutting of sweet potato var. VitAto around 30 cm in length were obtained from MARDI Rawang Station and used as planting materials. Actual experiment was conducted using Randomized Complete Block Design (RCBD) with four replications. The treatments were 0 (control), 10, 20, 40 and 80 ppm of PBZ (28% w/w of a.i traded as Deltat Cul-tar, Syngenta Ltd, U.K) diluted in 500 ml water before applied as soil drenching. They were applied on 20, 40 and 60 days after planting (DAP) modified based on experiment conducted by Gopi et al. (2007) and Jaleel et al. (2009). The PBZ was given in addition to the standard inorganic fertilizer application practice that recommended by Malaysian Agricultural Research and Development Institute (MARDI) currently used for VitAto cultivation on sandy soil and being adopted by farmers (MARDI, 2008). Chicken manure was applied a week before cultivation while NPK blue fertilizers were applied at 28, 35 and 49 DAP. Land preparation, cultivation and other agronomic practices were based on cultivation manual of VitAto on sandy tin tailing soil by MARDI (2008).

Growth analysis procedure

The experiment involved destructive growth analysis procedure for determination of dry mass production and leaf area at selected growth stages. The destructive growth analysis method by Evans (1972) and Hunt (1990) were used. The sequential growth analysis technique was used to ease in field operation by Jolliffe et al. (1982). One plant sample per replicate per treatment was harvested at each harvest period. The harvest periods corresponded to growth stages beginning from storage root initiation to storage root bulking and at maturity or consumable stage (final harvest). In this experiment, the harvests were at 30, 55, 77 and 99 DAP (corresponding to storage root initiation, early and middle bulking and maturity growth stages respectively). Plant samples were separated into leaves, stem, root and storage root. They were placed in the oven for 48 hours at 72° C for dry mass determination.

Leaf area, dry mass production and partitioning determination

The leaf area was determined using Adobe Photoshop CS6 which based on Jarou (2009). Sweet potato plant parts dry mass consisted of leaf, stem, root and storage root were measured using AL204 analytical balance (Mettler Toledo, United States). These data were obtained at 30, 55, 77 and 99 days after planting (DAP). The sum of all plant parts dry mass were used to compute total dry mass production. They dry mass partitioning data were calculated based on the dry mass production data using the following formula as described by several workers (Hunt, 1990; Bhagsari, 1990):

- Leaf mass ratio = Leaf dry mass/Total dry mass
- Stem mass ratio = Stem dry mass/Total dry mass
- Root mass ratio = Root dry mass/Total dry mass
- Storage root mass ratio = Storage root dry mass/Total dry mass
- Root to shoot ratio = (Root + Storage root dry mass)/ (Leaf + Stem dry mass)

Where, Lf_w , St_w and Rt_w and SRt_w are leaf, stem root and storage root dry mass (g) respectively, W = Total plant dry mass (g)

Statistical analysis

The data was analyzed using the analysis of variance (ANOVA) and Tukey's pairwise comparison to evaluate the statistical significance of multiple comparison data. The p values ≤ 0.05 was used to determine the level of significance. The relationship between variables was determined using Pearson correlation.

Result

Leaf area and dry mass production

The total, stem, root and storage root dry mass production showed a general increase from the initiation to maturity stages (Figure 1 a, c, d and e). In contrast, leaf area and leaf dry mass production were increased from the initiation to middle bulking (77 DAP) stages before declining thereafter (Figure 2 b and f). The leaf area for control was significantly higher than 20 and 40 ppm at the initiation stage but the 40 ppm PBZ treatment showed significant higher leaf area than the control at the maturity stage. The total dry mass production for control and 10 ppm were significantly higher than 80 ppm at the early and middle bulking stage (55 and 77 DAP respectively) but at the maturity stage 10 ppm was significantly higher than both control and 80 ppm PBZ treatments.

The leaf dry mass production for control, 10 and 20 ppm were significantly higher than 80 ppm at the early bulking stage (55 DAP) while 10 ppm PBZ was significantly higher than the control at the maturity stage. The control and 40 ppm had significant higher stem dry mass than 80 ppm treatment at the early bulking (55 DAP) and maturity stages respectively. The root dry mass for 20 ppm was higher than 40 ppm and 80 ppm PBZ treatments at the early bulking stage (55 DAP). However, 40 ppm showed significant higher stem dry mass than 20 ppm and 80 ppm PBZ treatments at the middle bulking (77 DAP) and maturity stages respectively. At the early bulking stage (55 DAP), the storage root dry mass for control was significantly higher than 80 ppm treatment. At the middle bulking stage (77 DAP) the 20 ppm was significantly higher than 40 ppm and 80 ppm PBZ treatments. However, at the maturity stage, 10 ppm was significantly higher than control and 80 ppm PBZ treatments.

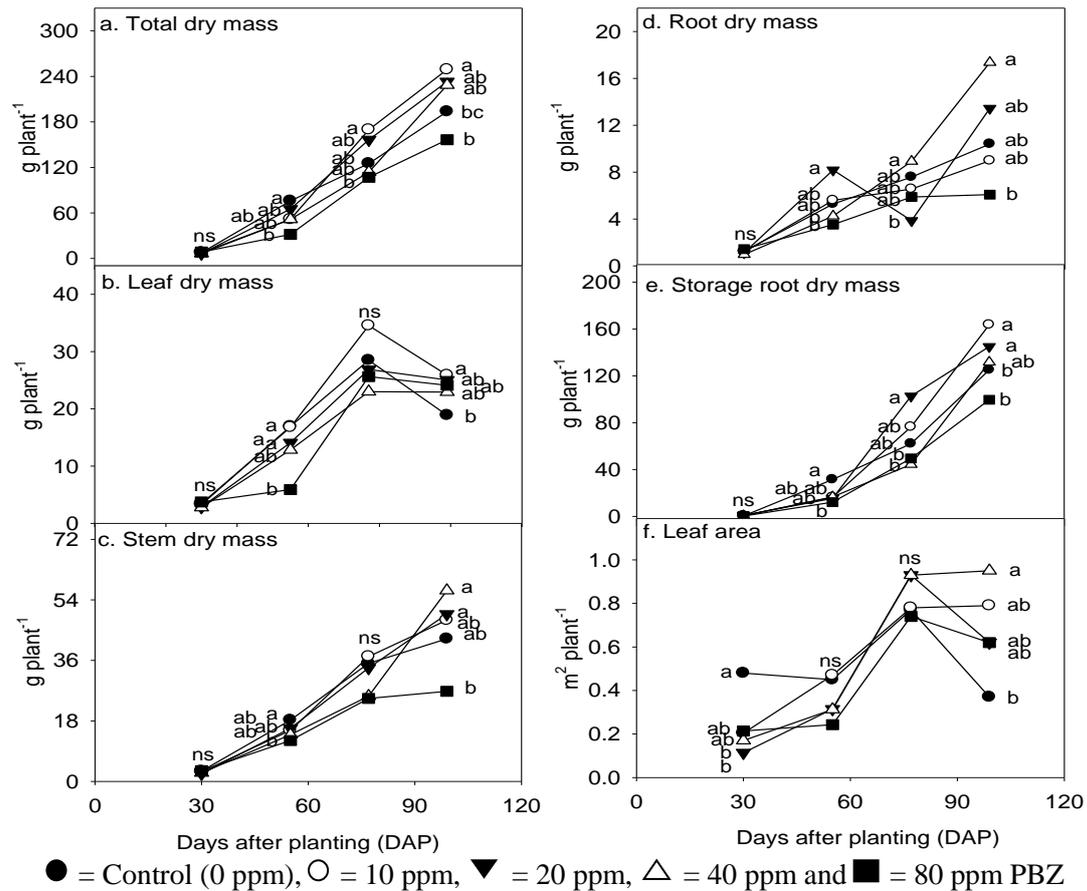


Figure 1. Dry mass of various plant parts and leaf area of VitAto as affected by various low concentrations of paclobutrazol treatments throughout the entire cropping period. Different letters follow different treatments at specific harvest time indicate a significant difference at $p \leq 0.05$ while ns indicate no significant. The values are the means of four replicates

Dry mass partitioning

The leaf, stem and root mass ratio were generally declined from the initiation to maturity stages (Figure 2 a, b and c). On the other hand, SRtMR and R/S Ratio were increased from the initiation to maturity stages (Figure 2 d and e). The LfMR for 80 ppm was significantly higher than 20 ppm PBZ at middle bulking stage (77 DAP) but no significant differences was observed at other growth stages. The StMR for 80 ppm was significantly higher than the control at the early bulking stage (55 DAP) while 40 ppm was significantly higher than 80 ppm PBZ at the maturity stage. The RtMR for 20 ppm PBZ was significant higher than the control treatment at the early bulking stage (55 DAP). However, no significant differences were observed at other growth stages. At the early bulking stage (55 DAP), the control treatment had significant higher SRtMR than 20 ppm while at the maturity stage, the 10 ppm was significantly higher than 40 ppm PBZ. The 10 ppm treatment also showed higher R/S ratio than 40 ppm PBZ at the maturity stage eventhough no significant differences were observed at other growth stages.

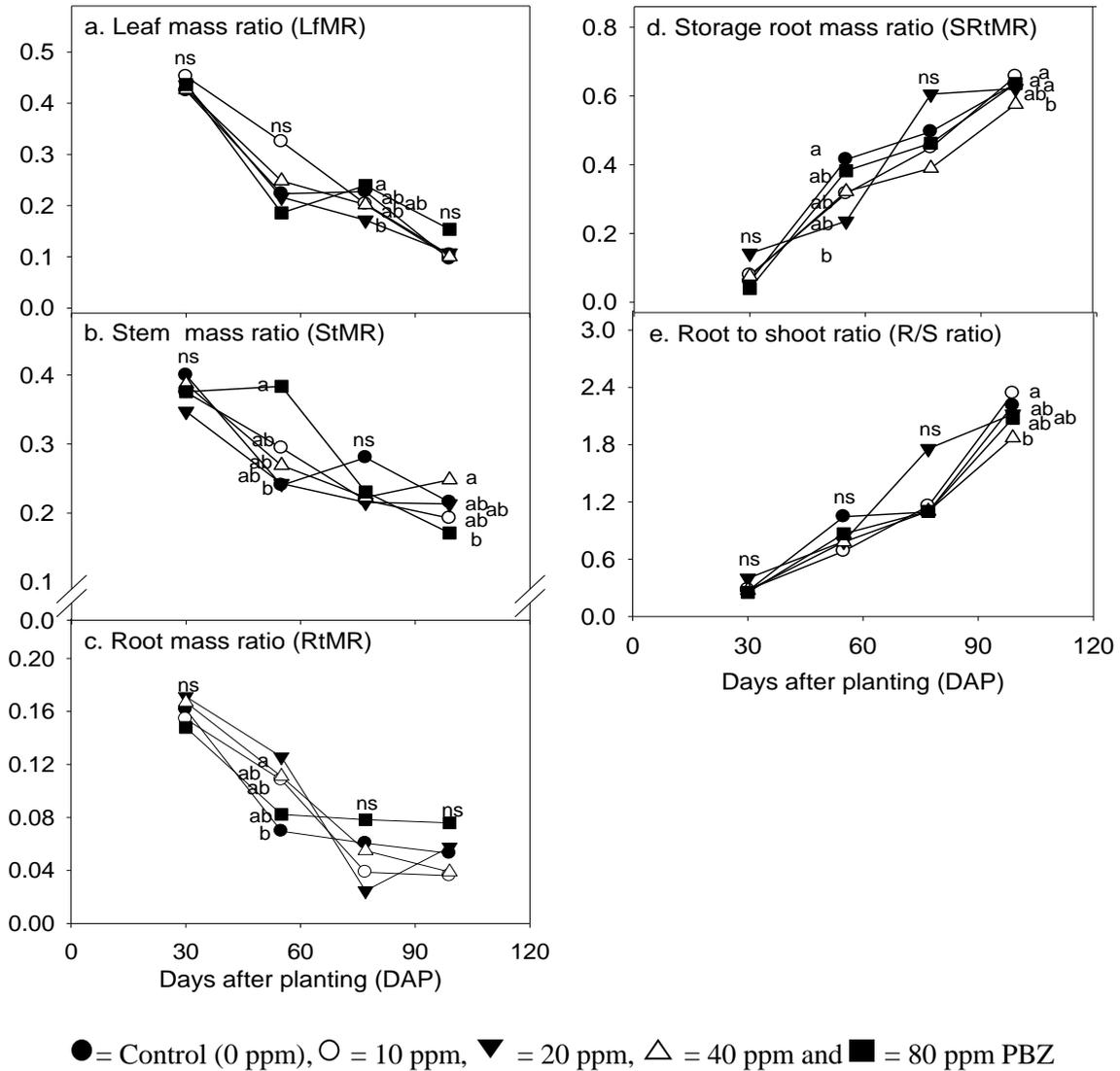


Figure 2. Dry mass partitioning of VitAto as affected by various low concentrations of paclobutrazol treatments throughout the entire cropping period. Different letters follow different treatments at specific harvest time indicate a significant difference at $p \leq 0.05$ while ns indicate no significant. The values are the means of four replicates

Correlation studies of various parameters related to dry mass production and partitioning throughout the entire cropping period

The correlation studies showed that, total dry mass was strongly correlated with leaf area, leaf, stem and storage root dry mass productions ($r = 0.68^{***}$, 0.84^{***} , 0.94^{***} and 0.98^{***} respectively) as presented in Table 3. The leaf dry mass showed strong correlations with leaf area, stem and storage root dry mass ($r = 0.75^{***}$, 0.82^{***} and 0.68^{***} respectively). The stem dry mass was also strongly correlated with storage root dry mass ($r = 0.88^{***}$).

The total dry mass production had strong positive correlation with SRtMR and R/S ratio ($r = 0.81^{***}$ and 0.87^{***} respectively). The total dry mass had strong negative correlation with LfMR and StMR ($r = -0.82^{***}$ and -0.73^{***} respectively). The LfMR was strongly correlated with StMR ($r = 0.78^{***}$) and negatively correlated with SRtMR and R/S ratio ($r = -0.93^{***}$ and -0.91^{***} respectively). The StMR was negatively correlated with both SRtMR and R/S ratio ($r = -0.88^{***}$ and -0.87^{***} respectively), and the SRtMR was strongly correlated positively with R/S ratio ($r = 0.94^{***}$).

Table 3. Correlation coefficient of various parameters related to dry mass production and partitioning as affected by various low concentrations of paclobutrazol treatments

Para.	Total DM	LfDM	StDM	SRtDM	LfA	LfMR	StMR	SRtMR	R/S
LfDM	0.84** *								
StDM	0.94** *	0.82** *							
SRtDM	0.98** *	0.68** *	0.88** *						
LfA	0.68** *	0.75** *	0.65** *	0.51** *					
LfMR	- 0.82** *	- 0.68** *	- 0.78** *	- 0.79** *	- 0.55** *				
StMR	- 0.73** *	- 0.72** *	- 0.61** *	- 0.66** *	- 0.54** *	0.78** *			
SRtMR	0.81** *	0.65** *	0.70** *	0.82** *	0.39**	- 0.93** *	- 0.88** *		
R/S	0.87** *	0.68** *	0.74** *	0.86** *	0.47** *	- 0.91** *	- 0.87** *	0.94** *	

Note: Para. = Parameters, LfDM = leaf dry mass, StDM = stem dry mass, SRtDM = storage root dry mass, LfA = leaf area, LfMR = leaf mass ratio, StMR = stem mass ratio, SRtMR = storage root mass ratio, R/S = root to shoot ratio.

*, **, *** indicate significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively while growth parameters without * indicate no significant correlations. The values are the means of four replicates.

Discussion

Dry Mass Production

Total biomass and leaf area

The total dry mass production showed positive response to 10 ppm PBZ in this experiment. High PBZ concentrations at 80 ppm generally reduced the total dry mass production lower than the control treatment. Therefore, the high concentrations of PBZ at 80 ppm were not suitable as supplement to inorganic fertilizer in VitAto cultivation. This finding indicated that, the higher the PBZ concentrations the lower the dry mass produced. Elfiza (2011) also found that high PBZ concentration (187.5 ppm) reduced sweet potato dry mass production compared to the control. Similar results on biomass reduction were observed in strawberry (Deyton et al., 1991), ornamental pepper (Grossi et al., 2005) and lettuce (Abed, 2018).

The total dry mass production showed no significant response to PBZ concentrations, except for 10 ppm which was significantly increased higher than control. Similar result was observed in cassava study using low PBZ concentrations around 10 to 30 ppm (Panyapruet et al., 2016). It showed that, the lower the PBZ concentrations used the higher the production of total dry mass obtained. Plant hormones or growth regulators are chemicals that involved in almost every aspects of plant growth metabolism which could be used to obtain the desired practical results for specific regulatory effect at a very low concentration (Dascaluc and Ralea, 2002; Gray, 2004) which also applicable to PBZ (Nivedithadevi et al., 2012). The 10 ppm PBZ was proven to give an increase in total dry mass production of *Catharanthus roseus* (Jaleel et al., 2008) and *Solanum trilobatum* (Nivedithadevi et al., 2012). The 20 ppm PBZ was shown to increase total dry mass production of elephant foot yam (Gopi et al., 2005) and carrot (Gopi et al., 2007).

The relationship between above and below ground parts

The leaf area, above and below ground dry mass production responded negatively to 80 ppm PBZ but showed positive response to 10, 20 and 40 ppm treatments. The 80 ppm PBZ treatment had a reduction effect while treatments from 10 to 40 ppm PBZ gave increasing effects to the plant growth. The finding from this study was different from other PBZ studies which indicated reduction in the leaf area and above ground dry mass but increased the below ground dry mass production as reported in crops such as elephant foot yam (Gopi et al., 2005), potato (Tsegaw, 2006), cassava (Gomathinayagam et al., 2007) and Chinese potato (Kishorekumar et al., 2007). The effect of 10, 20 and 40 ppm PBZ in increasing the dry mass production of most plant parts was similar to study in carrot (Gopi et al., 2007). The application of other triazole compounds at lower concentrations such as hexaconazole also increased both above and below ground parts of *Plectranthus forskholii* (Lakshmanan et al., 2007).

The preferred mode of action of triazole compounds is inhibiting gibberellin biosynthesis (Fletcher and Hofstra, 1988; Davis et al., 1988) and enhance an increase in assimilate translocation from above to below ground parts (Gomathinayagam et al., 2009; Borhan et al., 2017a; Kamran et al., 2018). Gibberellin reduced the sink strength in potato tuber to attract assimilate which further reduced the partitioning and accumulation of dry mass in tuber (Booth and Lovell, 1972). In addition, gibberellin is one of the factors that inhibited or delay potato tuber initiation (Vreugdenhil and Sergeeva, 1999; Davies, 2009). However, high PBZ concentrations would further inhibit the gibberellin biosynthesis and presumably reduced the growth of above ground parts especially leaves as found in this study. In general, reduction in leaf production would contribute to lower leaf area thus reduce the source size. The reduction in the source size eventually will result in limited assimilate production that can be translocated to storage root. Therefore, the PBZ concentrations above 40 ppm are not suitable to be applied to this crop which can reduce the storage root production. A cultivar such as VitAto with a four month growing period (Tan et al., 2010) is considered as a cultivar that is source limited due to a shorter vegetative growth period (Li and Kao, 1990). On the other hand, one of the roles of PBZ is to induced early tuberization of potato by shorten the vegetative growth period (Tsegaw, 2006). Under high concentrations of PBZ given to this cultivar, it will further limiting the production of leaves as a source organ.

As previously discussed, high PBZ concentrations resulted in inhibition of the gibberellin biosynthesis. However, the gibberellin biosynthesis must not be completely inhibited because it can provide negative influence on plant growth. Gibberellin is important in stimulating stem and root growth, regulate the

transition from juvenile to adult phases, fruit set and parthenocarpy (Taiz and Zeiger, 2010). The low PBZ concentrations (10, 20 and 40 ppm) used probably moderately inhibited gibberellin biosynthesis but not completely retard the growth of plant parts especially leaves. The lower the PBZ concentrations the higher were the leaves production which contributed to a higher leaf area (source size) and therefore more assimilate produced to contribute to the higher plant total dry mass production. The total dry mass production is important as one of the factors that can influence the storage root yield (Belehu, 2003). In this study, total dry mass production had a strong positive correlation with the storage root dry mass production which similar with the finding of Bourke (1984) and Borhan et al. (2017a). The storage root dry mass production was maximum at the maturity stage. The 10 ppm PBZ treatment had 163.6 g of storage root dry mass production which was 30.9% higher than control treatment. This treatment showed comparable value of storage root dry mass production with other sweet potato studies ranging from 124 to 155 g (Mortley et al., 1994), 143.9 to 204 g (Belehu, 2003) and 161.7 to 194.6 g (Borhan et al., 2017a) per plant basis.

Dry Mass Partitioning

Source-sink relationship

As indicated from the results, the assimilate translocation to the leaves (LfMR), stem (StMR) and root (RtMR) were higher at storage root initiation stage while the assimilate translocation to storage root (SRtMR) was higher at maturity stage as reported (Belehu, 2003). The LfMR, StMR and RtMR were negatively correlated with SRtMR in both experiments which in agreement with several researchers (Belehu, 2003; Kareem, 2013). As the SRtMR gradually increased towards maturity, the R/S ratio also showed a similar pattern. Beside the duration of crop growth period, the growth stages within the cropping cycle also influence the source and sink relationship. At the initiation stage, source capacity is more important than sink capacity thus, more assimilate was translocated to vegetative parts for leaves and stem production. However, when both source capacity (leaf area) and sink capacity (storage root number) were developing at storage root bulking and maturity stages, they are equally important in determining the yield production (Li and Kao, 1985; 1990).

The distribution of dry mass within the plant

In general, the PBZ treatments in both experiments enhanced the distribution of dry mass to various plant parts greater than the control treatment in all growth stages. It seemed, the application of PBZ increased the sink strength of plant parts to attract assimilate. Sink strength is the competitive ability or capacity of a sink organ to attract imported assimilates (Marcelis, 1996; Bihmidine et al., 2013). However, plant under high PBZ concentrations had lower leaf area (source limited) than the control treatment therefore contributed to lower source strength to produce assimilate which cause lower dry mass production in many plant parts. When source is limited, the growth of plant organ depends on source strength and may also depend on the sink organ's potential capacity and affinity to accumulate assimilates (Marcelis, 1996). In contrast, low PBZ concentrations; 10, 20 and 40 ppm generally had higher dry mass in plant parts probably through production of greater leaf area. The bigger source size can increase the source strength or capacity to produce more assimilates. Source strength or capacity refers to the rate of physiological process related to assimilate production such as the rate of photosynthesis per unit leaf area and chlorophyll content (Marcelis, 1996). Source strength depends on the source size such as leaf area and followed by source activity such as unit leaf rate (Marschner, 1995; Ansari, 2015).

Based on the finding of this study, low PBZ concentrations were more suitable to be applied as supplement to inorganic fertilizer in VitAto cultivation because it can increase both source and sink strength at vegetative and reproductive stage respectively. The 10 ppm PBZ treatment was considered optimal since it increased the sink strength of storage root to attract greater assimilate as shown by higher SRtMR than other treatments at maturity stage. The storage root is major sink component in sweet potato (Li and Kao, 1985; Eguchi et al., 1995) where, high proportion of assimilate will be translocated at the final growth phase from bulking to maturity stages (Belehu, 2003). The storage root mass ratio (SRtMR) is harvest index at maturity stage, which is the proportion of storage root dry mass production per plant total dry mass production (Bhagsari, 1990). The highest values of SRtMR in this experiment was 65% for 10 ppm PBZ. This treatment is 7.7% higher than the control treatments at maturity stage. The SRtMR values per plant basis from this experiment showed similar range with SRtMR from other sweet potato studies around 56 to 79% (Bourke, 1985), 0.49 to 0.71% at 170 DAP (Liu et al., 2013) and 65.0 to 77.1% (Borhan et al., 2017b).

Conclusion

Among various concentrations of PBZ used as supplement to the recommended inorganic fertilizer, the best treatment that can give positive effect to dry mass production and partitioning of VitAto was the 10 ppm PBZ. In general, the effects of PBZ showed that the lower the concentrations used, the higher the growth and yield of VitAto would be obtained. The lower the PBZ concentrations used the higher were the leaves production which contributed to higher leaf area (source size) and therefore more assimilate produced to contribute to the higher plant total dry mass production. High plant total dry mass production contributed to high assimilate translocation to the storage root, therefore, increase the storage root dry mass production which eventually increase the size of storage root.

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Economic evaluation of three planting patterns for intercropping cocoa with coconut

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Abstract

Intercropping is one from the best ways to improve productivity in agricultural land area. The aim of the study was to evaluate the economic suitability of three planting patterns of coconut to be intercropped with cocoa. This study is also aim to identify the most suitable planting pattern for intercropping coconut with cocoa. The studies consist of four treatments which is 2 rows of coconut with 4 rows of cocoa, 2 rows of coconut with 5 rows of cocoa, 2 rows of coconut with 6 rows of cocoa and cocoa monoculture as a control. Cocoa clones involved in each treatment are PBC 140, KKM 5, KKM 22, PBC 123, KKM 1 and BR 25. Actual yield for cocoa were recorded (from 2009 until 2014) each time of harvesting and pod and bean analysis were done twice in a year. Actual harvested coconuts were also recorded from 2009 until 2014. Actual yield (RM) for both coconut and cocoa were also calculated. In 2011, T1 give the higher cocoa and coconut revenue which is RM18,362.88 followed by T3 with the total revenue of RM18,066.08 T2 with the revenue of RM 17,358.08 lastly T4, with the revenue of RM 7,325.75. Differ in 2012, T3 give the higher revenue with RM 15,572.94, followed by T1, with RM 14,803.53, T2 with 14,630.13 and lastly T4 with RM 7,217.30 Based on the total revenue of both crops, intercropping with economic crops will give the better incomes especially for smallholders.

Keywords: Planting patterns, Coconut, Cocoa, Intercropping, Economic evaluation

Introduction

Cocoa tree or widely known as *Theobroma cacao* L. is a tree which are found more than 2,000 years ago by the local people of Central America which is living deep in the tropical rainforests. *Theobroma cacao* is one from twenty-two species in the genus of *Theobroma*. *Theobroma* means “food of the gods” in Latin and cacao derived from the Nahuatl (Aztec language) which is bitter and water. Basically, this cocoa fruits commonly used to produce chocolates, beverages, cocoa mass, cocoa powder, cocoa butter and the used were expand to produce cosmetic products.

Shade tree is any tree which are used specifically for its shade. Shade tree is a must in cocoa planting and require shade at every stages of growth. Immature cocoa require 25% of sunlight and light requirement, and it increases with the cocoa maturity. (Lee; et al., 2013). They are three types of shade commonly used in cocoa plantation which is permanent, semi-permanent and temporary shade. Based on specific requirement, coconut tree can be used as a shade tree

because it allow adequate light penetration and these trees will give economic return to the farmers.

Intercropping concepts were introduced to cocoa smallholders to enhance their productivity and profit. Beside the use of it sparse canopy, it also can increase smallholders income by selling both crops. Intercropping concepts is a planting two or more crops on the same piece of land. In cocoa intercropping planting system, cocoa was served as main crop and another economic crop is intercropped with suitable spacing. Smallholders can get better benefits by applying the concepts of intercropping commercialization through product value added using pragmatic agroforestry models, which are potential in generating high and stable income (Hashim et al., 2012).

Coconut tree or widely known as *Cocos nucifera* is one from the family of *Areaceae* (palm family). The term of 'coco' is derived from Portuguese and Spanish word with the meaning of "head" or "skull" that resemble facial features of coconut fruits. Coconuts are known for its great versatility, from traditional uses, for food to cosmetics. In Malaysia, it is known as "pokok seribu guna" which indicated it highly potential uses of all part of the tree. In coconuts fruits, it contains a large quantity of clear liquid in both mature and immature coconuts. This water is acts as natural energy, because it contain high mineral content and no fat content and low contents of carbohydrates, calories and sodium. (Yong et al., 2009). Coconut can also be processed to give oil from the kernel, charcoal from the hard shell and coir from the fibrous husk. The husks and leaves can be used as material to make a wide variety of products such as decorating and furnishing. The coconut also has cultural and religious significances especially in Hindu rituals.

According to statistics reported by MOA, in 2015, there is 81,695.7 ha of coconut planted area for both smallholders and estates in Malaysia, with the production of 504,621.2 Mt. With high potential uses especially in religious festival especially in Eid Mubarak and Hindu rituals, there is a promising incomes for smallholders. Therefore, this study was initiated to determine the suitability and economic perspective to intercrop cocoa with coconut planting. The trial was conducted with the objective to determine the best planting pattern for intercropping cocoa and coconut in order to enhance and maximize the profit gain by the smallholders.

Materials and Methods

This study was carried out at Cocoa Research and Development Centre, Jengka, Pahang. It consisted of four treatments which is two rows of coconut with four rows of cocoa, two rows of coconut with five rows of cocoa, two rows of coconut with six rows of cocoa and cocoa monoculture as a control. The stand/ha of cocoa is differ from each treatment. For T1- the stands/ha is 605, T2 with 677 stands/ha, T3 with 736 stands/ha and T4 with 1111 stands/h, while for coconut, T1- 181 stands/ha, T2 with 162 stands/ha and T3 with 147 stands/ha. For T1, the clone use was PBC 140, KKM 5, KKM 22 and PBC 123, while for T2, the clone use was KKM 1, KKM 22, PBC 123, PBC 140 and KKM 5. For T3 and T4, the clone use was KKM 5, KKM 22, PBC 140, KKM 1, PBC 123 and BR 25. The experimental design is randomized complete block design (RCBD) with three replications. The data was recorded throughout the year. Pandan coconut or known as aromatic dwarf planted in 2004 while cocoa

were planted in 2005. These variety were chosen because of its quality, high potential and plant height of six meter. Actual yield was recorded every time of harvesting. Economic cost analyses were also calculated and all data were analyzed using SAS for mean separations of the treatment effects.

Results and Discussions

Table 1 showed dry bean yield for four treatments from 2009 until 2013. From table showed, there is a significant difference between treatments at 5% significant level except for 2011. This data indicated that the planting patterns showed an effect on dry bean of cocoa. This is due to the competition for light, moisture and fertilizer between both plant and each tree. In 2009 until 2013, T3 showed highest yield, followed by T4, T2 and lastlt T1. Eventhough T1 showed lowest result in dry bean yield, but it showed better number of dry bean when divided into total number of stand as compared with T4.

Table 1. Data on cocoa dry bean yield performance (kg/ha/year) (2009 – 2013)

	2009	2010	2011	2012	2013
Two rows of coconut with four rows of cocoa	572.20 ^c	967.44 ^c	738.90 ^a	723.10 ^b	296.70 ^b
Two rows of coconuts with five rows of cocoa	762.11 ^b	1,136.87 ^{bc}	817.00 ^a	1,004.20 ^{ab}	341.71 ^b
Two rows of coconut with six rows of cocoa	967.48 ^a	1,462.46 ^a	851.00 ^a	1,223.90 ^a	452.98 ^a
Cocoa monoculture	777.05 ^b	1,245.57 ^b	885.00 ^a	1,045.40 ^{ab}	354.45 ^b
CV	11.66	7.781	18.66	22.03	10.25
F-value	9.71*	14.72*	0.50 ^{ns}	2.66*	9.48*

Column means followed by the same letter are not significantly difference ($P>0.05$, Duncan's Multiple Range Test)

Coconut fruits were also counted from 2011 until 2013. There was no significant difference between treatments in 2011 until 2013. In 2011, two rows of coconut with four rows of cocoa showed higher coconut fruits, followed by two rows of coconut with six rows of cocoa and lastly two rows of coconut with five rows of cocoa. In 2012 and 2013, two rows of coconut with four rows of cocoa show higher coconut yield, followed by two rows of coconut with five rows of cocoa and lastly, two rows of coconut with six rows of cocoa. This results were in line with the number of coconut stands in the treatments.

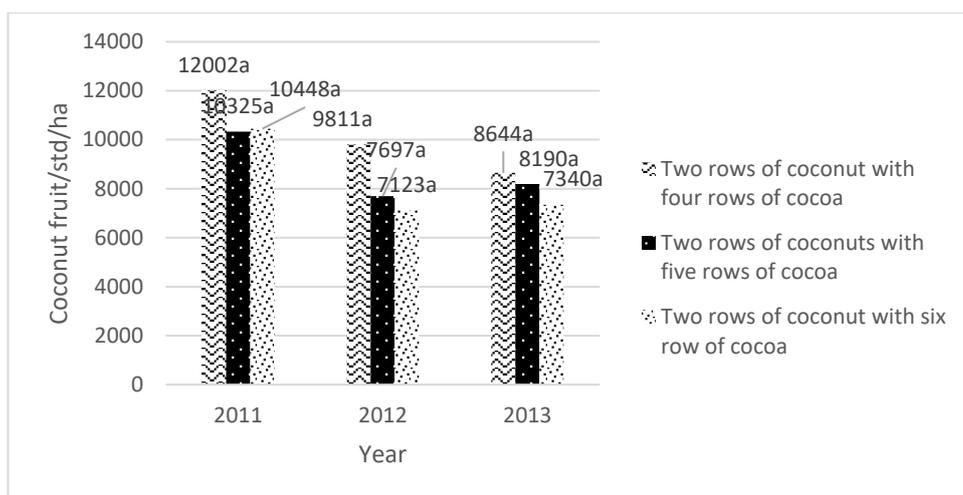


Figure 1. Mean counted coconut fruits at three different planting patterns of cocoa

Table 2, 3, and 4 showed the actual revenue for both crops from 2011 until 2013. Each coconuts were sold at RM1/fruits. In 2011, the average price of cocoa dry bean was RM 8,608/tonne, in 2012 was RM6,904/tonne and in 2013, the price of cocoa dry bean were RM6,493.00 (Malaysian Cocoa Board Statistic, 2016). There is a significant different between treatment in total revenue from 2011 until 2013. In 2011, two rows of coconut with four rows of cocoa showed better revenue, followed by two rows of coconut with six row of cocoa, two rows of coconuts with five rows of cocoa and lastly cocoa monoculture. In 2012, two rows of coconut with six row of cocoa showed better revenue followed by two rows of coconut with four rows of cocoa, two rows of coconuts with five rows of cocoa and lastly cocoa monoculture. In 2013, two rows of coconut with four rows of cocoa showed higher income, followed by two rows of coconut with five rows of cocoa, two rows of coconut with six rows of cocoa and lastly cocoa monoculture.

Table 2. Actual revenue (RM) for both cocoa and coconut in 2011.

Treatment	Cocoa	Coconut	Actual yield (RM)
Two rows of coconut with four rows of cocoa	6,360.88	12,002.00	18,362.88
Two rows of coconut with five rows of cocoa	7,033.08	10,325.00	17,358.08
Two rows of coconut with six row of cocoa	7,618.08	10,448.00	18,066.08
Cocoa monoculture	7,325.75	-	7,325.75

Table 3 Actual revenue (RM) for both cocoa and coconut in 2012.

Treatment	Cocoa	Coconut	Actual yield (RM)
Two rows of coconut with four rows of cocoa	4,992.53	9,811.00	14,803.53
Two rows of coconuts with five rows of cocoa	6,933.13	7,697.00	14,630.13
Two rows of coconut with six row of cocoa	8,449.94	7,123.00	15,572.94
Cocoa monoculture	7,217.30	-	7,217.30

Table 4.Actual revenue (RM) for both cocoa and coconut in 2013.

Treatment	Cocoa	Coconut	Actual yield (RM)
Two rows of coconut with four rows of cocoa	1,926.41	8,644.00	10,570.41
Two rows of coconuts with five rows of cocoa	2,218.72	8,190.00	10,408.72
Two rows of coconut with six row of cocoa	2,941.19	7,340.00	10,281.19
Cocoa monoculture	2,299.30	-	2,299.30

Table 5, 6 and 7 showed the economic cost analysis in 2011, 2012 and 2013. Based on table 5, in 2011 two rows of coconut with four rows of cocoa showed better profit compared to other treatments, followed by two rows of coconut with six rows of cocoa, two rows of coconut with five rows of cocoa, and lastly, cocoa monoculture. In 2012, two rows of coconut with six rows of cocoa showed higher profit, followed by two rows of coconut with four rows of cocoa, two rows of coconut with five rows of cocoa and lastly cocoa monoculture. The decreasing number of cocoa drybean is the main factor of decreasing total revenue two rows of coconut with four rows of cocoa. This might be due to the high number of both crops in the plot area which cause competition for fertilizer between both plant and effect the production of cocoa drybean. In 2013, two rows of coconut with four rows of cocoa showed higher profit followed by two rows of coconut with five rows of cocoa, two rows of coconut with six rows of cocoa and lastly cocoa monoculture. Cocoa monoculture showed net loss because of the single crop in the treatment compared to other treatments which have two crops in the treatment. This indicated that intercropping are needed and will assist farmer to get greater yield and better profit. Cocoa drybean in all treatment showed decreasing value since they are longest dry season in the year where it increase the cherelle wilt percentage (el-nino phenomenon).

To determine the effectiveness of intercropping system, several formulas have been developed to evaluate the productivity for both plants. The index that has been used was Land Equivalent Ratio or known as LER (Harwood, 1979, Mead and Stern, 1979). Land Equivalent Ratio (LER) can be defined as the relative land area required as a sole crop to produce the same yields as intercropping system (Mead and Willey, 1980). Value of 1.0 indicates that there is no difference in yield between both crops while if LER value greater than 1.0, indicates that there is yield advantage for both crops. Average yield for coconut fruits was 24,960/ha. (Hakim, 2011). From the index of LER, there was no significant difference in all treatments in 2011, while there is a significant different in 2012 to 2013 (Table 8). In 2011 until 2013, two rows of coconut with six rows of cocoa showed better LER value, followed by two rows of coconut with five rows of cocoa, two rows of coconut with four rows of cocoa and lastly cocoa monoculture.

Table 5. Economic Cost Analysis 2011

		Two rows of coconut with four rows of cocoa	Two rows of coconut with five rows of cocoa	Two rows of coconut with six row of cocoa	Cocoa monoculture
1.0	Input cost				
1.1	Fertilizer (Cocoa)	1,555.38	1,740.48	1,892.17	2,856.25
1.2	Fertilizer (Coconut)	1,938.87	1,735.34	1,574.66	-
1.3	Pesticide	72.00	71.40	75.15	94.55
1.4	Weedicide	214.00	210.97	220.35	263.96
	Total for input cost	3,780.25	3,758.19	3,762.33	3,214.76
2.0	Income for cocoa (RM)	6,360.88	7,033.08	7,618.08	7,325.75
2.1	Income for coconut (RM)	12,002.00	10,325.00	10,448.00	-
	Income	18,362.88	17,358.08	18,066.08	7,325.75
3.0	Net profit/loss	14,582.63	13,599.89	14,303.75	4,110.99

Table 6. Economic Cost Analysis 2012

		Two rows of coconut with four rows of cocoa	Two rows of coconut with five rows of cocoa	Two rows of coconut with six row of cocoa	Cocoa monoculture
1.0	Input cost				
1.1	Fertilizer (Cocoa)	1,742.40	1,949.76	2,119.68	3,168.00
1.2	Fertilizer (Coconut)	2,172.00	1,944.00	1,764.00	-
1.3	Pesticide	72.00	71.40	75.15	94.55
1.4	Weedicide	209.87	208.60	220.17	281.93
	Total for input cost	4,196.27	4,173.76	4,179.00	3,544.48
2.0	Income for cocoa (RM)	4,992.50	6,933.13	8,449.94	7,217.30
2.1	Income for coconut (RM)	9,811.00	7,697.00	7,123.00	-
	Income	14,803.53	14,630.13	15,572.94	7,217.30
3.0	Net profit/loss	10,607.23	10,456.37	11,393.94	3,672.82

Table 7. Economic Cost Analysis 2013

	Two rows of coconut with four rows of cocoa	Two rows of coconut with five rows of cocoa	Two rows of coconut with six row of cocoa	Cocoa monoculture
1.0	Input cost			
1.1	1,793.22	2,006.63	2,181.50	3,260.40
1.2	2,235.35	2,000.70	1,815.45	
1.3	88.00	89.59	97.39	147.02
1.4	228.25	225.04	220.17	281.93
	Total for input cost	4,321.96	4,314.51	3,689.35
2.0	1,926.41	2,218.72	2,941.19	2,299.30
2.1	8,644.00	8,190.00	7,340.00	-
	Income	10,408.72	10,281.19	2,299.30
3.0	6,225.59	6,086.76	5,966.68	(1,390.05)

Table 8. Data on Land Equivalent Ratio (2011-2013)

Treatment	2011	2012	2013
Two rows of coconut with four rows of cocoa	1.32±0.24 ^a	1.13±0.29 ^{ab}	1.24±0.03 ^b
Two rows of coconuts with five rows of cocoa	1.32±0.27 ^a	1.27±0.25 ^{ab}	1.25±0.05 ^b
Two rows of coconut with six row of cocoa	1.34±0.07 ^a	1.53±0.43 ^a	1.60±0.24 ^a
Cocoa monoculture	1.00±0.00 ^a	1.00±0.00 ^b	1.00±0.00 ^c
CV	15.49	16.92	8.67
FV	1.54 ^{ns}	4.00 [*]	9.77 [*]

Column means followed by the same letter are not significantly difference ($P>0.05$, Duncan's Multiple Range Test)

Conclusion

From this evaluation, it can be conclude that coconut can be intercropped with cocoa two rows of coconut with four, five or six rows of cocoa, where it give better cocoa and coconut yield, income for farmers and maximum number of Land Equivalent Ratio compared to cocoa monoculture.

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Learning styles of Livestock Production Program students at Universiti Malaysia Sabah

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Abstract

Students have different preference in learning styles. The awareness on students learning style could enhance teaching delivery and consequently contribute to better efficiency in education. This study was performed to determine the preferred learning styles of 64 students in the Livestock Production Program at Universiti Malaysia Sabah. Visual Auditory Reading Kinesthetic (VARK) questionnaire consisted of 16-point multiple choices was given to the first, third, and fourth year students of the program. Based on the assessment, many of the students were kinesthetic (K, 35%) compared with visual (V, 24%), auditory (A, 23%), and read/write (R, 18%). Among K students, male students were dominant (43%) compared to female students (28%). Other male students were V (24%), A (19%), and R (14%) whereas female students showed little polarization in learning styles (K, 28%; V, 22%; A, 28; R, 22%). Furthermore, students at different level of study had different learning styles. First year students were A (44%), while third and fourth year students were R (40%) and V (33%) respectively. Interestingly, most of the male students were R (33%) regardless of the level of study, except not for students who preferred K. There was a trend that K students changed learning styles as the level of study increased (first year, 44%; third year, 33%; fourth year, 22%). Finally, students were found to favor unimodal learning style (54%), followed by bimodal (32%), trimodal (14%), and surprisingly, none favoring quadrimodal. Both male and female student showed equally similar percentage of preference on unimodal styles of learning (male, 54%; female, 50%). In conclusion, kinesthetic is the regular learning style of the male students, but for the female students they equally prefer visual, auditory, read/write and kinesthetic. Further study need to be conducted to investigate the relationship between the students learning styles and lecturers' teaching styles.

Keywords: learning styles; learning preferences; VARK model.

Introduction

Learning styles are defined as a characteristic feature that determines cognitive and psychosocial behavior of learners, perceiving of knowledge, interaction and processing of information in different learning environments (Diwakar et al., 2018). Theories of learning styles suggest that individuals think and learn best in different ways. These are not differences of ability but rather preferences for processing certain types of information or for processing information in certain types of way (Willingham, et al., 2015). Learning styles refer to the way in which individuals approach learning situations to process information (Cassidy, 2004; Zoghi et al., 2010). Association between learning styles and learning achievement outcomes remains contentious (Norman, 2009); however, it has been generally accepted that individuals' learning styles have an impact on their performance and achievement of learning outcomes (Cassidy, 2004). In contrast, through cognitive aspect, learning style can be referred to various means in perception conception and information dealing out to form ideas and philosophies (Fleming and Baume, 2006).

There are several theories characterizing the stages and processes involved in deep or transformative learning. Dewey's model (Dewey, 1938) of interconnecting educative experiences to reflection, Kolb's model (Kolb, 1984) of experiential learning, Schon's model of (Schön, 1983) reflection as a

practice, Gibb's model of cross-enrichment of theory and practice to enhance reflection (Gibbs, 1988), Moon's model (Moon, 2013) of provoking interpretations to complex ideas look into sequential set of events and learning. Another study (Bransford et al., 1999) categorizes teaching strategies as lecture-based, skill-based, inquiry-based, and individual vs. group based and technology-enhanced learning.

The learning style preferences of undergraduate science students such as nursing (Rassool, 2007), pharmacy (William et al., 2013), medical (Baykan and Naçar 2007) agriculture students (Friedel and Rudd, 2006) have been previously reported. The study lead by Wehrwein et al., (2007), resolved that there is a variability of learning styles in a schoolroom are varying male and female refer different learning styles.

Few research is known on the learning styles of science students. In the present study, the learning styles of students taking Livestock Production Program at the faculty of Sustainable Agriculture, Universiti Malaysia Sabah (UMS) were explored using the VARK questionnaire. The questionnaire contains 16 multiple-choice questions with four possibilities to select an answer. Each possibility represented one of the four modes of perception. However, the students were allowed to select more than one answer to each question, in order to identify their poly modal modes of perception and learning.

Materials and Method

Location of study

The study was conducted at the Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, Sandakan Campus, Sabah during 2017 – 2018 academic year.

Sample population

A total of sixty-four students (26 males and 38 females) consisting of 22, 23 and 19 students from first, third and fourth year, respectively were purely volunteering participation in order to avoid biasness and student to lecturer obligation issue. The students completed the 16 multiple-choice VARK questionnaire online in the faculty's computer laboratory. Responses from the second year students were not able to be collected because they were participating in other program activity outside the campus.

Procedures

The purpose of the study was explained to the students. The surveying was done using the VARK questionnaire obtained from www.vark-learn.com/ (Fleming, 2015). Students were given about 10 minutes to answer the VARK online questionnaire. Although the survey was casual, but, no interaction between the students was allowed while answering the questionnaire. To avoid lecturer's opinion effects, no clarifications were also offered regarding any of the question. Hence, the students need to answer to the questions to the best of their interpretations. The using of the VARK questionnaire, written permission through e-mail had been obtained for using the VARK questionnaire from its developer.

Statistical analysis

The descriptive analysis of the data was carried out using Microsoft Excel sheet and score. Differences and learning styles between the male and female students were examined using Pearson correlation analysis. All statistical tests were judged at 5% level of significance.

Results and Discussion

The distribution of learning style preferences of the students is shown in **Figure 1**. Kinesthetic (K), visual (V), auditory (A) and read/write (R) were 35%, 24%, 23% and 18%, respectively. This trend resembled that of the nursing students in China studied by **Zhu et al. (2018)**. The nursing students

kinesthetic modality style was the predominant unimodal style (18.20%), while the read-write modality was the least popular (2.5%).

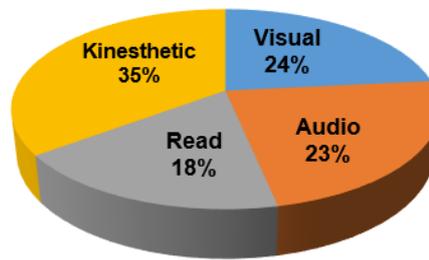


Figure 1. Distribution of students learning style

The percentages of K and V in the previous study (Table 1) contradicted with that of Zhu et al. (2018). In their study, the majority of the nursing students preferred the AK modality. Our result thus means that students who preferred K (learning by experience through physical movement) was also likely to prefer V (learning by looking at picture, graphs, videos and graphics) and for those preferred R (learning by words and texts) was also likely to prefer A (learning by listening method, by speaking or from music, discussion and explanation) (Table 1).

Table 1. Correlation coefficient (r) of learning styles of the students.

Parameters	Learning styles			
	Visual	Audio	Read/Write	Kinesthetic
Visual				
Audio	0.34 ^{ns}			
Read/Write	0.37 ^{ns}	0.51*		
Kinesthetic	0.49*	0.30 ^{ns}	0.30 ^{ns}	

* Significant at $p \leq 0.05$; **significant at $p \leq 0.01$; ns – not significant

The distribution of learning styles between male and female students are presented in Figure 2. Many of the male students were K (43%), and only in few more A (19%) and R (14%) compared to the female students (28% K, 28% A and 22% R). However, as also mentioned earlier, those preferred R were also likely to prefer A. The trend in our study similar to that of Horton et al. (2012), where R Female students was only $28.9 \pm 0.9\%$ slightly higher than R male students ($25.3 \pm 1.3\%$). The female students are likely to reading or writing also listening, and talking or discussing as an approach to learn something (Table 2). According to Miller (2001), this type of students can remember information through loud reading or pronouncing when understanding, particularly when learning something fresh. The students can then reinforce their retention of their information by listening again to the audio of the information, by teaching other persons, and by talking with the teachers. Audio students read certainly, describe cleverly, write story fluently, have good terminology, spell easily, like to write letters, and own strong ability in memorizing terms or details (Armstrong, 2004). In the previous study, K Male students were not associated with other learning styles

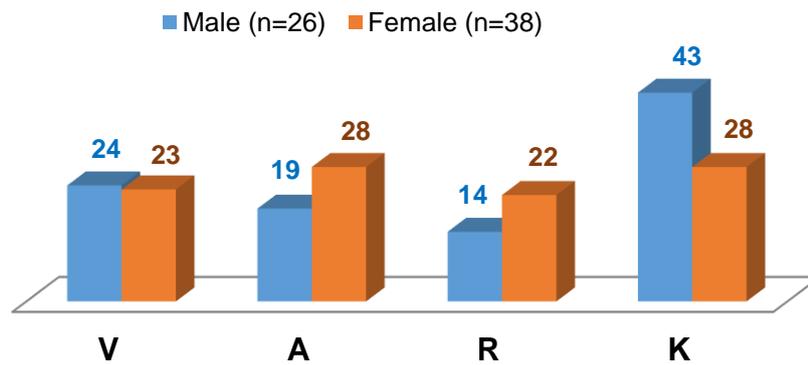


Figure 2. Percentage of learning style of the students based on gender. V = Visual; A= Audio; R = Read/Write; K = Kinesthetic.

Table 2. Correlation coefficient (r) of learning styles of the male students.

Parameters	Learning styles			
	Visual	Audio	Read/Write	Kinesthetic
Visual				
Audio	0.15 ^{ns}			
Read/Write	0.50 ^{ns}	0.63*		
Kinesthetic	0.42 ^{ns}	0.14 ^{ns}	0.07 ^{ns}	

* Significant at $p \leq 0.05$; **significant at $p \leq 0.01$; ns – not significant

Table 3. Correlation coefficient (r) of learning styles of the female students.

Parameters	Learning styles			
	Visual	Audio	Read/Write	Kinesthetic
Visual				
Audio	0.54 ^{ns}			
Read/Write	0.23 ^{ns}	0.43 ^{ns}		
Kinesthetic	0.60*	0.53 ^{ns}	0.73**	

* Significant at $p \leq 0.05$; **significant at $p \leq 0.01$; ns – not significant

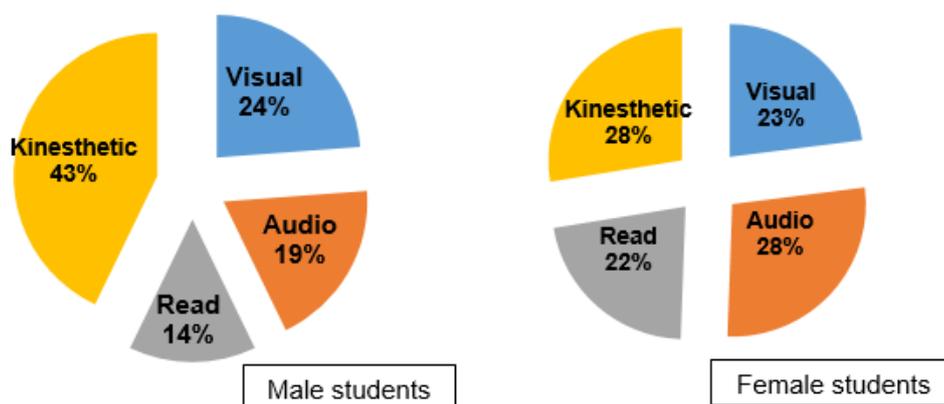


Figure 3. Percentage of learning style of the students based on gender.

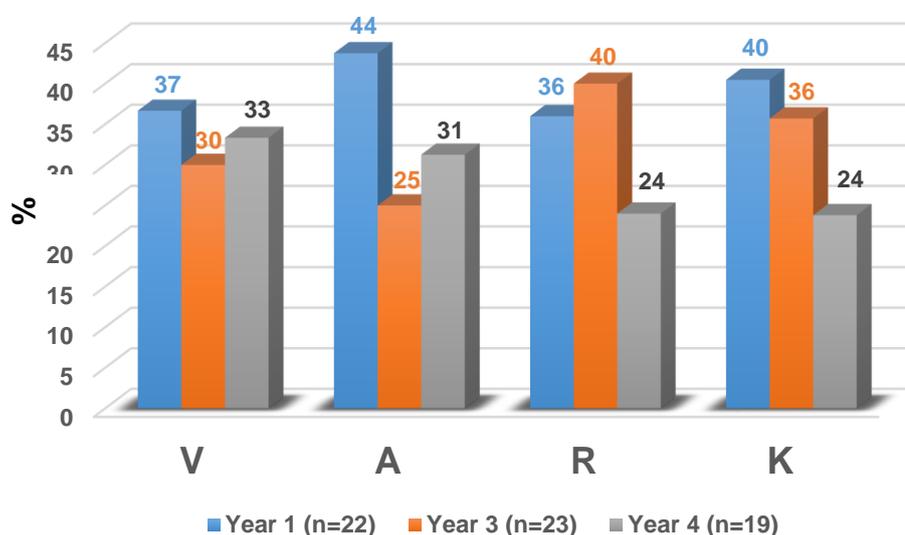


Figure 4. Percentage of learning styles based on students' level of study. V = Visual; A= Audio; R = Read/Write; K = Kinesthetic.

All first year students prefer audio (44%) and kinesthetic (40%) (Figure 4, 5) (Balasubramaniam and Indhu, 2016), it is same pattern in first year of male student for A (50%) and K (44%) (Figure 6) but not for the female students who is mostly preferred audio (42%) (Figure 7). The third year students preferred R or reading/writing (40%) (Figure 4, 5). That proportion was contributed more from the third year of female students (42%) (Figure 7). Final year students preferred to V (33%) (Figure 4, 5). Interestingly, many of the male students change learning styles from first to fourth year where the number of V, A and K students decreased during their study period except for R (33%) (Figure 6).

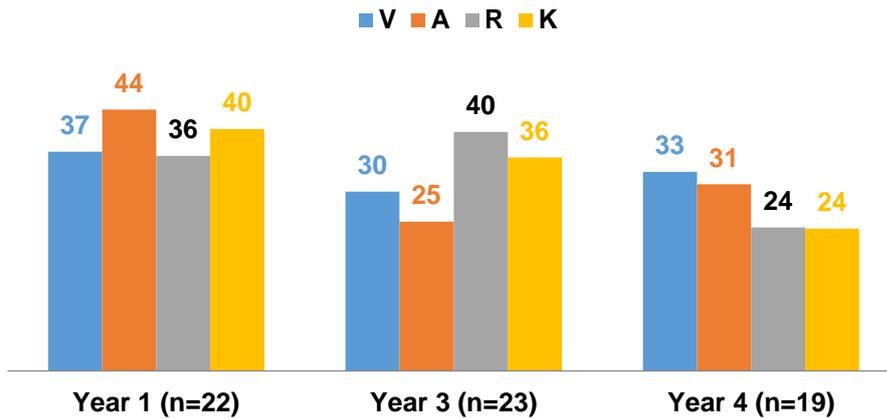


Figure 5. Percentage of students in each learning style based on students' level of study. V = Visual; A= Audio; R = Read/Write; K = Kinesthetic.

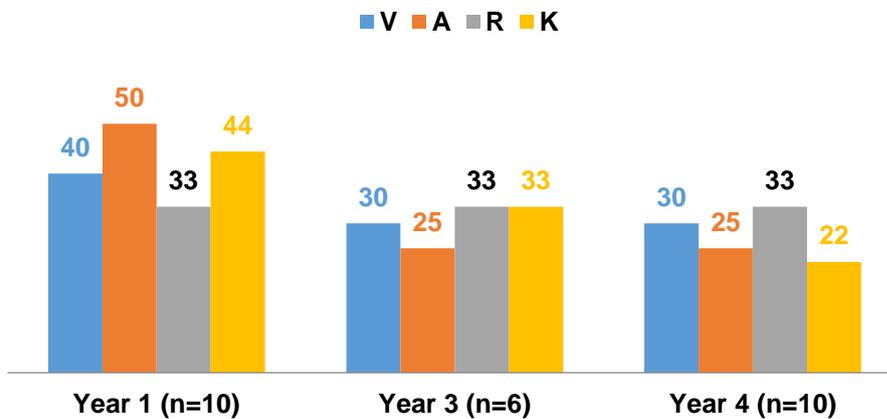


Figure 6. Percentage of male students based on learning style and students' level of study. V = Visual; A= Audio; R = Read/Write; K = Kinesthetic.

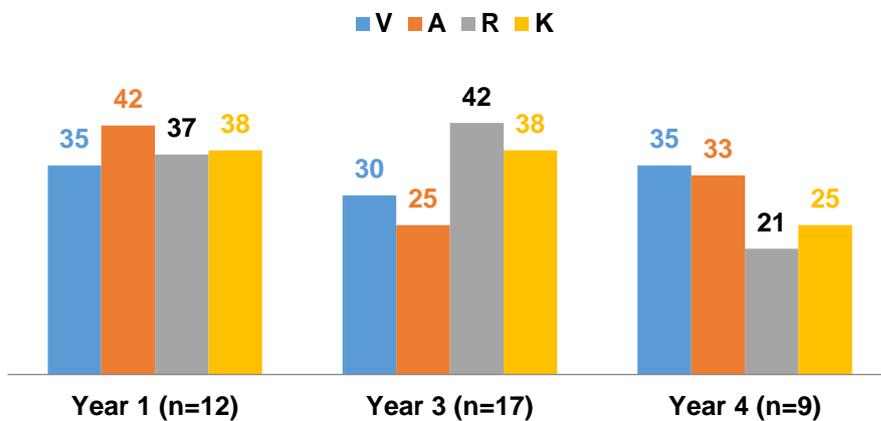


Figure 7. Percentage of female students based on learning styles and level of study. V = Visual; A= Audio; R = Read/Write; K = Kinesthetic

Overall unimodal learning style (students who's prefer only one of the VARK) was (54%) among the students, followed by bimodal (32%), and trimodal (14%); but none was quadrimodal (Figure 8a). Similar trend was observed for female (Figure 8c), and male students, but for the latter (Figure 8b). This result is opposite to that of the medical students (Lujan and DiCarlo, 2006a; Baykan and Nacar, 2007; Prithishkumar and Michael, 2014) and nursing students (Zhu et al., 2018) where more than 63% and 58.49% of them, respectively, was found to be multimodality. A study done among clinical students in a Malaysian Medical College also showed that 44% were unimodal and 56% were multimodal (Sinha et al., 2013). Another study by Wehrwein et al. (2007) revealed that 87.5% for undergraduate physiology male students in Michigan State University preferred multimodal instruction compared to only 45.8% only for female students. Multimodal students are reported to prefer information to arrive in a variety of modes. These students do not learn by simply listening to teaching session in a classroom, paying attention to the teacher, or remembering coursework (Lujan and DiCarlo, 2006b).

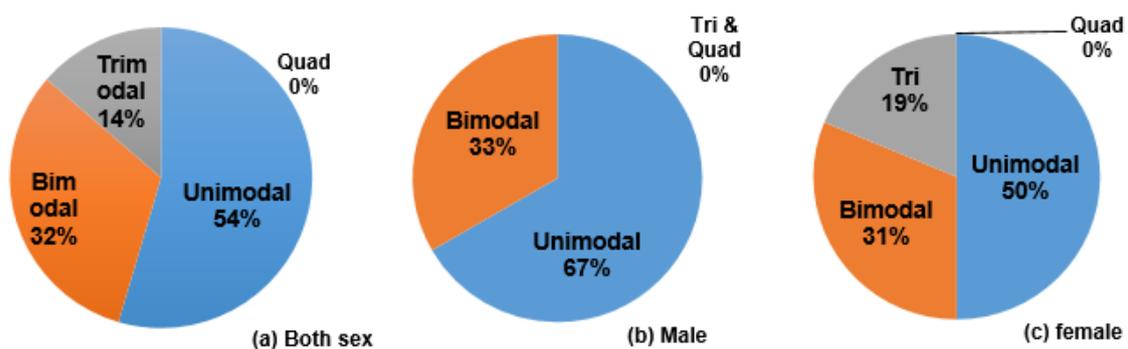


Figure 8. Diversity of learning modality based on gender.

When level of study was considered the learning style of the first and fourth year students was bimodal (45%) while that of the third year students was unimodal (31%) (Figure 9). Recent study by Balasubramaniam and Indhu (2016) revealed that first year medical students were multimodal (52%) rather than unimodal (48%). Interestingly, based on gender, third year male students were more likely to be unimodal (25%) (Figure 9b). Female students, regardless of level of study, were more diverse in learning styles (Figure 9c). Surprisingly, none of the students at any year was quadrimodal.

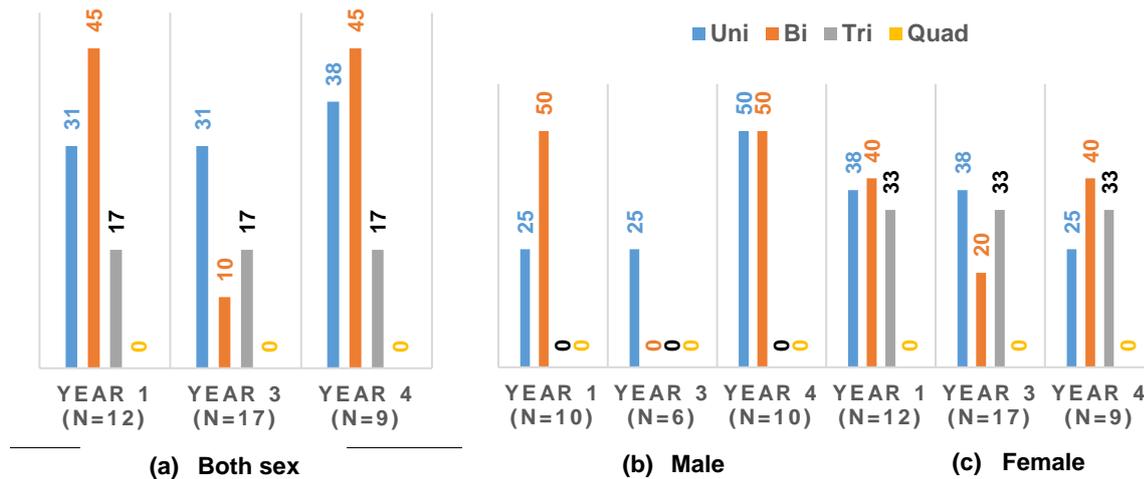


Figure 9. Diversity of learning modality based level of study. Uni = Unimodal; Bi = Bimodal; Tri = Trimodal; Quad = Quadramodal.

A learning preference is defined as the utmost ‘effective and efficient modality’, in which a learner has a regular preference to ‘perceive, practice, store and remember new information (Rourke et al., 2002). There is no particular best approach to teach, but teachers can vary their teaching styles to furnish to the learning style of each unique student (Fleming, 1995; Becker et al., 2007). Attentiveness of learning styles will help educators recognize and elucidate learning difficulties among students (Baykan and Nacar, 2007).

Conclusion

The common learning modality of the Livestock Production Program students in Faculty of Sustainable Agriculture is unimodal, either male or female students. The regular learning styles of the students is kinesthetic, but for the female students is almost equally distributed between visual, auditory, read/write and kinesthetic. In terms of learning styles based on level of study, first year students prefer audio (listening and speaking such as discussion and explanation); third year students prefer recite/write (words and texts); while fourth year students prefer visual (looking at pictures, graphs, videos) and there could not take complete note during presentation. Since the students have a wide variety of learning styles, teachers are recommended to combine the various teaching approaches to match the learning styles of the students. Further study need to be conduct to investigate the relationship between learning styles of students and teaching styles of lecturers.

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**Effect of different substrate on the morphology of *Halophila beccarii* Aschers.
(Hydrocharitaceae)**

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Abstract

Substrate affects the distribution of seagrass which the plants show variation in morphology depending on the type of substrate they inhabit. *Halophila beccarii* is one of the seagrass species inhabit river estuaries where different substrates can be found. The plant was collected from Lawas River Estuaries, Sarawak, and then replanted into three selected substrates; (T1) fine beach sand, (T2) loamy beach sand, and (T3) coarse beach sand. Each type of the substrates was segregated into different plastic trays (33 cm length x 26 cm width x 9 cm height) and submerged in a glass aquarium filled with seawater (25 psu). Each treatment (TX) was given four trays as replication. The trays were planted with 3-4 ramets of *H. beccarii* and left to grow for eight weeks. Selected measurements were taken on leaf length (LL), leaf width (LW), petiole length (PL) and the number of rosette leaves per shoot (LPS). The morphometric data were analyzed using one-way ANOVA tests. After eight weeks, the plant grew on substrate T2 displayed the highest morphological measurements and shoot density counted compared to others substrate types. *H. beccarii* shows selection toward substrate types by showing significant morphology variation. In the wild habitats, the plant inhabits estuarine areas with the dynamic type of substrate. The study showed that *H. beccarii* displays morphological variances according to types of substrate they grow.

Keywords: seagrass; laboratory culture; growth; morphology

Introduction

Halophila beccarii, as other seagrasses play an important role in marine ecology and biology in their habitat, as high primary and secondary producers (Bronwyn, 2006). Different substrate types, mineralogy, grain sizes and nutrient load affect the morphology of seagrass (Mellor *et al.*, 2007), distribution, growth, and survival (Bradley and Stolt, 2005; van Katwijk and Wijgergan, 2004). Unlike *Halophila ovalis* which is known to inhabit diverse sediment types at various habitat (den Hartog 1970; Hillman *et al.*, 1995), information about the effect of substrate types on the morphology of *H. beccarii* morphology is limited. As substrate play factor determined plant survival, distribution and morphology at natural habitat. Thus, the effect of different substrates on morphometric such leaf length, leaf width, petiole length and the number of leaves per shoot under laboratory were studied for a further understanding of *H. beccarii* behavior in wild habitats.

Materials and methods

Halophila beccarii samples were collected from Punang-Sari-Lawas River Estuary, Sarawak, East Malaysia. The samples were rinsed and cleaned from debris before being transported to the Research Center at Universiti Putra Malaysia, Bintulu Sarawak Campus. The culture system was set up using 90 cm length x 46 cm width x 60 cm height glass tank, filled with artificial seawater, fitted water pump with an external filter to provide water current and filtration (Figure 1). The water salinity was fixed at 25 psu, an increase in salinity, was corrected by adding the aged rainwater to maintain the salinity. The culture glass tank was placed under a shaded area with light regime fluctuated from 160 μ mol/m²/sec (10% of the ambient light intensity outside) to 240 μ mol/m²/sec (15% of the ambient light intensity outside) recorded between 12.00 noon to 2.00 pm. There were three types of substrates fine beach sand (T1), loamy beach sand (T2) and coarse beach sand (T3) collected from the nearby Tg. Batu Beach, Bintulu, Sarawak. For each substrate, there were four plastic trays (33 cm length x 26 cm wide x 9 cm height) used as replicates. The trays were filled with substrate, 4-6 cm thick (Figure 1). The plants were planted for eight weeks, using different treatments, the morphometric changes were then observed and recorded e.g., leaf length (LL), leaf width (LW), petiole length (PL) and the number of leaves per shoot counted (LS) (Figure 2.0). The data were analyzed using Duncan's Multiple Range Test (SPSS for Windows Release 10.0).

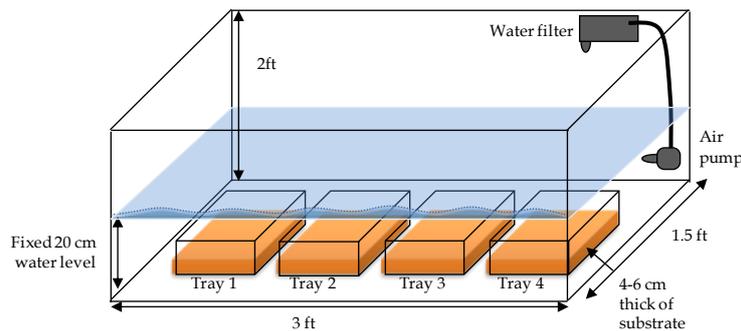


Figure 1.0. The layout of the experiments of culturing *H. beccarii* using different types of substrates.

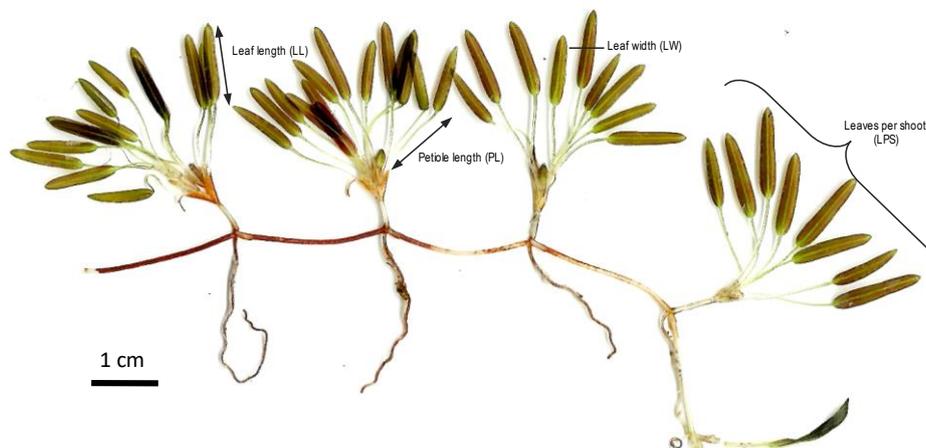


Figure 2.0 Selected morphometric of *Halophila beccarii* ramets (4 shoot).

Results and discussion

At studied area, *Halophila beccarii* was observed to inhabit mud flats proximity to a mangrove area. The observation in the study area showed there was a variation in substrate types, however, *H. beccarii* could only be found inhabiting the loamy substrate. A similar observation from the natural habitats, where plants are often found inhabiting substrates from sandy clay to mud which contains high silt or clay content (den Hartog, 1970; Parthasarathy *et al.*, 1991; Muta Harah *et al.*, 1999; Abu Hena, 2009). Under laboratory conditions, after eight weeks, *H. beccarii* showed higher shoot density with T2 (25.31 ± 6.33) compared to other substrates (Figure 3.0). Meanwhile, in terms of morphometric measurements, the plant showed a significant difference with T2 (14.55 \pm 1.78 mm leaf length, 14.07 \pm 2.36 mm leaf width, 3.13 \pm 0.46 mm petiole length and 7.00 \pm 1.06 no. of leaves per shoot). Samples grew with T1 and T3 showed diminished morphology (Figure 4.0). A previous laboratory study showed *Halophila ovalis* favored sandy substrate (Sidik *et al.*, 2010), as opposed to the current study where *H. beccarii* showed better morphology with loamy sand (T2). The substrate types differing in particle size and silt content may influence the aeration property of the substrates. Different seagrass species have different levels of tolerance to anoxic environments (Terrados *et al.*, 1999; Koch, 2001). Coarse sand consists of large particle size allowing for high oxygen diffusion which affects rhizome elongation. Both, coarse and fine sand types have the low nutrient hold capability, and thus reduces anchor capability that may affect plant growth (Koch *et al.*, 2000).

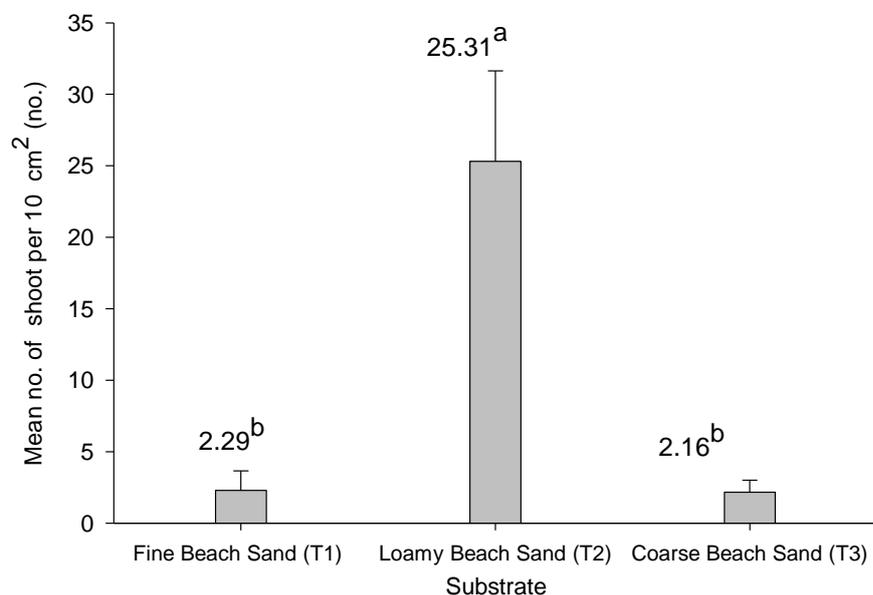


Figure 3.0 Shoot density between different substrates.

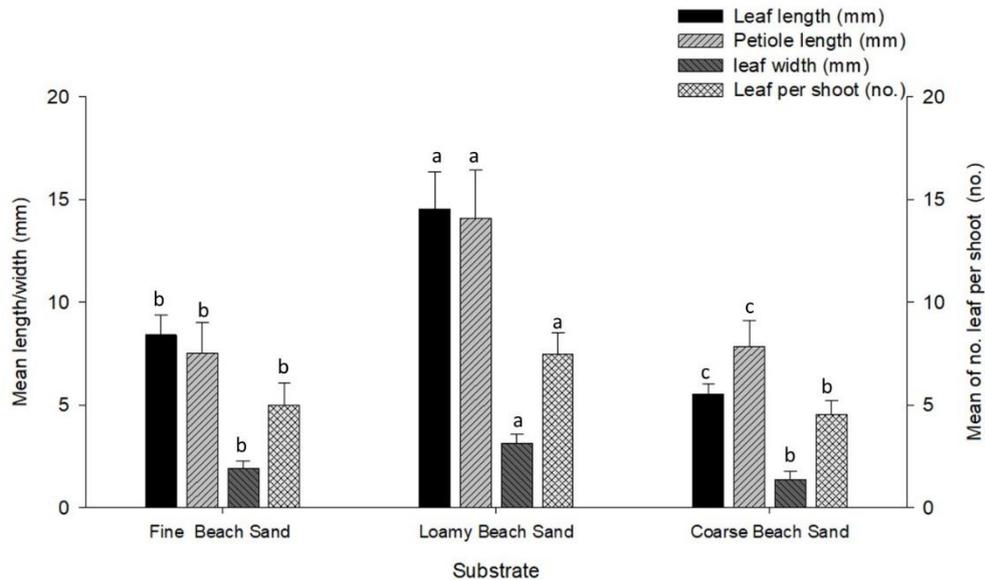


Figure 4.0 Effect of different substrate on the morphology of *H. beccarii*.

Conclusion

The plant showed favor to the substrate with high silt and clay at both laboratory and natural habitat. *Halophila beccarii* showed better growth of treatment T2, compared to other substrates. The substrate particles sizes, silt and clay content effect *Halophila beccarii* growth, and morphology at natural habitat.

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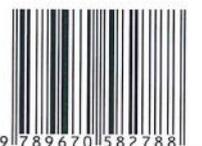
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