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PSAF 2021

THE 1ST POSTGRADUATE SEMINAR ON AGRICULTURE AND FORESTRY 2021

Enhancing Knowledge Through Research

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CAMPUS DIRECTOR'S FOREWORD

Assalamualaikum to all of you.

It is a great pleasure for me to extend a very warm welcome to all participants, to this 1st Postgraduate Seminar on Agriculture and Forestry: Enhancing Knowledge Through Research, organized by Universiti Putra Malaysia Bintulu Sarawak Campus. All of your contributions have helped to make the seminar outstanding and meaningful. Special thanks to organizing committee that work hard to make the seminar a success.

This seminar is a catalyst that draws the postgraduate students and researchers, to meet and discuss a range of agriculture and forestry issues. I would like to encourage all participants to make maximum use of this opportunities, to exchange and share views and opinions, and continue to keep abreast with the latest issues and development in all pertinent matters. Sharing of knowledge opens a new perspective on how we can look at things. This effort can definitely help us to fill the knowledge gap that we have been searching all this time. Events such as this seminar will play a vital role in addressing some current issues but in particular, it provides a platform for deliberations that can certainly enhance participants' research aptitude. I am sure all of us here has an exceptional knowledge in our own area of expertise and again, I would like to appeal to all participants to make full use of this opportunity to share and learn, in order to strengthen your research output to a higher level. As a fellow researcher, we discover new path of knowledge that may not be known before. These breakthrough in knowledge gap can be translated into an easier language for the community to understand. The livelihood of community around us can be improved by a proper transfer of knowledge and the process can be made smoother with more findings. Sharing of knowledge in this seminar will ultimately contribute to betterment of the community.

As it is being hosted digitally during our pandemic period, preparing for this momentous occasion takes a great deal of meticulous effort and time. I would like to applaud to the organizers in making this seminar a success. To all the aspiring participants, I wish all of you a fruitful and an engaging seminar where sharing of knowledge at this seminar could provide inspiring beneficial inputs to all of the participants. I hope that this first PSAF 2021 seminar would not be the last and will continue as an annual event for many years to come. Stay safe and stay healthy in this pandemic, don't forget to follow the SOP wherever and whenever you are.

Assalamualaikum warahmatullahi wabarakatuh.

Assoc. Prof. Dr. Shahrul Razid Sarbini
Campus Director
Universiti Putra Malaysia Bintulu Sarawak Campus

CHAIRMAN'S FOREWORD

It is my great pleasure to welcome you to the 1st Postgraduate Seminar on Agriculture and Forestry: Enhancing Knowledge through Research (PSAF 2021), held through a virtual platform considering the on-going COVID-19 pandemic.

The aim of this seminar is to provide a platform for disseminating scholarly postgraduate research findings in agricultural and forestry science. These areas include crop science, animal science, fisheries, and forestry. It also hopes to bring postgraduates together to share their experiences, ideas, and suggestions to improve their overall research and presentation skills.

The theme of this seminar is befitting these uncertain times, where the concerns of food production and food security are rising globally. Before the pandemic, hunger was already on the rise due to extreme climate events, climate change, pest and disease outbreaks, and geopolitical conflicts. The pandemic is estimated to dramatically exacerbate the level of acute food insecurity worldwide. As agricultural and forestry researchers, we play a pivotal role in alleviating this condition. Besides food production, agriculture and forestry sector also produces non-food products such as fibers, fuel, and other materials used in the manufacturing industry. I hope that this seminar will not only involve the dissemination of research findings, but also encourage postgraduate students to translate these valuable findings into practical and commercial applications.

I would like to express my sincere appreciation to the organizing committee for their tireless efforts and unwavering commitment to making this seminar a success. I wish all participants an enjoyable seminar with fruitful discussions and networks building even when we are gathered virtually.

Dr. Kwan Yee Min
Chairman of PSAF 2021
Universiti Putra Malaysia Bintulu Sarawak Campus

CHIEF EDITOR'S FOREWORD

Assalamualaikum W.B.T. and Salam We Love UPM!

It gives me great pleasure to welcome you to the e-Proceedings of the 1st Postgraduate Seminar on Agriculture and Forestry: Enhancing Knowledge through Research (PSAF 2021), which was organized by Universiti Putra Malaysia Bintulu Sarawak Campus. This seminar catalyzed postgraduate students and researchers to interact and discuss a variety of agricultural and forestry concerns.

The idea for organizing the PSAF 2021 originated at the beginning of the year after discussions with academics and researchers from UPMKB. Preparing for this momentous occasion required a considerable lot of painstaking effort and time because it was hosted digitally during our pandemic period.

As the Editor-in-Chief, I'd like to thank and express my appreciation to my co-editors as well as the contributing writers for editing their papers in accordance with the proceedings' standards. Last but not least, special appreciation is made to the PSAF 2021 organizing committee.

With the number of selected papers in this e-proceedings, the seminar has achieved its objectives: to bring together postgraduate students to share their findings and sustain the research culture in the university and industry. The research topics are based on fundamental research, advanced research methodologies, and applied research in the field of agricultural science and forestry.

I look forward to future intellectual contributions and thank you for your interest in reading the papers published here!

Ts. Dr. Fauziah Abu Bakar
Editor-in-Chief
Universiti Putra Malaysia Bintulu Sarawak Campus

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FORESTRY AND AGROFORESTRY

Physiochemical Properties of Organically Grown Red and Yellow-Fleshed Varieties of Watermelon Fruits

Tahir Dalorima[1*], Siti Zaharah Sakimin[2], Ramisah Mohd Shah[1]

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ABSTRACT

Organically produced fruits had garner positive traction worldwide. The present research studies the variability in mineral content, antioxidant capacity, Total Soluble Solids (TSS) content, lycopene content and other physiochemical properties of two watermelon varieties cultivated organically, thus the objective of this study is to compare the fruit quality of two watermelon varieties grown organically. There was an interaction between varieties and organic fertilisers in rind thickness, juice content and lycopene content. Results reveals that poultry manure had the thinnest significant rind size in new dragon variety (0.38 cm). Subsequently, Seaweed extract on new dragon variety had the significantly highest lycopene content (6.132). New dragon variety when tested with Poultry manure showed the highest significant percentage inhibition activity of antioxidant (53.93). Nutrient contents determined showed an interaction between varieties and organic fertilisers in Potassium (K), Sodium (Na), Iron (Fe) and Manganese (Mn). The Golden delight variety treated with poultry manure produced the significantly higher K content with mean values 1389 mg/100 g. Poultry manure and seaweed extract in both varieties maintains higher significant Fe content. Results for Na content reveals that, the Control in the Golden delight variety (58.97 mg/100 g) was lowest and varies significantly with other treatments. The control on both varieties was significantly lower when compared with poultry manure and seaweed extract when Mn content was determined. Subsequent results reveal significant positive correlation between physiochemical properties determined. The New dragon variety treated with poultry manure gave the best results.

Keywords: Watermelon; varieties; organic fertilisers; quality

INTRODUCTION

Human beings had known watermelon (*Citrullus lanatus*) for centuries and observed its nutritional benefits. Watermelon is an herbaceous creeping plant belonging to the family Cucurbitacea which thrives in the tropical region and has been cultivated for thousands of years in Africa and Asia [1]. There has been an increase in recent

years on consumer demand for high quality watermelon fruits and juice, coupled with growing awareness about health benefits of the fruit [2]. Watermelon is packed with some essential antioxidants in nature, for example the fleshy red part of watermelon fruit is a source of potent carotenoid antioxidant and lycopene that reduces man's risk of prostate cancer when combined with drinking green tea [3]. Fruits are an excellent source of essential antioxidant compounds and minerals which have properties of chemopreventive [4]. In organic cultivation, the soil becomes rich in nutrients, therefore, crops grow healthy, making the quality of the products more nutritious, tastier and contain substances that are good for health [5].

The total soluble solids (TSS) of a watermelon when treated with pigeon manure was high followed by poultry manure, cow dung, sheep manure and finally control group. High ash percentage was in cow dung followed by control group [6]. Watermelon cultivar shapah has high redness score on poultry manure as compared with other manures this was so because of the increase of lycopene content in the flesh [7]. Watermelon fruit quality properties such as fruit color and thickness of the rind were highest in crops treated with organic fertilisers and farm yard manure (6). Application of cattle manure led to thinner rind thickness in the range of 0-4 cm [8]. Zhang et al. [9] tested watermelon on vermicompost and reported that additional vermicompost could significantly increase the concentrations of complex sugars, lycopene, and soluble protein.

The use of organic fertilisers for the growth of crops supplies adequate quantities of nutrients to improve the crop [10]. There are differences in fruit quality between red-fleshed watermelon and yellow-fleshed watermelon [11]. However, there is a gap of information about fruit quality between the red fleshed and yellow fleshed watermelons grown with different organic fertilisers or organic farming systems. Subsequently, the objective of this study is to compare the fruit quality of two watermelon varieties grown organically.

MATERIALS AND METHODS

Watermelon crops were grown organically at the research farm of Universiti Malaysia Terengganu in Bukit Kor Marang, Terengganu. In this study, two varieties of watermelons, Golden Delight 363 (Yellow-fleshed) and New Dragon 117 (Red-fleshed) (F_1 hybrid) were used as planting materials and different organic manures viz., well decomposed poultry manure, vermicompost, goat manure, cow dung, seaweed extract, fish waste fertiliser, and Dolomites was use as the sources of plant nutrients. The research was a double factor experiment which involves eight treatments (T), of which different organic fertilisers were used as main factors, and two varieties (V) of watermelon as sub-factors, which was replicated four times. The experiment was layout in a Complete Randomize Design (CRD). Laboratory analysis of samples were conduction at the central laboratory complex of Universiti Malaysia Terengganu.

Determination of pH was carried out when fruits were harvested 10 weeks planting. The pH of the fruit was measured using a glass electrode pH meter after extracting the juice and sieving the juice. The pH meters was calibrated with a buffer solution at pH 4.0 and 7.0 before being use. Three samples were measured from each treatment and an average was calculated. The results were expressed as pH of fruit Juice.

Watermelon ash content was determined to all the replicated**,** Ash of a biological material is an methodical term for the organic residue that remains after the organic matter has been burnt off. The methods use by Inuwa et al. [12] was used. Crucibles were dried in an oven at 95 °C for 30 min, and was transferred into the desiccators to cool and weighed, each sample of 5 g were weighed into the crucible and heated in a muffle furnace set at 400 °C for 2 h after which the crucibles were transferred into desiccator to cool and be weighed. The percentage ash was then calculated using the formular below:

$$\text{Crude ash}(\%) = \frac{w_3 - w_1}{w_2 - w_1} \text{X } 100$$

Where;

W1 = Weight of empty crucible
W2 = Weight of empty crucible and sample before ashing
W3 = Weight of empty crucible and Sample after ashing

Samples for total soluble solids (TSS) content were determined with the aid of a digital refractometer and the results was expressed as ºBrix. All experiments were conducted at room temperature (± 28 °C) and four replications of all these measurements were carried out.

Lycopene Content was determined by measuring one grams of flesh tissue was added to 250 µL of acidic methanol (1% HCL w/v). The material was homogenized and then incubated at 4 °C for 1hour with moderate shaking. The suspension was clarified by centrifugation (14,000 r.p.m.) for five minutes. Absorption of extract at 530 nm and 657 nm wavelength was determined photometrically. The lycopene concentration was expressed as mg/100 g product. Quantification of anthocyanin was performed using the following equation:

$$\text{Anthocyanin} = (A_{530} - 0.25 \times A_{657}) \times M\text{-}1$$

Where;

A_{530} and A_{657} – Absorptions
M – Weight of plant material use for extraction (g)

The DPPH radical scavenging activity was measured by the method of Yang et al. [13]. Watermelon extract was dissolved in 10 mL of distilled water to a final concentration of 100 lg/mL. Two millilitre of 0.2 mM DPPH in ethanol was added to 1 mL of the PLFP solution. The absorbance was measured at 517 nm after 20 min of incubation at 25 °C. Distilled water was used as the control. The scavenging activity of DPPH radicals by the sample was calculated according to the following equation:

DPPH radical scavenging activity (%) = (1 absorbance of sample/absorbance of control) × 100.

The total moisture component of the samples is described as the juice content of the sample. Crucibles were oven dried at 90 °C for 30 min and transferred into desiccators to cool. After cooling, watermelon flesh was diced into cubes of 5 mm × 5 mm × 5 mm size. Each sample was weighed in the crucible and oven dried at 110 °C to a constant weight. Three replications of all measurements were carried out. The percentage juice content of each sample was then calculated as follows:

$$\text{Juice content } (\%) = \frac{w_3 - w_1}{w_2 - w_1} X$$

Where;

W1 = Weight of empty crucible
W2 = Crucible weight and sample before drying
W3 = Crucible weight and sample after drying

Watermelon fruits were cut into halves, exposing the rind and the flesh in a circumference. The thickness of the rind was measured with the aid of an electronic digital vernier callipers, replicated data were logged and the average was recorded in centimetre (cm).

The analysis of minerals were performed as per the methodology of Olayinka & Etejere [14]. Hundred grams of dry material was further processed for the wet digestion by the diacid (1 $HCLO_4$: 3 HNO_3) mixture and allowed to stand overnight. The samples were heated on hot plate until solid particles nearly disappears and heat until a clear colourless solution is obtained. Once digested, samples were further evaporated to near dryness. Once the reaction has subsided, samples were cooled and made up to 100 mL with distilled water. The solutions were allowed to stand overnight, filtered through a dry paper to remove silica without washing. The solution containing samples were retain and use for the analysis of minerals against the reagent blank by Atomic Absorption Spectrophotometer (AAS). Three replicates of samples from each treatment were use, and the average were computed, results was computed as mg 100 $g^{-1.}$ Other mineral elements such as sodium (Na) potassium (K) were determined by Flame Photometer (Bibby Scientific Limited, UK: Model No PFP7).

Statistical Analysis

All data were subjected to Analysis of Variance (ANOVA). The statistical package that was used is SAS version9. Treatment differences were separated using Least Significant Differences (LSD) Test. Correlation analyses were also carried out to establish the relationships between parameters measured for predictive purposes.

RESULTS

Effects on Qualitative Attributes

Table 1 Effects of different types of organic fertilisers on the growth and yield of two varieties of watermelon on qualitative attributes of watermelon fruits.

Factors	Rind thickness (cm)	TSS (Brix)	Juice content (%)	Lycopene content (mg/100g FW)	Antioxidant activity (% inhibition)	pH	Ash content (%)
Varieties (V)							
Golden Delight	1.28a	6.58b	91.22b	0.45b	25.06b	5.22a	0.43a
New Dragon	0.81b	7.51a	93.8a	5.20a	49.71a	5.21a	0.44a
$LSD_{0.05}$	0.07***	0.193***	0.336***	0.19***	0.706***	0.04^{NS}	0.219^{NS}
Organic Fertilisers (OF)							
Control	1.37a	6.51c	91.5b	1.76c	34.62e	5.33a	0.57a
Poultry Manure	0.72c	7.69a	93.67a	3.43a	41.40a	5.13b	0.32d
Goat Dung	0.96b	7.08abc	92.46b	2.74b	38.14bc	5.18b	0.45b
Cow Dung	1.09b	6.97bc	92.09b	2.96ab	37.25bcd	5.22ab	0.45b
Fish Waste Extract	1.05b	6.95bc	92.33b	2.99ab	35.71de	5.24ab	0.43bc
Dolomite	1.03b	7.06abc	92.31b	2.79b	36.06cde	5.22ab	0.42bc
Vermicompost	1.01b	6.75bc	92.13b	2.81b	37.32bcd	5.22ab	0.49ab
Seaweed Extract	1.13b	7.31ab	93.53a	3.13ab	38.60b	5.17b	0.36cd
$LSD_{0.05}$	0.225***	0.622**	1.084***	0.598***	1.278***	0.129**	0.070***
Interactions							
V × OF	*	NS	NS	*	*	NS	NS

Means followed by same letter(s) within a factor group in same column are not significantly different at $p<0.05$.

Table 1 reveals a significant interaction between watermelon varieties and organic fertilisers on rind thickness, lycopene content and antioxidant activity and results are presented in Figure 1. However, results for qualitative traits of watermelon varieties treated with different organic fertilisers shows that new dragon variety was significantly higher in total soluble solids (TSS) and juice content with mean values of 7.5 and 93.7, respectively. Poultry manure (7.68) was significantly different with the Control as regards TSS but results for other organic fertilisers remains unchanged. Juice content determined shows that Poultry manure and Seaweed extract gave the highest significant percentage of juice content with mean values of 93.67 and 93.53% respectively, results for the Control remains statistical the same

with the other treatments tested. pH value tested showed that Poultry manure, Goat dung and Seaweed extract differs significantly with the Control, however other organic fertilisers remain statistically the same with the control. Results for ash content in Poultry manure (0.32), and Seaweed extract (0.36%) shows higher significance as compared to other fertilisers, however the control gave the least significant mean value at $p<0.05$.

Interactions of Varieties and Organic Fertilizers on Rind Thickness, Lycopene Content and Antioxidant Activity

Figure 1 (A) shows a significant interaction between varieties and organic fertilisers in rind thickness, Poultry manure had the thinnest significant rind size in New Dragon variety (0.38). The control in Golden Delight variety had the thickest rind size (1.47 cm). However, New dragon variety tested with all the organic fertilisers had the thinnest rind size as compared to Golden Delight variety all through. The interaction effects of variety and organic fertilisers for lycopene content shows all organic fertilisers tested on Golden Delight variety are statistically the same and they vary significantly with the results of New Dragon variety. Meanwhile, Seaweed extract on New Dragon variety had the significantly highest lycopene content (6.132), followed by Poultry manure applied on the New Dragon variety (5.03), other tested organic fertilisers in New Dragon variety remained unchanged at $p<0.05$ (Figure 1 B). Results in Figure 1 (C) shows an interaction between varieties and organic fertilisers when antioxidant activity was determined. New Dragon variety when tested with Poultry manure showed the highest significant activity of antioxidant (53.93). Other results for New Dragon remain unchanged throughout the organic fertilisers tested.

Effects on Mineral Content

Results from Table 2 shows that there as an interaction between varieties and organic fertilisers in K, Na, Fe and Mn contents and results for interactions is presented in Figure 2. The mean separation for minerals of watermelon varieties treated with different organic fertilisers is shown in Table 2. New dragon variety had the highest significant determined Calcium (Ca), Magnesium (Mg), Sodium (Na), Iron (Fe) and Manganese (Mn) with mean value of 116.9 mg/100 g, 77.5 mg/100 g and 69.5 mg/100 g, respectively. Meanwhile, Cu and Zn do not vary statistically between varieties at $p<0.05$. Results for organic fertilisers shows that Poultry manure and Seaweed extract had higher significant Ca and Mg content. There was no significant difference between organic fertilisers when Copper (Cu) content was determined. Zinc (Zn) content determined showed no significant differences between varieties and organic fertilisers tested at $p>0.05$.

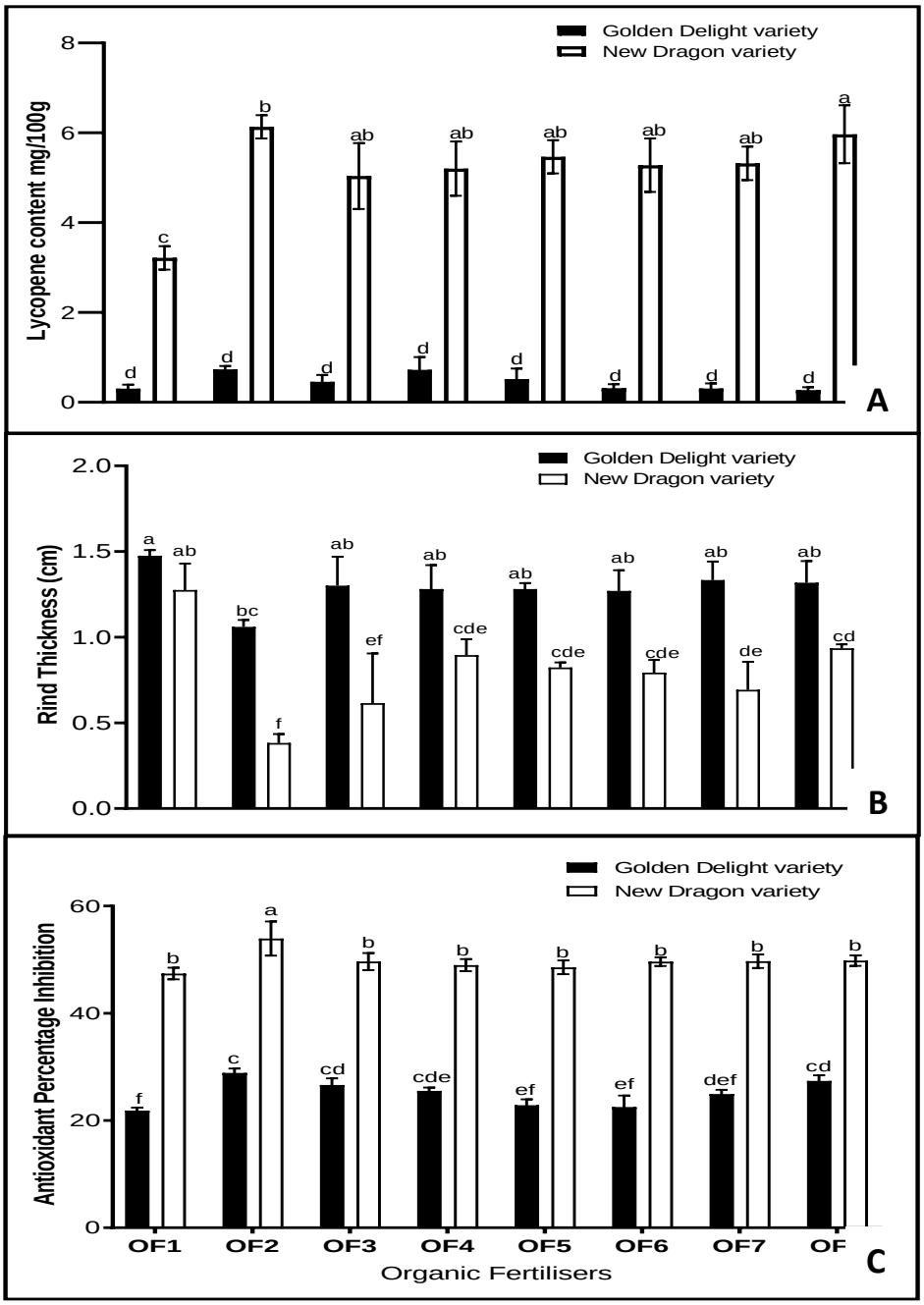

Figure 1 Interaction between varieties and organic fertilisers on rind thickness (A), lycopene content (B) and antioxidant activity (C). Bars indicate ± SE and different letters represent the statistical significance at $p < 0.05$. OF1: Control, OF2: Poultry manure, OF3: Goat dung, OF4: Cow dung, OF5: Fish waste extract, OF6: Dolomite, OF7: Vermicompost, OF8: Seaweed extract.

Table 2 Effects of different types of organic fertilisers on the growth and yield of two varieties of watermelon on mineral contents (mg/100 g).

Factors	Ca	Mg	K	Na	Cu	Fe	Mn	Zn
Varieties (V)								
Golden Delight	106.35b	74.91b	1190.9b	61.85b	1.0099a	3.727b	1.235b	4.250a
New Dragon	116.96a	77.57a	1273.1a	69.54a	0.9895a	3.768a	1.264a	4.257a
$LSD_{0.05}$	1.56***	1.038***	19.29***	1.338***	0.025^{NS}	0.0076***	0.005***	0.012^{NS}
Organic Fertilisers (OF)								
Control	102.98abc	73.92c	1186.7c	61.85b	0.9816a	3.665c	1.209c	4.216b
Poultry Manure	118.00a	79.06a	1359.2a	74.42a	0.9976a	3.789a	1.278a	4.275a
Goat Dung	111.73bc	75.64c	1214.7c	63.93b	1.0239a	3.754b	1.253b	4.253a
Cow Dung	110.05bc	75.72c	1204.4c	64.56b	1.0207a	3.753b	1.243b	4.2654a
Fish-Waste Extract	113.55c	76.22bc	1198.9c	62.25b	0.9558a	3.752b	1.238b	4.257a
Dolomite	112.13bc	74.77c	1206.3c	61.68b	1.0015a	3.738b	1.255b	4.264a
Vermicompost	110.31bc	75.72c	1208.0c	63.07b	1.0135a	3.745b	1.243b	4.249ab
Seaweed Extract	112.50bc	78.88ab	1277.6b	73.80a	1.0030a	3.781a	1.277a	4.254a
$LSD_{0.05}$	5.031***	3.35***	62.23***	4.318***	0.08^{NS}	0.025***	0.017***	0.038^{NS}
Interactions								
V × OF	NS	NS	*	***	NS	**	***	NS

Means followed by same letter(s) within a factor group in same column are not significantly different at $p<0.05$.

Interaction Between Varieties and Organic Fertilizers on Potassium, Iron, Sodium and Manganese Content

Results in Figure 2(A) shows the interaction between varieties and organic fertilisers in determining potassium (K), the Golden Delight variety treated with poultry manure produced the significantly higher K content with mean values 1389 mg/100 g. There was interaction in Iron (Fe), the control in both varieties was significantly lower as compared with other treatments. Poultry manure and seaweed extract in both varieties maintains higher significant Fe content. More so, differences between other treatments are not significant, although New Dragon variety in all organic fertilisers remains promising (Figure 2B). There was an interaction between varieties and organic fertilisers in determining Sodium (Na) which showed that, the Control in the Golden Delight variety (58.97) was lowest and varies significantly with other treatments, however there is no significant difference between varieties in organically treated fruits (Figure 2C). Figure 2(D) shows results for Manganese (Mn) content tested, there was an interaction between varieties and organic fertilisers. New Dragon variety on all the organic fertilisers do not vary significantly, although Golden delight variety on poultry manure and seaweed extract are at the same level with New Dragon variety statistically. The control on both varieties was significantly lower when compared with poultry manure and seaweed extract.

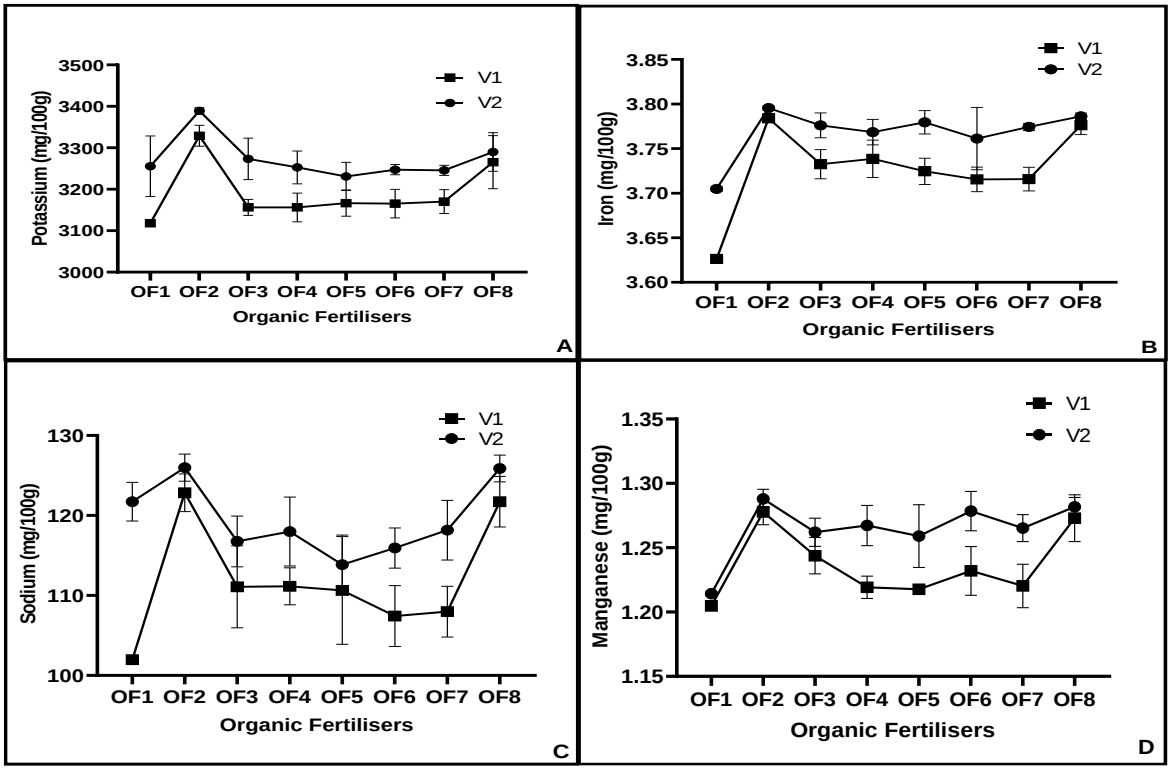

Figure 2 Interaction between varieties and organic fertilisers on potassium (A), iron (B), sodium (C) and manganese content (D). Bars indicate ± SE and different letters represent the statistical significance at $p<0.05$. OF1: Control, OF2: Poultry manure, OF3: Goat dung, OF4: Cow dung, OF5: Fish waste extract, OF6: Dolomite, OF7: Vermicompost, OF8: Seaweed extract.

Correlations Between Parameters

Results from the correlation matrix (Figure 3) shows that, ash content has significant positive correlation with pH (r^2=0.894), and significant negative correlation with TSS (r^2= -0.771) and juice content (r^2=-0.662), ash content has no significant correlation with rind thickness, lycopene, and DPPH. However, DPPH has statistically significant positive correlation with TSS, lycopene and juice content with r^2=0.768, 0.973 and 0.842 respectively, more so there is a significant correlation between DPPH and RT (r^2=-0.746) and no significant correlations with pH and ash content. The juice content had positive significant correlation with TSS (r^2=0.954) and lycopene (r^2=-0.0849), furthermore it correlates negative negatively with pH and rind thickness at $p<0.05$ with r^2=-0.674 and -0.787 respectively. Lycopene content had positive significant relationship with TSS and correlated negatively with rind thickness. There is a negative significant correlation in pH with TSS (r^2=-0.774) and positive significant relationship RT (r^2=0.731). Finally, the RT had a negative significant relationship with TSS (r^2=-0.865).

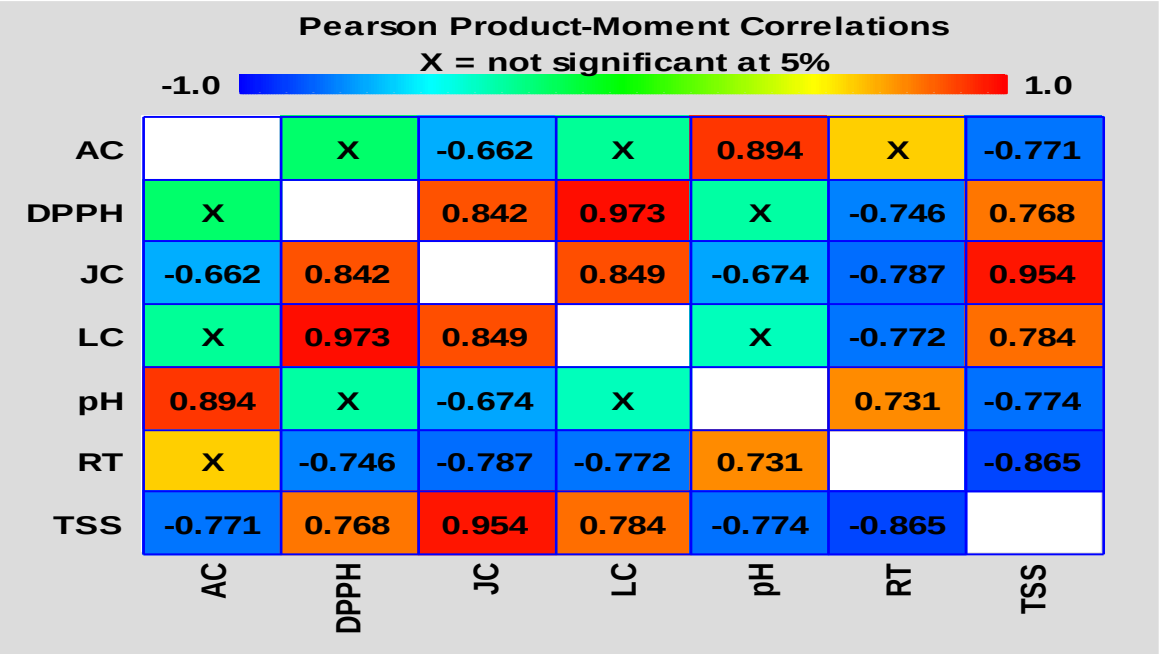

Figure 3 Linear correlations between biochemical parameters. AC: Ash content, DPPH: radical scavenging activity, JC: Juice content, RT: Rind thickness, TSS: Total soluble solids.

DISCUSSION

The watermelon rind is the whitish flesh the fruit left behind after the bright colour flesh. Results from this research showed that New dragon variety had the thinnest rind, and the control gave the thickest rind, these result is in concordance with a similar findings Audi et al. [8], who argued that cattle manure application led to thinner fruit rind in watermelon cultivation. All organic fertilisers tested showed improvement compared with the control. It is evident from this study that New dragon variety had the thinnest rind size compared with Golden delight variety, this agrees with a study in which it was reported that rind thickness watermelon is due to its genetic trait [15]. Rind thickness seems to be propotional to fruit size as yellow crimson watermelon hybrid produces the thickest rind, followed by giza hybrid and the least rind thickness was in the envy hybrid [16]. In the current study, the control gave the thickest rind, thicker rind in the control could be attributed to less nutrients and moisture content in the soil which hinders fruit biomass (flesh) formations. This argument agrees with the findings Jannoura et al. [18] who reported that soil amended with organic fertilisers improves fruit biomass accumulation in pea plants.

Maturity standards for melons are ten based on the level soluble solids [19]. According to Maynard et al. [20], sweetness is one the prime quality factors in watermelon fruit and it is related to total soluble solids (TSS). From this study, New Dragon variety had the highest TSS content measured. This is in agreement with the observations Yau et al. [21] where they reported that amongst the several varieties melons tested, the red fleshed variety produced the highest TSS content.

Data from this study shows statistical difference between the organic fertilisers tested and they are superior to the control, this is in accordance with the arguments [22], they verified that applying nitrogen rich organic fertilisers provided significant increment in TSS content melon fruits. Evidently, organic matter increases the TSS watermelon. In a study conducted by Pereira & Mitra [23] in guava and Patil et al. [24] in banana whom reported that the increased fruit quality parameters like TSS are due to the application different organic fertilisers.

Watermelon is a very juicy fruit, with a juice content over 90% [21], results from this study reveals that juice content ranges from 92.21-93.8%. These fruit juice content percentages were in agreement to the value recorded by Hayes [25]. Although higher juice content was recorded in New Dragon variety treated with poultry manure and seaweed extract there was no interaction effects between varieties and fertilisers. This might be attributed to the physiological properties watermelon fruit, because unripe fruits could produce more water content than some ripe fruits, this observation is in agreement with the reports Canet [26], that unripe watermelon has the high moisture content. However, there was increase in lemon juice content when organic fertilisers were applied [27].

Lycopenes are carotenoid that has antioxidant properties, and it gives fruits and vegetables their red colour [28]. Varieties of watermelon varied greatly in lycopene content, ranging from 0.2 to 10 mg/100 g [29] and this is in consistent with findings from this study. From the present study the red fleshed variety New Dragon variety had the highest lycopene content, this result confirm the findings Perkins-Veazie et al. [30] were they reported that several red fleshed watermelon produced high lycopene contents. The lycopene content New Dragon variety red-fleshed watermelon in this study was varied greatly with that Golden Delight variety yellow-fleshed watermelon (Table 1). These findings show that lycopene is abundant in the flesh red-fleshed watermelon, while lycopene is scarce in yellow-fleshed watermelon. This observation is in agreement with the study Tadmor et al. [31] where less lycopene was found in the yellow-fleshed watermelons studied. There was an interaction between variety and organic fertilisers used, when poultry manure and seaweed extract were added to New Dragon variety there was significant higher lycopene content recorded, this agrees with the findings Massri & Labban [6] where they asserted that poultry manure improve lycopene content watermelon fruits. Additionally, when organic fertilisers were applied to strawberry plant, increase in lycopene content was recorded [32]. Fertilizer types had significant effect on tomato fruit lycopene content [33].

DPPH radical was used in the evaluation free radical scavenging activity watermelons. According to the results obtained, the New Dragon variety had statistically significant antioxidant activity, these varieties high antioxidant activity can be due to the presence high phenols, flavonoids, lycopene, or other reducing agents that reduce the oxidised state antioxidant compounds. Similar observation

has been recorded in watermelon [2,11]. There was significant interaction between varieties and organic fertilisers used, when poultry manure and seaweed extract were applied to New Dragon variety, there was significant increase in antioxidant activity in the watermelon fruit. These results are in agreement with previous a study, that nitrogen rich fertiliser application has important effects on the antioxidant content bush tea [34]. Organic fertiliser had a positive effect on the production total antioxidants in red flesh sweet potato [35]. The lowest levels antioxidants scavenging activities watermelon fruit extract were observed in the absence organic fertiliser, it is sufficed to suggest that elements in the organic matters have correlation with increasing the scavenging activities watermelon fruit. These observations are agreement with the findings several researchers, who reported that mineral nutrition has little or no effect on the improving the polyphenols and antioxidants in some plants [36].

There was no significant difference between the varieties tested in this study, this agrees with the findings Massri & Labban [6] where they reported that the pH value for different watermelon varieties was very close. For organic fertilisers tested all the organic fertilisers were statistically higher than the untreated fruits, the control performed the least in regards to fruit pH, this might be in connection with the stage ripeness fruit, fruit parameter such as the pH value is related to the maturity stage the fruit [37]. Azarmi et al. [38] reported that vermicompost improves cucumber juice pH. Watermelon juice had higher pH values (between 6.28 and 6.53), with the application different organic fertilisers [6].

Treatments with high ash contents are expected to assist peristaltic movement as well as speed up metabolic processes necessary for improvement growth and development. Results from this study shows that the two varieties tested do not vary statistically when tested for ash content, this observation somewhat contradicts the findings Olayinka & Etejere [14] that cucumber contained higher amount ash when compared with watermelon. Almost all organic fertilisers tested were significantly different with the untreated fruits in this study. This study's results are in contradictions with the findings Yossif & Ibrahim [39], where they reported that ash content were not significantly affected by organic fertilisers tested on Rhode grass. However, organic fertiliser improved ash percentage dwarf bean [40]. However results from this study is similar with findings of Massri & Labban [6] where they compared with ash content between three varieties watermelon.

There are higher accumulations macro and micronutrients in the fruits watermelon [41]. Results from this study reveals that Ca is significantly higher in New Dragon variety than in Golden Delight variety, this might be attributed to the higher synthesis properties as genetic trait red fleshed watermelons. Calcium content for organic fertilisers treated and untreated fruits did not vary significantly this might be Ca uptake by the crop is minimal because low calcium content in organic fertilisers treated soil. Calcium content watermelon did not show any significant difference

amongst treatment [12]. Magnesium (Mg) content in watermelon flesh obtained from this study was higher in New Dragon variety, and in organic fertilisers like the poultry manure and seaweed extract. Poultry manure is associated with higher Mg content, this is in agreement with the findings Abdul-Hamid et al. [42] who stated that higher magnesium content in the soil is related to high uptake Mg by kenaf plant. Potassium (K) content in this study shows that when New dragon variety treated was with poultry manure a significant spike in K content was observed, this is concordance with the study Ayoola et al. [43] whom reported that poultry manure improves the potassium content *Cucumis sativus* fruit. There was an interaction between varieties and organic fertilisers when K content was determined, and poultry manure and seaweed extract are highly significant in the New Dragon variety. This agrees with the findings Lee [44] who reported that mineral contents crops can be enhanced by soil fertilization with mineral rich materials. Sodium (Na) content in this study reveals that poultry manure and seaweed extract gave the significant higher results when applied on New Dragon variety, this might be so because poultry manure and seaweed extract has high mineral content. Increase Na concentration in plant tissue is one the primary plant responses to excess minerals in the soil [45].

The red flesh watermelon is reported to have higher Na content when compared with other cultivars, this assertion is in agreement with the findings Olayinka & Etejere [14] where they reported that red fleshed watermelon has high Na content. Copper (Cu) content measured showed no significant different between treatments, Cu is among the heavy metals considered phytotoxic to crops. The watermelon crop seems to have a mechanism whereby Cu concentration does not exceed certain levels, this argument is supported by a study conducted by Gupta & Kalra [46] on cereals. Iron is an essential mineral used to transport oxygen around the body in the form haemoglobin. Iron (Fe) content tested shows an interaction effect between New Dragon variety with poultry manure and seaweed extract, the Control varies significantly with treated fruits and other treatments remain unchanged between each another. The chemical characteristics the organic fertilisers used varies greatly and one the main sources variation is in the concentration Fe contain in them. In this study it is evident that the watermelon fruit had a share Fe residue from the soil and results from this study within the required consumption value [47]. Manganese (Mn) was higher in New Dragon variety when treated with poultry manure and seaweed extract, however the untreated fruits was significantly lower amongst tested treatments, this study was in agreement with the findings Abdul-Hamid et al. [42] on kenaf and Watanabe et al. [48] in tomato fruits. Zinc deficiencies are normally corrected by soil applications zinc compounds [49]. No significant interaction was recorded in varieties for Zinc (Zn) content tested, however the control varies statistically with tested organic fertilisers, this is an indicator that the organic fertilisers influence Zn content in tested watermelon fruits. This is in agreement with the findings Aghili et al. [50] on bread wheat treated with green manures.

CONCLUSION

The current study showed that different organic fertilisers had different influences on two varieties watermelon. Results for qualitative attributes reveals that New Dragon variety was superior in rind thickness, TSS, juice content, lycopene content and antioxidants. However, new dragon had the thinnest rind thickness, poultry manure, goat dung and dolomite had the highest TSS. According to results for juice content and lycopene content determined shows that poultry manure and seaweed extract produced the highest results. Antioxidant activity determined reveals that the new dragon variety remains superior. For juice pH and ash content determined the results shows that the Control produced the least results. In an interaction, Golden Delight variety had the highest lycopene content and antioxidant activity. Minerals contents of watermelon varieties treated with different organic fertilisers shows that, New Dragon variety had the highest significant Ca, Mg, K, Na, Fe and Mn content. Results for organic fertilisers tested shows that poultry manure and seaweed extract had higher Ca, Mg, K and Na. There was an interaction between variety and organic fertilisers, results reveals that the New Dragon variety had the highest K content, poultry manure in the Golden Delight variety and New Dragon variety produced the highest Fe content, Na and Mg determined shows that poultry manure, seaweed extract, dolomite and cow dung remain highest in both varieties. There was significant positive correlation between ash content and pH. Antioxidant activities correlates with TSS, lycopene content and juice content. Lycopene content and juice content correlates with TSS. In overall, the results showed that watermelon New Dragon variety produced the best qualitative traits in watermelon fruit.

REFERENCES

1. Koocheki A, Razavi SMA, Milani E, Moghadam TM, Abedini M, Alamatiyan S, et al. Physical properties of watermelon seed as a function of moisture content and variety. Int Agrophysics. 2007;21(4):349–59.
2. Nagal S, Kaur C, Choudhary H, Singh J, Bhushan Singh B, Singh KN. Lycopene content, antioxidant capacity and colour attributes of selected watermelon (*Citrullus lanatus* (Thunb.) Mansfeld) cultivars grown in India. Int J Food Sci Nutr. 2012;63(8):996–1000.
3. Holden JM, Eldridge AL, Beecher GR, Buzzard IM, Bhagwat S, Davis CS, et al. Carotenoid content of US foods: an update of the database. J Food Compos Anal. 1999;12(3):169–96.
4. Morais DR, Rotta EM, Sargi SC, Bonafe EG, Suzuki RM, Souza NE, et al. Proximate composition, mineral contents and fatty acid composition of the different parts and dried peels of tropical fruits cultivated in Brazil. J Braz Chem Soc. 2017;28(2):308–18.
5. Wakui Y. Organic farming technology in Japan. Pilot Proj Better Farm Income by Org Veg Prod Koibuchi Coll Agric Nutr MITO. 2009;
6. Massri M, Labban L. Comparison of Different Types of Fertilizers on Growth , Yield and Quality Properties of Watermelon (Citrllus lanatus). Agric Sci. 2014;5(may):475–82.
7. Zubairu A, Gimba ASB, Mamza WJ, Highina BK. Proximate Analysis of Dry Watermelon (Citrullus lanatus) Rind and Seed Powder. Available online wwwjsaercom J Sci Eng Res 473 J Sci Eng Res [Internet]. 2018;5(3):473–8. Available from: www.jsaer.com

8. Audi W, Aguyoh JN, Gao-Qiong L. Yield and Quality of Watermelon as affected by Organic and Inorganic Nitrogen Sources. Asian J Agric Food Sci. 2013;1:180–5.
9. Zhang N, Bao Y, Xie Z, Huang X, Sun Y, Feng G, et al. Efficient characterization of tetraploid watermelon. Plants. 2019;8(10):1–10.
10. Lory JA, Massey R, Joern B. Using Manure as a Fertilizer for Crop Production.
11. Sim Choo W, Yen Sin W. Ascorbic Acid, Lycopene and Antioxidant Activities of Red-fleshed and Yellow-fleshed Watermelons. Pelagia Res Libr Adv Appl Sci Res [Internet]. 2012;3(5):2779–84. Available from: www.pelagiaresearchlibrary.com
12. Inuwa HM, Aina VO, Gabi B, Aimola I, Thompson V. Determination of differences in nutrient composition of *Citrullus vulgaries* (water melon) fruits after plucking. Br J Dairy Sci. 2011;2(2):27–30.
13. Yang B, Zhao M, Shi J, Yang N, Jiang Y. Effect of ultrasonic treatment on the recovery and DPPH radical scavenging activity of polysaccharides from longan fruit pericarp. Food Chem. 2008;106(2):685–90.
14. Olayinka BU, Etejere EO. Proximate and chemical compositions of watermelon (*Citrullus lanatus (*Thunb.) Matsum and Nakai cv red and cucumber (Cucumis sativus L. cv Pipino). Int Food Res J. 2018;25(3):1060–6.
15. Abdel-Al ZE. The effects of irrigation frequency and cultivars on premature cracking and blossom-end rot of watermelon fruits grown in the Sudan. In: III Africa Symposium on Horticultural Crops 49. 1973. p. 185–92.
16. Abdel-Mawgoud AMR, Tantaway AS, Hafez MM, Habib HAM. Seaweed extract improves growth, yield and quality of different watermelon hybrids. Res J Agric Biol Sci. 2010;6(2):161–8.
17. Jannoura R, Joergensen RG, Bruns C. Organic fertilizer effects on growth, crop yield, and soil microbial biomass indices in sole and intercropped peas and oats under organic farming conditions. Eur J Agron [Internet]. 2014;52:259–70. Available from: http://dx.doi.org/10.1016/j.eja.2013.09.001
18. Jannoura R, Bruns C, Joergensen RG. Organic fertilizer effects on pea yield, nutrient uptake, microbial root colonization and soil microbial biomass indices in organic farming systems. Eur J Agron [Internet]. 2013;49:32–41. Available from: http://dx.doi.org/10.1016/j.eja.2013.03.002
19. Kader AA, Holcroft DM. Postharvest: An Introduction to the Physiology and Handling of Fruit, Vegetables and Ornamentals. Horttechnology. 2018;
20. Maynard DN, Dunlap AM, Sidoti BJ. Sweetness in diploid and triploid watermelon fruit. REPORT-CUCURBIT Genet Coop. 2002;25:32–5.
21. Yau EW, Shamsudin R, Muda N, Chin NL, Hassan O. Physico-chemical compositions of the red seedless watermelons (Citrullus lanatus). Int Food Res J. 2010;17(2):327–34.
22. Lima PS, Rodrigues VLP, de Medeiros JF, de Aquino BF, da Silva J. Yield and quality of melon fruits as a response to the application of nitrogen and potassium doses. Rev Caatinga. 2007;20(2).
23. Pereira LS, Mitra SK. Studies on organic along with inorganic nutrition in guava. Indian Agric. 1999;43(3–4):155–60.
24. Patil MP, Hulamani NC, Athani SI, Patil MG. Response of new tomato genotype Megha to integrated nutrient management. Adv Agric Res India. 1998;9:39–42.
25. Hayes GD. Food engineering data handbook. Longman Scientific & Technical; 1987.
26. Canet W. Determination of the moisture content of some fruits and vegetables by microwave heating. J Microw Power Electromagn Energy. 1988;23(4):231–5.
27. El-Shiekh AF. Effect of some biological fertilizers on productivity and postharvest fruit quality of'Adalia'lemons grown in the united Arab emirates. In: III International Conference on Postharvest and Quality Management of Horticultural Products of Interest for Tropical Regions 1047. 2013. p. 45–50.
28. Suwanaruang T. Analyzing Lycopene Content in Fruits. Agric Agric Sci Procedia [Internet]. 2016;11:46–8. Available from: http://dx.doi.org/10.1016/j.aaspro.2016.12.008

29. Perkins-Veazie P, Collins JK. Flesh quality and lycopene stability of fresh-cut watermelon. Postharvest Biol Technol. 2004;31(2):159–66.
30. Perkins-Veazie P, Collins JK, Pair SD, Roberts W. Lycopene content differs among red-fleshed watermelon cultivars. J Sci Food Agric. 2001;81(10):983–7.
31. Tadmor Y, King S, Levi A, Davis A, Meir A, Wasserman B, et al. Comparative fruit colouration in watermelon and tomato. In: Food Research International. 2005. p. 837–41.
32. Zuo Y, Zhang J, Zhao R, Dai H, Zhang Z. Application of vermicompost improves strawberry growth and quality through increased photosynthesis rate, free radical scavenging and soil enzymatic activity. Sci Hortic (Amsterdam) [Internet]. 2018;233(October 2017):132–40. Available from: https://doi.org/10.1016/j.scienta.2018.01.023
33. Ojo MA. Impact of organic and inorganic fertilizers on growth , fruit yield , nutritional and lycopene contents of three varieties of tomato (*Lycopersicon esculentum* (L .) Mill) in Ogbomoso , Nigeria. 2015;14(31):2424–33.
34. Daniel MI, Mudau FN, Mashela PW, Soundy P. Seasonal responses of total antioxidant contents in cultivated bush tea (Athrixia phylicoides L.) leaves to fertilizer rates. Med Aromat Plant Sci Biotechnol. 2007;1(1):77–9.
35. Koala M, Hema A, Somé K, Palé E, Sérémé A, Belem J, et al. Evaluation of Eight Orange Fleshed Sweetpotato (OFSP) Varieties for Their Total Antioxidant , Total Carotenoid and Polyphenolic Contents. J Nat Sci Ressearch. 2013;3(4):67–73.
36. Alka G, Anamika S, Ranu P. A review on watermelon (*Citrullus lanatus*) medicinal seeds. ~ 2222 ~ J Pharmacogn Phytochem [Internet]. 2018;7(3):2222–5. Available from: http://www.globinmed.com.
37. Juroszek P, Lumpkin HM, Yang R-Y, Ledesma DR, Ma C-H. Fruit quality and bioactive compounds with antioxidant activity of tomatoes grown on-farm: comparison of organic and conventional management systems. J Agric Food Chem. 2009;57(4):1188–94.
38. Azarmi R, Giglou MT, Taleshmikail RD. Influence of vermicompost on soil chemical and physical properties in tomato (Lycopersicum esculentum) field. African J Biotechnol. 2008;7(14):2397–401.
39. Yossif AM, Ibrahim YM. Effect of fertilizers (urea, farmyard and chicken manure) on growth and yield of rhodes grass (Chloris Gayana L. Knuth.). Univers J Plant Sci. 2013;1(3):85–90.
40. Olfati JA, Khasmakhi-Sabet SA, Shabani H, Peyvast G. Alternative Organic Fertilizer to Cow Manure for French Dwarf Bean Production. Int J Veg Sci. 2012;18(2):190–8.
41. Grangeiro LC, Cecílio Filho AB. Acúmulo e exportação de macronutrientes pelo híbrido de melancia Tide. Hortic Bras. 2004;
42. Abdul-Hamid H, Yusoff M-H, Ab-Shukor N-A, Zainal B, Musa M-H. Effects of different fertilizer application level on growth and physiology of Hibiscus cannabinus L.(Kenaf) planted on BRIS soil. J Agric Sci. 2009;1(1):121.
43. Ayoola OT, Makinde EA, Programme E, Plantation M. Complementary Organic and Inorganic Fertilizer Application: Influence on Growth and Yield of Cassava/maize/melon Intercrop with a Relayed Cowpea. Aust J Basic Appl Sci. 2007;1(3):187–92.
44. Lee J. Effect of application methods of organic fertilizer on growth, soil chemical properties and microbial densities in organic bulb onion production. Sci Hortic (Amsterdam). 2010;124(3):299–305.
45. Meneguzzo S, Navari-Izzo F, Izzo R. NaCl effects on water relations and accumulation of mineral nutrients in shoots, roots and cell sap of wheat seedlings. J Plant Physiol. 2000;156(5–6):711–6.
46. Gupta UC, Kalra YP. Residual effect of copper and zinc from fertilizers on plant concentration, phytotoxicity, and crop yield response. Commun Soil Sci Plant Anal. 2006;37(15–20):2505–11.
47. Péneau S, Dauchet L, Vergnaud A-C, Estaquio C, Kesse-Guyot E, Bertrais S, et al. Relationship between iron status and dietary fruit and vegetables based on their vitamin C and fiber content. Am J Clin Nutr. 2008;87(5):1298–305.

48. Watanabe T, Nishijima O, Nakamura H. Residue analysis of metals in crops, 2: Manganese residue in tomato by Mancozab application. Bull Agric Chem Insp Stn. 1975;
49. Takkar PN, Walker CD. The distribution and correction of zinc deficiency. In: Zinc in soils and plants. Springer; 1993. p. 151–65.
50. Aghili F, Gamper HA, Eikenberg J, Khoshgoftarmanesh AH, Afyuni M, Schulin R, et al. Green manure addition to soil increases grain zinc concentration in bread wheat. PLoS One. 2014;9(7):e101487.

Antioxidant Activity, Total Phenolic Content, Total Flavonoid Content and Phytochemical Screening of *Cyperus iria*, *Cyperus distans*, *Fimbristylis miliacea*, *Fimbristylis dichotoma*, and *Fimbristylis globalise*

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ABSTRACT

Weed from the Cyperaceae family includes 3000 species and 220 were identified as weeds. Although this family has been identified as one of the invasive weeds in the world, the existence of secondary metabolites shows that this weed can exert various biological activities. This study focuses on the phytochemistry, antioxidant, total phenolic content (TPC), and total flavonoid content (TFC) of 5 weeds in the Cyperaceae family. The methanol extracts of five species; *Cyperus iria, Cyperus distans, Fimbristylis miliacea, Fimbristylis dichotoma*, and *Fimbristylis globulosa* were screened for phytochemical, TPC, TFC, and antioxidant activity. Phytochemical screening showed the presence of saponin, terpenoid, phenolic, and steroid. Antioxidant activity was determined using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) test. The extracts have significant antioxidant activity with values of IC_{50} = 309.07 µg/mL (*C. iria*); 113.19 µg/mL (*C. distans*); 250.67 µg/mL (*F. miliacea*); 213.72 µg/mL (*F. dichotoma*) and 623.82 µg/mL (*F. globulosa*). The antioxidant activity of the extracts was positively associated with the total phenolic and flavonoid contents.

Keywords: Antioxidant; Cyperaceae; phytochemistry; total flavonoid content; total phenolic content

INTRODUCTION

The Cyperaceae family has been broadly studied for showing various bioactive compounds of pharmacological interest. This family is known as sedges, and it is known as the third largest monocotyledons family which can be found in Tropical

Asia and Tropical South America. Several species of the genus *Cyperus* have been reportedly used as food, natural drugs, building materials, traditional mats, and ornamental plants, while some hold significant uses in land management. As for the genus *Fimbristylis*, it is also has been used in folk medicine. Despite its undesirable presence as weeds in rice fields. *Fimbristylis miliacea* is a grass-like herb with a fibrous root system and a widely distributed rice-fields weed. However, the sedges were always being thrown away as they disturbed the growth of the crops. Previous studies showed that there were some secondary metabolites in these sedges which can be further used in insecticidal and antimicrobial activity. Hence, this study aims to identify the secondary metabolites in *Cyperus iria, Cyperus distans, Fimbristylis miliacea, Fimbristylis dichotoma, and Fimbristylis globulosa*.

MATERIALS AND METHODS

Crude Extracts

The solvent used was methanol. The pulverized plant sample was extracted with methanol by maceration. The methanolic extract was concentrated in a rotary evaporator.

Antioxidant Test

The crude extracts will be tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical were determined by UV spectrophotometry at 518 nm [1]. Different concentrations of the plant extracts will be prepared using analytical methanol (1, 3, 7, 10, 20, 30, 40, 50, 80, and 100 µg/mL). Vitamin C will be used as an antioxidant standard. The same amount of methanol and DPPH were mixed to prepare the blank solution. The radical scavenging activity was calculated using the following formula:

$$\% \text{ inhibition} = ((Ab\text{-}Aa)/Ab) \times 100$$

in which Ab is the absorption of the blank sample and Aa is the absorption of the extract.

Determination of Total Phenolic Content (TPC)

The number of total phenolics were determined with Folin-Ciocalteu reagent [2]. Gallic acid was used as the standard and the results were expressed as mg/g gallic acid equivalents (GAE). 1 mL of standard solution of concentration 0.01, 0.02, 0.03, 0.04 and 0.05 mg/mL of gallic acid were prepared in methanol. The concentration of 0.1 and 1 mg/mL of plant extract was also prepared in methanol and 0.5 ml of each sample was introduced into test tubes and mixed with 2.5 mL of 10 folds dilute Folin- Ciocalteu reagent and 2 mL of 7.5% sodium carbonate. The tubes were covered with parafilm for 30 minutes at room temperature and the absorbance was read at 760 nm using a UV-Visible spectrophotometer.

Determination of Total Flavonoid Content (TFC)

The aluminium chloride method was used to determine flavonoids. Quercetin was used as standard and flavonoid contents were measured as quercetin equivalent [3]. 1 mL of standard or extract solution (20, 40, 60, 80,100 mg/L) was taken into a 10 mL volumetric flask, containing 4 mL of distilled water. 0.3 mL of 5% $NaNO_2$ was added to the flask. After 5 min, 0.3 mL 10% $AlCl_3$ was added to the mixture. At the sixth minute, 2 mL of 1M NaOH were added, and the volume was made up to 10 mL with distilled water. The absorbance was read at 510 nm using a UV-Visible spectrophotometer.

Phytochemical Screening

The powdered crude extracts were subjected to phytochemical analysis for the presence of saponins, flavonoids, terpenoids, and tannins using the standard qualitative procedures following Trease and Evans (1989), Harborne (1998), and Ekpo and Etim (2009) with little modification [4]. For saponins, 0.5 g of the extract was added and mixed with Fehling's solution and then 5% of sodium trioxocarbonate solution was later added. The mixture was then boiled. The pink precipitate indicated the presence of saponins. For terpenoids, 0.5 g powdered samples were soaked with chloroform and the solution was then filtered into a new test tube. 3 mL sulphuric acid was added to the solution. The reddish-brown coloration indicated the presence of terpenoids. For flavonoids, 0.5 g of the extract and few pieces of magnesium strips were mixed with concentrated HCl. An orange faint colour of effervescence solution indicated the presence of flavonoids. For tannins, 0.5 g of the plant extract was stirred with 1 mL of distilled water, filtered and ferric chloride solution or reagent was added to the filtrate. A blue-black or blue-green precipitate was taken as evidence for the presence of tannins.

RESULTS AND DISCUSSION

All five species had significant scavenging effects with increasing concentrations in the range of 100-500 ppm when compared with Ascorbic acid and BHA. Antioxidant assay of *C. iria, C. distans, F. miliacea, F. globulosa,* and *F. dichotoma* extracts displayed the ability to inhibit DPPH free radical formation. The IC_{50} value 25.63 µg/mL was obtained for Ascorbic acid and 52.23 µg/mL was obtained for BHA as the standard and for *C. iria* was 309.07 µg/mL, *C. distans* was 113.19 µg/mL, *F. miliacea* was 250.67 µg/mL, *F. globulosa* was 623.82 µg/mL and *F. dichotoma* was 213.72 µg/mL. The lower IC_{50} suggests that it has a higher antioxidant capacity. The result showed that Ascorbic acid showed the strongest antioxidant activity followed by BHA, *C. distans, F. dichotoma, F. miliacea, C. iria, and F. globulosa.* Our antioxidant result analyses showed the presence of antioxidants and DPPH radical scavenging activity, which is denoted in phenolic compounds from plants and responsible for the radical scavenging activity [5].

Table 1 IC_{50} Value (µg/mL).

	IC_{50} (µg/mL)
BHA	52.23
Vitamin C (Ascorbic acid)	25.63
C. iria	309.07
C. distans	113.19
F. miliacea	250.67
F. globulosa	623.82
F. dichotoma	213.72

Total phenolic and flavonoid contents of these weeds were compared. There was a positive linear correlation between antioxidant activity, total phenolic content, and total flavonoid content for *Cyperus iria, Cyperus distans, Fimbristylis miliacea, Fimbristylis dichotoma, and Fimbristylis globulosa*. These results suggested that the phenolic compounds and flavonoids compounds contributed significantly to the antioxidant capacity of the investigated weed species. The results on phytochemical screening showed that *F. miliacea* has a positive reaction towards saponin; while all three species except *C. iria* and *F. globulosa* has weakly positive reaction towards terpenoid; *F. dichotoma* has a positive reaction towards phenolic including other *C. iria, C. distans, and F. miliacea* which have weak positive reactions; while only 3 species showed weak positive reactions towards flavonoid which are *C. iria, F. dichotoma, and F. globulosa*.

Table 2 Total phenolic and flavonoid contents.

Plant extracts	**Total phenolics (mg gallic acid equivalent/g dried extract)**	**Total flavonoid (mg quercetin equivalent/g dried extract)**
C. iria	6.836	176
C. distans	21.649	32.25
F. miliacea	5.421	-
F. dichotoma	13.228	23.5
F. globulosa	62	84.75

CONCLUSION

From the results above, we can conclude that these five species of the Cyperaceae family have the potential to be exploited and used for many purposes. Further studies need to be done to understand more about their abilities as the results in antioxidant activity, TPC, TFC, and phytochemical screening indicated that this species can be used in insecticidal and antimicrobial activity.

Table 3 Result on phytochemical screening according to different compounds.

Phytochemical compound	*Cyperus iria*	*Cyperus distans*	*Fimbristylis miliacea*	*Fimbristylis dichotoma*	*Fimbristylis globulosa*
Saponin	-	-	++	-	-
Terpenoid	-	+	+	+	-
Phenolic	+	+	+	++	-
Flavonoid	+	-	-	+	+

(+) weakly positive reaction, (++) positive reaction, (+++) strongly positive reaction, (-) absent.

ACKNOWLEDGEMENT

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REFERENCES

1. Dehpour, A. A., Ebrahimzadeh, M. A., Fazel, N. S., & Mohammad, N. S. (2009). Antioxidant activity of the methanol extract of *Ferula assafoetida* and its essential oil composition. *Grasasy aceites*, 244-249.
2. Bhalodia, N. R., Acharya, R. N., & Shukla, V. J. (2011). Evaluation of in vitro antioxidant activity of hydroalcoholic seed extracts of *Cassia fistula* Linn. *Free Radicals and Antioxidants*, 68-76.
3. Olajire, A. A., & Azeez, L. (2011). Total Antioxidant Activity, Phenolic, Flavonoid and Ascorbic Acid Contents of Nigerian Vegetables. *African Journal of Food Science and Technology*, 22-29.
4. Sule, W. F., Okonko, I. O., Omo-Ogun, S., Nwanze, J. C., Ojezele, M. O., Ojezele, O. J., Olaonipekun, T. O. (2011). Phytochemical properties and in-vitro antifungal activity of *Senna alata* Linn. crude stem bark extract. *Journal of Medicine Plants Research*, 176-183.
5. Oladipupo, A. A. (2014). Screening and antioxidant activity of ethanolic extract of *Azadirachta indica*. *Journal of Applied Chemistry*, 46-49.

The Characterization of *Carica papaya* WRKY Transcription Factors in Response to Salinity Stress

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ABSTRACT

Carica papaya is an economically important plant that is highly susceptible to abiotic stresses, which have a deleterious impact on agricultural yield and quality. As such, improving resistance to these stresses is the primary objective of this research. WRKY transcription factors (TFs) are involved in plant defence to stresses. Overexpression of certain WRKY TF helped plants to overcome stresses. Therefore, this research aims to isolate and study the roles of *WRKY* TFs from *C. papaya* under salinity stress by overexpressing the *WRKY* gene in *Arabidopsis thaliana*. Total RNA was extracted from plants exposed to high salinity conditions, followed by RT-PCR of *WRKY2* and *WRKY4*. The purified PCR products were cloned into pCAMBIA1304 and confirmed by sequencing. The pCAMBIA1304-*WRKY* recombinant vector was transformed into *Agrobacterium tumefaciens* LBA4404. The amplification of *WRKY2* and *WRKY4* from total RNA showed that these genes are expressed only under salinity stress, and no amplicons were obtained from the control sample. The pCAMBIA1304-WRKY recombinant vector was successfully constructed and transformed into *A. tumefaciens* LBA4404. The presence of the vector was confirmed using colony PCR. The recombinant vector will be transferred into *A. thaliana* using the flower dip method. Transgene will be screened and validated using PCR until T3 generation. The T3 plants will be subjected to salinity stress treatment followed by physiological and biochemical analyses. Demonstrating the importance of *WRKY* gene response to abiotic stresses will enable us to engineer stress-tolerant transgenic crops in the near future to improve growth and productivity under unfavourable conditions.

Keywords: *Carica papaya*; *WRKY*; *Arabidopsis thaliana*; salinity stress' pCAMBIA1304

INTRODUCTION

Carica papaya, commonly known as papaya, is the third most cultivated tropical crop popularly consumed worldwide, and it is economically important due to its high

nutritive value and pharmacological benefits. In recent years, there is an increase in global demand for *C. papaya*, and therefore there is a need to increase fruit production and quality. However, papaya plants are easily affected by various environmental stresses such as drought, salinity, and low temperature. The higher salinity level of soil or water is one of the main environmental stresses, causing crop yield loss worldwide. Most salinity-affected lands are due to the accumulation of salt in soil from weathering of rocks containing soluble salts and seawater encroachment due to rising sea levels. The high salt level in the soil can affect many plants' physiology, morphology, and biochemistry, including papaya.

To overcome this problem, scientists are working together to develop salt-tolerant plants. WRKY TF is one of the vital TFs expressed by plants during stress conditions [1]. As such, the mechanism of salinity tolerance involving TFs has to be understood. Although many studies have been done on WRKY TF in various other plants such as rice, maize, cotton, tobacco, and so on, less progress has been made on understanding the function of *WRKY* proteins from *C. papaya* in response to salinity stresses. Therefore, this study aims to isolate and functionally characterize *WRKY* genes from *C. papaya* and study the roles of *WRKY* TFs under salinity stress by overexpressing the *WRKY* gene in *Arabidopsis thaliana*.

MATERIALS AND METHODS

Plant Material Preparation and Salinity Treatment

The seeds of *C. papaya* were obtained from fresh Hong Kong papaya fruit purchased from Jaya Grocer, Ipoh. *C. papaya* seeds pre-treated with 200 mg/mL of gibberellin acid (GA3) for 24 hours were rinsed using distilled and sown into the pot (12 cm × 12 cm) containing moistened organic soil. The pots were labelled accordingly and allowed for germination to occur at room temperature. Plants were watered daily with distilled water. Salinity stress treatment was performed on a 1-month-old plant by subjecting the plants to 200 mM sodium chloride, while control plants were watered using distilled water.

RNA extraction and *WRKY* gene amplification

Total RNA was extracted using the CTAB method [2], DNase treatment was using RQ1 RNase-Free DNase (Cat.# M6101) (Promega Corporation) [3], and Reverse transcription-polymerase chain reaction (RT-PCR) was using Deoxy HiSpec Reverse Transcriptase(Eastern Biotech Co., Ltd.) [4].

WRKY2 gene was amplified using the primer set WRKY2_F (5'-CCATGGATGTTTTACATGCAG-3') and WRKY2_R (5'-AGATCTCTACCTTCTCTTCGA-3') [1]. The primer set WRKY4_F (5'-CCATGGATGGAGAAGTAC-3') and WRKY4_R (5'-

AGATCTTTATACAATCAGGTTGAG-3') [1] was used for the amplification of *WRKY4* gene. PCR was performed at 95 °C for 3 min to denature the template and 30 cycles of denaturation at 95 °C for 45 s, annealing for 45 s at 50 °C and elongation at 72 °C for 1 min. The PCR tubes were incubated for an additional extension for 5 min at 72 °C, and the PCR products were resolved using agarose gel electrophoresis. The PCR products were confirmed using DNA sequencing by Apical Scientific Sdn. Bhd.

Constructs preparation and Transformation

WKRY2 and *WRKY4* genes were cloned into pGEM®-T Easy vector using TA cloning based on the manual of pGEM®-T Easy Vector Systems (Promega Corporation) [5]. The pGEM®-T Easy Vector-WRKY genes were transformed into DH5-α *E. coli* competent cells using CaCl2 chemical transformation [6]. The positive transformants were confirmed using colony PCR, and plasmid pGEM®-T Easy-WRKY was extracted from the positive transformants using Biobasic EZ-10 Spin Column Plasmid DNA Miniprep Kit. The plasmids pGEM®-T Easy-WRKY and pCAMBIA1304 were digested using restriction enzymes BglII and NcoI [7], followed by ligation using T4 DNA ligase. The recombinant vector pCAMBIA1304-*WRKY* was transformed into *A. tumefaciens* LBA4404 using freeze and thaw method [8].

RESULTS AND DISCUSSION

Pre-treatment of *C. papaya* seeds was performed to enhance the germination rate. Seeds treated with GA3 showed the highest germination rate, about 90% (Table 1). Significantly, the maximum germination rate was also observed in a previous study by [9]. GA3 enhances germination by activating cytological enzymes. Besides, it also hydrolyzes amylase and protease by acting in the embryo, which ultimately induces germination [10]. The lowest germination rate was observed in seeds treated with KNO3 (52.5%). Previous studies reported similar results where papaya seeds were pre-treated with KNO3, which has a low effect on the germination of papaya seeds [11,12].

Table 1 Comparison of pre-treatment used to induce germination of *C. papaya* seeds.

Pre-treatment	T1		T2		T3		Mean (%)
	Germination rate	%	Germination rate	%	Germination rate	%	
GA_3 (200 mg/L)	36/40	90.0	35/40	87.5	37/40	92.5	90.0
NAA (1 mg/L)	32/40	80.0	29/40	72.5	32/40	80.0	77.5
KNO_3 (200 mg/L)	25/40	62.5	17/40	42.5	21/40	52.5	52.5

Following the treatment, control plants were thriving, and the leaves were greenish (Figure 1). In contrast, the leaves of salinity-treated samples became yellowish and withered (Figure 2). The stress-treated plants were not as healthy as control plants. Due to salinity stress, the plants tend to have stunted growth and reduction in the leaf area [13]. Plants undergo deleterious effects such as low osmotic potential, nutrient imbalance, etc., caused by salinity stress [14].

Figure 1 *C. papaya* control plant samples after 28 days of salinity treatment. Plants are healthy with greener leaves.

Figure 2 Salinity treated *C. papaya* plant samples after 28 days of salinity treatment. Plants are unhealthy with yellow leaves and marginal chlorosis.

The *WRKY2* (1000 bp) and *WRKY4* (450 bp) genes were successfully amplified from salinity-treated papaya leaf samples. Sequence analysis using homology search in NCBI Genbank confirmed that the *WRKY2* gene showed 99.88% similarity and is closely related with *C. papaya* probable WRKY transcription factor 2 (LOC110820643) whereas, *WRYK4* gene showed higher similarity to *C. papaya* probable WRKY transcription factor 75 (LOC110816683).

The *WRKY* genes were successfully cloned into pGEM®-T Easy and transformed into *E. coli* competent cells. The genes were then transformed into the plant expression vector, pCAMBIA1304 and the positive transformant was identified through colony PCR. The pCAMBIA1304 and recombinant vector pCAMBIA1304-*WRKY2* were successfully transformed into *Agrobacterium tumefaciens* LBA4404 cell (Figure 3).

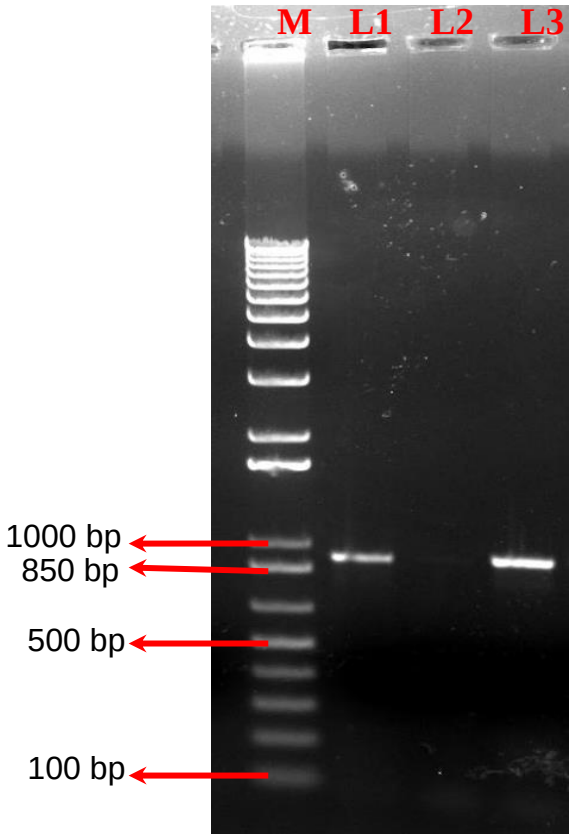

Figure 3 Amplification of *WRKY*2 genes from *A. tumefaciens* LBA4404 cell using colony PCR upon transformation.

CONCLUSION

The construction and transformation of the recombinant plasmid into *A. tumefaciens* LBA4404 for Arabidopsis transformation is successful. Subsequently, generation of transgenic *A. thaliana* and salinity stress tolerance assay followed by physiological changes and oxidative stress analysis will be conducted.

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REFERENCES

1. Pan, L. J., & Jiang, L. (2014). Identification and expression of the WRKY transcription factors of *Carica papaya* in response to abiotic and biotic stresses. *Molecular Biology Reports, 41*(3), 1215-1225. https://doi.org/10.1007/s11033-013-2966-8
2. Yang, G., Zhou, R., Tang, T., & Shi, S. (2008). Simple and efficient isolation of high-quality total RNA from *Hibiscus tiliaceus*, a mangrove associate and its relatives.

Preparative Biochemistry and Biotechnology, *38*(3), 257–264. https://doi.org/10.1080/10826060802164991

3. Deoxy HiSpec Reverse Transcriptase kit. (2011). Retrieved July 4, 2019 from Yeastern Biotech Co., Ltd Product Manual: http://si.secda.info/yb_md/wp-content/uploads/2017/07/Deoxy-HiSpec-Reverse-Transcription.pdf
4. RQ1 RNase-Free DNase kit. (2018). Retrieved June 13, 2019 from Promega Product Manual: https://worldwide.promega.com/-/media/files/resources/protocols/product-information-sheets/g/rq1-rnase-free-dnase-protocol.pdf?la=en
5. pGEM®-T Easy Vector Systems. (2018). Retrieved August 29, 2019 from Promega Product Manual: file:///C:/Users/windows%207/Downloads/pGEM%20T%20and%20pGEM%20T%20Easy%20Vector%20Systems%20TM042%20(1).pdf
6. Chang, A. Y., Chau, V. W., Landas, J. A., & Pang, Y. (2017). Preparation of calcium competent *Escherichia coli* and heat-shock transformation. *JEMI methods*, *1*, 22-25. https://jemi.microbiology.ubc.ca/node/127
7. Assembly of Restriction Enzyme Digestion Technical Manual. (2011). Retrieved August 14, 2019 from Promega Product Manual: https://www.promega.com/-/media/files/resources/protocols/technical-manuals/101/restriction-enzymes-protocol.pdf
8. Al-Hajaj, M., & Al-Dallee, Z. (2018). Cloning and transformation human glucocerebrosidase gene in *Agrobacterium tumefaciens* LBA4404 strain. *International Journal of Biosciences, 12*(4), 313-330. http://dx.doi.org/10.12692/ijb/12.4.313-330
9. Rana, G., Deb, P., Dowarah, B., & Sushmitha, K. (2020). Effect of Seed pre-treatment on seed germination and seedling growth of papaya. *International Journal of Current Microbiology and Applied Science*. *9*(4), 1066-1071. https://doi.org/10.20546/ijcmas.2020.904.126
10. Choudhary, R. C., Kanwar, J., Agarwal, H., & Prakash, O. (2020). Effect of GA3 and growing media on seed germination of papaya (*Carica papaya* L.) cv. Pusa Nanha. *IJCS*, *8*(5), 1423-1425. https://doi.org/10.22271/chemi.2020.v8.i5t.10500
11. Mahajan, G., Mutti, N. K., Jha, P., Walsh, M., & Chauhan, B. S. (2018). Evaluation of dormancy breaking methods for enhanced germination in four biotypes of *Brassica tournefortii*. Scientific Reports, *8*(1) 1-8. https://doi.org/10.1038/s41598-018-35574-2
12. Zanotti, R. F., dos Santos Dias, D. C. F., Barros, R. S., da Silva, L. J., & Sekita, M. C. (2014). Germination of "Solo" papaya seeds treated with plant hormones. *Journal of Seed Science, 36*(1), 94-99. https://doi.org/10.1590/S2317-15372014000100012
13. de Lima-Neto, A. J., Cavalcante, L. F., Mesquita, F. D. O., Souto, A. G. D. L., dos Santos, G. P., dos Santos, J. Z., & de Mesquita, E. F. (2016). Papaya seedlings irrigation with saline water in soil with bovine biofertilizer. *Chilean journal of agricultural research*, *76*(2), 236-242. http://dx.doi.org/10.4067/S0718-58392016000200014
14. Ashraf, M., & Harris, P.J.C. (2004). Potential biochemical indicators of salinity tolerance in plants. *Plant Science 166*(1), 3-16. https://doi.org/10.1016/j.plantsci.2003.10.024

Isolation and Characterization of Photosynthetic Bacteria (PB) From Rice Straw

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ABSTRACT

Photosynthetic bacteria (PB) have been extensively used in agriculture to promote plant growth and improve crop quality. This study focuses on isolating photosynthetic bacteria from rice straw and molecular characterizing photosynthetic bacteria. Isolation of bacteria was conducted by suspending the cells into the NA agar by using the serial dilution method. Gram stain test has been done and followed by aerobic dark and micro-aerobic screening for another step. UV-Vis spectrophotometer was used to determine the growth rate for each sample strain on the first day under two conditions with different wavelengths (500 nm-600 nm). Furthermore, in order for the required accuracy and precision to be attained, Polymerase Chain Reaction (PCR) Amplification of 16S rRNA Amplification using primers 8F and 805R showed the sizes of band that can be used at band 797 bp. The DNA band is observed through gel electrophoresis and visualized with ID Rodeo blue LED illuminator for the imaging process. From all the observation, red bacteria were type of PB which is *Rhodopseudomonas* genus. This PB may give rise to a novel biological agent with a dual function in disease management while promoting plant growth.

Keywords: photosynthetic bacteria (PB); effective microorganisms; *Rhodopseudomonas*; disease management; plant growth promoting

INTRODUCTION

Photosynthetic bacteria (PB) are unique microorganisms that use the sun as an energy source. Besides that, PB is one of the important microorganisms that can contribute to replenishing oxygen in the earth and the previous study, reported that categorized in prokaryotic organisms and comprises cyanobacteria, green sulfur bacteria, green non-sulfur bacteria, purple sulfur bacteria, purple non-sulfur bacteria and prochloron [1]. The previous research determined that these microorganisms may had a huge impact on why the world evolved the way it did and may show potential for life in places deemed uninhabitable, including extreme climates. In this study, these experiments were done by obtaining PB resources from rice straw [2]. Organic matter is the best source for obtaining PB and the current study believed

that rice straw as the primary source of PB [3]. However, the experiment focuses on identifying PB, which will be formulated and applied to plants that are not resistant to sunlight.

The general objectives of this study:
1. To isolate photosynthetic bacteria from rice straw.
2. To characterize photosynthetic bacteria from rice straw.

MATERIALS AND METHODS

Sample collection and Isolation of Bacteria

Rice straw samples were collected from Bintulu Sarawak. Collected samples were isolated and cultured using Nutrient Agar (NA) and incubated one week at room temperature.

Gram Staining Test

Media with single colonies of bacteria were selected to further test gram staining negative or positive bacteria under 100x magnification to see 2mm of the sample [4].

Primary and secondary screening

Samples were incubated into G5 broth for primary and primary screening within 24 hours, and bacterial growth was measured using a spectrophotometer at a wavelength of 60 nm [5].

Bacterial identification

DNA Extraction

Primer sets were designed to target specific 16S ribosomal DNA (rRNA) sequences of photosynthetic bacteria [6]. Materials of DNA extraction were provided by BioTake Corporation, which is Buffer RB, Buffer CB, Buffer IR, Buffer WB, Buffer EB, isopropanol, and proteinase. The kit was provided with a fast way to isolate pure DNA of bacteria.

Gel extraction and Polymerase Chain Reaction (PCR) purification

The PCR was used to amplify the bacterial DNA for the preparation of DNA sequencing. The universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3’) and 805R (5′-GACTACCAGGGTATCTAAT-3’) were used following into another step which is initial denaturation, denaturation, annealing and extension. The DNA band

was observed through gel electrophoresis and visualized with ID Rodeo blue LED illuminator for the imaging process.

RESULTS AND DISCUSSION

Colony Forming Unit/g (Bacteria)

Figure 1 showed a few single colony growths after one week of incubation with a yellowish colour. Bacteria colonies grew on the plate and were isolated onto the new NA agar media and incubated at room temperature for 24 hours. Figure 2 shows the streaking cultured results after 24 hours. The colour of the growing colonies was red, which is a very similar characteristic of PB [7].

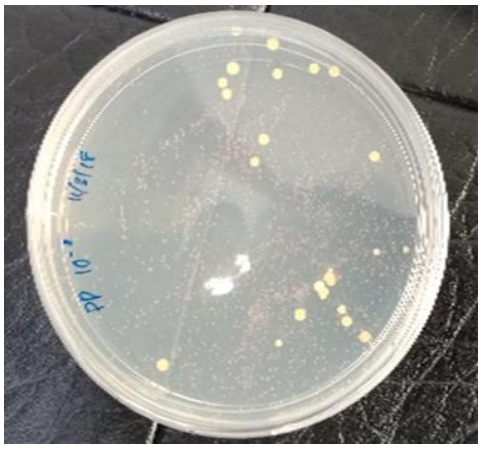

Figure 1 Spreading colony on NA.

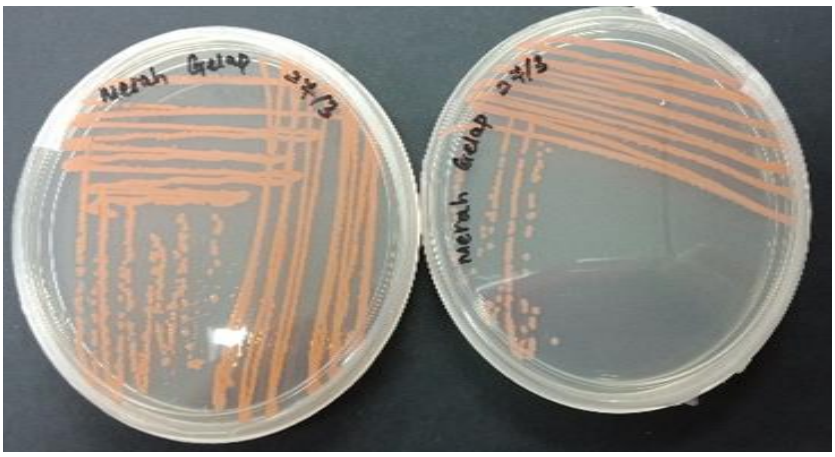

Figure 2 Morph culturally of bacteria after incubation.

Gram Staining Test

Figure 3 showed the observation for PB based on previous studies achieved very similar to PB, which is gram-negative, elongated, rod-shaped and non-spores forming [8].

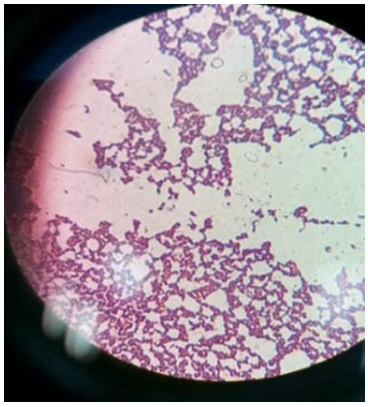

Figure 3 Gram-stained cell (100x) microscope.

Screening in Aerobic Dark and Micro-Aerobic Light Condition

Tables 1 and 2 showed that red bacteria keep growing under light and dark conditions within 10 days. This showed the presence of red bacteria within these two conditions as PB characteristics [9].

Table 1 Growth rate of bacterial isolates under anaerobic dark condition after 10 days of incubation.

Light	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Red bacteria	1.7543	1.3097	1.3885	1.4007	1.4545	1.4564	1.4692	1.4486	1.4923	1.5200

Table 2 Growth rate of bacterial isolates under aerobic light condition after 10 days of incubation.

Dark	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Red bacteria	1.0671	1.1560	1.1713	1.1522	1.1692	1.1769	1.2030	1.1862	1.9852	2.0122

Polymerase Chain Reaction (PCR) Amplification of 16S rRNA

The DNA band was observed through gel electrophoresis and visualized with ID Rodeo blue LED illuminator for imaging (Figure 4). Red bacteria were sent for sequencing to BioTake Corporation. Hence, red bacteria was the type of PB, which is *Rhodopseudomonas* genus.

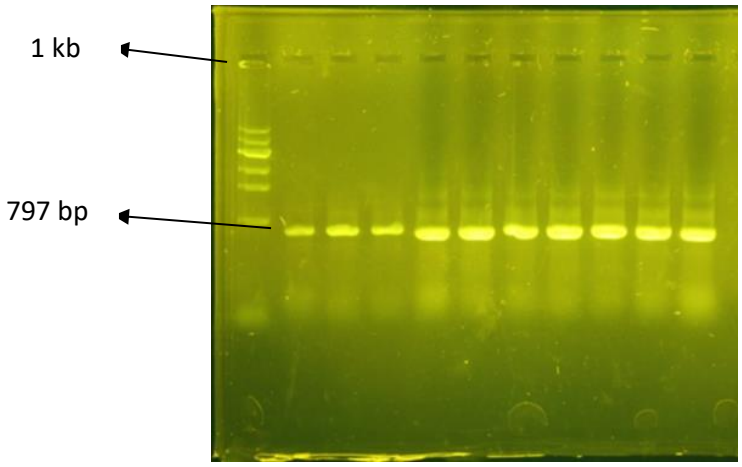

Figure 4 Amplification obtained with purified DNA isolates red (R) colony.

CONCLUSION

This PB may give rise to a novel biological agent with a dual function in disease management while promoting plant growth. This study shows there is a great opportunity in the agricultural industry. The previous finding reported that some PB is important to improve the quality and quantity of yields in agriculture [10]. In the future, PB might be used in all sectors, such as the medical sector, food industry, and oil and gas industry [11]. Therefore, the opportunity to research this PB is prevalent.

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REFERENCES

1. Myrsini, Sakarika., Janne, Spanoghe., Yixing, Sui., Eva, Wambacq., Oliver Grunert., Geert, Haesaert., Marc, Spiller., Siegfried E., Vlaeminck. (2019). Purple non-sulphur bacteria and plant production: benefits for fertilization, stress resistance and the environment. 21August 2019 https://doi.org/10.1111/1751-7915.13474Citations: 15.
2. Amirreza, T., & Shahabaldin, R. (2017) Application of photosynthetic bacteria for removal of heavy metals, macro-pollutants and dye from wastewater. Department of Civil Engineering, Jami Institute of Technology, Isfahan, IranbDepartment of Environmental Engineering, Faculty of Civil Engineering, Universiti Teknologi Malaysia.
3. Alberts, B., Johnson, A., & Lewis, J. (2002) Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Chloroplasts and Photosynthesis. Available from: https://www.ncbi.nlm.nih.gov/books/NBK26819/

4. J, Xu., Y, Feng., Y, Wang., & X, Lin. (2013) Characteristics of purple nonsulfur bacteria grown under Stevia residue extractions. Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing, Jiangsu 210008, China. 2013/0092: received 14 January 2013, revised 29 June 2013 and accepted 30 June 2013.
5. Hong, L, G., & Fu-Li Z. (2014) The effects of composite photosynthetic bacterial inoculant PS21 on the biochemical characteristics of wheat seedlings under tetrabromobisphenol A stress. Biotechnol Biotechnol Equip. 2015 Mar 4; 29(2): 289–298. Published online 2015 Jan 13. doi: 10.1080/13102818.2014.999298
6. Laurie, A., Achenbach., Jennifer, C., & Michael, T, M,.(2001)Photosynthetic and Phylogenetic Primers for Detection of Anoxygenic Phototrophs in Natural Environments. Appl Environ Microbiol. 2001 Jul; 67(7): 2922–2926. doi: 10.1128/AEM.67.7.2922-2926.2001
7. Tan, K, S., Sujjat, A, A., & Julian, R. (2014) Isolation and Characterization of Purple Non-Sulfur Bacteria, Afifella marina, Producing Large Amount of Carotenoids from Mangrove Microhabitats, Borneo Research Institute, University Malaysia Sabah, https://doi.org/10.4014/jmb.1308.08072
8. Wei, H., Y., Okunishi, S., Yoshikawa., Takeshi., Kamei., Yuto., Maeda., & Hiroto. (2016). Isolation and Characterization of a Purple Non-Sulfur Photosynthetic Bacterium Rhodopseudomonas faecalis Strain A from Swine Sewage Wastewater. Biocontrol Science. 21. 29-36. 10.4265/bio.21.29.
9. Oliver, B., Francois, M., Morel., & Xinning, Z. (2018) The purple non-sulfur bacterium Rhodopseudomonas palustris produces novel petrobactin-related siderophores under aerobic and anaerobic conditions. Environ Microbiol. 2018 May;20(5):1667-1676. doi: 10.1111/1462-2920.14078. Epub 2018 Mar 26.
10. Mieko, H, T., Kumiko, M., & Keiji N. (2016) A Screening Method for the Isolation of Polyhydroxyalkanoate-Producing Purple Non-sulfur Photosynthetic Bacteria from Natural Seawater. Enzyme Research Team, Biomass Engineering Research Division, RIKEN Center for Sustainable Resource Science, Wako, Japan. https://doi.org/10.3389/fmicb.2016.01509
11. Asker, D., Beppu, T., & Ueda, K. (2007) Unique diversity of carotenoid-producing bacteria isolated from Misasa, a radioactive site in Japan. Appl. Microbiol. Cell Physiol. 77:383-392.
12. Kai J, L., Sook, K, L., & Chi T, L. (2020) Development of a low-cost culture medium for the rapid production of plant growth-promoting *Rhodopseudomonas palustris* strain PS3. https://doi.org/10.1371/journal.pone.0236739, University of Technology, SWEDEN.
13. Nancy, M., Heidi, S., Aronson., Diana, P., Bojanova., Jayme, F., Buska., Michael, L., Wong, S, Z., & Donato, Giovannelli. (2019) Microbial Life Under Stress: Biochemical, Genomic, Transcriptomic, Proteomic, Bioinformatics, Evolutionary Aspects and Biotechnological Applications of Poly-Extremophilic Bacteria.
14. Pin, S., Xinqiu, T., Chenggang, L., Deyong, Z., Ju'e, C., Songbai, Z., Xuguo, Z., Qingpin, Y., Jing, Z., Zhuo, Z., Yong, L. & Xiangyang, L. (2017) Photosynthetic bacterium *Rhodopseudomonas palustris* GJ-22 induces systemic resistance against viruses. Microb Biotechnol. 2017 May; 10(3): 612–624.
15. Rani, N, M. (2006) The Study of Hydrogen Production Using Photosynthetic Bacteria Rhodospirillum Rubrum. umpir.ump.edu.my.

Effect of *Azolla* on Growth Performance of Choy Sum (*Brassica chinensis* var. *Parachinensis*)

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ABSTRACT

Azolla has been used as a soil amendment (fresh or composted) due to high nitrogen (N) content (3-6% N by dry weight). *Azolla* incorporation in rice fields can increase the yield up to 47%. However, the effect of fresh and composted *Azolla* on the growth performance of leafy vegetables is still lacking being studied. Therefore, this study aimed to determine the effect of fresh and composted *Azolla* on the growth performance of choy sum. Polybag trial with the arrangement of the completely randomised design was conducted throughout two planting seasons. The soil treatments were i) non-amended (control), ii) 150 kg/ha 15:15:15 NPK fertilizer (0.7 g/polybag), iii) fresh *Azolla* 3, 6, 9% and iv) composted *Azolla* 1, 2, 3% of soil weight. At harvest, the fresh and dry weight (shoot and root), leaf area, and root length were observed. The data were analysed using ANOVA of Statistical Analysis System (SAS) version 9.4, SAS Institute, Cary, NC. The choy sum treated with composted *Azolla*3% showed a better performance in shoot fresh, and dry weight, dry root weight and leaf area than the other treatments, followed by NPK fertilizer and composted *Azolla* 2%. An evident decline in the growth performance of choy sum was reported in the second planting season. Composted *Azolla* at a higher rate can enhance the uptake of nutrients from the soil by crop; hence, improving the choy sum growth performance.

Keywords: Soil amendment; soil fertility; *Azolla microphylla*; compost

INTRODUCTION

In parallel with the current growth in agricultural production, farming activities are also considered a major cause of environmental harm. Huge amounts of synthetic nutrients applied into the soil every year are not consumed by plants due to nutrients running off from crop fields [1]. This has greatly affected the soil quality and fertility over the long run and indirectly will reduce the crops yield. Besides, Malaysia's soils seem to be highly weathered due to the tropical environment as high rainfall and

temperature are recorded throughout the year [2]. Thus, this has caused the leaching of plant nutrients and the accumulation of sesquioxides. Therefore, restoration of soil nutrients and organic matter by applying good soil amendment can play a huge role in maintaining soil fertility to promote plant growth [3].

Azolla is the world's smallest pinnate-shaped macrophyte that floats on water surfaces that has multi-uses in the agriculture sector [3]. It is a unique aquatic fern due to its symbiotic association with the nitrogen-fixing cyanobacteria, *Trichormus azollae*. Its high fixed atmospheric nitrogen (N) and other nutrients content renders it a perfect additional fertilizer for crops, either in fresh, dried, or composted [4]. Therefore, farmers have used this fern in paddy fields throughout the past years, especially in China, India, Bangladesh, and Vietnam [3].

Basically, after four weeks of incorporation, 50% of the applied *Azolla* decomposed, producing organic matter and releasing nutrients into the soil [5]. *Azolla* can sustain soil N supply by returning N to the soil in quantities roughly equal to those extracted from soil by the paddy; hence, increasing the paddy yield [6]. Similar results were obtained when *Azolla* was used in red spinach [7]. Not only that, enhancement of soil texture, chemical availability, and microbial population was reported as well when *Azolla* was used as soil amendment [8]. Furthermore, due to its invasive characteristic, *Azolla* tends to form dense mats on the water surface, improving the physical and chemical conditions of the water simply by accumulating the toxic substances in water, including heavy metals [9]. *Azolla* also has a good source of protein, essential minerals, and vitamins suitable for being used as an alternative feed for animals [10].

Azolla effectiveness on crop production has been proven by several studies which were widely conducted on paddy and several studies on other grains crops and vegetables. Fresh *Azolla* is less common to be used in other crops planted apart from paddy. Therefore, a study was conducted to determine the effect of fresh and composted *Azolla* on leafy vegetables, choy sum, especially on clayey soil. *Azolla* was used as a soil amendment to determine the effectiveness of this fern on the growth performance of choy sum.

MATERIALS AND METHODS

This experiment was conducted in a greenhouse at the Faculty of Sustainable Agriculture, Universiti Malaysia Sabah. The soil used in this experiment was collected from the plow layer (the top 15 cm of soil) of palm oil estate at the faculty farm, located about 1 km from the experimental site. The soil was air-dried and ground before use. Its texture was clay, a low fertility soil that was slightly alkaline (pH 7.29). The soil contained about 2.44% of total carbon, 2.55% of total nitrogen, and 125 cmol/kg of cation exchange capacity. Each polybag was filled with 1 kg of soil.

The composted *Azolla* was prepared according to the method suggested by [11] with slight modifications to fit in the study conditions. *Azolla* was cleaned and washed before being sun-dried for further use. The dried and fresh *Azolla* were mixed at 50% and 30% by weight, respectively, and 20% w/w of molasses, in a black bin. Then, the moisture was maintained throughout the composting period (1-2 weeks). If the material temperature was increased, the material was turned to introduce oxygen for the further composting process. The matured compost was harvested once the temperature remained constant even after turning and then the compost was dried at room temperature at 27-32 °C until minimum moisture content (75%) was achieved. The composts were compressed and squeezed to determine the compaction formed. The compost is ready to be used when there was no more water found from the composts upon compression and the compaction formed were loosely attached and easily crumbled

Polybag trial with an arrangement of the completely randomized design was conducted throughout two planting seasons. *Azolla* was applied on the soil as a soil amendment in the form of fresh biomass or compost. The soil was treated with i) non-amended ii) 150 kg/ha 15:15:15 NPK fertilizer (0.7 g/polybag) applied after the first and second week of transplanting iii) fresh *Azolla* – 3, 6, 9% and iv) composted *Azolla* 1, 2, 3% of soil weight was applied at the beginning of planting season. Fresh *Azolla* contained 40.94% and 6.29% whereas composted *Azolla* contained 36.42% and 8.26% of total carbon and total nitrogen, respectively. After *Azolla* had been incorporated into the soil, the soil was incubated in the greenhouse for a week before transplanting. Seedlings at the 21-days age were transplanted, and the fresh and dry weight (shoot and root), leaf area, and root length were observed at harvest. The data were analysed by using ANOVA of Statistical Analysis System (SAS) version 9.4, SAS Institute, Cary, NC.

RESULTS AND DISCUSSION

Table 1 shows there were significant interaction effects between soil amendments and planting seasons on shoot fresh and dry weight, root dry weight, and leaf area of choy sum. Meanwhile, there was no significant interaction effect between soil amendments and planting seasons on root fresh weight and root length of choy sum. Composted *Azolla* - 3% showed the highest values in shoot fresh weight and leaf area of choy sum, followed by the application of recommended NPK fertilizer and composted *Azolla* - 2%. Undeniably, the fertiliser application is reliable, but, in this study, the fertilizer treatment results were comparable with the increasing rate of composted *Azolla* treatments. However, the fresh *Azolla* treatments did not differ in all growth parameters compared to the control (non-amended soil).

The low carbon (C): N ratios in *Azolla* make this fern decompose fast; hence, quick N releases further use by the crops. The mineralization of soil organic matter contributed to a constant release of ammonium-nitrogen and nitrate-nitrogen in the

soil [11]. The availability of sufficient N maintained the green part of leaves and assisted in protein formation such as protoplasm, chloroplasts, and enzymes, benefitting photosynthetic activity and crop growth performance [12,13]. Moreover, compost is a more stable organic matter that releases nutrients slowly [14]. Hence, the evidence increased in the fresh shoot weight and leaf area of composted *Azolla* treatments.

Table 1 Main and interaction effect of non-amended soil, 150 kg/ha 15:15:15 NPK and different application rates of fresh (3, 6, 9%) and composted (1, 2, 3%) *Azolla* on the shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, root length and leaf area of choy sum during two planting seasons.

Factor	**Weight (g)**				**Root length (cm)**	**Leaf area (cm^2)**
	Fresh shoot	**Dried shoot**	**Fresh root**	**Dried root**		
Soil amendments (SA)						
Non-amended	1.29c[z]	0.21c	0.78a	0.05c	5.71b	9.49bc
15:15:15 NPK[y]	4.44ab	0.58a	0.73a	0.15a	11.05a	20.19a
Fresh *Azolla* – 3%	1.21c	0.20c	0.22a	0.06c	4.03b	9.41bc
Fresh *Azolla* – 6%	1.20c	0.13c	0.19a	0.05c	5.47b	8.26c
Fresh *Azolla* – 9%	1.03c	0.19c	0.26a	0.06c	5.92b	9.53bc
Composted *Azolla* – 1%	1.34c	0.18c	0.19a	0.05c	5.24b	11.54bc
Composted *Azolla* – 2%	3.44b	0.40b	0.37a	0.07bc	8.27ab	13.47b
Composted *Azolla* – 3%	4.92a	0.57a	0.45a	0.10b	6.49b	20.05a
F test significance	***	***	ns	***	*	***
Planting season (PS)						
1	2.78a	0.34a	0.26a	0.07a	6.58a	13.86a
2	1.94b	0.27a	0.54a	0.08a	6.46a	11.63b
F test significance	**	ns	ns	ns	ns	*
SA × PS	***	***	ns	***	ns	***

[y] 150 kg/ha 15:15:15 NPK fertilizer (0.7 g/polybag)

[z] For each factor, means within a column followed by the same letter are not significantly different by LSD at P≤0.05.

*, **, *** Significant at P≤0.05, P≤0.01, P≤0.001, respectively.

[ns] Non-significant at P>0.05

Although the root length of choy sum treated with *Azolla* treatments did not significantly differ from the control, a slight increase can be observed in the composted *Azolla* treatments compared to the fresh *Azolla* treatments and control. The organic soil C contributed by composted *Azolla* enhanced the soil aeration and porosity by loosening the soil, making it easier for roots to penetrate and absorb more nutrients [15]. Principally, the organic matter applied will attach with the clay particles, building up the soil micro- and macro-aggregates [14]. Besides, *Azolla* application led to increased soil microbiological activities such as mineralization by microbes [7,9].

The choy sum growth performance was better in the first planting season than the second planting season. This can be explained by the uptake of nutrients by choy sum in the first planting season. Hence, the lower available nutrient in the soil can be uptake by choy sum, as the *Azolla* treatments were only applied at the beginning of the experiment.

CONCLUSION

Azolla plays a role in heightening the good side of clay soil. The results showed that incorporating composted *Azolla* – 3% has similar results on the growth performance of Choy sum with the NPK fertilizer treatment. The choy sum's growth performance also was observed to increase in composted *Azolla* – 3% than the control and fresh *Azolla* treatments. Therefore, a bigger scale study incorporating *Azolla* as soil amendment should be conducted to strengthen the results obtained in this study.

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REFERENCES

1. da Costa, P. B., Beneduzi, A., de Souza, R., Schoenfeld, R., Vargas, L. K., & Passaglia, L. M. P. (2013). The Effects of Different Fertilization Conditions on Bacterial Plant Growth Promoting Traits: Guidelines for Directed Bacterial Prospection and Testing. *Plant and Soil*, *368*(1–2), 267–280. https://doi.org/10.1007/s11104-012-1513-z
2. Anda, M., Shamshuddin, J., Fauziah, C. I., & Omar, S. R. S. (2008). Mineralogy and factors controlling charge development of three oxisols developed from different parent materials. *Geoderma*, *143*(1–2), 153–167. https://doi.org/10.1016/j.geoderma.2007.10.024
3. Kollah, B., Patra, A. K., & Mohanty, S. R. (2016). Aquatic *microphylla Azolla*: A perspective paradigm for sustainable agriculture, environment and global climate change. *Environmental Science and Pollution Research*, *23*(5), 4358–4369. https://doi.org/10.1007/s11356-015-5857-9

4. Lumpkin, T. A., & Plucknett, D. L. (1980). *Azolla*: Botany, physiology and use as a green manure. *Economic Botany*, *34*(2), 111–153. https://doi.org/10.1007/BF02858627
5. Watanabe, I., Ventura, W., & Mascarifia, G. B. (1992). Mineralization of *Azolla* N and its availability to wetland rice. *Soil Science and Plant Nutrition*, *38*(3), 505–516. https://doi.org/10.1080/00380768.1992.10415082
6. Cissé, M., & Vlek, P. L. G. (2003). Influence of urea on biological N_2 fixation and N transfer from *Azolla* intercropped with rice. *Plant and Soil*, *250*, 105–112. https://doi.org/10.1023/A:1022830423154
7. Widiastuti, D. P., Davis, J. G., & Gafur, S. (2018). *Azolla* fertilizer as an alternative n source for red spinach production on alluvial and peat soils in West Kalimantan, Indonesia. *International Nitrogen Initiative Conference*, 1–4. Melbourne, Australia. Retrieved from https://www.researchgate.net/publication/328899361_Azolla_fertilizer_as_an_alternative_N_source_for_red_spinach_production_on_alluvial_and_peat_soils_in_West_Kalimantan_Indonesia
8. Bhuvaneshwari, K., & Kumar, A. (2013). Agronomic potential of the association azolla – anabaena. *Science Research Reporter*, *3*(1), 78–82. Retrieved from https://www.researchgate.net/publication/305298251_Agronomic_potential_of_the_association_Azolla_-Anabaena
9. Pinero-Rodríguez, M. J., Fernández-Zamudio, R., Arribas, R., Gomez-Mestre, I., & Díaz-Paniagua, C. (2021). The invasive aquatic fern *Azolla filiculoides* negatively impacts water quality, aquatic vegetation and amphibian larvae in Mediterranean environments. *Biological Invasions*, *23*(3), 755–769. https://doi.org/10.1007/s10530-020-02402-6
10. Chatterjee, A., Sharma, P., Ghosh, M. K., Mandal, M., & Roy, P. K. (2013). Utilization of *Azolla microphylla* as feed supplement for crossbred cattle. *International Journal of Agriculture and Food Science Technology*, *4*(3), 207–214. Retrieved from https://www.ripublication.com/ijafst_spl/ijafstv4n3spl_06.pdf
11. Jumadi, O., Hiola, S. F., Hala, Y., Norton, J., & Inubushi, K. (2014). Influence of *Azolla* (*Azolla microphylla* Kaulf.) compost on biogenic gas production, inorganic nitrogen and growth of upland kangkong (*Ipomoea aquatica* Forsk.) in a silt loam soil. *Soil Science and Plant Nutrition*, *60*(5), 722–730. https://doi.org/10.1080/00380768.2014.942879
12. Bharali, A., Baruah, K. K., Bhattacharyya, P., & Gorh, D. (2017). Integrated nutrient management in wheat grown in a Northeast India soil: Impacts on soil organic carbon fractions in relation to grain yield. *Soil and Tillage Research*, *168*, 81–91. https://doi.org/10.1016/j.still.2016.12.001
13. Yoshida, S. (2009). Mineral Nutrition of Rice. In *Fundamentals of Rice Crop Science* (pp. 111–176). International Rice Research Institute (IRRI), Manila, Philiipines.
14. Razavipour, T., Moghaddam, S. S., Doaei, S., Noorhosseini, S. A., & Damalas, C. A. (2018). *Azolla* (*Azolla filiculoides*) compost improves grain yield of rice (*Oryza sativa* L.) under different irrigation regimes. *Agricultural Water Management*, *209*, 1–10. https://doi.org/10.1016/j.agwat.2018.05.020
15. Lestari, S., Mutryarny, E., & Susi, N. (2019). *Azolla mycrophylla* fertilizer for sustainable agriculture: compost and liquid fertilizer applications. *International Journal of Scientific and Technology Research*, *8*(7), 542–547. Retrieved from http://www.ijstr.org/final-print/july2019/Azolla-Mycrophylla-Fertilizer-For-Sustainable-Agriculture-Compost-And-Liquid-Fertilizer-Applications.pdf

Effect of Biopriming with Food Waste Bokashi Leachate on *Basella rubra* L. Seed Germination and Root Growth Performance

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ABSTRACT

Basella rubra L. is a type of spinach, which is edible with high nutrient composition. It is also known to be an antioxidant. However, initial germination and root growth remain an issue due to hard exterior seed coating. Thus, some may germinate within 10-21 days, and some may not work at all. Inhibited growth may lead to vegetative propagation and micropropagation, which fundamentally reduce the growth and yield. *Basella* seed treated with Bokashi leachate was found to improve seed germination and root growth. Thus, a study used food waste EM Bokashi leachate (0%, 0.067%, 0.1%, 0.2%) with priming duration (6 and 12 hours). The experiment was conducted in a Completely Randomized Design (CRD) with 3 replications. Based on the study results, seed germination and root growth significantly hasten the mean germination rate and root projection area until day 7 of germination by priming duration and Bokashi leachate concentration. However, germination percentage had no significant improvement by leachate. Long priming duration significantly reduced the root development due to the seed may loss of desiccation tolerance. The concentration of leachate and priming duration had no significant interaction. To improve the germination and root growth performance, 6 hours of seeds priming duration or 0.2% (1:500) of food waste Bokashi leachate was recommended to soak the *Basella rubra* seeds.

Keywords: Malabar spinach; organic farming; seed dormancy; seed soaking

INTRODUCTION

Basella spp. can be propagated by seeds or 20cm cuttings with the method of direct or transplanting [1]. The best germination temperature is between the range of 18-24 ℃ [2], and the days to emergence are 14 to 21 [3]. Due to the hard seed coat, germination under *in vitro* or *ex vitro* is a bottleneck [4]. Hence, physically and chemically, pre-treatment is used to improve the germination process. Physically, the seed is scarified by using a sharp or sandpaper and cut through the hard seed.

Basella rubra L. (Basellaceae) seed will propagate *in vitro* and improve to 70% with 2% of urea [4]. The biopriming of sunflowers has significantly improved seed germination and growth performance [5]. In tomatoes, seed priming with Bokashi leachate increased (13%) stem diameter of transplants, allowing plant nutrients uptake [6]. The objective of this study was to determine the effect of food waste EM Bokashi leachate on *Basella rubra* L. seed germination and root development.

MATERIALS AND METHODS

Study Site and Experimental Design

The experiment was carried out at University Putra Malaysia (UPM). The experiment was conducted at a Completely Randomized Design (CRD) with 3 replications of 100 seeds. There were 4 levels of concentration of Bokashi leachate which are 0%, 0.067% (1:1500), 0.1% (1:1000) and 0.2% (1:500) with 2 levels of priming duration (6 and 12 hours). Then, seeds were collected, rinsed with plain water and dried prior to germination. Seeds were rolled in a kitchen towel with a food wrapper and supported with 1 cm height of tap water. The water level was maintained at 1 cm and refilled daily. In total, 24 experimental units.

Bokashi Leachate Preparation

The Bokashi preparation method was modified based on [7]. One (1) part (20 mL) of EM·1 and molasses were dissolved in 45 parts (900 mL) water, prepared as a mixture. Subsequently, one (1) part of the mixture was then mixed with two (2) parts (1.8 kg) of rice bran and kept in a garbage bin (50 × 45 cm) and covered with a black garbage bag for two weeks; and finally, sun-dried. Bokashi bucket was self-made using two (2) garbage bins: top bin with 26 ø2 mm holes and bottom bin with a tap. Cropped 2 cm collected food waste consisted of raw and cooked plant and animal-based roughly in the ratio of 3:2. The waste was arranged with 1cm of Bokashi bran and 5 cm of small pieces of food waste. Each layer was compacted and covered tightly. The leachate was harvested on day 4 of fermentation.

Seed Germination Test

Daily counting of seedlings on germination test was conducted until day 7. The data were subjected to R-program statistical software under the package “germination metrics” for the germination traits [8].

Root Morphology

Fresh roots at day 7 were quantified using Root Scanner (Epson Expression 1680) with root scanning analysis software, version Win-Rhizo 2007d.

Statistical Analysis

Data recorded were subjected to two-way Analysis of Variance (ANOVA) using R statistical software. When F values were significant at the $p<0.05$ level, treatment means were compared and separated using Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

No Significant Interaction Effect Between Priming Duration and Concentration on The Seed Germination and Root Growth Performance

Based on the results, leachate concentration and priming duration had no significant interaction ($p>0.05$) on all the parameters. Similarly, biopriming of *Brassica rapa* had no significant interaction between concentration and priming duration [9]. There is no significant effect on seed priming duration and salinity level on seed germination percentage and speed [10].

Short Priming Duration Improved Seed Germination Performance

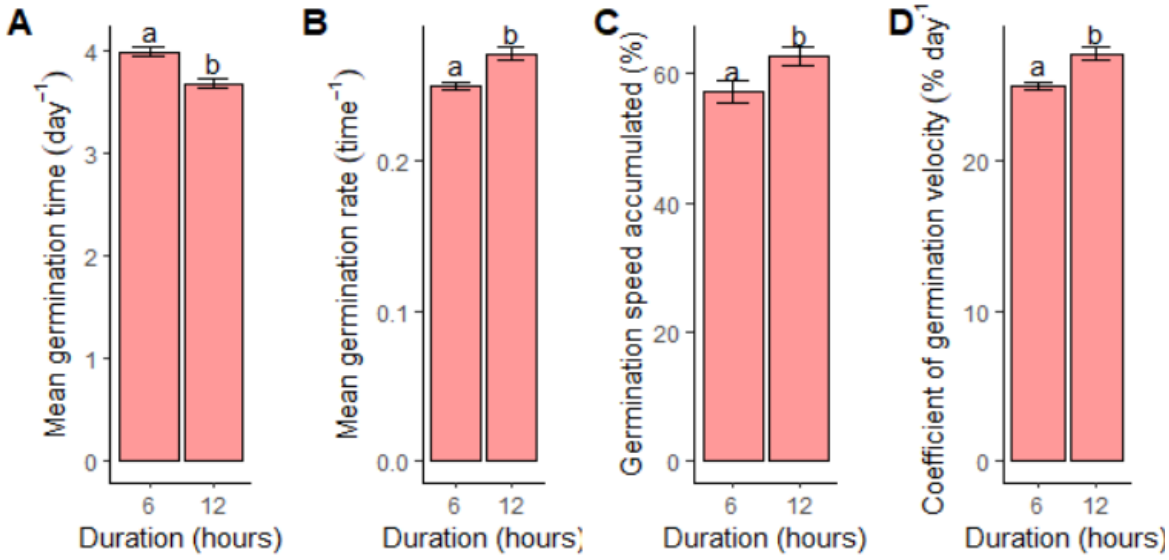

Figure 1 The effect of priming duration of the seedlings. Means ± standard error with different letters is significantly different at $P<0.05$ using DMRT.

The concentration of Bokashi leachate to soak seeds had no significant effect on the germination percentage. The germination percentage of bitter ground is significantly reduced in a longer priming duration (16 hours) (Saleem et al., 2016). The 12 hours seed priming duration has significantly enhanced the mean germination time. However, 6 hours of seed priming duration significantly improved the mean germination rate ($time^{-1}$), germination speed accumulated (%) and coefficient of the velocity of germination (% day^{-1}) (Figure 1). The coefficient of the

germination velocity in 12 hours was significantly higher than 6 hours priming duration.

Seed Germination Performance Not Affected by Bokashi Leachate Concentration

No significant difference in Bokashi leachate concentration was shown in the seed germination performance. In contrast, different concentrations of chitosan seed priming significantly affect the germination index and mean germination time but germination percentage [11].

Long Biopriming Duration Deleterious the Initial Root Growth Performance

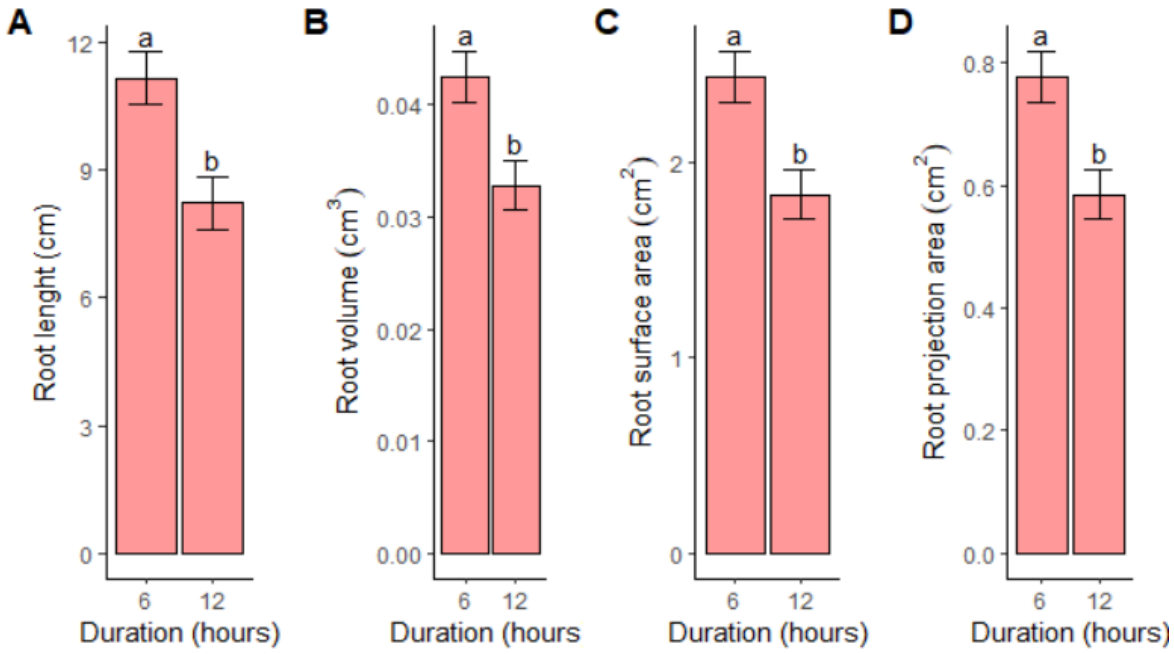

Figure 2 The effect of priming duration (hours) on seedlings.

Seed priming within 6 hours showed a significant improvement in root length, root projection area, root volume and root surface area compared to others during the early growth (Figure 2). long priming duration (12 hours) negatively affected the root growth performance and mean germination time. Comparably, 12 hours priming duration of pistachio reduced the root dry weight [12]. The seed may cause loss of desiccation tolerance in long priming duration [13].

High Concentration Enhanced Initial Root Growth Performance

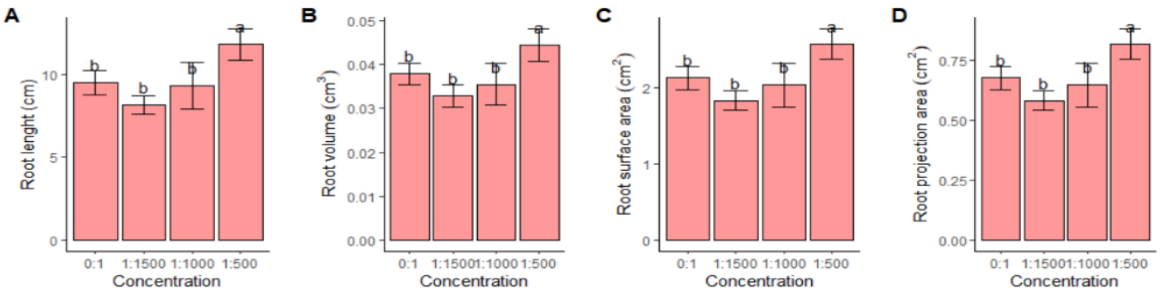

Figure 3 The effect of concentration of Bokashi leachate (%) on seedlings.

Root growth characteristics, including taproot and lateral root in the early phase of plant growth, were improved by seed priming [14]. Seed priming with 0.2% of Bokashi leachate showed a significant improvement in root length, root projection area, root volume and root surface area compared to others during the early growth (Figure 3).

CONCLUSION

Long biopriming duration (12 hours) significantly affected root growth development. Based on the results, treatment of 6 hours of seeds biopriming duration or 0.2% (1:500) of food waste Bokashi leachate was recommended to prime the *Basella rubra* seeds to enhance the seed germination and root growth performance in the early stage. The Bokashi leachate concentration could be increased to obtain more superior germination and root growth performance for future study.

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REFERENCES

1. Lin et al. (2009). Discovering indigenous treasures: promising indigenous vegetables from around the world. *AVRDC - The World Vegetable Center*, 319. Link: https://worldveg.tind.io/record/36750
2. A. Chaurasiya, R. Kumar Pal, P. Kumar Verma, A. Katiyar, N. Kumar, and C. Author. (2021). An updated review on Malabar spinach (*Basella alba* and *Basella rubra*) and their importance. *Journal of Pharmacognosy and Phytochemistry*, 10(2). doi: 10.22271/phyto.2021.v10.i2p.13974
3. Cornell University. (2006). Explore Cornell - Home Gardening - Flower Growing Guides Growing Guide. Link: http://www.gardening.cornell.edu/
4. S. S. Kumar & P. Giridhar (2021). *In vitro* micropropagation of *Basella rubra* L. through proliferation of axillary shoots. *Plant Cell, Tissue and Organ Culture*, 144 (2), (477–483)

Feb. doi: 10.1007/s11240-020-01960-w

5. A. Moeinzadeh, F. Sharif-Zadeh, M. Ahmadzadeh, & F. H. Tajabadi. (2010). Biopriming of sunflower (*Helianthus annuus* L.) seed with *Pseudomonas fluorescens* for improvement of seed invigoration and seedling growth. *Australian Journal of Crop Science*, 4(7), 564–570), 2010. Doi: http://www.cropj.com/moeinzadeh_4_7_2010_564_570.pdf
6. M. Olle. (2020). Short communication: The improvement of the growth of tomato transplants by bokashi tea. *Agraarteadus*, 31(1), 70–73. doi: 10.15159/jas.20.1
7. D. M. Christel. (2017). The Use of Bokashi as A Soil Fertility Amendment in Organic Spinach Cultivation. Link: https://scholarworks.uvm.edu/graddis/678
8. J. Aravind, S. Vimala Devi, J. Radhamani, S. R. Jacob, & K. Srinivasan. (2019) germination metrics: Seed Germination Indices and Curve Fitting. Link: https://aravind-j.github.io/germinationmetrics/articles/Introduction.html
9. J. M. Chin, Y. Y. Lim, & A. S. Y. Ting. (2021). Biopolymers for biopriming of *Brassica rapa* seeds: A study on coating efficacy, bioagent viability and seed germination. *Journal of the Saudi Society of Agricultural Sciences*, 20(3), 198–207, Apr. 2021, doi: 10.1016/j.jssas.2021.01.006.
10. N. M. Seyed & E.-M. Mahjoobeh. (2016). Effect of hydro-priming duration on germination and early seedling growth of rapeseed under salinity stress. *African Journal of Agricultural Research*, 11(43) 4395–4400. doi: 10.5897/ajar2016.11269
11. Y. J. Guan, J. Hu, X. J. Wang, & C. X. Shao. (2009). Seed priming with chitosan improves maize germination and seedling growth in relation to physiological changes under low temperature stress. *Journal of Zhejiang University: Science B*, 10(6), 427–433. doi: 10.1631/jzus.B0820373.
12. A. Esmaeilpour & P. van Damme. (2016). Evaluation of seed soaking times on germination percentage, germination rate and growth characteristics of pistachio seedlings. *in Acta Horticulturae*, 1109, 107–112. doi: 10.17660/ActaHortic.2016.1109.17.
13. S. Paparella, S. S. Araújo, G. Rossi, M. Wijayasinghe, D. Carbonera, & A. Balestrazzi. (2015). Seed priming: state of the art and new perspectives. *Plant Cell Reports*, 34(8). Springer Verlag, 1281–1293. doi: 10.1007/s00299-015-1784-y.
14. S. Blunk et al. (2019). Seed priming enhances early growth and improves area of soil exploration by roots. *Environmental and Experimental Botany*, 158, 1–11. doi: 10.1016/j.envexpbot.2018.11.003.

Phytochemical screening, Antioxidant activity, Total Phenolic Content and Total Flavonoid Content of Ethanol Extract of *Melastoma malabathricum, Clidemia hirta, Chromolaena odorata* and *Ageratum conyzoides*

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ABSTRACT

The ethanol extract of the leaves of *Melastoma malabathricum, Clidemia hirta, Chromolaena odorata,* and *Ageratum conyzoides* were assessed for chemical constituents and possible potential for antioxidant activity. These plants were categorized under broad-leaved weeds that were widely spread in the open land area. These weeds came from two different families: Melastomataceae for *M. malabathricum* and *C. hirta* and Asteraceae for *C. odorata* and *A. conyzoides*. Screening of the weeds was performed using standard methods and revealed various secondary metabolites such as saponins, terpenoids, phenols, tannins, and flavonoids. Antioxidant activity was validated by the DPPH radical scavenging assay of *M. malabathricum, C. hirta, C. odorata,* and *A. conyzoides* crude ethanol extract. Total phenolic content and total flavonoid content were determined by using the spectrophotometric method. Results obtained showed the weeds have good antioxidant activity with an IC50 value of *M. malabathricum* (192.01 µg/mL), *C. hirta* (161.37 µg/mL), *C. odorata* (505.12 µg/mL), and *A. conyzoides* (1068.59 µg/mL). The antioxidant, total phenolic content, and total flavonoid content of these weeds were positively correlated. The study indicates that these weeds possess a valuable phytochemicals compound, contain good antioxidants properties, total phenolic content, and total flavonoid content. These findings are of great importance because of the availability of the weeds for testing on bioactivities.

Keywords: Antioxidant; *Melastoma malabathricum; Clidemia hirta; Chromolaena odorata; Ageratum conyzoides.*

INTRODUCTION

Weeds are recognized as the unwanted and undesirable plant that grows at an open land area [1]. This study used broadleaves weeds from two different families: Melastomataceae for *M. malabathricum* and *C. hirta* and Asteraceae for *C. odorata* and *A. conyzoides*. Phytochemicals found in plants in plant chemicals have protective and preventive properties against diseases [2]. Bioactive chemicals play an important role in plant's protection, such as antibacterial, antiviral, antifungal, and insecticidal agents [3]. These weeds have potential and can benefit humans with their good antioxidant and positive relation with total phenolic content and total flavonoid content. These weeds were selected to study their phytochemical constituents which can prove their contribution to several bioactivities due to the antioxidant properties, and also their correlation with total phenolic and total flavonoid content. Thus, the present study aims to screen the phytochemicals present in the leaves of *M. malabathricum*, *C. hirta*, *C. odorata* and *A. conyzoides* and to determine the antioxidant activity, total phenolic, and total flavonoid content.

MATERIALS AND METHODS

Preparation of Extracts

Melastoma malabathricum, C. hirta, C. odorata and *A. conyzoides* leaves were collected, and all of them were washed under running tap water, dried under the shaded area, then were dried at 50 °C for 24 hours using the oven. The dried samples were prepared into powder using a mechanical blender and grinder. One hundred grams of powders of selected weed leaves were soaked in 500 mL of 95% ethanol at room temperature (27±1 °C) from day 1 to day 7. The extracts were then filtrated through Whatman No. 1 filter paper before evaporating the residual solvent using a rotary evaporator. The crude extracts were kept at 4 °C in a chiller until used.

Phytochemical Screening

Phytochemical screening of selected weeds leaves was tested with modification for the presence of saponins, terpenoid, phenolic, tannins, and flavonoid. The qualitative results were expressed as (+) for the presence and (-) for the absence of phytochemicals.

Determination of Antioxidant Activity

The antioxidant activity was determined by its DPPH radical scavenging activity. Ascorbic acid and Butylated hydroxyanisole (BHA) were used as standard controls [4]. The percentage inhibition was calculated using the equation. IC_{50} values denote the concentration of the sample which is needed to scavenge 50% of DPPH free

radicals [5]. DPPH radical scavenging activity (%) was calculated using the following equation:

DPPH radical scavenging activity (%) =

[(Absorbance of control - Absorbance of the test sample) / (Absorbance of control)] × 100

Determination of Total Phenolic Content

Total phenolic content was measured using Folin Ciocalteu's method with absorbance measured at 750 nm UV-Visible spectrophotometer instrument. The extracts were done in triplicates. The blank was performed using a reagent blank of distilled water. The standard for this method was using Gallic acid, and the standard gallic acid was plotted as the calibration curve. The expression of total phenolic contents data was in mg of gallic acid equivalent weight (GAE)/ g of dry mass [6]. Total phenolic contents of the extracts were expressed in (GAE)/g extract, using the regression curve equation.

Determination of Total Flavonoid Content

The Aluminum chloride method was used for flavonoid determination [7]. In this method, the Quercetin was used as a standard and the flavonoid contents were expressed as quercetin equivalent. Standard quercetin was plotted as the calibration curve. The absorbance was noted at 510 nm using a UV-Visible spectrophotometer. Total flavonoid contents of the extracts were expressed in mg Quercetin equivalents (QE)/g extract using a regression curve equation.

RESULTS AND DISCUSSION

Phytochemical Screening

Saponins, terpenoids, phenols, tannins, and flavonoids were present in the samples. The chemical compounds present in plants are the biologically active constituents. These biologically active compounds play a major role in various properties of plants like antioxidant, antimicrobial, anti-cancer, and insecticidal activity [8].

Table 1 Qualitative phytochemical screening.

	M. malabatrichum	***C. odorata***	***A. conyzoides***	***C. hirta***
Saponins	+++	+	+	+++
Terpenoids	+++	+++	+	-
Phenols	+++	+++	+++	+++
Tannins	+++	+++	+++	+++
Flavonoids	+++	++	++	+++

Antioxidant Activity

The scavenging effect of *M. malabathricum, C. hirta, C. odorata* and *A. conyzoides* with Ascorbic acid and BHA were compared. The weeds extract at the used concentrations showed the potential effect of DPPH activity as a percentage of free radicals' inhibition. The measured IC50 result indicates better antioxidant activity. Therefore, the lower IC50 suggests that it has a higher antioxidant capacity.

Table 2 IC_{50} Value (µg/mL).

	IC_{50} (µg/mL)
Ascorbic acid	155.85
BHA	174.1
M. malabatrichum	192.01
C. hirta	161.37
C. odorata	505.12
A. conyzoides	1068.59

Total Phenolic and Flavonoid Contents

Total phenolic and flavonoid contents of these weeds were compared. There was a positive linear correlation between antioxidant activity, total phenolic content, and total flavonoid content for *M. malabathricum, C. odorata* and *A. conyzoides*. These results suggested that the phenolic compounds and flavonoids compounds contributed significantly to the antioxidant capacity of the investigated weed species.

Table 3 Total phenolic and flavonoid contents.

Plant extracts	**Total phenolics (mg gallic acid equivalent/g dried extract)**	**Total flavonoid (mg quercetin equivalent/g dried extract)**
M. malabatrichum	343.9	442.64
C. hirta	524.99	507.92
C. odorata	79.57	462.08
A. conyzoides	201.89	644.72

CONCLUSION

Weeds are plants that are usually neglected for studies as they are considered unimportant plants. The presence of secondary metabolites in such weeds can be useful as the weeds can be exploited and used for several beneficial purposes. Qualitative phytochemical screening, IC_{50} value for antioxidant activity, total phenolic and flavonoid contents can contribute to several bioactivities such as insecticidal activity and antimicrobial in the future findings.

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REFERENCES

1. Zimdahl, R. L. (2007). Weeds–the beginning. In 'Fundamentals of weed science'.
2. Breslin, A. (2017). The chemical composition of green plants. *Sciencing, Leaf Group Ltd*, *76*.
3. Hajlaoui, H., Trabelsi, N., Noumi, E., Snoussi, M., Fallah, H., Ksouri, R. and Bakhrouf, A. (2009). Biological activities of the essential oils and methanol extract of two cultivated mint species (*Mentha longifolia* and *Mentha pulegium*) used in the Tunisian folkloric medicine. *World Journal of Microbiology and Biotechnology*, 25(12), 2227-2238.
4. Dehpour, A. A., Ebrahimzadeh, M. A., Fazel, N. S. and Mohammad, N. S. (2009). Antioxidant activity of the methanol extract of *Ferula assafoetida* and its essential oil composition. *Grasasy aceites*, 60(4), 405-412.
5. Shekhar, T. C. and Anju, G. (2014). Antioxidant activity by DPPH radical scavenging method of *Ageratum conyzoides* Linn. leaves. *American Journal of Ethnomedicine*, 1(4), 244-249.
6. Bhalodia, N. R., Acharya, R. N., & Shukla, V. J. (2011). Evaluation of in vitro Antioxidant Activity of hydroalcoholic seed extratcs of *Cassia fistula* linn. *Free Radicals and Antioxidants*, *1*(1), 68-76.
7. Olajire, A. A., & Azeez, L. (2011). Total antioxidant activity, phenolic, flavonoid and ascorbic acid contents of Nigerian vegetables. *African Journal of Food Science and Technology*, *2*(2), 22-29.
8. Shreshtha, S., Anushi, J., Joshi, A. N., Joshi, N., & Anupma, H. (2017). Study of total phenol, flavonoid contents and phytochemical screening of methanolic crude extracts of two weed plants. *Annals of Plant Sciences*, *6*(6), 1645-1648.

Development of Corn Starch Biofilm Reinforced with Cellulose Nanofiber Isolated from Banana Pseudostem

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ABSTRACT

Starch-based biofilms have the disadvantages of hydrophilicity, poor mechanical properties, low water vapour barrier property and low freeze stability during biofilms production. Therefore, this research investigates the application of cellulose nanofibers (CNF) as the reinforcing agents in the biofilms composite. CNF were isolated from banana pseudostem waste via alkaline treatment followed by acid-chlorite treatment and acid hydrolysis. Biofilms were then developed from a mixed suspension of corn starch, glycerol and extracted CNF using the casting process. Starch films without CNF acted as a control. The biofilms were then characterized by gravimetric analysis and digital thickness gauge meter. Based on the results obtained, the developed starch reinforced with CNF biofilms possesses a 1.67% lower moisture content, greater thickness and 5.91% higher water absorption abilities than the control starch-based film. In general, chemical treatments on banana pseudostem waste can produce CNF, and it is potentially applicable to be used as reinforcing agents in developing biofilms.

Keywords: Banana pseudostem; cellulose nanofibers; starch-based films; biofilm; agricultural waste

INTRODUCTION

Banana pseudostem is responsible for supplying and carrying nutrients from the soil to fruit in a banana plant. After the banana fruit has been matured and processed, this pseudostem will generally go into waste biomass because the plant will become obsolete for the next harvest [1]. Besides, after each ton of banana fruit is harvested, 10% needs to be rejected and generated approximately 4 tons of biomass waste. Every 60 kg of banana harvested produces 200 kg of discarded waste stems [2]. The biomass waste is coming from the root, pseudo-stem, rotten fruit, peel, fruit-

stem, and rhizome. One hectare of banana farms will generate 220 tons of biomass waste [3]. The remainder is considered as waste and needs to manage accordingly.

Cellulose is an abundant polymer in nature and occurs in various plants & living organisms -environmentally friendly, cheap & biodegradable [4]. The use of cellulose nanofibers as support components in matrices increases thermo-mechanical properties limits the exposure of polymers to water and retains biodegradability. In general, mixing cellulose nanofibers with polysaccharides (such as starch) enhances the properties [5].

This study aims to obtain nano size of fiber from banana trunk through which includes the extraction of CNF from banana pseudostem. Second, to study the morphology of the banana pseudostem cellulose nanofiber (CNF). Finally, the objective is to study banana pseudostem cellulose nanofiber films (BCNF). This research aims to investigate the application of cellulose nanofibers (CNF) as the reinforcing agents in the biofilms composite

MATERIALS AND METHODS

Materials

Banana stem fibres were obtained by scraping banana pseudo-stems, which was collected from banana orchard, Universiti Putra Malaysia Bintulu Sarawak Campus. Analytical grade sodium hydroxide, sodium chloride, acetic acid, sulphuric acid, and glycerine were purchased from Jayachem, Sarawak. Corn starched was purchased from a local vendor.

Nanocellulose Extraction

Banana trunk has been further dried in the force convection oven at 60˚C for 2 days. Banana trunk was grinded using household blender for 30 second with pulse in every ten second. The grinded banana trunk was sieved using laboratory test sieve ASTM E11.

20 grams of fiber with size <180 µm was was immersed with hot distilled water for 12 hours. Next, the fiber was rinsed with water, and the water was removed by filtration step using cotton mesh (soy filter mesh). It was further dried in the oven for a day at a temperature 60 °C. The fibers was later treated with a solution of 200 ml of 2.5 mol L-1 sodium hydroxide and was sterilized by autoclaving at 121 °C for 15 min. Sodium hydroxide solution was removed from fiber by rinsing it with water by centrifugation for 15 min at 10,000 r.p.m.

Next, the fibre went to a bleaching stage. This step was done by soaking 200 mL of sodium chlorite in $NaClO_2$ solution under acidic conditions (pH 4–5) at 70 °C.

Bleaching agent residue was removed from fiber by centrifuge. and the fibre was rinsed with distilled water properly. This procedure was repeated (5-7 times) until the pH of the treated fibre was 7.0. [6]. The treated fiber was dried at 37 °C and stored before the acid hydrolysis process begun. Firstly, H_2SO_4 solution was added with 10 g of treated cellulose fibre. The mixture was again suspended in water with high agitation. It was then put in dialysis tube (Supelco) and submerged in water for 7 days to washed out the residual acid. The cellulose nanofiber was stored in the refrigerator until further use.

Developments of Biofilms

Biofilms with CNF (BCNF) were prepared by dissolving corn starch and CNF in 100 mL of distilled water with stirring. After the solution was completely dissolved, glycerin was added as plasticizer and the mixture was heated slowly to a mild boiling. Five milliliters of the film mixture was pipetted into petri dishes (100 mm diameter by 15 mm depth). The petri dishes were placed for 24 hours in an oven (Memmert) set at 60 °C. The method was repeat for the preparation of control biofilms (BC) without the additional of CNF.

Gravimetric analysis of biofilm

Physical characterization of the prepared biofilms was done by conducting few analyses like the thickness, moisture content, the swelling studies, and density the films. The results are shown as mean ± standard deviation.

RESULTS AND DISCUSSION

The initial alkaline and bleaching treatments aimed to remove lignin from the middle lamellae and separate the cellulose fibers [7]. Along these first steps of the chemical treatment used to isolate CNFs from the banana pseudostem (Figure 1), the material changed from dark brown to pale brown. The image of CNF is shown in Figure 1 below.

Figure 1 Cellulose nanofibers.

A significant change in physical appearance caused by CNF films was observed on the samples, as shown in Figure 2.

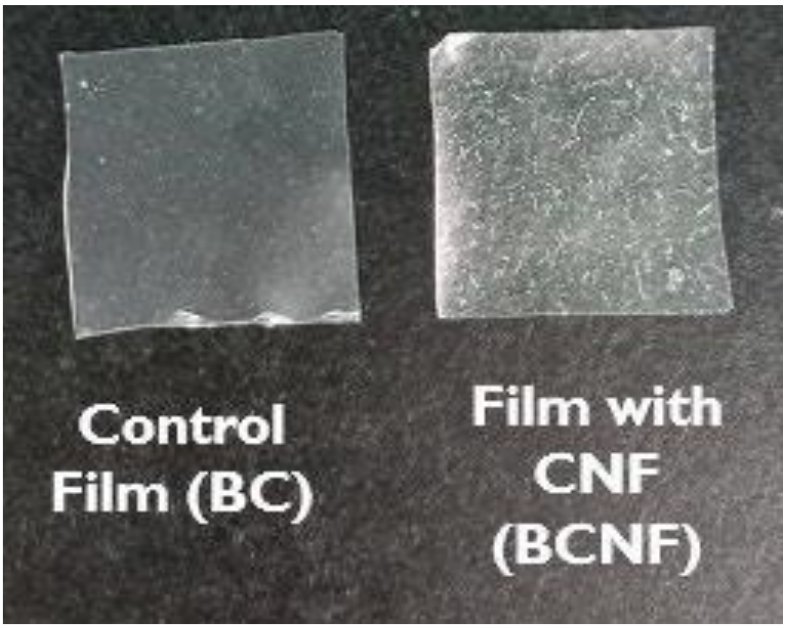

Figure 2 (Left) Control film; (Right) Film with CNF.

Table 1 summarized the physical characterization analyses of both films. The difference in thickness value achieved for the films with the availability of the cellulose nanofibers in the BCNF films. Addition of cellulose nanofibers elicited a statistically significant difference in the density value of the prepared films, with BC showing higher density value than the BCNF. The incorporation of cellulose nanofibers to the BCNF films has affected the density.

Lastly, incorporation of cellulose nanofibers also increased the moisture content and the swelling abilities of the BCNF biofilm as cellulose nanofibers tend to be hydrophilic due to presence of multiple hydroxyl groups which enables it to interact with water molecules [8].

Table 1 Results of gravimetric analysis of biofilms.

Sample	Thickness (μm)	Density (g/cm^3)	Moisture content (%)	Swelling (%)
BC	49±1	2.45±0.07	17.7±0.2	52.8±0.6
BCNF	195±2	0.72±0.05	21.1±0.4	53.7±0.9

CONCLUSION

The biofilms from the CNF of banana pseudostem were successfully formed. Film reinforced with CNF present higher moisture content and swelling abilities as the cellulose are hydrophilic. Besides, starch and cellulose nanofibers isolated from

banana pseudostem have high potential reinforcing materials in biodegradable films, respectively. Lastly, further analysis should be performed to the biofilms produced in this work to confirm its applicability in food packaging industry.

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REFERENCES

1. Subagyo, A., & Chafidz, A. (2020). Banana Pseudo-Stem Fiber: Preparation, Characteristics, and Applications. In Banana Nutrition - Function and Processing Kinetics (pp. 1–19).
2. Jamaluddin, M.F., Jailani, S. and Zainol, N., 2011. Factorial Analysis on Acetic Acid Production Using Banana Stem Waste. International Journal of Chemical and Environmental Engineering, 2(4).
3. Subagyo, A., & Chafidz, A. (2020). Banana Pseudo-Stem Fiber: Preparation, Characteristics, and Applications. In Banana Nutrition - Function and Processing Kinetics (pp. 1–19).
4. Pelissari, F.M., Andrade-Mahecha, M.M., do Amaral Sobral, P.J. and Menegalli, F.C., 2017. Nanocomposites based on banana starch reinforced with cellulose nanofibers isolated from banana peels. Journal of Colloid and Interface Science, 505, pp.154-167.
5. Espinosa, E., Tarrés, Q., Domínguez-Robles, J., Delgado-Aguilar, M., Mutjé, P., & Rodríguez, A. (2018). Recycled fibers for fluting production: The role of lignocellulosic micro/nanofibers of banana leaves. Journal of Cleaner Production, 172, 233–238. https://doi.org/10.1016/j.jclepro.2017.10.174.
6. Fazeli, M., Keley, M., & Biazar, E. (2018). Preparation and characterization of starch-based composite films reinforced by cellulose nanofibers. International Journal of Biological Macromolecules, 116, 272–280. https://doi.org/10.1016/j.ijbiomac.2018.04.186.
7. Andrade-Mahecha, M. M., Pelissari, F. M., Tapia-Blacido, D. R., & Menegalli, F. C. (2015). Achira as a source of biodegradable materials: Isolation and characterization of nanofibers. Carbohydrate Polymers, 123, 406-415.
8. Srivastava, K.R., Dixit, S., Pal, D.B., Mishra, P.K., Srivastava, P., Srivastava, N., Hashem, A., Alqarawi, A.A. and Abd_Allah, E.F., 2021. Effect of nanocellulose on mechanical and barrier properties of PVA–banana pseudostemfibercomposite films. Environmental Technology & Innovation, 21, p.101312.

Detection and Resistance Profile of *Vibrio alginolyticus* Isolated from Farmed Asian Seabass (*Lates calcarifer*) in Terengganu, Malaysia

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ABSTRACT

Vibriosis is one of the diseases that infect Asian seabass, Lates calcarifer. Misuse of antibiotic in treating bacterial diseases cause the increasing of antibiotic resistance. Thus, the purposes of this study were: (1) to isolate and identify *Vibrio alginolyticus* in farmed Asian seabass, L. calcarifer in Terengganu; and (2) to determine antibiotic resistance pattern of *V. alginolyticus* isolates. Using aseptic technique, kidney and liver were inoculated onto thiosulphate citrate bile salt agar (TCBS) and CHROMagar Vibrio. Polymerase chain reaction (PCR) was done for further confirmation using primers targeting *pyrH* gene and specific *V. alginolyticus*. Then, antibiotic susceptibility test was done using 18 types of antibiotic discs namely ampicillin, cefotaxime, cefotetan, chloramphenicol, ciprofloxacin, erythromycin, cefepime, gentamicin, kanamycin, cephalothin, nalidixic acid, rifampicin, streptomycin, tetracycline, oxytetracycline trimethoprim/sulfamethoxazole, vancomycin and Vibriostat O129. Multidrug resistance (MDR) level also was calculated. As a result, 15 isolates were confirmed as *V. alginolyticus*. Most of the isolates were at least resistance to more than three antimicrobials, with vancomycin (80%) followed by ampicillin (73%). MDR was ranging from 0.05 to 1, with 9 difference of antibiotic resistance patterns. 66% of the isolates showed the MDR value of higher than 0.2 which means the isolate were continuously exposed to antibiotics. This study demonstrates the distribution of multidrug resistance strains that could be of concern to the seabass farmers in Terengganu, Malaysia. In addition, data from this study can be further used in fish disease management plan.

Keywords: Vibriosis; *pyrH* gene; multidrug resistance; antibiotic resistance patterns

INTRODUCTION

Vibrio alginolyticus is a Gram negative, positive for sucrose fermentation, salt tolerance, able to grow in different temperature and has a large geographic distribution especially in bathing area [1]. It is considered as a major problem in aquaculture and there are reports of multiple antibiotic resistance in *V. alginolyticus* in Malaysia particularly in fish like grouper [2], oyster [3], catfish [4] and farmer seahorse [5]. It also related with raw shellfish consumption and contributing to human disease infections like a severe gastrointestinal and extraintestinal [3]. The application of antibiotic was used to decrease and control the Vibrio infection. Antibiotics are convenience, affordable, and high efficiency. However, abusive and excessive of antibiotic led to emergence of multi drug resistances and has emerge of 'super resistant bugs' [6]. Prolonged exposure or treatment might cause high toxicity to the fish and antibiotics accumulation in fish muscle [4]. The purpose of this study was to isolate and identify *Vibrio alginolyticus* in farmed L. calcarifer, Asian seabass in Terengganu, Malaysia. In addition, the antibiotic susceptibility test was conducted to determine multidrug resistance and resistance patterns of *V. alginolyticus* isolates.

MATERIALS AND METHODS

Fish Sampling

In November 2020, a total of ninety Asian seabass samples with average weight of 500 g were collected from three different fish farm in Terengganu namely Kampung Fikri, Kuala Setiu (5°38'37.4"N 102°45'14.6"E); Kg. Kuala Ibai, Kuala Terengganu (5°16'32.5"N 103°09'45.3"E) and Kg. Beris Chawat, Sungai Besut (5°48'51.5"N 102°33'24.1"E). The fish were farmed in net caged of freshwater water. All the fish samples were transported in an ice box to the Aquatic Animal Health laboratory, Faculty of Veterinary Medicine, UMK for further examination.

Isolation of *Vibrio* spp.

The fish were dissected in a sterile condition. Loopful of kidney and liver were streaked separately onto thiosulphate-citrate-bile salt-sucrose (TCBS, Oxoid, England) and CHROMagar Vibrio (CHROMagarTM, France) agar. All inoculated plates were incubated at 35 °C for 24 to 48 h. The potential isolates of *Vibrio spp.* were further identified using Gram staining, oxidase, and catalase test. All the bacteria were kept in TSB with 50% glycerol for a long-term storage.

DNA Extraction and Molecular Identification

The DNA template of isolated bacteria were extracted using boiling cell method [7] with slight modification. Polymerase Chain Reaction (PCR) assay was used in this

study. Primers *pyrH* gene detection and species specific of *V. alginolyticus* were used. The amplification of a 440bp DNA fragment of *pyrH* gene (F: 5'-GATCGTATGGCTCAAGAAG-3'; R: 5'-TAGGCATTTTGTGGTCACG-3') and 324bp of *V. alginolyticus* specific primer (F: 5'- TCCGTGGTGCAGGCCTTGCT; R: 5'-TCAACTTTCGTCGCTTTTAGT-3') (this study). The PCR mixture reaction (Qiagen, USA) with 10 µL final volume consisted of 5 µL of TopTaq Master Mix, 0.2 µl (10 pmol) of each primer, 1 µL of CoralLoad solution, 2.6 µL of nucleus free water and 1 µL of DNA template. The *pyrH* PCR amplification was done according to Amalina et al. (2019). PCR amplification of *V. alginolyticus* specific primer was as follow, initial denaturation at 95°C for 3 min; 30 cycles of 95°C for 30 s, annealing temperature 60°C for 30 s, 72°C of extension for 30 s, and final extension at 72°C for 10 min. The amplification was performed using thermocycler Bio-Rad C1000 Thermal Cycler (USA). Then, 5 µL of PCR product were added to agarose gel in 1.5%, stained with ethidium bromide, electrophoresed at 100 V for 42 min and visualized under UV light using Gel DocTM E2 Imager (BioRad, USA).

Antibiotic Susceptibility Test

The isolates of *V. alginolyticus* from molecular identification were proceed with antibiotic susceptibility test (AST). By using disc diffusion technique, the bacterial suspensions were spread onto Mueller-Hinton agar (MHA, Oxoid, UK) plates. After a minute, places the antibiotic disc onto agar and incubated at 35 °C for 18-24 h. The inhibition zone of agar was measured and recorded using according to the guideline from Clinical and Laboratory Standard Institute. The following antibiotics were used namely ampicillin (AMP, 10 µg), cefotaxime (CTX, 30 µg), cefotetan (CTT, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), erythromycin (E, 15 µg), cefepime (FEP, 30 µg), gentamicin (CN, 10 µg), kanamycin (K, 30 µg), cephalothin (KF, 30 µg), nalidixic acid (NA, 30 µg), rifampicin (RD, 5 µg), streptomycin (S, 10 µg), tetracycline (TE, 30 µg), oxytetracycline (OT, 30µg) trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 µg), vancomycin (VA, 30 µg) and Vibriostat O129 (O129, 10 µg).

The multiple antibiotic resistance (MAR) index of isolates was calculated as x/y, where x represents the number of antimicrobials to which a particular isolate is resistant, and y represents the number of antimicrobials to which the isolate is exposed [9].

RESULTS AND DISCUSSION

In this study, 180 isolates were presumptive of *Vibrio alginolyticus* on TCBS agar which form large and yellow (sucrose-fermenting) colonies while colourless colonies on CHROMagar™ Vibrio (table 1). Liver, kidney and spleen are organs used to isolate Vibrio infection [4]. This study reports the Vibrio infection specifically about

V. alginolyticus in farmed Asian seabass in Terengganu, Malaysia. The sampling period was conducted in pre-monsoon when water temperature was fluctuated. The high temperature hit may trigger an environmental stressor to suppress immune systems [10]. The increasing of mortalities and frequent outbreak of fish were correlated with a country's climate vulnerability [11].

This present study did not isolate any of *V. alginolyticus* from Kuala Setiu. While 3 and 12 isolates of *V. alginolyticus* from Kuala Ibai and Sungai Besut, respectively. This might be due to a shallow of estuaries ecosystems and may influence the infection of bacterial. Almeida et al. (2001) mentioned that bacterial abundance was two to three times lower at high tide than at low tide and two time higher in brackish than in marine water.

Using PCR, 37 out of 180 isolates were detected as *Vibrio* spp. using *pyrH* gene. Then, only 15 out of 37 isolates were further identified as *Vibrio alginolyticus* using specific species primer (Figure 2). Amalina, et al. (2019) stated that in identification of *Vibrio spp.*, *pyrH* gene provided a better resolution and more suitable for a rapid determination. Gram staining showed *V. alginolyticus* is rod-shaped and Gram-negative bacteria.

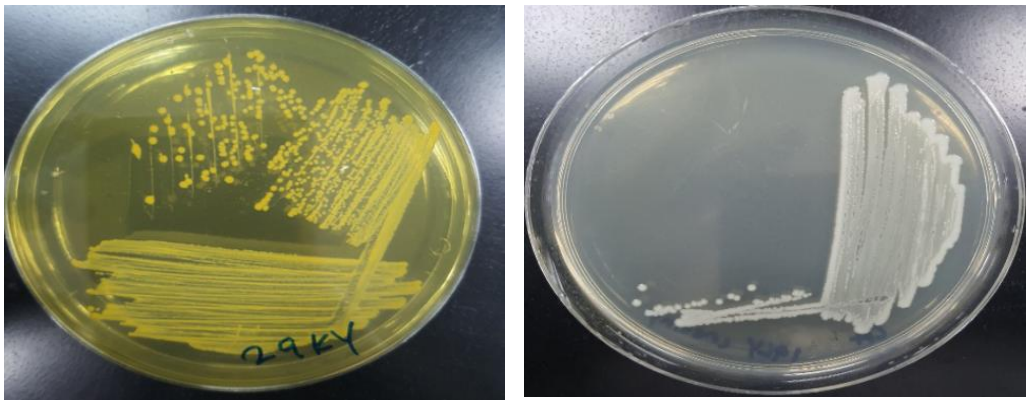

Figure 1 *Vibrio alginolyticus* isolates on TCBS agar (left) showed medium and yellow colonies, CHROMagar (right) showed the medium and white colonies.

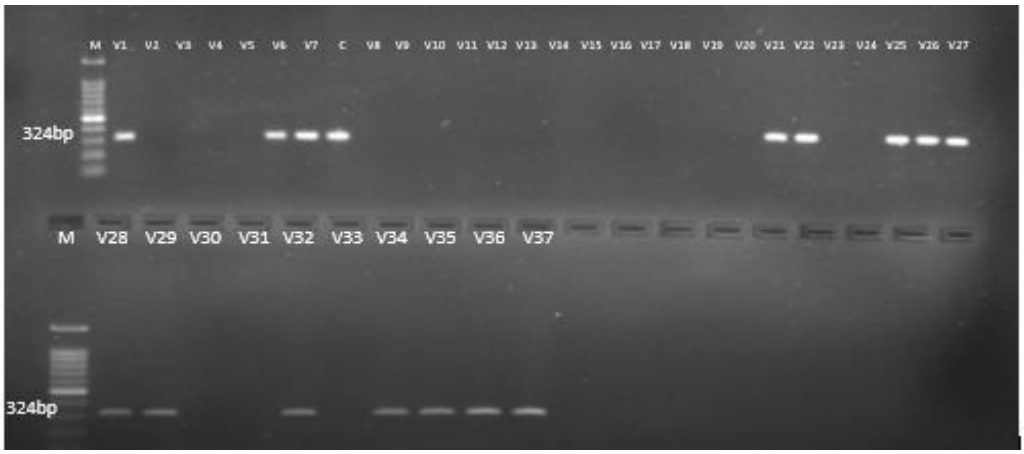

Figure 2 Gel electrophoresis of *Vibrio alginolyticus* using specific primer.

Table 1 Multidrug and antibiotic resistance pattern results of *V. alginolyticus.*

Patterns	Resistance pattern	Strain	MDR value	Place
I	CTX, S, SXT	VAT 1	0.16	
II	AMP, E, NA, RD, VA	VAT 2	0.38	Kuala Ibai
III	CTX, FEP	VAT 3	0.11	
IV	AMP, C, CN, CIP, CTT, CTX, E, FEP, K, KF, NA, OT, RD, S, SXT, TE, VA	VAT 4, VAT 5, VAT 6, VAT 7, VAT 13, VAT 14, VAT 15	1.0	
V	NA, VA	VAT 8	0.27	Sungai
VI	AMP, KF, VA	VAT 9	0.16	Besut
VII	AMP, VA	VAT 10	0.11	
VIII	OT	VAT 11	0.05	
IX	AMP, KF, RD, S, VA	VAT 12	0.33	

In this study, nine resistance patterns were identified for *V. alginolyticus* isolated from Terengganu (table 1). *Vibrio alginolyticus* isolates showed resistance to vancomycin (80%), ampicillin (73%) and Vibriostat O129 (66%). On the other hands, more than 50% of *V. alginolyticus* isolates were susceptible to chloramphenicol, gentamicin, ciprofloxacin, cefotetan, kanamycin and trimethoprim/sulfamethoxazole. Multiple drug resistance index range for Kuala Ibai between 0.11 to 0.38 and Sungai Besut between 0.05 to 1.0, the isolates that have MDR value more than 0.2 posed the high risk of sources contamination. Based on MDR index, the mean of value was 0.57 mean, the *V. alginolyticus* isolate have been exposed to antibiotic for a long period. Deng et al. (2020) mentioned that vancomycin, amoxicillin and furazolidane has more than 50% of resistance prevalence.

The resistance of Vibrio species to vancomycin and ampicillin are not a new report cases [4]. Mohamad et al. (2019) stated that the isolated pathogens from hybrid grouper in Malaysia were found resistance to ampicillin and vancomycin but sensitive to oxytetracycline and tetracycline. Another study reported that *V. alginolyticus* isolated from cockle were resistance against penicillin, ampicillin, vancomycin and erythromycin [15]. Similarly, all *V. alginolyticus* bacterial isolated from oyster at coastal area of Korea were resistance to ampicillin and vancomycin and cephalothin [3]. In China, *V. alginolyticus* strain from farmed seahorse appeared to be highly resistance to 19 out of 47 antibiotics including vancomycin and ampicillin [5].

Even though this study were not studying about plasmid, Amalina et al. (2019) revealed that the resistance reaction toward vancomycin and ampicillin found that it was related to the mediated chromosome since the isolates were remained after plasmid curing. Vancomycin and ampicillin (aminopenicillins) were included in

inhibits cell wall synthesis mechanisms of antibiotics interference. Thus, the bacteria acquire resistance to antibiotics and gain a survival advantage in the host [15]. Shahimi et al. (2021) added that the high percentage of antibiotics resistance may be due to continued used of medicated feeds in animal and leads to dissemination of virulent and resistant pathogens into the environment and affect the other microorganisms.

CONCLUSION

As a conclusion, this current study successfully identified 15 isolates of *Vibrio alginolyticus* isolated from farmed Asian seabass in Terengganu using in-house species-specific PCR primer. Most of the isolates were resistant to ampicillin and vancomycin with 9 different antibiotic resistance patterns. Continuous monitoring of antibiotic resistance in *Vibrio spp.* should be done for proper preventative measures in fish disease management plan. This research also will serve as a base for further studies in virulent gene and antibiotic resistant mechanisms in *Vibrio spp.*

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REFERENCES

1. Ahmed, O. B., & Dablool, A. S. (2017). Quality Improvement of the DNA extracted by boiling method in Gram negative bacteria. *International Journal of Bioassays*, 6(4), 5347–5349. doi.org/10.21746/ijbio.2017.04.004
2. Ali, J., Rafiq, Q. A., & Ratcliffe, E. (2018). Antimicrobial resistance mechanisms and potential synthetic treatments. In Future Science OA, 4(4), 1–10. doi.org/10.4155/fsoa-2017-0109
3. Almeida, M. A., Cunha, M. A., & Alcântara, F. (2001). Loss of estuarine bacteria by viral infection and predation in microcosm conditions. Microbial Ecology, 42(4), 562–571. doi.org/10.1007/s00248-001-0020-1
4. Amalina, N. Z., Santha, S., Zulperi, D., Amal, M. N. A., Yusof, M. T., Zamri-Saad, M., & Ina-Salwany, M. Y. (2019). Prevalence, antimicrobial susceptibility and plasmid profiling of *Vibrio spp*. isolated from cultured groupers in Peninsular Malaysia. BMC Microbiology, 19(1), 1–15. doi.org/10.1186/s12866-019-1624-2
5. Amalina, N. Z., Dzarifah, Z., Amal, M. N. A., Yusof, M. T., Zamri-Saad, M., Al-Saari, N., Tanaka, M., Mino, S., Sawabe, T., & Ina-Salwany, M. Y. (2019). Recent update on the prevalence of Vibrio species among cultured grouper in Peninsular Malaysia. Aquaculture Research, 00, 1–9. doi.org/10.1111/are.14275
6. Chuah, L. O., Effarizah, M. E., Goni, A. M., & Rusul, G. (2016). Antibiotic Application and Emergence of Multiple Antibiotic Resistance (MAR) in Global Catfish Aquaculture.

Current Environmental Health Reports, 3(2), 118–127. doi.org/10.1007/s40572-016-0091-2

7. Deng, Y., Xu, L., Chen, H., Liu, S., Guo, Z., Cheng, C., Ma, H., & Feng, J. (2020). Prevalence, virulence genes, and antimicrobial resistance of Vibrio species isolated from diseased marine fish in South China. Scientific Reports, 10(1), 1–8. doi.org/10.1038/s41598-020-71288-0
8. Kang, C., Shin, Y., Jang, S., Jung, Y., & So, J. (2016). Antimicrobial susceptibility of *Vibrio alginolyticus* isolated from oyster in Korea. Environmental Science and Pollution Research. doi.org/10.1007/s11356-016-7426-2
9. Krumperman, P. H. (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of faecal contamination of foods. Applied and Environmental Microbiology, 46(1), 163–170. doi.org/10.1007/s11356-014-3887-3
10. Mohamad, N., Mohd Roseli, F. A., Azmai, M. N. A., Saad, M. Z., Md Yasin, I. S., Zulkiply, N. A., & Nasruddin, N. S. (2019). Natural Concurrent Infection of Vibrio harveyi and *V. alginolyticus* in Cultured Hybrid Groupers in Malaysia. *Journal of Aquatic Animal Health*, 31(1), 88–96. doi.org/10.1002/aah.10055
11. Mustapha, S., Mustapha, E. M., & Nozha, C. (2013). *Vibrio alginolyticus*: An Emerging Pathogen of Foodborne Diseases. *International Journal of Science and Technology*, 2(4), 302–309. www.researchgate.net/publication/236214804
12. Reverter, M., Sarter, S., Caruso, D., Avarre, J. C., Combe, M., Pepey, E., Pouyaud, L., Vega-Heredía, S., de Verdal, H., & Gozlan, R. E. (2020). Aquaculture at the crossroads of global warming and antimicrobial resistance. Nature Communications, 11(1), 1–8. doi.org/10.1038/s41467-020-15735-6
13. Shahimi, S., Elias, A., Abd. Mutalib, S., Salami, M., Fauzi, F., Nurul, N. A., Abd. Ghani, M., & Azuhairi, A. (2021). Antibiotic resistance and determination of resistant genes among cockle (*Anadara granosa*) isolates of *Vibrio alginolyticus*. Environmental Science and Pollution Research, 28(32), 44002–44013. doi.org/10.1007/s11356-021-13665-4
14. Xie, J., Bu, L., Jin, S., Wang, X., Zhao, Q., Zhou, S., & Xu, Y. (2020). Outbreak of vibriosis caused by *Vibrio harveyi* and *Vibrio alginolyticus* in farmed seahorse Hippocampus kuda in China. Aquaculture, 523, 735168. doi.org/10.1016/j.aquaculture.2020.735168
15. Yang, B., Zhai, S., Li, X., Tian, J., Li, Q., Shan, H., & Liu, S. (2021). Identification of *Vibrio alginolyticus* as a causative pathogen associated with mass summer mortality of the Pacific Oyster (*Crassostrea gigas*) in China. Aquaculture, 535. doi.org/10.1016/j.aquaculture.2021.736363

Mahsheers in Malaysia: A Review on Feed for *Tor tambroides* (Empurau) Cultured in Captivity

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ABSTRACT

The Malaysian masheer (*Tor tambroides*) that belongs to Cyprinidae family is a high potential freshwater aquaculture industry in Sarawak. However, this freshwater species population trend seems to decrease due to overfishing and captive breeding. As *Tor tambroides* has slow growth from 380 to1250 g for an approximate period of three years while cultured in captivity, a review was undertaken to obtain a comprehensive understanding on feeds and feeding efficiency of this highly prized fish. This review exploited various research on *T. tambroides* that encapsulate the fields of morphology, distribution, farming techniques, industry, market status and importantly the required nutrition for *Tor tambroides* cultured in captivity. It is hoped that this review can be a reference tool to aquaculturist and nutritionist who may be interested in culturing this species to mass production scale. The review also includes recommendations and future perspectives of *T. tambroides* for aquaculture while accommodating conservation endeavours of wild Malaysian mahseer fish.

Keywords: Malaysian masheer; *Tor tambroides*; feed; farming techniques

INTRODUCTION

Tor species, generally known as masheer, are widespread and found extensively at famous freshwater ecosystem throughout the Asian region. To date, there are approximately 16 species of *Tor* that had been identified in 1999. However, the number of species identified had increased to 30 species in 2007. In Malaysia waters, there were only three local species identified namely *Tor tambroides*, *Tor duoronensis* and *Tor tambra*. *Tor tambroides* can be detected at the headwaters of most state's major river systems while the other species are mostly found in medium to large-sized rivers (Figure 1). These Masheer species were discovered mostly at Peninsular Malaysia namely state of Kelantan, Negeri Sembilan, Pahang and Perak respectively and also in Sarawak.

PHYLOGENETIC TREE

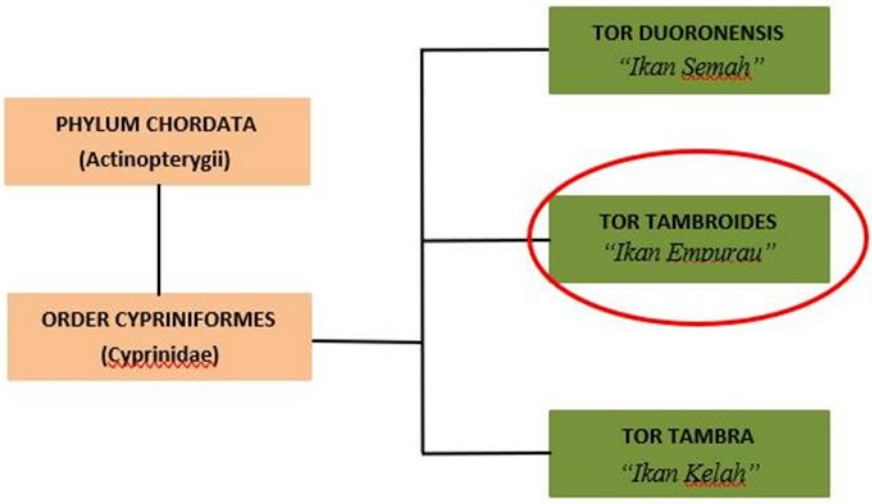

Figure 1 Phylogenetic Tree for Tor species reported in Malaysia

Tor tambroides grows significantly slower than other freshwater cyprinids, although this species can reach a similar ultimate body size as other widely cultivated freshwater cyprinid species. In the natural habitat, it takes a year to achieve 500–600 g [5], whereas in pond culture, it takes around 2 years and 9 months to achieve an average size of 800 g (range 380–1250 g). The slow and low growth rate of this species especially those cultured in captivity usually embarked on poor health status such as infection to the fish as well as high management cost for fish farmers. The most ultimate problem often faced by the local farmers is the high cost of feed for this species as there is no commercial diet specifically designed for faster growth performance without detrimental effects on the cultured fish.

FEEDING ECOLOGY PROFILE

The analysis of feeding ecology is indeed an important way of understanding the roles of fish in their environments. To date, research on diet and nutritional intake of freshwater fish is still ongoing since it is the foundation for the establishment of a successful fisheries management programs based on fish capture and culture. A fish must acquire both macro and micronutrients from its diet. The availability of prospective feeds reflects the physical environment, in which a species range is determined by its capacity to detect, acquire, and digest food. This reflects the importance of exploiting the flora and faunas of the designated natural habitat for fish specifically *T. tambroides.*

DIETARY REQUIREMENT

Dietary protein has a significant impact on the rate of growth of cultured fish. Due to the high cost of protein ingredients, accurate information on the protein requirements of fish is crucial for rare species in aquaculture [1]. *Tor tambroides* is an omnivorous feeder, in natural habitats Malaysian masheer scavenges on molluscs, small fish, freshwater crustacean and plant. In addition to that, *Shorea macrophylla* also known as “buah engkebang” by the locals in Sarawak is known to be one of food resources for Malaysian masheer. The consumption of *Shorea* fruit is widely considered to lead to the special taste of Malaysian mahseer. Although many claimed that *T. tambroides* unique taste hailed from this jungle fruit, nutritive information of this fruit on the nutrition requirement of Tor species is still lacking.

Although the protein requirement of *T. tambroides* is still understudied, protein requirement for Malayan masheer fry is knownto be higher than tilapia, catfish and common carp. Another species of masheer (*T. putitora*) have a protein requirement around 45%. The best performance of *Tor putitora* fed with diet with 23 mg protein/Kj compared to P/E ratio of 19 or 26 mg protein/Kj [1]. In 2008, [1] reported that fingerling of *T. tambroides* with weight of 20g and 55g need requirement of an estimated 48% protein for maximum growth rate. [2] found similar result in which 40% dietary protein yielded the significantly lowest feed conversion ratio (2.19 ± 0.163) in *T. tambroides* fingerlings, by using different level protein diets to 30%, 35%, 45%, and 50%. However, different spawning trials may affect FCR and growth rate.

Malayan masheer is also categorized as semi-fatty fish that contains 4.6%-5.2% of muscle crude lipid, while fatty fish such as Rainbow trout and Chum salmon contain 5%-8% lipid inedible tissue [3]. Another finding from [1] found that 15% dietary lipid for diet formulation under 40% protein requirement noteworthy did not affect increased growth and feed utilization efficiency compared to protein concentration with 48%. Optimum dietary lipid for Malayan masheer should not more than 5%. If the total lipid concentration is higher than 5% (10% and 15%) for *Tor tambroides* will increase dietary gross energy at 1kJ per g diet because there is no interaction between lipid and protein and any extra dietary energy in the feed will be deposited as fat in the visceral cavity, liver, and abdominal activity [1,4]. Most of the fish's body lipid is deposited by dietary carbohydrate [5;6]. For Malaysian mahseer fingerlings, dietary n-3 to n-6 PUFA ratio of less than 0.3. [7] proposed crude palm oil as the best lipid source for the Malaysian mahseer fingerling due to its high 16:0 and 18:1n-9 levels, as well as the moderate 18:2n-6 level, which aided in the efficient use of energy sources. This lipid source regulates muscle tissue to maintain high concentrations of n-3 and n-6 LC-PUFA (long-chainpoly unsaturated fatty acids). In addition, inclusion of 0% *engkebang* oil as feed shows that *T. tambroides* juveniles maintained the most muscle n-6 and n-3 polyunsaturated fatty acids as compared to 1.25, 2.5, 3.75, and 5% *engkebang* oil [6]. The application of *Shorea macrophylla*

or the "*engkebang* fruits" oil as dietary lipid could cause special taste without negative consequences on whole body proximate composition or growth performance in Malaysian mahseer.

Carbohydrates seem to be the cheapest source of energy for humans, fish, and other animals. In comparison to fat and protein requirements, carbohydrate requirements have not been thoroughly researched. It is generally known that appropriate levels of carbohydrates should have been included in fish feed to improve energy availability, though this should be done with cautiousness for carnivorous fish such as trout and salmon, which are less well adapted to digest complex carbohydrates. In fish diets, a lack of carbohydrates stimulates protein and lipid catabolism. However, adequate levels of easily digested carbohydrates stimulate protein sparing. The effects of different dietary carbohydrate levels on *Tor tambroides* fingerlings growth in terms of body weight, liver histology, and feed efficiency levels have been studied [8]. Four isonitrogenous has been suggested by [8] (40% crude -1 protein) and isocaloric (17.6 kJg) diets that included different concentration of carbohydrate by corn starch (30%, 25%, 20% and 15%) respectively. The liver histology of fingerlings treated with higher carbohydrate levels revealed hypertrophy and mild hepatic steatosis. They reached the conclusion that dietary carbohydrate concentrations of 20% and 25% provided the best growth performance among 15% to 30%. The optimal dietary carbohydrate level for mehseer fingerlings was determined using a second order polynomial regression analysis model at 23.4%. The best carbohydrate for *Tor tambroides* fingerling determines of corn starch, tapioca starch, sago, taro.

FARMING TECHNIQUE OF *Tor tambroides*

According to Department of Fisheries Malaysia (DOF), this species is yet to be farmed commercially as the suitable farming methods are yet to be discovered and tested on the ground. However, there are many aquaculture companies for *Tor tambroides* especially in Sarawak. The commercialisation of culturing *T. tambroides* in captivity has received intensive support including research fund of some RM4.5 million in 2011 from the Government of Malaysia for more research on this species. This indicates the strong intention of the Government to simultaneously preserve wild fish stocks while improving quality of this species cultured in captivity.

Tor spp is known as a relatively slow growing fish in captivity compared to other freshwater finfish species. In Malaysia, *T. tambroides* has more aquaculture potential compared to *T. duoronensis* because *Tor tambroides* indicated higher growth rate. Furthermore, the growth and health of Empurau culture are also affected by physiochemical environment [9], microbiological manipulated by physiochemical and farming technique. In addition, the application of pond techniques is known to make an impact on the growth rate of *Tor* spp as compared to cage techniques.

Results from 2014 research conducted by [9] at the Fisheries Research and Production Centre (IFRPC) Serian, Sarawak exhibited tolerable healthy range for fish growth as shown in Table 1 in terms of physicochemical characteristics, including total ammonia nitrogen (TAN), biochemical oxygen demand (BOD), dissolved oxygen (DO) pH, and temperature.

Table 1 Physiochemical characteristic at Fisheries Research & Production Centre (IFRPC) in Serian, Sarawak [9].

POND	PH	Temperature	DO (mg/L)	BOD (mg/L)	TAN (mg/L)
P1	7.95	28.9	5.6	3.2	0.00
P2	9.15	29.5	5.2	3.4	0.46
P3	8.40	27.7	4.8	2.8	2.14
P4	7.41	28.6	5.4	3.2	0.14
P5	9.16	28.5	5.7	3.2	0.04
P6	9.00	29.2	5.5	4.2	0.00
P7	7.62	26.4	5.6	3.3	1.01
Average	**8.38**	**28.4**	**5.4**	**3.33**	**0.54**

The average pH found in the study was 8.38, that was still within the acceptable range for empurau fish. In fact, the largest fish farms were often grown in water with pH ranging from 7.0 to 9.0. However, any pH value below than pH 4.8 and higher than pH 10.8 may be hazardous to pond aquatic creatures. Under both natural and artificial conditions, temperature played a significant impact in some physiological processes, including the release of impulses for the breeding mechanism for fish. The average temperature based on this research was 28.4°C. High production may be caused by temperatures between 30°C and 35°C. The total amount of dissolved oxygen required by microorganisms for biodegradation of organic matter is known as biochemical oxygen demand (BOD). It's a typical metric for measuring organic contaminants in water. The average Dissolved Oxygen (DO) was lower compared to natural environment for Empurau fish. In comparison, Table 2 [10] shows the water quality requirements of Empurau based on natural environment reading at Seturan, Langap and Loreh, Indonesia.

Table 2 Natural environment physiochemical characteristic at Seturan, Langap and Loreh, Indonesia [11].

Water quality parameters	Range
Temperature (ºC)	25.26-27.30
pH	6.81-7.09
Dissolved oxygen (mg/L)	6.30-8.34
Conductivity (2mS/cm)	0.051-0.118
Velocity (m/sec)	0.27-0.86
Water colour	Clear to turbid

[10] found that microbiological factors were manipulated by physiochemical, in which 11 Enterobacteriaceae were isolated by using API 20E identification kit. *Enterobacter cloacae*, *Erwinia* spp, *Serratia odorifera*, *Citrobacter freundii*, *Citrobacter braakii*, *Buttiaxella agrestis, Enterobacter cloacae*, *Proteus vulgaris*, Vibrio fluvialis, Brucella spp., Cedecea davisae were among the isolated *Enterobacteriacae*. The presence of some of these bacteria, such as *Citrobacter freundii*, has been related to information health issues because they are potentially pathogenic microorganisms that have been implicated as aetiological agents of animal and human diarrhoea [10]. Furthermore, *Citrobacter freundii* has the potential to cause disease to empurau culture [10]. The study concluded that physiochemical factors could manipulate the microbiota in fishponds, orchestrating the growth of the empurau culture.

INDUSTRY AND MARKET STATUS OF EMPURAU IN SARAWAK

Empurau has a high economic value in Malaysia, specifically in Sarawak, where the price per kilogramme (kg) of fish can reach RM800 depending on grade and size [12]. The price also depends on types of fish whether wild fish or farmed from aquaculture industry. In most cases, wild empurau fetches higher market value than the semi-wild stock [13]. This fish became an instant hit among consumers that any restaurant in Sarawak mainly Chinese restaurant would offer a menu of empurau that could cost more than RM 1300 a dish [12]. The continuous exploitation of supply of empurau to aquaculturist has affected the population dynamics of this fish in the wild. Number of wild empurau remains low and continues to decrease due to overfishing. This is further hampered by the fish's modest growth rate when cultured in captivity that makes it difficult to rear the species and this had marked up the price of empurau.

Juveniles or Fingerling of *Tor tambroides* and *Tor douronesis* also show an increase in demand of juveniles for aquaculture and aquarium industry. In 2008 it was reported that the price of empurau juvenile can fetch a handsome price up to RM24 with a total length of 0.07 m [14].

CONCLUSION

In conclusion, the review found that *T. tambroides* can fetch a handsome price at both juvenile or at adult phase due to its unique taste and pricey value. Nevertheless, many gaps need to be seriously looked into by researchers to further understand the feeding nature of this fish so that suitable fish feed can be developed to meet the growing market for *T. tambroides* aquaculture.

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REFERENCES

1. Ng, C. K. (2004). King of the rivers: mahseer in Malaysia and the region. Inter Sea Fishery (M). SDN BHD, Kuala Lumpur. 170 pp. Ovenden, J. R., 1990. Mitochondrial DNA and marine stock assessment: a review. Aust. J. Mar. Freshw. Res. 41, 835-853.
2. Ng, W. K., N. Abdullah, & S. S. De Silva. (2008). The dietary protein requirement of the Malaysian mahseer, *Tor tambroides* (Bleeker), and the lack of protein- sparing action by dietary lipid. Aquaculture, 284(1-4), 201-206.
3. Misieng, J. D., M.S. Kamarudin, & M. Musa. (2011). Optimum dietary protein requirement of Malaysian mahseer (*Tor tambroides*) fingerling. Pakistan Journal of Biological Sciences, 14, 232–235.
4. O¨ zogul, Y. & F. O¨ zogul. (2007). Fatty acid profiles of commercially important fish species from the Mediterranean, Aegean and Black Seas. Food Chemistry 100, 1634-1638.
5. Ng, W. K. & V. C. Andin. (2011). The Malaysian mahseer, *Tor tambroides* (Bleeker), requires low dietary lipid levels with a preference for lipid sources with high omega-6 and low omega-3 polyunsaturated fatty acids. Aquaculture, 322: 82-90.
6. Ishak, S. D., M.S. Kamarudin, E. Ramezani-Fard, & Y.A. Yusof. (2016). Effects of varying dietary carbohydrate levels on growth performance, body composition and liver histology of Malaysian mahseer fingerlings (*Tor tambroides*). Journal of Environmental Biology, 37: 756–764.
7. Kamarudin, M. S., M. L. Bami, A. Arshad, C. R. Saad, & M. Ebrahimi. (2018). Preliminary study of the performance of crude illipe oil (*Shorea macrophylla*) as a dietary lipid source for riverine cyprinid *Tor tambroides*. Fisheries science, 84: 385–397.
8. Bami, M. L., M. S. Kamarudin, C. R. Saad, A. Arshad, & M. Ebrahimi. (2017). Effects of palm oil products on growth performance, body composition and fatty acid profile of juvenile Malaysian mahseer (*Tor tambroides*). Journal of Oil Palm Research, 29: 387–400.
9. Ishak, S. D., M. S. Kamarudin, E. Ramezani-Fard, & Y. A. Yusof. (2016). Effects of varying dietary carbohydrate levels on growth performance, body composition and liver histology of Malaysian mahseer fingerlings (*Tor tambroides*). Journal of Environmental Biology, 37: 756–764.
10. Lee, K. S., S. Lihan, F. F. G. Dasthagir, K. M. Mikal, F. Collick, & N. K. Hua. (2014). Microbiological and physicochemical analysis of water from Empurau fish (*Tor tambroides*) farm in Kuching, Sarawak, Malaysian Borneo. International Journal of Scientific & Technology Research, 3: 285–292.
11. Rachmatika, I., Nasi, R., Sheil, D. & Wan, M. (2005). A First Look at the Fish Species of the Middle Malinau. Center for International Forestry Research (CIFOR), Bogor, Indonesia, 34-35.
12. Entri, D. A. (2013). Determination of *Tor Tambroides* (empurau) Growth Rate Using Different Feed System (Doctoral dissertation, Universiti Malaysia Sarawak).
13. Harith, M. N. & Hassan, R. (2011). Blue-green algae and nutrient concentrations in two *Tor tambroides* aquaculture ponds differing in construction. Journal of Tropical Biology & Conservation (JTBC).
14. Joseph, M. (2018). Ternak Ikan EMPURAU, Termahal DI Malaysia. Berita Harian. https://www.bharian.com.my/wanita/lain-lain/2018/09/471239/ternak-ikan-empurau-termahal-di-malaysia.

Analysis of Genetic Diversity in Five Captive Population of Asian Seabass (*Lates calcarifer*) for Selective Breeding in Malaysia

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ABSTRACT

Asian seabass or *Lates calcarifer* is an important commercial fish in the Indo-Pacific region. It is classified as a remarkable species from both wild fishery and aquaculture contexts. Unfortunately, undesirable characters are common due to inbreeding in aquaculture. In Malaysia, limited to no publication concerning the genetic diversity of captive *Lates calcarifer* had been done to diminish this problem. This study aims to contribute to the baseline data on the genetic diversity in 5 captive populations of Asian Seabass for selective breeding in Malaysia, which can be used to access the future breeding program of captive seabass. The study's objectives are to determine the best breeds of Asian seabass (*L. calcarifer*) in 5 captive populations for selective breeding and to analyse the population genetic structure of the Asian seabass in aquaculture captive based on mitochondrial cytochrome oxidase subunit I (COI). Mitochondrial CO1 sequences were conducted on 146 samples from five hatcheries in different states of Peninsular Malaysia. Out of 15 haplotypes obtained, 6 shared and 9 (60%) unique haplotypes were defined to indicate a high number of population-specific haplotypes. Haplotype diversity (Hd) range was 0.301 to 0.709, and nucleotide diversity ranging from 0.01 to 4.8%. The genetic distance range was 0 to 10%. In general, the populations were divided into two mtDNA lineages. Mismatch distribution analysis revealed that *L. calcarifer* populations underwent complex changes with analysis of the CO1 dataset in each population in the overall population.

Keywords: *Lates calcarifer*; captive; CO1 mtDNA; genetic distance

INTRODUCTION

The demands for aquaculture products rocketing due to frequent and extreme climate change and human-induced environmental pollution which endanger natural fishery stocks and decrease capture production [1]. In Australia and Southeast Asia, there is substantial interest in improving aquaculture of barramundi or Asian seabass (*Lates calcarifer*) production efficiency through selective breeding [2]. However, to date, little effort has been made. The population genetics of this fish

are poorly known even though its economic importance increases worldwide. In Malaysia, limited to no publication concerning the genetic diversity of captive *Lates calcarifer* had been conducted. This study aims to contribute to the baseline data on the genetic diversity in 5 captive populations of Asian Seabass (*L. calcarifer*) for selective breeding in Malaysia, which can be used later to access the selective breeding of captive sea bass. The study's objectives are to determine the best breeds of seabass (*L. calcarifer*) in 5 captive populations to maximize the genetic diversity in the base stock and to analyse the population genetic structure of the *Lates calcarifer* in aquaculture captives based on mitochondrial cytochrome oxidase subunit I (COI).

MATERIALS AND METHODS

A total of 150 samples of *L. calcarifer* were collected from five Malaysian commercial hatcheries; two on the East coast (Kelantan, n = 30; Terengganu, n = 30), one on the West coast (Selangor, n = 30), one in Northern Territory (Perak n = 30), and one in Southern territory (Johor, n = 30) (Figure 1). Juvenile specimens (3 to 4 cm) were chosen and identified based on morphological characteristics according to the description of [3]. The muscle tissues were removed from the whole fish and were preserved in 95% ethanol. These samples are then stored at -20 °C for further analyses. The 609 bp fragment of the cytochrome c oxidase 1 gene was amplified with a pair of universal primers, specifically FishF1 and FishR1. After run electrophoresis on 1% agarose gels approximately for 30 minutes at 75 V followed by a 1-hour soak, amplified products were viewed and photographed under UV light. Software used for the data analysis were Chromas 2.6.6, MEGA7, DnaSP program version 6.0, and Arlequin 3.1.

RESULTS AND DISCUSSION

Out of 15 exhibited haplotypes, 9 were population private that were not shared by other populations. More than half (60%) of the population haplotype were specific indicate that dispersal among seabass individuals was restricted [4]. Other than that, a high number of specific haplotypes might also happen due to the independent origin of haplotypes through mutation [5]. This unique haplotype could be useful for stock identification by being a population-specific marker. Haplotype 1 (Lc1) was shared among all the seabass populations (most abundant, n = 96), suggesting it as the ancestral haplotype. Haplotype sharing might be caused by common ancestral origin and subsequent gene flow among populations [6]. Based on the four basic classifications of demographic history by Grant and Bowen (1998), Terengganu population with a small value of both (h < 0.5 and p < 0.5%) were categorized in the first group where recent population bottleneck or founder event by single or a few mtDNA lineages had to happen [7]. Population from Perak (h = 0.7, p = 4%) exhibit the large h and p clustered the population in the fourth category. This displays the incidence of a large stable population with long evolutionary history

or secondary contact between differentiated lineages in this population. Besides, phylogenetic analysis using Neighbor-Joining (NJ) tree also divided 15 haplotypes into two distinct lineages with strong bootstrap support.

Table 1 Distribution of 15 observed haplotypes, nucleotide diversity, number of haplotypes, haplotype diversity and number of polymorphic sites among populations of *Lates calcarifer*.

Haplotypes	**GenBank Accession Numbers**	**Populations**				
		Kelantan (n=27)	**Terengganu (n=30)**	**Perak (n=29)**	**Selangor (n=30)**	**Johor (n=30)**
L. calcarifer 1	MZ540093	0.4814	0.9666	0.4827	0.8333	0.5000
L. calcarifer 2	MZ540094	0.3703	-	0.2413	0.0666	0.1000
L. calcarifer 3	MZ540095	0.0370	-	-	-	-
L. calcarifer 4	MZ540096	0.0370	-	-	-	-
L. calcarifer 5	MZ540097	0.0370	-	-	-	-
L. calcarifer 6	MZ540098	0.0370	-	-	-	-
L. calcarifer 7	MZ540099	-	0.0333	-	-	0.0666
L. calcarifer 8	MZ540100	-	-	0.1379	-	0.2333
L. calcarifer 9	MZ540101	-	-	0.0344	-	-
L. calcarifer 10	MZ540102			0.0344	-	-
L. calcarifer 11	OK184465	-	-	0.0344	-	-
L. calcarifer 12	MZ540103	-	-	0.0344	-	-
L. calcarifer 13	MZ540104	-	-	-	0.1000	-
L. calcarifer 14	MZ540105	-		-	-	0.0666
L. calcarifer 15	MZ540106	-	-	-	-	0.0333
Nucleotide diversity (PiJC)		0.0475	0.0001	0.0389	0.0125	0.0237
Number of haplotypes		6	2	7	3	6
Haplotype diversity (Hd)		0.6496	0.0667	0.7094	0.3012	0.6989
Number of polymorphic sites		57	1	60	55	57

Number under each population indicate the frequencies of individuals with that haplotype in each population. n = sample size.

Genetic distance values were also performed. The genetic divergence between all population values was 0% - 10%. Detail examination on their taxonomic status is needed because there was high chance of conspecific populations or even a valid species and merit additional study concerning their specific status when genetic divergence values were between 2% to 11% status [8].

CONCLUSION

Conservation of Asian seabass at its natural variation level is required as it display generally wide range of genetic diversity and evident genetic distance between population. Analyses using larger sample sizes and inclusion of hypervariable nuclear markers (Microsatellite) is needed for a more detail result.

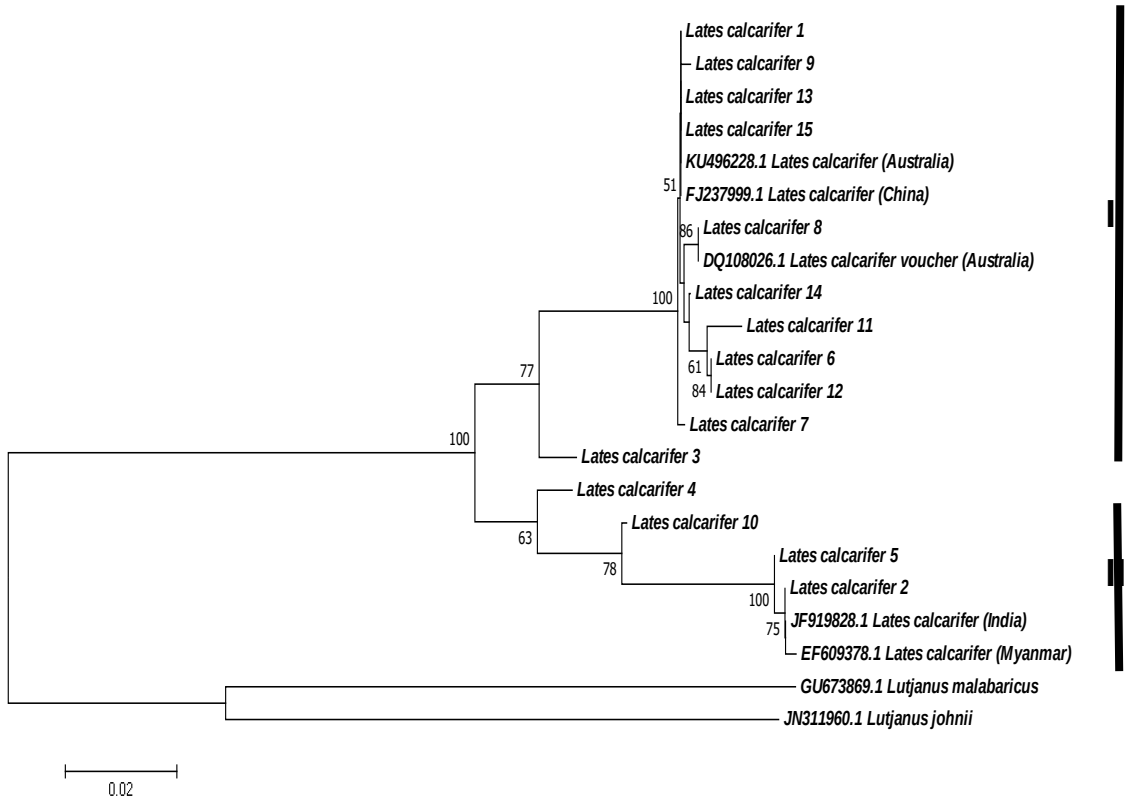

Figure 1 Neighbor-Joining (NJ) tree showing relationships among cytochrome c oxidase (COI) haplotypes of *Lates calcarifer, Lutjanus malabaricus* and *Lutjanus johnii.* The number at each node represents the bootstrap value (%) based on 1000 pseudo replications of the NJ analysis.

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REFERENCES

1. Barange, M., Merino, G., Blanchard, J. L., Scholtens, J., Harle, J., Allison, E. H., Allen, J. I., Holt, J., & Jennings, S. (2014). Impacts of climate change on marine ecosystem production in societies dependent on fisheries. *Nature Climate Change*, *4*(3), 211–216. https://doi.org/10.1038/nclimate2119
2. Loughnan, S. R., Smith-Keune, C., Jerry, D. R., Beheregaray, L. B., & Robinson, N. A. (2016). Genetic diversity and relatedness estimates for captive barramundi (Lates calcarifer, Bloch) broodstock informs efforts to form a base population for selective breeding. *Aquaculture Research*, *47*(11), 3570–3584. https://doi.org/10.1111/are.12807
3. Peninsula, M. (n.d.). *Fishes of Terengganu*.
4. Chenoweth, S. F., Hughes, J. M., Keenan, C. P., & Lavery, S. (1998). Concordance between dispersal and mitochondrial gene flow: Isolation by distance in a tropical

teleost, Lates calcarifer (Australian barramundi). *Heredity*, *80*(2), 187–197. https://doi.org/10.1038/sj.hdy.6882920

5. Sahoo, L., Mohanty, M., Meher, P. K., Murmu, K., Sundaray, J. K., & Das, P. (2019). Population structure and genetic diversity of hatchery stocks as revealed by combined mtDNA fragment sequences in Indian major carp, Catla catla. *Mitochondrial DNA Part A: DNA Mapping, Sequencing, and Analysis*, *30*(2), 289–295. https://doi.org/10.1080/24701394.2018.1484120
6. Das, S. P., Swain, S., Jena, J., & Das, P. (2018). Genetic diversity and population structure of Cirrhinus mrigala revealed by mitochondrial ATPase 6 gene. *Mitochondrial DNA Part A: DNA Mapping, Sequencing, and Analysis*, *29*(4), 495–500. https://doi.org/10.1080/24701394.2017.1310852
7. Shugar, D., & Zielińska, Z. (1983). Obituary: Professor Michael Laskowski Sr. *Acta Biochimica Polonica*, *30*(2), 113–114.
8. Bradley, R. D., & Baker, R. J. (2001). A test of the genetic species concept: Cytochrome-b sequences and mammals. *Journal of Mammalogy*, *82*(4), 960–973. https://doi.org/10.1644/1545-1542(2001)082<0960:ATOTGS>2.0.CO;2

Length-Weight, Length-Length Relationships, and Condition Factor of Four Carangidae Species in Kuala Selangor

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ABSTRACT

The Carangidae family of the ray-finned fish comprises economically and ecologically important species such as scads, travellies, and runners. The length-weight relationship, length-length relationship, and condition factor were described for four Carangidae species collected in Kuala Selangor waters**.** The fish samples were collected at fish landing jetties, and four times sampling was conducted from January to February 2020. The samples were obtained from local fishers who operated fishing vessels using trawl nets. At each time sampling, around 6 kg of unsorted fish samples were collected and identified. The length-weight relationship of the fish samples was calculated using the conventional formula $W = aL^b$. A simple linear regression model was constructed to estimate the length-length relationships. The Fulton's condition factor was estimated using the formula $K = W(100) / L^3$. A total of 241 samples were collected of four species which are *Carangoides malabaricus*, *Decapterus macrosoma*, *Megalaspis cordyla* and *Selaroides leptolepis*. Three species displayed negative allometric growth with *b* value ranging from 2.56 to 2.83. *Decapterus macrosoma* showed positive allometric growth ($b > 3$) with r^2 of 0.9521. The length-length relationship findings revealed that the variables were highly correlated ($r^2 > 0.95$). The Fulton's condition factor for all species showed higher than 1 which indicated robustness of the fish towards its environment. This study provides valuable basic information of the Carangidae fish population that can be contributed to the management and monitoring programme of these species in that area.

Keywords: Length-weight; length-length; condition factor; Carangidae

INTRODUCTION

Carangidae fishes are one of the important sources of protein and commonly caught by local fishermen. These diverse and economically important fishes recorded approximately 146 species from 32 genera worldwide and can be found in tropical and subtropical waters around the world. [1] recognized 42 species and 18 genera in Malaysia. These fishes are also commonly known as trevallies, scads, jacks, pompanos, and amberjacks, and these fishes can grow up to 2 meters long.

The length-weight relationship (LWR) and length-length relationship (LLR) of fish are essential for fisheries management and stock assessment [2]. These relationships are also important for biological studies in terms of calculating the production of biomass, estimation of fish growth rates between seasonal changes, and indicating the morphological differentiation between species from different regions.

The length-weight relationship can be stated in a formula which makes it easier for predicting the fish weight at a certain particular length by using the formula to display the relationship of both body parameters. The length-length relationships are the method that utilized preferred length to estimate and compare fish growth in population, as the length can be measured swiftly than weight [3].

The condition factor (CF) can be defined as the well-being of the fish, and the higher the CF value represents a better general fish condition in that area. In other words, the heavier the fish at a certain length, the better the condition demonstrated in that fish population. The good condition portrays when the CF value is one or greater than 1, and less than 1 indicates the bad condition [4]. The CF of fish can be influenced by age and sex of the fish, season, food availability, water quality and environmental condition. LWR, LLR and CF are important for the fishery industry as they may provide the best sizes of fish in terms of length and weight for the harvesting period for particular species [5].

The objective of this study was to provide baseline information for LWR, LLR, and CF for four species of Carangidae sampled in Kuala Selangor. The estimated growth values of these species might contribute to the valuable information for future research in terms of comparative fish growth in this study area.

MATERIALS AND METHODS

The samples were collected at the fish landing jetties in Kuala Selangor, located on the West Coast of Peninsular Malaysia. The sampling was done from January to February 2020. All fish samples were weighed and measured for the total length (TL) and standard length (SL). The TL and SL were measured using a graduated plastic measuring board, and the bodyweight (BW) using scale balance. All measurements were measured to the nearest 0.01 g and 0.1 cm, respectively. The fish species identification was based on the taxonomic keys by [6].

The fish samples' length-weight relationship (LWR) was calculated using the conventional formula $W = aLb$ [7]. This formula was log-transformed to estimate the parameters '*a*' and '*be* by using the least-square method as $Log\ W = log\ a + b\ log\ L$, where W is the weight of fish in gram (g), L is the total length of fish in centimetres(cm), 'a' is the intercept of the regression curve and '*b*' is the regression coefficient. The length-weight relationship shows an isometric growth when $b = 3$,

and when the value of b is different from 3, weight growth is allometric ($b > 3$; $b < 3$). The strength of variability between weight and length was calculated by determining coefficient (r^2).

A simple linear regression model (e.g., $TL = a + b\ SL$) was constructed to estimate the relationship of TL vs SL and SL vs TL. Fulton's condition factor was estimated using the formula $K = W(100) / L3$ as described by [8].

RESULTS AND DISCUSSION

A total of 62 *Carangoides malabaricus*, 46 specimens of *Decapterus macrosoma*, 78 specimens of *Megalaspis cordyla*, and 55 specimens of *Selaroides leptolepis* were collected during the sampling period. All species' total length and weight recorded ranged from 12.5 to 33.1 cm and 22.3 to 407.2 g. The length-weight relationship (LWR) of combined sex Carangidae fishes are presented in Table 1. The Linear regression was significant for all species ($P < 0.05$). The *b* values for each species caught ranged from 2.558 for *S. leptolepis* to 3.186 for *D. macrosoma*, and the coefficient of determination (r^2) recorded at 0.951 and 0.952, respectively, showed a strong correlation between the variables.

Table 1 Length-weight relationships parameters for four Carangidae species sampled in Kuala Selangor

Species	n	Regression parameters				
		a	95% CI of a	b	95% CI of b	r^2
Carangoides malabaricus	62	0.0033	0.0013-0.0455	2.8643	2.7210-3.2880	0.9711
Decapterus macrosoma	46	0.0059	0.0034 - 0.0103	3.1855	3.0041-3.3668	0.9521
Megalaspis cordyla	78	0.0143	0.0109 - 0.0424	2.8561	2.7162-3.1581	0.9724
Selaroides leptolepis	55	0.0387	0.0252 - 0.0595	2.5581	2.3990-2.7170	0.9513

n, number of specimens sampled; *a*, intercept; *b*, slope coefficient; *CI*, confident interval, r^2, coefficient of determination.

The *b* value recorded for *D. macrosoma* indicated positive ($b > 3$) allometric growth was also reported by other authors, except study done in Kenya by [9] recorded negative ($b < 3$) allometric growth, meaning that the length increase is faster than the weight gain. Negative allometric growth was recorded for *C. malabaricus* ($b = 2.8643$), as the *b* value is below '3' with a strong r^2 of 0.9711. *M. cordyla* displayed negative allometric growth ($b = 2.856$) that is consistent with other finding [10], however, a study done in China recorded positive allometric growth [11]. *S. leptolepis* also showed negative allometric growth ($b = 2.543$) with r^2 of 0.951. A similar observation of negative allometric growth was made along the Indonesian

coast. Nevertheless, positive allometric growth was recorded in the west and east coasts of Peninsular Malaysia [12]. Based on the Bayesian length-weight prediction by [13], only *S. leptolepis b* value was below the expected range (*b* = 2.5581), which may occur because of the low individual count or sample size collected for this species. The length-length relationship (LLR) for all species were highly significant ($P < 0.05$) with high coefficient of determination values ($r^2 > 0.95$) (Table 2). The linear regression for LLR was the best fit for *D. macrosoma* ($r^2 = 0.982$), and the lowest was recorded by *M. cordyla* with a coefficient of determination $r^2 = 0.951$.

Table 2 Comparison of length-length relationships parameters for four Carangidae species sampled in Kuala Selangor.

Species	**n**	**Equation**	***a***	***b***	**95% CI of *b***	***r²***
Carangoides malabaricus	62	TL = *a* + *b* SL	0.7931	1.1365	1.1035-1.1874	0.972
		SL = *a* + *b* TL	1.1672	0.9451	0.8063-0.9875	
Decapterus macrosoma	46	TL = *a* + *b* SL	-1.9403	1.2162	1.1880 – 1.2444	0.982
		SL = *a* + *b* TL	1.7431	0.8154	0.7965-0.8343	
Megalaspis cordyla	78	TL = *a* + *b* SL	2.0781	1.0146	0.95315-1.13014	0.951
		SL = *a* + *b* TL	-0.2561	0.9029	0.8262-0.9796	
Selaroides leptolepis	55	TL = *a* + *b* SL	-0.9976	1.2806	1.2500 – 1.3112	0.981
		SL = *a* + *b* TL	0.8657	0.775	0.7565 – 0.7935	

n, sample size; TL, total length; SL, standard length; *a*, intercept; *b*, slope coefficient; CI, confident interval, r^2, coefficient of determination.

Results obtained for the LWR and LLR in this study may be influences by several factors such as season, environmental condition, food availability, stomach fullness, gonad weight, sexes, and age group of the fish [14]. It can be said that the positive growth obtained for *D. macrosoma* showed that the study area might provide a favourable condition, or this species can tolerate more environmental changes as compared to the other three species. Although the negative growth pattern is displayed by *C. malabaricus*, *M. cordyla*, and *S. leptolepis*, the *b* value recorded for

this species is within the documented values of 2.5 to 3.5. Therefore, a larger sample size is essential to confirm these growth pattern values.

The overall Fulton's condition factor for all four species in the study area was greater than 1. The results are in agreement with other findings recorded in Indonesia and India. This indicates that the fishes are healthy, even though this value might be influenced by climate changes, food viability, feeding habits of the fish, and environmental factors [15]. Besides, this shows that the study area may be a good place for these fishes as food providers.

CONCLUSION

Generally, *Decapterus macrosoma* has faster growth than *Carangoides malabaricus*, *Megalaspis cordyla*, and *Selaroides leptolepis*. It can also be said that *D. macrosoma* grows heavier as it increases in length, and *C. malabaricus*, *M. cordyla*, and *S. leptolepis* become slenderer as it increases in length Kuala Selangor waters. The length-weight relationships (LWR) and length-length relationships (LLR) information of these fishes could be the baseline study or future references for the fishery management in Kuala Selangor. However, an upcoming study focusing on samples size, sex of the individuals, sampling season, and water parameters will give a better understanding of the recent findings' LWR and LLR variations.

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REFERENCES

1. Mohsin, A. K. M., & Ambak, M. A. (1996). *Marine fishes and fisheries of Malaysia and neighbouring countries*, Universiti Pertanian Malaysia Press, Serdang.
2. Petrakis, O. & Stergiou, K. I. (1995). Weight-length relationships for 33 fish species in Greek waters. *Fisheries Research,* 21, 465-469. https://doi.org//10.1016/0165-7836(94)00294-7
3. Ali, K., & Bahar, B. (2008). Length-weight relationship of the bouge *Boops boops* (Linneaus, 1758) in Izmir Bay (Aegean Sea of Turkey). *Belgian Journal of Zoology*, 138(2), 154-157. Retrieved from https://www.researchgate.net/
4. Abobi, S. M. 2015. Length-weight models and relative condition factors of nine (9) freshwater fish species form the Yapei Scretch of the White Volta, Gahana. *Elixir Applied Zoology*, 79, 30427-30431. Retrieved form https://www.elixirpublishers.com/index.php?route=articles/category&path=294_454

5. Froose, R. (2006). Cube law, condition factor and weight-length relationship: history, meta-analysis and recommendations. *Journal of Applied Ichthyology*, 22(4), 241-253. https://doi.org/10.1111/j.1439-0426.2006.00805.x
6. Matsunuma, M., Motomura, H., Matsuura, K., Shazili, N. A. M., Ambak, M. A. (2011). Fishes of Terengganu – east coast of Malay Peninsula, Malaysia. National Museum of Nature and Science, University Malaysia Terengganu and Kagoshima University Meseum.
7. Pauly, D. (1983). Some simple methods for the assessment of tropical fish stocks. FAO Fisheries Technical paper, (234), FAO, Rome, Italy.
8. Worthington, G. H. & Richardo, C. K. (1930). Scientific results of the Cambridge expedition to the East African lakes No15: the fish of Lake Rudolf and Lake Baningo. Zoological *Journal of the Linnean Society,* 267, 353-389. https://doi.org/10.1111/j.1096-3642.1936. tb00472.x
9. Akinyi, O. J., Sigana, D. A. O., Ondu, V. W., Wambiji, N., Ong'anda, H., & Orembo, B. (2018). Length-weight relationship of selected teleost fishes from Kilifi Country, Kenya. *WIO Journal of Marine Science*, 17(1), 125-135. Retrieved form https://www.ajol.info/index.php/wiojms/article/view/176417
10. Simon K. D., Moumita, D., & Mazlan A.G. 2014. Length-weight relationship and trophic level of hard-tail scad *Megalaspis cordyla*. *Science Asia*,*40*(5),317-322. https://doi.org/10.2306/scienceasia1513-1874.2014.40.317
11. Wang, X. H., Qiu, Y. S., Zhu, G. P., Du, Y. F., Sun, D. R., & Huang, S. L. (2011). Length-weight relationships of 69 fish species in the Beibu Gulf, northern South China Sea. *Journal of Applied Ichthyology*, 27, 959–961. https://doi.org/10.1111/j.1439-0426.2010.01624.x
12. Isa, M. M., Arshad, A. H., & Basir, S. (1997). Distribution, abundance and biological studies of economically important fishes in the South China Sea, Area I: East coast of Peninsular Malaysia. In Proceedings of the First Technical Seminar on Marine Fishery Resources Survey in the South China Sea, Area I: Gulf of Thailand and Peninsular Malaysia, 24-26 November 1997, Bangkok, Thailand (pp. 147-155). Samutprakan, Thailand: Training Department, Southeast Asian Fisheries Development Center. Retrieved from http://repository.seafdec.org/handle/20.500.12066/4310
13. Froese, R., Thorson, J., & Reyes Jr, R.B. (2014). A Bayesian approach for estimating length-weight relationships in fishes. *Journal of Applied. Ichthyology*, 30(1),78-85. https://doi.org/10.1111/jai.12299
14. Karakulak, F.S. Erk, H. & Bilgin, B. (2006). Length-weight relationships for 47 coastal fish species form the northern Aegen Sea, Turkey. *Journal of Applied Ichthyology*, 22, 274-278. https://doi.org/10.1111/j.1439-0426.2006.00736.x
15. Lambert, Y., & Dutil, J. (1997). Condition and energy reserves of Atlantic cod (*Gadus morhua*) during the collapse of the northern Gulf of St. Lawrence stock. *Canadian Journal of Fisheries and Aquatic Sciences,* 54 (10), 2388-2400. https://doi.org/10.1139/f97-145

The mtDNA D-loop Marker Identifies the Genetic Variability of Indochina's *Batagur affinis*

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Abstract

A population genetics study of the critically endangered Southern river terrapin (*Batagur affinis*) was carried out in Peninsular Malaysia, with a focus on four population regions: Pasir Gajah, Kemaman (KE), Terengganu; Bukit Pinang (BP), Kedah; Bota Kanan (BK), Perak; and Bukit Paloh, Kuala Berang (KB), Terengganu. There's the first report on *B. affinis* genetic diversity, phylogenetic relationship, and matrilineal hereditary structure in Malaysia. To assess the genetic heterogeneity of Malaysian Southern river terrapin in subspecies, we sequenced and analysed D-loop mitochondrial DNA (mtDNA) sequences in 120 samples from the Malaysia peninsula, East and West Coast. In *B. affinis*, 46 polymorphic sites have defined six haplotypes. The *B. a. edwardmolli* (Population 4) in Pasir Gajah, KB, Terengganu, has a single maternal lineage. By contrast, populations from KE, Terengganu, BP, Kedah, and BK, Perak have variations that distinguish them from the rest. Low genetic diversity was detected, with a significant genetic differentiation among populations**.**

Keywords: Southern river terrapin; Malaysia; four populations; low genetic diversity

INTRODUCTION

South Asian freshwater turtle *Batagur affinis* Cantor, 1847 (also known as the Southern river terrapin) is a freshwater turtle found in large rivers throughout the country. Furthermore, the terrapin can be found in large rivers throughout Southeast Asia, including Sumatra, Malaysia Peninsula, Singapore, Thailand, Vietnam, and Cambodia [1]. According to the organisation, [2] lists it as one of the world's 25 most in danger of extinction freshwater turtles and tortoises. Thus, it is classified as critically endangered by the International Union for Conservation of Nature (IUCN) [3]. Minor morphological differences, nesting ecology, colouration, three nuclear DNA markers, and three mitochondrial (mtDNA) were used to distinguish two

subspecies of *B. affinis* populations. The western nominate population (*B. affinis affinis*) and the eastern subspecies is *B. affinis edwardmollis* [1].

As a result, because no previous research on mtDNA D-loop sequence variability in Malaysian Southern river terrapins had been conducted, this work was precious in assessing the current genetic variety of *Batagur affinis* within two subspecies in Malaysia. Therefore, this conservation genomic analysis will assist decision-makers in making decisions about the conservation, utilisation, and exploitation of Indochina's *Batagur affinis* genetic resources.

MATERIALS AND METHODS

This study comprised 120 *Batagur affinis* individuals from four population regions crossing the East and West Malaysia Peninsula: Pasir Gajah, Kemaman (KE), Terengganu (4.2524° N, 103. 2957° E); Bukit Pinang (BP), Kedah (4.2221° N, 100.4370° E); Bota Kanan (BK), Perak (4.3489° N, 100.8802° E); and Bukit Paloh, Kuala Berang (KB), Terengganu (5.0939° N, 102.7821° E). A total of 30 individuals from the *B. affinis* population were sampled at each location. The field permit approval number is B-00335-16-20, which was rewarded by the Department of Wildlife and Parks, Peninsular Malaysia. Blood was drawn using two venipuncture techniques: the subcarapacial venous plexus (SVP) and the jugular vein.

We use 5'-TTTTTCCCCTAGCATATCACCA-3' (forward) and 5'-AGTTGCTCTCGGATTTAGGG-3 (reverse) primer set [4]. PCR amplification for gene D-loop fragments was carried out in a Go Taq Flexi PCR (Promega, Madison, USA) reaction mixture having 2 µL DNA template, 0.5 µL primer, 5 µL 5x PCR buffer, 2 µL x 25 mM MgCl2, 0.5 µL dNTP, 0.2 µL Taq DNA polymerase, and 14.3 µL distilled water (ddH2O). Initial denaturation at 94°C for 3 minutes was followed by 25 cycles of denaturation at 94°C for 35 seconds. Means while, the primer annealing at 60°C for 1 minute 30 seconds, extension at 72°C for 2 minutes, and a final extension at 72°C for 2 minutes. As a result, mtDNA D-loop sequences from various haplotypes were deposited in GenBank under the accession numbers from MZ555651 to MZ555656.

The ClustalW algorithm program by MEGA X [5] was used for multiple sequence alignment, and DnaSP 6.12.03 [6] summarised the haplotype distribution of the collected data. In addition, DnaSP version 6.12.03 was used to identify polymorphic sites (PS) or variable sites [7]. Finally, at the population level, Arlequin 3.5 was used to determine haplotype diversity (Hd) and nucleotide diversity (π) [8].

The mtDNA D-loop Sequence for both genes (*B. a. affinis* and *B. a. edwardmolli*) were analysed in MEGA X. Where's to produce phylogenetic trees with a confidence level of 1000 bootstrap replicates. Previously, the Model Test was conducted to determine the best model for tree construction using the identical MEGA X software.

The complete mitochondrial sequence of *Batagur affinis edwardmolli* (MN069309), *Batagur borneoensis* (HQ329672), *and Dermochelys coriacea* (KU883273) were selected from GenBank as the outgroup to construct the phylogenetic trees [4] [9] [10].

Phylogenetic analysis among populations was performed using MEGA X software, and the tree was built using the Maximum Parsimony method by [11] to determine the Maximum Parsimony (MP).

RESULTS AND DISCUSSION

Table 1 shows, six D-loop haplotypes were found, each definite by 46 Polymorphic Sites (PS) or substitutions number inside the 651 bp fragments. These PSs were composed of 43 transitions and three transversions at positions 1, 11, and 23. The Hd was low in each population, ranging from 0 to 0.53. The π was relatively low in all populations, ranging from = 0 to 0.012.

Table 1: Sample size, number of haplotypes, number of polymorphic sites, haplotype diversity and nucleotide diversity of different areas of *Batagur affinis*

Population*	No. of Haplotype	Sample Size	No. of Polymorphic Site (PS)	Haplotype diversity (Hd)	Nucleotide Diversity (π)
1	2	30	1	0.1287	0.0002
2	3	30	43	0.1908	0.0123
3	3	30	2	0.5356	0.0009
4	1	30	0	0.0000	0.0000

* 1: Kemaman, Terengganu (*B. a. edwardmolli*), 2: Bukit Pinang, Kedah (*B. a. affinis*), 3: Bota Kanan, Perak (*B. a. affinis*), 4: Kuala Berang, Terengganu (*B. a. edwardmolli*).

Thus, phylogenetic analysis of haplotypes strongly supported monophyletic status between *B. a. affinis* and *B. a. edwardmolli* (Fig. 1). The phylogenetic results further divided the *B. affinis* haplotypes into two groups (cluster I and cluster II). However, cluster I was supposed to be inhabited only by the Terengganu region (*B. a. edwardmolli*). But surprisingly, the *B. a. edwardmolli* was a minority population in Kedah, Malaysia, which found them together with H2 (*B. a. edwardmolli* KE09) and related to H4 (*B. a. affinis* B31). This means the *B. a. affinis* subspecies dominant in the Kedah and Perak region are present in cluster II (H3. H5 and H6) with significant bootstrap value.

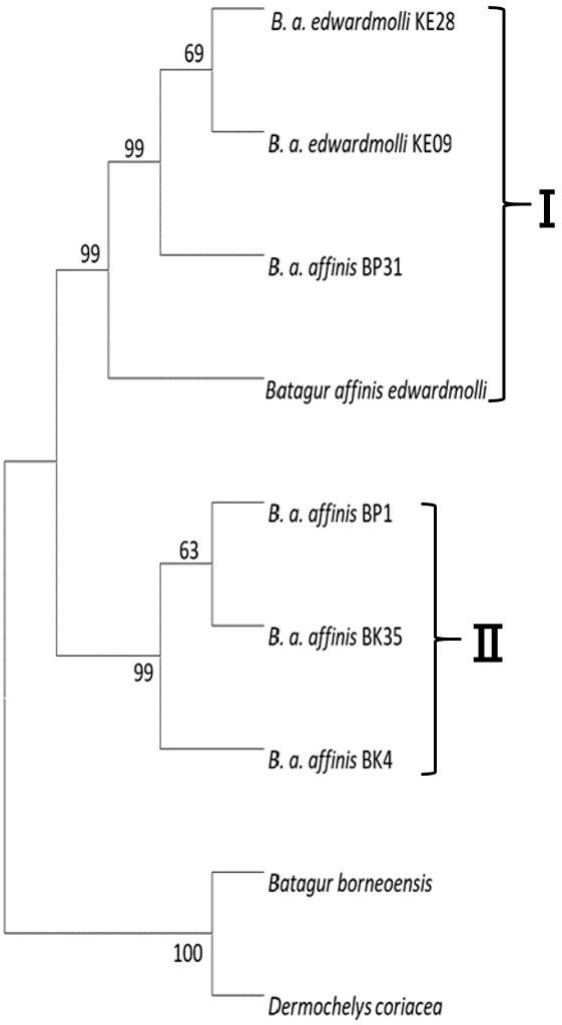

Figure 1: Maximum Parsimony (MP) tree showing the relationship between the D-loop haplotypes of *Batagur affinis*, *Batagur borneoensis* and *Dermochelys coriacea*. The number at each node represents the bootstrap value percentage based on 1000 pseudoreplication for the MP analysis.

Some haplotypes were shared between the regions, indicating that the same populations formed the regions. The six haplotypes observed in the terrapins' D-loop region (compared to [4]) are due to the large number of samples collected and the high substitution rate observed in the D-loop region. When compared to other *Batagur sp* populations, Hd is 0.214 on average. Similarly, the same 0.2 on Cambodian *B. a. edwardmolli* [4]. The current study found that low variability with a combination of low Hd and high π can indicate rapid demographic expansion from a small adequate population size [12].

CONCLUSION

Our study found low genetic variety and significant genetic differentiation among *B.affinis* populations. The findings of this study add to our understanding of the genetic status of Malaysian Southern river terrapin. This information was helpful for conservation programs and could be used to prioritise breeding plans towards sustainability.

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REFERENCES

1. Moll, E. O., Platt, S. G., Chan, E. H., Horne, B. D., Platt, K., Praschag, P., Chen, P. N., & Van dijk, P. P. (2015). *Batagur affinis* (Cantor 1847) - Southern River terrapin, Tuntong. *Chelonian Research Monographs*, 5(8), 090.1-17. https://doi.org/10.3854/crm.5.090.affinis.v1.2015
2. Turtle Conservation Coalition (2011). Turtles in trouble: the world's 25+ most endangered tortoises and freshwater turtles-2011. IUCN/SSC Tortoise and Freshwater Turtle Specialist Group, Turtle Conservation Fund, Turtle Survival Alliance, Turtle Conservancy, Chelonian Research Foundation, Conservation International, Wildlife Conservation Society, San Diego Zoo Global, Lunenburg, MA.
3. Horne, B. D., Chan, E. H., Platt, S. G., & Moll, E. O. (2016). *Batagur affinis*. https://doi.org/10.2305/IUCN.UK.2016-1.RLTS.T170501A13 15041.en.
4. Çilingir, F.G., Seah, A., Horne, B.D., Som, S., Bickford, D.P., & Rheindt, F. E. (2019). Last exit before the brink: Conservation genomics of the Cambodian population of the critically endangered southern river terrapin. *Ecology and Evolution, 9*(17), 9500-9510.
5. Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution, 35*(6), 1547.
6. Librado, P., & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics, 25*(11), 1451-1452.
7. Rozas, J., Sánchez-DelBarrio, J.C., Messeguer, X., & Rozas, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics, 19*(18), 2496-7.
8. Excoffier, L,, Laval, G., & Schneider, S. (2005). Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics, 1*, 117693430500100003.
9. Reid, B. N., Le, M., McCord, W. P., Iverson, J. B., Georges, A., Bergmann, T., Amato, G., Desalle, R., Naro-Macile, E. (2011). Comparing and combining distance-based and character-based approaches for barcoding turtles. *Molecular Ecology Resources, 11*(6), 956-967.

10. Vella N. & Vella A. (2016). The first genetic analyses of the leatherback turtle, *Dermochelys coriacea* from a stranding in central Mediterranean. Rapp. Comm. int. Mer Médit, 41.
11. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA 5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, *28*, 2731-2739.
12. Avise, J. C. (2000). Phylogeography: The history and formation of species. (pp. 447). Cambridge: Harvard University Press.

Knowledge on Threatened Species among Penan and Berawan Community in Gunung Mulu National Park, Sarawak, Malaysia

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ABSTRACT

Penan and Berawan indigenous communities have customary access rights to hunt animals and collect forest products at Gunung Mulu National Park (GMNP) to sustain their livelihood. However, their level of knowledge on the types of animals and plants that are allowed to be hunted or collected is a question that needs to be addressed. A poor knowledge of these threatened species might cause the extinction of these vulnerable species. Therefore, this study aims to examine the Penan and Berawan level of knowledge on threatened native animal and plant species in GMNP. A validated pictorial questionnaire was distributed to these 67 communities who lived at Kampung Sungai Melinau, Kampung Batu Bungan, Kampung Long Iman and Kampung Long Terawan using a face-to-face approach. They were asked to identify the native species that needed to be conserved. Most respondents (98.51%) had poor knowledge (mean score, 26.46%) about local threatened species even though they lived close to a UNESCO World Heritage Site. This may be due to lack of exposure to these species in the community. Thus, the findings of this study attempted to explore the situation from the bottom level which portrayed the lack of environmental awareness programmes being implemented in GMNP. It is suggested that the relevant stakeholders should emphasize on species knowledge among the community so that the existing policies can be enhanced and work towards biodiversity conservation.

Keywords: Endangered; knowledge; Mulu; biodiversity; IUCN; UNESCO

INTRODUCTION

Knowledge on species is critical for understanding biodiversity and the issues that surround it where it comprehends the interactions between species and their environment [1]. Familiarising oneself with species can help build a sense of connection to the natural environment [2]. The species can instil a sense of place and belonging in humans which indicates that it contributes to the authenticity of places and can help people develop a sense of attachment to their surroundings [3]. The relationship between sense of place, belonging and attachment should be cultivated in the Penan and Berawan who are indigenous to the World Heritage Site, Gunung Mulu National Park (GMNP) towards conserving biodiversity in the area. In contrast, low knowledge about the local environment might point to a lack of awareness on the importance of conserving animal and plant species which influences their well-being [4].

Traditional livelihoods of the Penan and Berawan communities rely on their customary access rights to hunt animals and collect forest products in GMNP. However, a lack of knowledge about these species could result in their extinction in the area. This could instigate the extinction of species in Mulu if the endangered category were hunted. Thus, the knowledge of threatened animals and plants species that must be conserved in GMNP, is a crucial aspect to be addressed. It is still not known whether these local communities are aware of the conservation status of such species. Therefore, this study aims to investigate the knowledge level of indigenous communities of Penan and Berawan on threatened species that require attention in GMNP.

MATERIALS AND METHODS

Research Area

This study was conducted in the settlement areas nearby GMNP, namely Kampung Sungai Melinau, Kampung Batu Bungan, Kampung Long Iman and Kampung Long Terawan, Mulu, Sarawak in March 2021. A validated pictorial questionnaire was distributed to 67 indigenous communities of Penan and Berawan through a face-to-face approach purposively. The respondents are aged more than 18 years old and have grown in the community.

Threatened Species Identification Test

Respondents were given the pictorial questionnaire and asked to identify which the most native animals that need to be conserved. The test consisted of 35 animals and 9 plant species photos with its common and scientific name is native to GMNP. the respondents were allowed to select any species to be saved in GMNP regards to their own knowledge to avoid biases. The scores were calculated based on the

percentage of the species they selected correctly according to the current IUCN Red List of Threatened Species categorization (Figure 1). In addition, the demographic background also was included in the section.

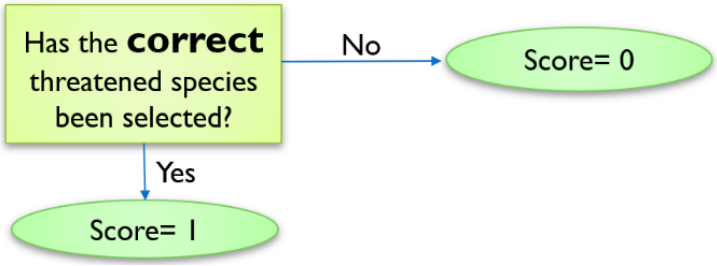

Figure 1 The basic codebook used for scoring.

Score Calculation per Individual

The number of correct identifications will indicate the threatened species knowledge levels by calculating the identification score per individual. According to IUCN (2021), there are three categories identified as threatened species with global extinction, namely Critically Endangered (CR), Endangered (EN) and Vulnerable (VU) [5]. Thus, the CR is the most threatened species that need to be conserved and should be given more attention in GMNP.

The score per individual was calculated as: -

$$\text{Individual's threatened species knowledge (\%)} = \frac{\text{Number of correct selections} \times 1}{\text{Total number of threatened species} \times 1} \times 100\%$$

Where, total number of threatened species (CR, EN, VU) is 22.

From the calculation, the value for an individual's threatened species knowledge is based on Bloom's cut off point, good (80%-100%), moderate (50%-79%) and poor (<50%) [6].

RESULTS AND DISCUSSION

Demographic Profile of Respondents

All respondents are Penan and Berawan, who aged more than 18 years old. Most of them are self-employed and engage in agriculture, fisheries, and tourism due to biodiversity resources benefits in the GMNP. The demographic profile of respondents is as presented in Table 1.

Table 1 Demographic profile of respondents.

Demographic background		**Frequency (n)**	**Percentage (%)**
Gender	Male	45	67.2
	Female	22	32.8
Ethnicity	Penan	22	32.8
	Berawan	45	67.2
Marital status	Married	47	70.1
	Single	19	28.4
	Others: Divorced	1	1.5
Age	19-25	6	9.0
	26-30	8	11.9
	31-39	14	20.9
	40-50	13	19.4
	Above 50	26	38.8
Education level	No formal education	7	10.4
	Primary school	16	23.9
	Secondary school	39	58.2
	Undergraduate (First degree, diploma, and certificate)	5	7.5
Income	Less than MYR2500	62	92.5
	More than MYR2500	5	7.5
Occupation	Government	4	6.0
	Private	14	20.9
	Self-employed	36	53.7
	Unemployed	9	13.4
	Student	2	3.0
	Retiree	2	3.0

Note: MYR1.00= USD 0.24

Knowledge on Threatened Species among Penan and Berawan Community

Table 2 shows the level of knowledge of respondents on endangered species which based on Bloom's cut off point. Most respondents (98.1%) have poor knowledge on threatened species with overall mean score, 26.46% even though they live close to UNESCO World Heritage Site, GMNP. Knowledge about species among laypeople is much lower than professionals as found in some studies [7]. This may be due to a lack of exposure to knowledge about species among them. In addition, respondents' knowledge about species is also further explained in Table 3. Most respondents chose pitcher plants namely *Nepenthes muluensis* and *N. lowii* as the most plant species that need to be saved. This species is less significant than other species due to its non-endangered status according to the IUCN Red List category.

However, *N. muluensis* is endemic to Borneo Island and cannot be found anywhere else which might be a reason why the respondents perceived it as one of the most plants that need to be conserved.

Table 2 Threatened species knowledge among the indigenous community of Penan and Berawan.

Level of knowledge	Score (%)	Number of respondents, (n)	Percentage of respondents (%)
Good	80-100	0	0
Moderate	50-79	1	1.49
Poor	0-49	66	98.51

Similar to *Buceros rhinoceros* or hornbill, most respondents chose this species as the most important animal to be conserved compared to the others in the GMNP. This might be because the species has become iconic to Sarawak Territory and perceived that the number of species is declining due to several threats such as poaching and the destruction of natural habitats. In addition, they are aware that *B. rhinoceros* is listed as totally protected animals in Sarawak and enacted under the Wildlife Protection Ordinance 1998. Respondents will possess good knowledge if all five topmost species selected are from the endangered (EN) category. However, there is only three chosen endangered species which ranked top three in this study.

Table 3 Five topmost species selected to be conserved in GMNP.

Species	IUCN Red List status	Number of respondents
*Buceros rhinoceros**	EN	50
Rhabdotorrhinus corrugatus	EN	47
Manis javanica	EN	44
Nepenthes muluensis	LC	44
Nepenthes lowii	VU	43

*Totally protected animals of Sarawak under the Wildlife Protection Ordinance 1998.

However, it is noted that the respondents stated that several species are not in the pictorial questionnaire, which is crucial to be conserved, including *Sus sucrofa* (common name: Wild boar), *Koompassia excelsa* (common name: tualang tree), *Amaurornis phoenicurus* (common name: white-breasted waterhen), *Gracula religiosa* (common name: common hill myna). Based on the IUCN Red List Category, most of these species are identified as not very significant to be

emphasized in the context of conservation due to its category that ranges from Not evaluated (NE) to Least concern (LC).

Based on this study, some respondents also mentioned that keruing trees (genus: Dipterocarpus) also need to be conserved in this GMNP. However, they did not specify any particular species for the keruing tree which indicates that they still know its conservation value. Keruing tree consist of several species that have different conservation status in Malaysia. However, in GMNP, the native keruing species is *Vatica maingayi* (common name: Keruing Babi) which has Critically endangered (CR) status according to IUCN [8], while endangered (EN) according to Sarawak Plant Red List [9].

CONCLUSION

The Penan and Berawan communities have poor knowledge of threatened species in GMNPthat would instigate the extinction of these animals and plants if not taken into account by relevant stakeholders in overcoming the awareness issue that could lead to the severe hunting of these endangered species. GMNP, through the cooperation of Sarawak Forestry Corporation and Borsarmulu Park Management, can enhance engagement with local communities by organizing environmental programmes to improve their species literacy, particularly on the IUCN Red List. In addition, it can improve communication and education strategies about native biodiversity that are consistent with prior knowledge. To engage people in biodiversity conservation and garner public support, it has been recognized that increasing public awareness of biodiversity is a good first step.

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REFERENCES

1. Somaweera, R., Somaweera, N., Shine, R. (2010). Frogs under friendly fire: how accurately can the general public recognize invasive species? *Biological Conservation*,143(6),1477-1484. https://doi.org/10.1016/j.biocon.2010.03.027.
2. Cox, D.T.C., Gaston, K.J. (2015). Likeability of garden birds: importance of species knowledge & richness in connecting people to nature. PLoS One 10 (11), 1–14. https://doi.org/10.1371/journal.pone.0141505
3. Standish, R.J., Hobbs, R.J., Miller, J.R. (2013). Improving city life: options for ecological restoration in urban landscapes and how these might influence interactions between people and nature. Landscape Ecology, 28 (6), 1213–1221.https://doi.org/10.1007/s10980-012-9752-1.
4. Louv, R. (2005). Last Child in the Woods: Saving Our Children from Nature-deficit Disorder. Atlantic Books, London.
5. IUCN. (2021). *IUCN Red List of Threatened Species*. International Union for Conservation of Nature and Natural Resources. https://www.iucn.org/resources/conservation-tools/iucn-red-list-threatened-species
6. Seid, M. A., & Hussen, M. S. (2018). Knowledge and attitude towards antimicrobial resistance among final year undergraduate paramedical students at University of Gondar, Ethiopia. *BMC Infectious Diseases*, *18*(1), 1–8. https://doi.org/10.1186/s12879-018-3199-1
7. Hooykaas, M. J. D., Schilthuizen, M., Aten, C., Hemelaar, E. M., Albers, C. J., & Smeets, I. (2019). Identification skills in biodiversity professionals and laypeople: A gap in species literacy. *Biological Conservation*, *238*(February), 108202. https://doi.org/10.1016/j.biocon.2019.108202
8. Ashton, P. (1998). *Vatica maingayi*. The IUCN Red List of Threatened Species 1998: e.T33149A9762739. https://dx.doi.org/10.2305/IUCN.UK.1998.RLTS.T33149A9762739.en
9. Julaihi, L.C.J., Julia, S. & Vilma, B. (2016). *Sarawak Plant Red List Dipterocarpaceae: Series II: Anisoptera, Cotylelobium, Hopea, Parashorea, Upuna & Vatica*. Sarawak Forestry Corporation Sdn. Bhd., Malaysia. pp. 105.

Utilization of Indigenous Food Aroma Enhancing Plants Among the Community in Bintulu, Sarawak

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ABSTRACT

Sense of aroma essentially improves the perceptions of the consumers towards the food. The aroma can stimulate the appetite and interact with the flavour complex to provide a palatability experience. The use of natural sources as aroma enhancers, especially indigenous plants, has been significantly practised from earlier generations. Still, some plants have not been commercialized and have rarely been consumed by the community recently. The present study aims to identify the plants used as food aroma enhancers and determine their consumption method. In order to record the plants' utilization, an ethnobotanical study was conducted around the Bintulu area. Face-to-face interviews with 136 respondents were conducted in a semi-structured manner from December 2020 to February 2021. A total of 16 species from 12 different families. Leaves were the dominant part used by the community for aroma enhancing purposes. The majority of the plants used were to help cut the foul smell from the protein ingredient. However, the mode of cooking varies based on the traditional knowledge passed down by the elders. This study is able to record outcomes about the indigenous food aroma enhancer plants, which can be a reference in the future. In addition, other communities can be introduced with the food aroma enhancer as an alternative in their cooking and open the commercialization chances for indigenous plants to be widely known.

Keywords: Ethnobotany; fragrance; food plant; traditional knowledge

INTRODUCTION

Over time, the utilization of indigenous plants in cooking has become more diverse. In culinary, indigenous plants can act as flavouring agents, food additives, colouring agents and medicine [5]. Indigenous plants that help to enhance the flavour and aroma of a dish are usually called spices or herbs by the natives. Flavour is divided into two aspects which are taste and aroma [1]. Aroma is an important aspect of the food industry as the volatile compound produced by plants stimulates the taste receptor and gives positive signals that enhance the human palate. The traditional knowledge of aroma enhancers in food, medicine, and food preservatives is usually

kept by the natives and is less known by other communities. This knowledge also seems to decline in Sarawak as the younger generation relies more on artificial aroma enhancers. In order to gain knowledge about the use of natural aroma enhancers from plant sources, this study was designed to obtain preliminary information on the use of plants as aroma enhancers by local communities especially in Bintulu, Sarawak. Thus, the objectives of this study were to identify food aroma enhancing plants used by local people in Bintulu, Sarawak and determine the consumption methods of food aroma enhancing plants used by the local people.

MATERIALS AND METHODS

An ethnobotanical study was conducted around the local farmers market ("Tamu') and various native settlement areas in Bintulu Division. 136 respondents participated in this field survey conducted in semi-structured form from November 2020 until May 2021. Information was collected based on the respondents' answers to the survey questionnaire. Questionnaires consisted of three sections: personnel information, basic information about the plants, and the potential of the plant species as food aroma enhancers. Each respondent was briefed prior to filling out the questionnaire. Due to the selective and simplest method of choosing a sample, the random sampling method is easy to use by giving all units the same probability of selection [7]. The species identification was conducted immediately after the survey with assistance from the survey respondents via a transect survey and a brief interview session. Plant species were identified and described based on morphology comparison and description [2,3,4,6]. The utilization of plants as food aroma enhancers was analysed using the Statistical Package for Social Science (SPSS), IBM ®V22.0 Software. Data was analysed descriptively to show frequency.

RESULTS AND DISCUSSION

About half of the respondents (47.06%) consists of Iban communities, followed by the Melanau communities (18.36%), Malay community (12.50%) and Kenyah (11.03%), whereas other ethnicities comprised less than 10.00% (Figure 1). Generally, Iban communities residing in rural areas have easy access to forest and wild areas. The result showed that female respondents (61.03%) had more knowledge of food flavour and the aromatic plant consumed daily than male respondents (38.97%).

In this study, 16 species from 11 different families were identified as indigenous food aroma enhancers used by the community in Bintulu (Table 1). Zingiberaceae are the biggest families, which are represented by four species. Followed by Poaceae and Lamiaceae with two species, Myrtaceae, Rutaceae, Achariaceae, Pandanaceae, Polygonaceae, Maranthaceae, Olacaceae, Apiaceae Umbilliferae, and Liliaceae represented by one species only.

Table 1 Food aroma enhancer plants used by the local people in Bintulu, Sarawak.

No.	Scientific name	Family	Local name	Edible part	Mode of consumption
1.	*Alpinia galanga* (L.) Willd.	Zingiberaceae	Lengkuas	Rhizome	Mixed with ginger during margination of chicken or fish for “pansuh” dish.
2.	*Apium graveolens var. secalinum* Alef	Umbilliferae	Daun sup	Young leaves	Chopped and added to chicken or vegetable soup.
3.	*Coriandrum sativum* L.	Apiaceae	Ketumbar	Young leaves	Chopped and added to chicken or meat dishes.
4.	*Curcuma longa* L.	Zingiberaceae	Kunyit	Rhizome	Blend together with onion, lemongrass, chillies, garlic and cooked together to make “masak lemak daging cili padi”.
5.	*Cymbopogon citratus* (DC.) Stapf	Poaceae	Lemongrass	Stem (tiller)	Blend to make herbal juice for mother after childbirth. Pound and cooked together with chicken or fish dishes.
6.	*Cymbopogon nardus (*L.) Rendle	Poaceae	Serai wangi	Stem (tiller)	Pound or chop with seafood or chicken to make tom yam dish.
7.	*Etlingera coccinea* (Blume) S.Sakai & Nagam	Zingiberaceae	Tepus	Pith	Pound or chop the pith and mixed together during marination of chicken or fish for “pansuh” dish.

8.	*Etlingera elatior (*Jack) R.M. Sm*.*	Zingiberaceae	Kantan	Flower; shoots	Used during marination of “pansuh” dish. Mix together with onion, anchovies, and chillies as “kerabu”.
9.	*Murraya koenigii* (L.) Spreng	Rutaceae	Daun kari	Young leaves	Added together in chicken, meat, or fish curry.
10.	*Ocimum basilicum* L.	Lamiaceae	Kemangi	Young leaves	Slice and add into dishes to eliminate the foul aroma of protein source.
11.	*Pandanus odorus* Roxb.	Pandanaceae	Pandan	Young leaves	The leaves were washed, tied and boiled together to get extract for making dessert.
12.	*Pangium edule* Reinw	Achariaceae	Kepayang	Young leaves	The young leaves were chopped and fermented with fish or meat as ‘pekasam’.
13.	*Persicaria odorata* (Lour.) Sojak	Polygonaceae	Kesum	Young leaves	Leaves were washed and stewed together with fishes in sour spicy gravy.
14.	*Phacelophrynium maximum* Blume	Maranthaceae	Lung/isip/itip	Young leaves	The leaves used to wrap the mashed rice called ‘nubaq layaq’
15.	*Scorodocarpus borneensis* Becc.	Olacaceae	Kesinduk	Young leaves	Stir-fry with onion, chillies, and garlic for fish or chicken dishes also for soup dishes.
16.	*Syzgium polyanthum* (Wight) Walp.	Myrtaceae	Bungkang	Young leaves	Added into soup or ‘pansuh’ to eliminate the foul smell of chicken or fish.

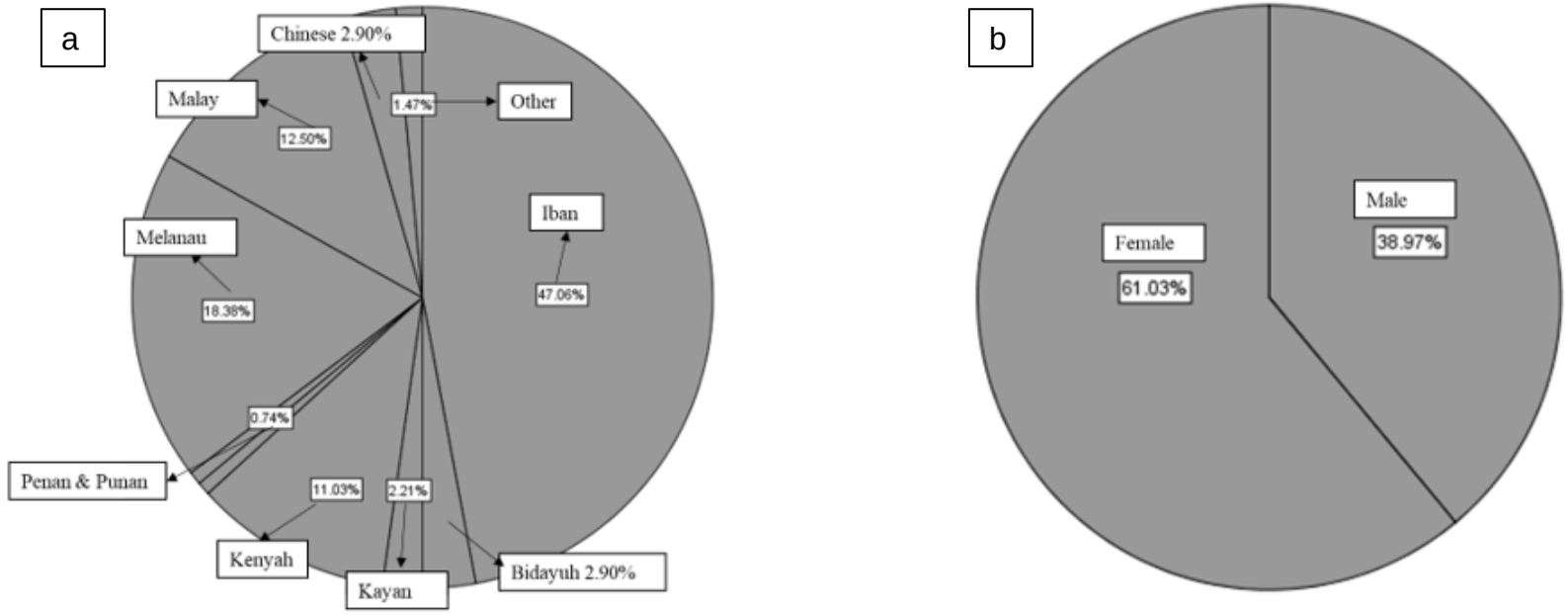

Figure 2 Demographic of local people in Bintulu, Sarawak: a) ethnic and b) gender.

CONCLUSION

All species identified in this study possessed different modes of consumption in the cuisine of Bintulu local people. It was recommended to study the volatile compound that is responsible for enhancing food aroma inside the species in the future.

REFERENCES

1. Belitz, H. -D., Grosch, W. & Schieberle, P. (2009). Aroma compounds In *Food Chemistry* (pp. 340-400), Springer, Berlin, Germany.
2. Det. P.A., Lau, C.Y., Umar, S., Brooke, P., Razili, R.M., Ismawi, H. & Liew, S.M. (2013). Edible Wild Plants in Sarawak. Kuching: Department of Agriculture Sarawak.
3. Ismail, S. (2000). Sayuran Tradisional Ulam dan Penyedap Rasa (pp.11-18). Penerbit Universiti Kebangsaan Malaysia, Bangi.
4. Jansen, P.C.M., Lemmens, R.H.M.J. & Oyen, L.P.A. (1993). Plant Resources of South-East Asia: Basic List of Species and Commodity Grouping. Pudoc, Wageningen. (pp.372).
5. Kunnumakkara, A. B., Koca, C., Dey, S., Gehlot, P., Yodkeeree, S., Danda, D., Sung, B. & Aggarwal, B. B. (2009). Traditional uses of spices: an overview. *Molecular Targets and Therapeutic Uses of Spices*. 1-25.
6. Rukayah, A. (2000). Ulam dan Sayuran Tempatan Semenanjung Malaysia, Dewan Bahasa dan Pustaka, Kuala Lumpur. (Malay).
7. Saupi, N., Ainul Asyira, S., Zakaria, M. H., Sarbini, S. R. & Yusli, N. A. (2020). An ethnobotanical study of indigenous leafy vegetables among local communities in Bintulu, Sarawak, Malaysia. *Borneo Journal of Resource Science and Technology,* 10(2): 155-165.

Effect of Photoperiod Regimes on The Cultivation of *Nannochloropsis* sp. in Palm Oil Mill Effluent for Lipid Production

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ABSTRACT

Microalgae require a photoperiod (light:dark) regime for effective photosynthesis. Photoperiod regimes affect the development and metabolic processes of microalgae. The purpose of this research was to investigate the impacts of continuous illumination vs. photoperiod conditions on growth and biomass content in *Nannochloropsis* sp., with an emphasis on bioactive components such as lipids. The impacts of photoperiod regimes on the development of *Nannochloropsis* sp. were investigated to maximize microalgal output in massive culture systems and lipid content. *Nannochloropsis* sp. was grown aseptically in 10% POME for 14 days under four different photoperiod regimes (24:00, 18:06, 12:12, and 06:08 h light:dark). The findings revealed that varying photoperiods had a significant impact on *Nannochloropsis* sp. growth and lipid accumulation. *Nannochloropsis* sp. showed improved cell growth under 18:06 h light: dark after 14 days of flask cultivation, with the highest dry biomass of 0.712 g L^{-1} and recovered 29.5% lipid. By altering the photoperiod, this work shows an alternative strategy to stimulate the development and intracellular lipid accumulation of *Nannochloropsis* sp.

Keywords: Microalgae; *Nannochloropsis* sp.; POME; photoperiod; growth; lipid

INTRODUCTION

Nannochloropsis sp. has a lot of potential as a source of bioactive components because it grows rapidly and is easy to cultivate even in hostile environments [1]. *Nannochloropsis* sp. has a higher lipid content (of DW) than most other microalgal strains, confirming its superiority [2]. Nonetheless, cultivating *Nannochloropsis* sp.

for the production of high-value bioactive components like lipid is expensive as it needs a massive amount of nutrients and water. As to tackle this issue, wastewater sources such as palm oil mill effluent (POME) which was found to hold a large concentration of carbohydrates, proteins, nutrients (nitrogen and phosphorus), lipids, and minerals, making it the most excellent raw material for the cultivation of *Nannochloropsis* sp. At the same time, *Nannochloropsis* sp. cultivation in POME can treat the POME by reducing the organic load of effluent owing to metabolism and microalgae uptake of wastewater components [3].

However, various environmental factors including pH, temperature, salinity, and light influence the lipid profiles of *Nannochloropsis* sp. Among these, light is the fundamental source of energy and a crucial element in photosynthesis, and it is required for the growth of unicellular microalgae [4]. According to Khoeyi et al. [5], the amount of energy available for photosynthetic organisms to carry out their metabolic activities is controlled by the quantity and quality of light. Photosynthesis is categorized into two: a light-dependent photochemical phase and a light-independent biochemical dark phase [6]. The components created in the light-dependent phase namely ATP and NADPH are utilized to produce metabolic compounds necessary for growth in the dark phase [7]. The duration of the photoperiod influences the growth and biorhythm of photosynthesis in algal cells, which varies from species to species [8]. Algal growth, reproduction, and lipid deposition are all influenced by the photoperiod, or light:dark (L:D) cycle [9]. Hence, the concept of this research is to evaluate the impacts of photoperiod regimes on the development and lipid accumulation of *Nannochloropsis* sp. cultivated in 10% POME.

MATERIALS AND METHODS

Microalgae Sample Collection, Isolation, and Identification

Microalgae samples were collected from Pantai Teluk Cempedak (3°55’33’’N, 103°22’23”E), Kuantan, Pahang, Malaysia. A 0.5 µm plankton net made of bolting silk cloth was used to collect the microalgae sample. A sterile inoculation loop was dipped into the sample and streaked over the Conway medium agar plate. Individual cluster cells were examined under a fluorescent microscope for distinguishing traits. With the use of standard manuals, the isolated microalgae were recognized. Out of a total of six microalgae species isolated and discovered, *Nannochloropsis* sp. was chosen due to its ability to develop quickly.

Nannochloropsis sp. Pre-cultivation

The microalga was pre-cultured until it reached the stationary growth phase in a 250 mL Erlenmeyer flask containing 100 mL Conway media. To begin growth, 100 mL of this pure culture was inoculated in a 1 L Erlenmeyer flask containing 500 mL of

fresh Conway medium. Before scaling up, the culture was allowed to multiply and was monitored regularly. The microalgae cultures were illuminated with fluorescent light at an intensity of light of 15 mol m^{-2} s^{-2}, with continuous sterile aeration.

Cultivation of *Nannochloropsis* sp. Under Different Photoperiod Regimes

Pre-cultured *Nannochloropsis* sp. in exponential phase was employed as inoculum. The inoculum was cultured into a 2 L flask with a working volume of 1 L containing 10% POME (v/v). Four distinct photoperiod regimes (24:00, 18:06, 06:18 and 12:12 h L:D) were used. As for the dark phase, the microalgal culture flasks were wrapped in aluminium foil and kept in dark enclosed boxes. The algae cultures were aerated with sterile-filtered air continuously and maintained at 25±2 °C. The initial optical density (OD) value of the cells was within the range of 0.469-0.475 for all treatments. Each treatment was carried out in triplicate.

Nannochloropsis sp. Growth Determination

The growth of *Nannochloropsis* sp. was evaluated in terms of OD and dry biomass weight (DCW). The OD of *Nannochloropsis* sp. culture was determined using a spectrophotometer at a wavelength of 680 nm. Every three days for 15 days, the DCW was determined gravimetrically. Aliquots of microalgae culture were collected regularly, and the samples were centrifuged for 10 minutes at 6,000 r.p.m. The pellet was retrieved and placed in a dried and pre-weighed glass petri dish. The samples were dried overnight at 60 °C before being weighed. The dry weights were expressed as g L^{-1}.

Determination of Total Lipid

Cells were harvested using a centrifuge (Refrigerated Centrifuge 5810R) at 6500 rpm for 15 minutes, then washed twice with distilled water. Cell biomass was then dried using a freeze dryer to a constant weight. A solvent-based approach was used to extract the lipid of *Nannochloropsis* sp. The total lipid content was calculated as:

$$Lipid\ yield\ (\%) = \frac{Weight\ of\ extracted\ lipid}{Weight\ of\ dry\ biomass} \times 100 \qquad (1)$$

Analysis of Lipid Composition

Extracted lipids were transformed into fatty acid methyl esters (FAME) via the transesterification method. Gas chromatography-mass spectroscopy (GC-MS) was employed to examine FAME.

RESULTS AND DISCUSSION

Nannochloropsis sp. Isolation and Identification

Six microalgae species, including *Chlorella* sp., *Amphora* sp., *Gyrosigma* sp., *Tetraselmis* sp., *Spirulina* sp., and *Nannochloropsis* sp., were discovered and traced down in Teluk Cempedak, Kuantan, Pahang, Malaysia. *Nannochloropsis* sp. had the best visibility and growth during the preliminary cultivation when compared to other microalgae. *Nannochloropsis* sp. was selected as a potential oleaginous model microalga for this study since it has a greater lipid content than other microalgal strains. Figure 1 shows the identified *Nannochloropsis* sp. microalgae strain under a fluorescent microscope.

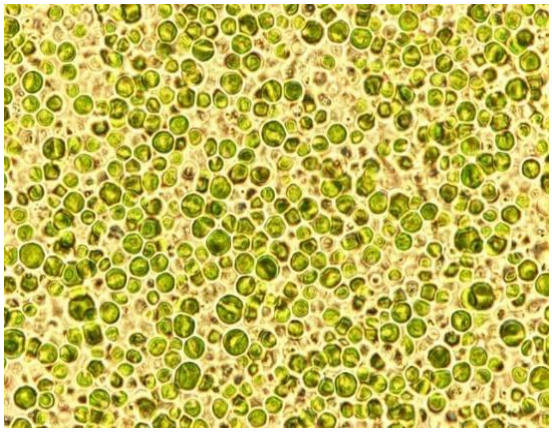

Figure 1 Identified *Nannochloropsis* sp. microalgae strain.

Cultivation of *Nannochloropsis* sp. Under Different Photoperiod Regimes

Figure 2 shows a graph that has been interpreted from the absorbance of the *Nannochloropsis* sp. that was being cultivated in 10% POME under 24:00, 18:06, 12:12, and 06:18 h (L:D) at 680 nm from day 0 to 14. The cell concentration of *Nannochloropsis* sp. in all the cultures yielded an OD value in the range of 0.469-0.475 at inoculation (day 0). During cultivation, the cultures went through four distinct stages: lag, exponential, stationary, and death.

Although all photoperiods demonstrated positive growth, the 18:06 h (L:D) cycle exhibited great growth compared to 24:00, 12:12, and 06:18 h (L:D). The maximal OD, DCW, and lipid content of 1.221, 0.712±0.023 g L^{-1}, and 29.5% respectively were achieved under 18:06 h (L:D) cycle, demonstrating that a drop in light durations from 24:00 to 18:06 h (L:D) had a positive influence on the production of biomass and lipid accumulation. This is because, continuous illumination caused photo-inhibition, which inhibits ongoing biomass development as surplus light is unable to be captured into the photosynthetic system [5]. This finding was in line with the findings of [6] and [10], who found that *Nannochloropsis* sp. and *Isochrysis galbana*

respectively generated higher cell densities under an 18:6 h (L:D) cycle than under 24:0 and 12:12 h (L:D) cycles. However, when compared to the lipid content reported by [6] the lipid content acquired in this study was 1.8% lower.

The culture subjected under the 06:18 h (L:D) cycle, on the other hand, showed the least growth. This is because the *Nannochloropsis* sp. cells were subjected to a long period of darkness, causing photo-limitation, which occurs when cells are exposed to insufficient light. As a result, *Nannochloropsis* sp. cells cannot store enough energy to keep them going during extended periods of darkness [11]. Even though dark is required for the biochemical stage to produce vital compounds for development [12] yet Shin et al. [13], believe that a longer dark period will slow down photosynthetic activity.

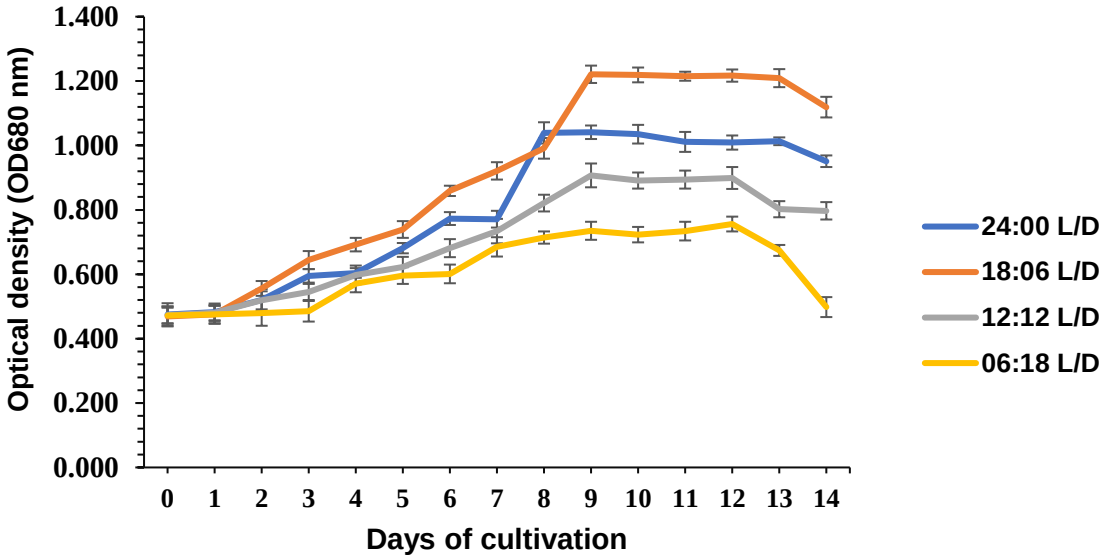

Figure 2 *Nannochloropsis* sp. growth curve cultivated under 24:00, 18:06, 12:12, and 06:18 h L:D.

Analysis of Fatty Acid Methyl Ester (FAME)

GC-MS was used to evaluate the fatty acid content of *Nannochloropsis* sp. using the lipid extract obtained from biomass exposed to 18:06 h L:D. The most prevalent fatty acids found in *Nannochloropsis* sp. were oleic acid (C18:1), linoleic acid (C18:3), palmitic acid (C16:0), and stearic acid (C18:3). The most common fatty acids detected in microalgal lipid acid are oleic acid, linoleic acid, and palmitic acid, according to a prior study [14].

CONCLUSION

The light conditions employed in this experiment had a profound impact on the development and lipid content of *Nannochloropsis* sp. The rate of microalgae growth can be enhanced or reduced based on the length of time they are exposed to light. This finding showed that an 18:06 h L:D light regime favoured growth and lipid accumulation in *Nannochloropsis* sp.

ACKNOWLEDGEMENT

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REFERENCES

1. Govindan, N., Maniam, G. P., Yusoff, M. M., Mohd, M. H., Chatsungnoen, T., Ramaraj, R., & Chisti, Y. (2020). Statistical optimization of lipid production by the diatom Gyrosigma sp. grown in industrial wastewater. *Journal of Applied Phycology*, *32*(1), 375-387. https://doi.org/10.1007/s10811-019-01971-x
2. Paramasivam, P., Palanisamy, K. M., Jayakumar, S., Govindan, N., Rahim, M. H. A., & Maniam, G. P. (2021). Lipid production from *Nannochloropsis* sp . grown in palm oil mill effluent. *International Conference of Sustainable Earth Resources Engineering 2020*, *641*, 2–9. https://doi.org/10.1088/1755-1315/641/1/012021
3. Palanisamy, K. M., Paramasivam, P., Maniam, G. P., Rahim, M. H. A., Ramaraj, R., & Govindan, N. (2019). Palm oil mill effluent as a potential medium for microalgae *Chlorella* sp. cultivation for lipid production. *Maejo International Journal of Energy and Environmental Communication*, *1*, 1–7.
4. Jayakumar, S., Yusoff, M. M., Rahim, M. H. A., Maniam, G. P., & Govindan, N. (2017). The prospect of microalgal biodiesel using agro-industrial and industrial wastes in Malaysia. *Renewable and Sustainable Energy Reviews*, *72*, 33–47. https://doi.org/10.1016/j.rser.2017.01.002
5. Khoeyi, Z. A., Seyfabadi, J., & Ramezanpour, Z. (2012). Effect of light intensity and photoperiod on biomass and fatty acid composition of the microalgae, *Chlorella vulgaris*. *Aquaculture International*, *20*(1), 41–49. https://doi.org/10.1007/s10499-011-9440-1
6. Wahidin, S., Idris, A., & Shaleh, S. R. M. (2013). The influence of light intensity and photoperiod on the growth and lipid content of microalgae *Nannochloropsis* sp. *Bioresource Technology*, *129*, 7–11. https://doi.org/10.1016/j.biortech.2012.11.032
7. Al-Qasmi, M., Raut, N., Talebi, S., Al-Rajhi, S., & Al-Barwani, T. (2012). A review of effect of light on microalgae growth. *Proceedings of the World Congress on Engineering*, *1*(2), 1–7. https://doi.org/10.1002/9781118608760.ch8
8. Krzemińska, I., Pawlik-Skowrońska, B., Trzcińska, M., & Tys, J. (2014). Influence of photoperiods on the growth rate and biomass productivity of green microalgae. *Bioprocess and Biosystems Engineering*, *37*(4), 735–741. https://doi.org/10.1007/s00449-013-1044-x
9. Babuskin, S., Radhakrishnan, K., Babu, P. A. S., Sivarajan, M., & Sukumar, M. (2014).

Effect of photoperiod, light intensity and carbon sources on biomass and lipid productivities of *Isochrysis galbana*. *Biotechnology Letters*, *36*(8), 1653–1660. https://doi.org/10.1007/s10529-014-1517-2

10. Fakhri, M., Sanudi, Arifin, N. B., Ekawati, A. W., Yuniarti, A., & Hariati, A. M. (2017). Effect of photoperiod regimes on growth, biomass and pigment content of *Nannochloropsis* sp. BJ17. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences*, *19*(2), 263–267.
11. Han, S., Jin, W., Abomohra, A. E., Tu, R., Zhou, X., He, Z., & Chen, C. (2019). Municipal wastewater enriched with trace metals for enhanced lipid production of the biodiesel-promising microalga *Scenedesmus obliquus*. *BioEnergy Research*,*12,* 1127–1133. https://doi.org/10.1007/s12155-019-10042-5
12. Ponraj, M., & Din, M. F. M. (2013). Effect of light/dark cycle on biomass and lipid productivity by *Chlorella pyrenoidosa* using Palm Oil Mill Effluent (POME). *Journal of Scientific and Industrial Research*, *72*(11), 703–706.
13. Shin, H. Y., Ryu, J. H., Bae, S. Y., Crofcheck, C., & Crocker, M. (2014). Lipid extraction from *Scenedesmus* sp. microalgae for biodiesel production using hot compressed hexane. *Fuel*, *130*, 66–69. https://doi.org/10.1016/j.fuel.2014.04.023

Genetic Diversity of Crystal Longan Fruit (*Pometia pinnata* J.R. Forst & G. Forst) in Bintulu

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ABSTRACT

Pometia pinnata, also known as Crystal longan fruit, is an underutilized species of the Sapindaceae family. This species is not commonly known internationally but extremely popular among the local people of Sarawak. However, limited scientific studies have been reported on this species, including their genetic diversity. Therefore, the present study aims to evaluate the genetic diversity of *P. pinnata* at Bintulu by morphology and molecular approaches. Five genotypes of *P. pinnata*, medium purple, big purple, red, rainbow, and green, were recorded during the field survey. Observation through the morphological approach indicated no significant differences (p<0.05) among the vegetative and floral characteristics among the genotypes. However, the fruit variables were varied among genotypes. A maximum parsimony tree generated two major clades of *P. pinnata* accessions grouped well with the reference sequences. Green genotypes were arranged in a single sub-clade while medium and big purple, rainbow and red genotypes were arranged separately into different subclades. Under this subclade, the big purple genotype was distantly arranged compared to the medium purple, red and rainbow. Proper classification of *P. pinnata* genotypes is important for future breeding work and conservation.

Keywords: ITS primer; Kasai; Matoa; Phylogenetic; Sapindaceae

INTRODUCTION

The Sapindaceae are the plants in the order Sapindales commonly produce arillate fruit, with sweet aromatic, juicy flesh that can be eaten fresh or developed as processed products [1]. The family comprises 1900 species, with the world's most known species of lychee and longan. *Pometia pinnata,* also known as Crystal longan, Kasai or Matoa, is an underutilized species of this family and popular among the local people of Sarawak [2]. Based on the observation, there are five genotypes of *P. pinnata* commonly found at local markets at Bintulu and fruit farms that are medium purple, big purple, red, rainbow and green. This species has considerable confusion concerning the taxonomy due to external fruit colouration [3-4]. To date, no published data available on the genetic diversity of *P. pinnata* genus presence in

Sarawak. Therefore, the present study aims to evaluate the genetic diversity of *P. pinnata* at Bintulu through morphology and molecular approach.

MATERIALS AND METHODS

The sampling was conducted randomly at 40 locations in Bintulu, Sarawak. Thirty-one (43) quantitative and (27) qualitative data on the leaves, stems, flowers, fruits and seeds were measured. Genomic DNA was extracted based on the standard procedure by NucleoSpin® Plant II. The PCR amplification was carried out using ITS primer sets following the optimized protocol by Ramaiya et al. [5].

RESULTS AND DISCUSSION

Generally, the vegetative and flower variables were not significantly different (p<0.05) between the genotypes. However, the distinct variation was recorded in the fruit parameters. The leaves were unipinnate and varied from oblique to oblongolate to elliptical shape. The plant, suitable to be planted as landscape trees as it is very attractive with new flush, were very colorful from orange, red to light green and the matured leaves were in dark green (Figure 1). Based on the observation the flower of *P. pinnata* are monecious where both the bisexual flower, dichogamy and male flower with rudimentary ovary presence in the same inflorescence. Proportionally there were 2 or 3 matured male flowers with 1 or 2 matured female flowers on the single pedicel. Based on the observation of the fruits of *P. pinnata* the fruits were globose to ellipsoid shape. Upon ripening, fruits of *P. pinnata* genotypes possessed green, red, and purple exocarp colours. The PCA results studied *P. pinnata* genotypes into three main clusters. The purple big genotypes were highly correlated with rind weight, pulp and seed weight and size.

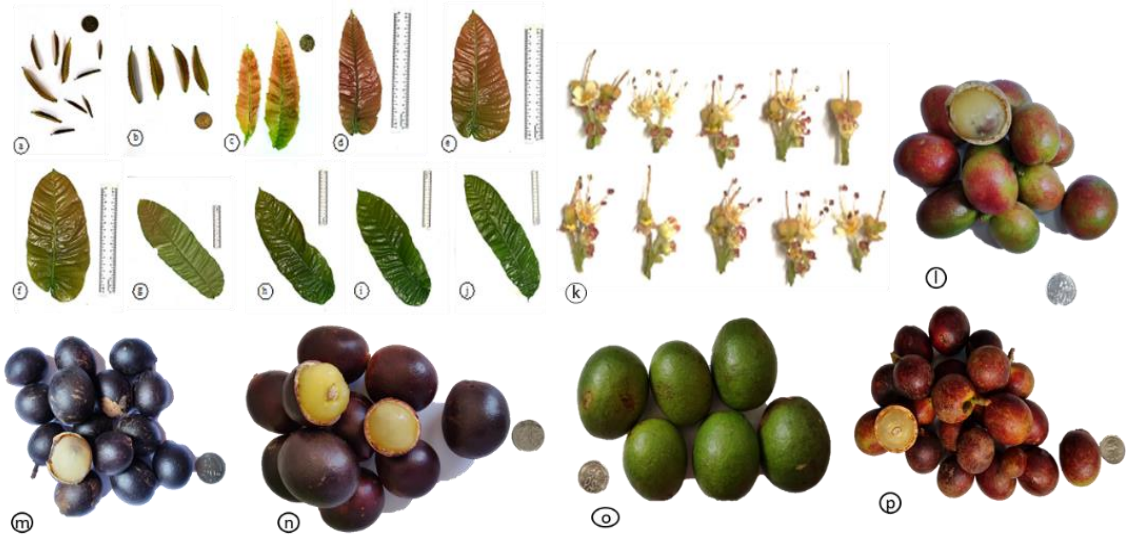

Figure 1 Flowers, leaves, and fruit morphology of *Pometia pinnata* at Bintulu.

PCR sequences for *P. pinnata* were ~700 base pairs using ITS primers. The phylogenetic tree obtained using Maximum Parsimony revealed two major clades.

Clade 1 comprised all the *P. pinnata* accessions along with reference sequences from China and Indonesia. Meanwhile clade 2 consisted of outgroup species from Sapindaceae family; rambutan, longan and lychee. Green genotype was arranged in a single sub-clade while medium and big purple, rainbow and red genotypes were arranged separately into different subclades. Under this subclade, the big purple genotype was distantly arranged compared to the medium purple, red and rainbow.

CONCLUSION

This study considers the first finding reported on the genetic diversity of *P. pinnata* in Sarawak. Morphological clustering by PCA clearly separates the genotypes based on the fruit characteristics into three main clusters. The genetic diversity of *P. pinnata* genotypes further supported the morphological clustering. ITS marker is useful to clearly separate the *P. pinnata* genotypes into specific grouping based on their reproductive characteristics.

REFERENCES

1. Huang, X., Subhadrabandhu, S., Mitra, S.K., Ben-Arie, R., and Stern, R.A., 2005. Origin, History, Production, And Processing. Litchi and Longan: Botany, Production and Uses. CABI Publishing, UK.
2. Furray, A., Ahmad, U., and Widodo, S., 2018. Study of Quality Parameter of Yellow Matoa (*Pometia pinnata*). *IOP Conf. Series: Material Science and Engineering,* 557.
3. Thomson, L.A., and Thaman, R.R., 2006. *Pometia pinnata*. Traditional Trees of Pacific Islands: Their Culture, Environment, and Use. Permanent Agriculture Resources. Hawaii: *Permanent Agriculture Resources,* 591-606.
4. Azlah, M., Chua, L.S., Rahmad, F., Abdullah, F., and Alwi, S.R.W., 2019. Review on Techniques for Plant Leaf Classification and Recognition. *Computers,* 8, 1-22.
5. Ramaiya, S.D., Bujang J.S., Zakaria M.H, Genetic diversity in *Passiflora* species assessed by morphological and ITS sequence analysis. *The Scientific World Journal*, 2014

Nutritional and Minerals Profiling of Banana (*Musa acuminata*), Orange (*Citrus reticulata*) and Watermelon (*Citrullus lanatus*) Waste Powder

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ABSTRACT

Fruit by-products are being studied as a non-conventional alternative source of nutritional and mineral content that might be employed as functional food components. Therefore, the objective of this study was to investigate the nutritional and mineral composition of banana (*Musa acuminata*), orange (*Citrus reticulata*), and watermelon (*Citrullus lanatus*) peels with different drying methods i.e., freeze- and oven-drying. The results revealed that orange peels powder was highly effective in preserving the fruit peel powder for a longer storage time because of the lower moisture content with 0.93% wet weight for the freeze-dried method. Meanwhile, oven-dried orange peels showed 1.82% wet weight, respectively. In this case, both drying techniques are adequate for minimizing post-harvest peel losses and industrial deterioration since they do not affect peel nutrients negatively. Besides, the carbohydrate content ranged from 47.74 to 83.04% dry weight, whereas crude fiber was presented in larger proximate quantities with 10.78 to 22% dry weight. A broad range of mineral content was recognized, as calcium and sodium being the most abundant compound with 826.42 to 3239.17 mg/100 g. Overall, the results revealed that all these fruit wastes could be exploited for the nutrient and value-added potential in food formulations due to their inexpensiveness, natural, safe, and environmentally friendly resources.

Keywords: Fruit waste powder; freeze-dried; mineral; oven-dried; proximate

INTRODUCTION

Fruit processing generates large amounts of peels, pomaces, and seeds [1]. Valuable compounds of these materials can be recovered and converted into food ingredients. Fruits are proven as a good source of minerals (calcium, iron, and zinc), fiber, vitamins, and other bioactive substances. Therefore, due to economic market price and huge availability, fruit remains from industry was currently in particular

demand. The proximate composition and mineral contents of three non-seasonal fruits waste powder (FWP) which are banana (*Musa acuminata*), orange (*Citrus reticulata*), and watermelon (*Citrullus lanatus*), with two different drying methods (i.e., freeze and oven-dried) were evaluated in this study.

MATERIALS AND METHODS

Fruit obtained from the local supermarket was prepared by boiling the cleaned peel cuts in hot water (± 90°C) for 30 minutes to inactivate any potential microorganism and enzyme reaction. The fruit peels were separately dried by using the freeze and oven drying method. After that, the fruit peels were powdered and analyzed for moisture, ash, crude protein, crude fat, crude fiber, carbohydrate, and minerals such as calcium (Ca), zinc (Zn), sodium (Na), potassium (K), magnesium (Mg), copper (Cu) and phosphorus (P) according to standard methods of Association of Official Analytical Chemists [2,3].

RESULTS AND DISCUSSION

The proximate composition showed that these FWP contains significantly high fiber (10.78 to 22%) and carbohydrate (47.74 to 83.04 %) contents, whereas the moisture (0.93 to 11.06%), ash (3.62 to 18.63%), fat (0.20 to 5.26%), and proteins (0.51 to 1.54%) are respectively low. Celestino [4] stated that products with lower moisture content are commonly more resistant to deterioration and chemical modifications by organisms. Thus, all FWP in this study were remarkable for mitigating microbial growth and longer shelf life. A broad range of mineral contents was noted among the FWP where calcium (Ca) and sodium (Na) were shown in larger quantities with 826.42 to 2727.54 mg/100 g. Besides, the result also showed that orange peels contain high carbohydrates, calcium, and copper composition compared to other FWP, as well as not exceed the standard limit provided by Recommended Dietary Allowances (RDA) in order to ensure consumer safety. In short, both drying technologies were found adequate for reducing the perishability of fruit waste and industry losses, as do not reducing fruit peel nutrients. However, it is not advised to use an oven-drying method as it might alter the fiber structure. It is important to remark that all the differences might be due to fruit varieties and geographical factors.

CONCLUSION

Overall, this study confirmed the presence of a potentially valuable source of nutrients and essential minerals in fruit waste such as banana, orange, and watermelon peels. The orange peels were efficient in the preservation of FWP for a prolonged storage period due to lower moisture content. Both drying methods efficiently minimize post-harvest losses of peels as fruit waste and less damage to the industry. Besides, this study recommended that fruit peels should not be

discarded but can be utilized in the preparation of healthy and nutritious items after proper processing. Therefore, lead the way for utilization of bio-wastes as they will serve as low-cost, natural, safe, and environmentally friendly raw materials. This study data also contributes to the knowledge of non-seasonal cultivated fruit nutrient composition.

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REFERENCES

1. Galanakis, C. M. (2013) Emerging technologies for the production of nutraceuticals from agricultural by-products: A viewpoint of opportunities and challenges. *Food and Bio-products Processing*, *91*(4), 575-579.
2. AOAC, (1995). *Official Method of Analysis of the Association of the Analytical Chemists.* Maryland, USA: Association of Official Analytical Chemists.
3. AOAC, (2005). *Official Method of Analysis of the Association of the Analytical Chemists*. Maryland, USA: Association of Official Analytical Chemists.
4. Celestino, S. M. C. (2010). Temperatura de bulbo úmido. In *Princípios de Secagem de Alimentos* (pp.11-15). Embrapa and Planaltina: Brazil Publisher.

Nutritional and Health Benefits of Indigenous Fruit of *Artocarpus odoratissimus* Blanco. in Sarawak

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ABSTRACT

In Sarawak, *Artocarpus odoratissimus* is commonly known as Terap. It is a native fruit to Borneo Island and is believed to be the most delicious tropical fruit by the local people. Although this fruit is widely appreciated as a possible food source for the local communities, its nutritional and phytochemical attributes have not been adequately investigated, particularly in Sarawak. This study aims to evaluate the nutritional profiling of *A. odoratissimus* fruits from Sarawak. AOAC standard protocols were used to determine the proximate compositions, mineral content, sugar and vitamin content of the flesh, fatty acid compositions of the seeds, and phytochemical properties. The nutritional profiling of each part of the fruit is varied. The results show that the flesh and seeds of *A. odoratissimus* are high in carbohydrate content with 82.70±0.36% and 59.20±0.13%, respectively. In comparison, the skin and pedicel are high in ash content (5.57±0.11% and 5.79±0.41%, respectively). Potassium was the major mineral component found in all parts of the fruit ranging from 905.61±18.89 mg/100 g (seeds) to 1210.40±28.00 mg/100 g (flesh). The flesh part predominantly consisted of non-reducing sugars of fructose (26.7±0.70 g/100 g) and glucose (25.38±0.45 g/100 g) and was rich in vitamin B1 (11.07±0.31 mg/100 g). Overall, this study supports the ethnobotanical uses of *A. odoratissimus* by the local communities, and this indicates that the fruit is gaining visibility in nutraceutical and pharmaceutical industries that could enhance the product development of this fruit.

Keywords: Food plant; indigenous fruit; nutritious; product development

INTRODUCTION

In general, indigenous plants are fruits and vegetables that grow naturally on the land or were introduced from one location to another by natural processes or human domestication a long time ago. Various ethnicities and indigenous peoples around the world have traditionally exploited indigenous plants allowing locals to benefit from their usage. Dabai, Terung Asam, Embang, and Midin were amongst the most promising indigenous species, which are growing more popular locally and

internationally [1]. Other promising plants include Terap (*Artocarpus odoratissimus*), an indigenous fruit species known for its odour gaining popularity in the local fruit market. Various researchers are working to uncover its potential. The state government of Sarawak believed that this fruit has great potential for product development and commercialization due to the increasing demand and attributes of *A. odoratissimus* fruit. However, there was limited documentation on the nutritional and phytochemical investigations on *A. odoratissimus* fruit, particularly in Sarawak. Therefore, this study aims to evaluate the nutritional and phytochemical properties of *A. odoratissimus* fruit parts.

MATERIALS AND METHODS

The fruits of *A. odoratissimus* were collected at the local market Pasar Utama Bintulu, Sarawak. The fruits were cleaned, and the flesh, seed, skin, and core parts were separated. The oven-dried samples were used for analyses of the proximate and mineral composition following the Association of Official Analytical Chemists [2]. The freeze-dried samples were used for the analysis of phytochemical compositions. Total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant activity (TAA) of the fruit parts were determined spectrophotometrically.

RESULTS AND DISCUSSION

The proximate composition shows that the seeds of *A. odoratissimus* possessed good protein content (21.89±0.01%) compared to the flesh (14.59±0.19%). This fruit could be an alternative source of protein for the rural people. The seeds also contain higher crude fat 18.23±0.20%. Additionally, higher carbohydrate content was recorded in the flesh ranged 82.27-83.41%. A higher concentration of K was recorded in the flesh (1210.40±28.00 mg/100 g). Significantly higher Ca was found in the core (1300.97±23.51 mg/100 g), whereas Mg content was found to be higher in the skin parts (263.52±7.02 mg/100 g). The Na values obtained for the flesh and seed are comparatively lower than the non-edible parts. Copper was found in trace quantities in all the fruit parts ranging 0.89-2.49 mg/100 g. The concentrations of Cu were found to be within the limits of recommended value by Malaysian Food Regulations [3]. Fructose comprised a larger portion in the flesh followed by glucose and the least amount was sucrose. *Artocarpus odoratissimus* flesh contains an excellent vitamin B complex content, which is Thiamin followed by Niacin, Folic acid, and Riboflavin, and vitamin C. Higher TPC was recorded in the core, followed by the skin as compared to the flesh. Higher TFC was observed in the skin, while a lower value was recorded in the flesh.

CONCLUSION

Findings revealed new and additional information on nutritional and phytochemical properties of *A. odoratissimus* fruits that support the ethnobotanical uses of this fruit since long ago by the local people. All edible and non-edible parts of this indigenous *A. odoratissimus are* gaining importance as food sources and health promoters. It could enhance this fruit's downstream application or product development to meet the State's mission in popularizing this crop.

REFERENCES

1. Runi, S. P., Aurelia, D. C., Khairunnisa, O., & Haniza, R. (2018). Indigenous plants of Sarawak: Utilization & potential. *Proceeding of the International Conference on Sustainable Agriculture* (p. 15). Sabah: Universiti Malaysia Sabah (Sandakan Campus).
2. AOAC. (2000). *Official method of analysis of the Association of the Analytical Chemists.* Maryland, USA: Association of Official Analytical Chemists.
3. Malaysian Food Regulations 1985.

The Hidden Gold Commodity of Sarawak Forest: *Durio* Species and Their Uniqueness

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ABSTRACT

Borneo is recognized as the land of diversity and rich heritage of flora as it is the widest island in the world. Sarawak, one of the parts of Borneo Island, is enriched with wild fruit tree species, including home for 16 unique *Durio* species that are diverse in shape, taste, and smell that holds great promise for future domestication. Despite much research on commonly consumed durian species, a dearth of information is available for wild durians present in Sarawak. Therefore, the present study aims to examine the edible *Durio* species present at Sarawak, their unique characteristics, and also their distribution pattern. Random sampling has been conducted at the center, and East zones of Sarawak, and six wild edible *Durio* species, i.e., *D. dulcis* (Kulit merah), *D. graveolens* (Isu), *D. testudinarum* (Durian kura-kura), *D. kutejensis* (Nyekak), *D. oxleyanus* (Durian daun) and *D. zibethinus* have been recorded. Findings revealed the wild *Durio* species are widely found on a low hill, ridge slopes, undulating land in mixed dipterocarp forests, and along the riverbank. Among the six species, *D. kutejensis* (Nyekak) and *D. graveolens* (Isu kuning and Isu oren) are domesticated and widely sold at local tamu markets in Sarawak. *Durio dulcis* (Tutong), *D. oxleyanus* (Durian daun), and *D. graveolens* (Isu merah) are still found in the forest. In general, there was a wide range of variation in fruit characteristics, including fruit and flesh colour (white, yellowish, yellow goldish, orange, red, and dark red), fruit odour (odourless, fragrant, and sharp fragrant), the thickness of flesh (thin, medium and thick), sweetness (from bitter to very sweet), flesh texture, fruit size, and shape.

Keywords: Borneo; *Durio* spp., taste preference, Sarawak; wild durians

INTRODUCTION

Malaysia, like other Southeast Asian countries, has tropical rainforests with a diverse range of fruit-bearing plants. Durian is popular as the “king of fruits", with its enormous size, strong odour, and intimidating thorn-covered husk. In the Malaysian National Agro-food Policy (2011-2020), durian has been regarded as the new agricultural wealth resource [1]. The genus *Durio* consists of 30 species, and 23 species have been recorded in Malaysia. Out of these, some species are restricted and can only be found in Sabah and Sarawak. Sarawak itself holds about 16 species

of *Durio*; *D. acutifolius, D. affinis, D. carinatus, D. crassipes*, *D. dulcis, D. excelsus, D. grandiflorus, D. graveolens, D. griffithi, D. kutejensis, D. lanceolatus, D. lissocarpus, D. testudinarum, D. oblongus, D. oxleyanus* and *D. zibethinus* [2] and seven from its are edible species [3]. Some wild durians are partially domesticated and can only be found in primary or secondary forests [4]. Despite the fact that the majority of these trees are wildly growing the some of them hold a commercial value as it is likely to be varied from the *D. zibethinus* in terms of shape, taste, scent, colour, and texture, that could offer a lot of potential for the future durian industry.

MATERIALS AND METHODS

Random sampling has been conducted throughout the center and East zone in Sarawak (Figure 1). *In-situ* and *ex-situ* observations on the morphological characters have been done on vegetative and reproductive parts that include 42 quantitative and 70 qualitative characteristics. Identification of plant morphology was based on Descriptors for Durian (*Durio zibehtinus* Murr) [5].

Figure 1 Sampling locations of *Durio* species.

RESULTS AND DISCUSSION

We had discovered about six wild edible *Durio* species, which are *D. testudinarum* (Durian kura-kura), *D. graveolens* (Isu kuning, Isu merah, Isu oren), *D. oxleyanus* (Durian daun), *D. kutejensis* (Nyekak or Pakan), *D. dulcis* (Durian Tutong) and *D. zibehtinus.* Most of the wild edible durians are found in the primary or secondary forest. *Durio kutejensis* (Nyekak) and *D. graveolens* (Isu kuning and Isu oren) are widely cultivated and domesticated by the local people in Sarawak. *Durio dulcis* (Tutong), *D. oxleyanus* (Durian daun) and *D. graveolens* (Isu isi merah) still remain in the primary or secondary forest. *Durio graveolens* was observed to have three flesh colours, *i.e.,* yellow, orange, and red. *Durio dulcis can only* be found in the primary forest and is the tallest (~50 m) among the other wild *Durio* species. The

wild edible *Durio* species have a smaller size and weight (10-20 cm, 0.5-1.2 kg, respectively) than the wild *D. zibethinus* (length and diameter 15-25 cm; weight 1.0-1.8 kg). Most of the wild edible durians have a creamy taste and soft texture. *Durio dulcis* has an extremely pungent smell, while the others have a slightly pungent smell. The wild edible durians are also attractive due to various exocarp and flesh colours.

CONCLUSION

Overall, distinct regional demands for different species/varieties reflect local idiosyncrasies in consumer tastes. These durian species are diverse in shape, taste, smell, colour, and texture compared to the established clones from *D. zibethinus* and hold great promise for future domestication.

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REFERENCES

1. Safari, S., Athirah, N. F., Sunthralingam, C., Jamaludin, J. A. and Mustaffa, R. (2018). Durian as new source of Malaysia's agricultural wealth. *FFTC Agricultural Policy Articles*, 1.
2. Peng, J. S. M. (2019). Volatile esters and sulfur compounds in durians & a suggested approach to enhancing economic value of durians. *Malaysian Journal of Sustainable Agriculture*, *3*, 5-15.
3. Kueh, H. S. (2003). Indigenous fruits of Sarawak. Kuching: Sarawak Forest Department.
4. Lim, T. K. (2012). *Edible Medicinal and Non-Medicinal Plants.* pp. 550-583. Dordrecht, The Netherlands: Springer.
5. Bioversity International (2007). Descriptors for durian (*Durio zibethinus* Murr.). Rome: Bioversity International.

Acceleration of Lipid Accumulation in Oleaginous Diatom *Navicula* sp. Under Nitrogen Limitation

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ABSTRACT

Diatom biomass gain attention globally as a source of lipid due to their high growth rate and biomass composition which can compete with fossil fuel for biodiesel production. Accumulations of natural lipid can be enhanced by various stress factors in diatoms. In this study, the effect of different nitrogen concentration on biomass and lipid production was investigated by cultivation of oleaginous diatom *Navicula* sp. for biodiesel production. The cultivation performed in phases where initially the culture was cultured in standard media for seven day and followed in different concentration of nitrogen such as limitation (0.35 mM), standard as media (1.76 mM), Repletion (3.5 mM) and free of nitrogen (0 mM) as second phase for eight days. The cells were grown under light intensity of 150 µmol photon $m^{-2}s^{-1}$(12:12 h day: light period) temperature (21±1 °C). Diatom *Navicula* sp. showed highest growth and biomass production (734±15 mg/L) in nutrient f/2 standard media and extracted 19% of lipid. However, 24% of lipid yield was extracted which is significantly high from the biomass (528±10 mg/L) cultured in limited nitrogen media. Nitrogen limitation enhanced the fatty acid as saturated fatty acid C16:0, unsaturated fatty acid C16:1 and polyunsaturated fatty acid C18:3. *Navicula* sp., have potential to accumulate lipid in nitrogen limitation condition biodiesel production.

Keywords: Biodiesel; biomass; diatom, *Navicula* sp.; nitrogen limitation

INTRODUCTION

Diatoms (Bacillariophyta) are unicellular photosynthetic creatures with silica cell walls. It can also trap CO_2, grow in a wide range of aquatic settings, and store oil

at a far higher density than other plants (around 50 wt % of biomass). Diatoms have a brief life cycle and do not require arable land [1,2,3].

Lipid production from diatom must be extremely efficient and cost-effective in order to be commercially viable, because biodiesel is such a low-value product [4-7]. Therefore, the lipid-producing diatom strain chosen should have a high lipid content as well as a high cell growth rate which include *Thalassiosira pseudonana, Chaetoceros affinis, Navicula saprophila* and *Skeletonema marinoi* have high oil content, ranging from 20% to 50% by weight, making them suitable for biofuel generation [3-8].

The key elements that govern lipid accumulation in microalgae include light irradiation, temperature, and other chemical stimuli such as pH, salinity, mineral salts, and nitrogen deficiency [1,4,9]. Among that, macronutrient deprivation is one of the most popular strategies due to its great efficiency [7,10]. As a result, diatom lipid content and fatty acid production are critical criteria for producing high-quality biodiesel fuel with the appropriate qualities [11-12]. Continuous nitrogen deprivation enhances the lipid and carbohydrate content of microalgae, but also slows their development rate, lowering their overall productivity [6].

Continuous nitrogen deprivation enhances the lipid and carbohydrate content of microalgae, but also slows their development rate, lowering their overall productivity [6]. To address this issue, a two-stage culture approach has recently gained popularity, in which microalgae are grown in a nutrient-rich medium to obtain large cell biomass and then transferred to a nutrient-deficient medium to promote lipid and carbohydrate accumulation [12]. Therefore, this study is to investigate the biomass and lipid productivity, *Navicula* sp. cells grown in f/2 medium with different concentration of nitrogen where it is limited, excess and free from the standard f/2 conventional medium. The fatty acid content profile of the diatom also was analyzed to see if the produced diatom lipid could be used to make biodiesel.

MATERIALS AND METHODS

Strain and Culture Medium

Navicula sp. culture was obtained from Algae Culture Collection Centre and Laboratory, Universiti Malaysia Pahang. The culture was maintained in natural seawater supplemented with f/2 nutrients. The *Navicula* sp. inoculum for the experiment was preculture in 1000 ml sterilised (20 min at 120 °C) Erlenmeyer flasks with 500 mL f/2 media under light intensity of 150 µmol photon $m^{-2}s^{-1}$, temperature (21±1 °C) and with shaking (80 r.p.m.). The growth of culture was monitored by taking absorbance reading using Genesys UV-VIS Spectrophotometer.

Experimental Conditions

The experiment was conducted in artificial seawater with f/2 nutrients with 120 µM $Na_2SiO_3.5H_2O$ to grow the culture. 10% of the actively growing culture of *Navicula* sp. was inoculated in 500 ml of standard f/2 culture medium in 1000 ml Erlenmeyer flask. All the cells were grown at 21±1°C, 150 µmol photon $m^{-2}s^{-1}$, of light intensity with 12:12 h dark: light cycle. The growth of culture was monitored for the first 7 days by taking optical density of culture in 1 mL of cuvette using a spectrophotometer at 680 nm. On day eight media was added in culture where in early stationary phase, with different concentration as limited (0.20 mM), standard concentration as control (1.76 mM), repleted (3.5 mM) and free of nitrogen (0 mM) in f/2 medium. The growth of culture was monitored again for the next 8 days. On day 15, grown cells were harvested by centrifugation at 7826 ×*g* for 10 min and dried at 70 °C in a heat chamber for 18 h.

Lipid extraction and Fatty Acid Methyl Ester Analysis

Lipids were extracted by Bligh and Dyer assisted with Ultrasound technique. Dry biomass was weighed and mixed in 50 mL of hexane (1:8, w/v) solution in falcon tube. The mixture was vortexed for 30 s. The tube was placed in a water bath at 70±2°C, 1000-watt power for 60 min and disrupted the cells. The mixture was centrifuged at 3293 ×*g*, 10 °C for 5 min and collected the supernatant. Repeated the step by adding 50 mL of hexane solvent with same biomass, until it turns to colourless. The extracted lipid was trans esterified by using hexane/methanolic-KOH (2:1, 1%) [13]. The gas chromatography mass spectrometry (GC-MS) uses for the detection of fatty acid methyl esters content.

RESULTS AND DISCUSSION

Effect of Nitrogen Concentration in Different Concentrations of *Navicula* sp.

The effect of nitrogen limitation and repletion in diatom *Navicula* sp. with growth, biomass and lipid production were investigated. Before harvesting the cells, the morphological structure of cells was examined under light microscope and found changes on the boat shaped diatom based on their environment of media.

The growth begins to deviate after the media is added in with different concentrations of nitrogen in the following days of experiment as shown in Figure 1a. Continuous nitrogen limitation enhances the lipid and carbohydrate content of microalgae, but it drastically reduces biomass production. Highest biomass yield obtained from standard nitrogen media (734±12 mg/L) However, Nitrogen limitation reduced the biomass production and averagely obtained (528±15 mg/L) after 15 days cultivation. The least growth and biomass production found in repleted media due to excess amount nutrients cause toxic effects to the cells. As a result, it

inhibited the growth and biomass production of *Navicula* sp. Previous studies reported that, *Navicula* sp. obtained 369 mg/l biomass after six days of batch culture [13-15]. In response to nitrogen deficiency, the diatom was able to boost light intake and divert carbon metabolism toward lipid synthesis [15].

The Bligh and Dyer method assisted with ultrasound technique highly suited for lipid extraction from the dry biomass of *Navicula* sp. Highest lipid (24%) was obtained from the biomass harvested from the limited nitrogen media and followed by control media (21%). The lowest lipid (4%) obtained from the repleted nitrogen media due to limited growth of cells. In nitrogen free media found lower lipid yield (18%) even though obtained highest biomass production. Many studies have found that when nitrate levels are low, marine diatoms produce more lipids [11-15].

Figure 1 Growth curve (A) and biomass and lipid production (B) of *Navicula* sp. cultivated in different concentrations of nitrogen.

Fatty Acid Composition Analyses

Gas chromatography mass spectrometry analysis revealed the fatty acid methyl ester which is present in *Navicula* sp. extracted and trans esterified lipids. Significant criteria for improved biodiesel quality are composition of fatty acids of marine diatoms is carbon number between C14:0, C16:0, C16:1, C18:0, C18:1, and C20:5 (n-3) (14-15). Palmitic acid (C16:0) was the most abundant fatty acid in the repleted nitrogen medium (45.91%), followed by mono-saturated palmitoleic (C16:1) at 33.78% in free of nitrogen media. Highest polyunsaturated fatty acid (C20:4) found from free of nitrogen media at 12.67%.

CONCLUSIONS

This study highlighted the potential of *Navicula* sp. has the source for biofuel production at optimum conditions. The effect of nitrogen was compared between the first and second phase of cultivation. Nitrogen limitation influences the accumulation of lipid in cells (obtained 24% of cell dry weight). Nitrogen limitation also enhances the biomass and lipid productivity in the cells.

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REFERENCES

1. Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances*, *25*(3), 294-306.
2. Enamala, M. K., Enamala, S., Chavali, M., Donepudi, J., Yadavalli, R., Kolapalli, B., & Kuppam, C. (2018). Production of biofuels from microalgae-A review on cultivation, harvesting, lipid extraction, and numerous applications of microalgae. *Renewable and Sustainable Energy Reviews*, *94*, 49-68.
3. Govindan, N., Maniam, G. P., Yusoff, M. M., Chatsungnoen, T., Ramaraj, R., & Chisti, Y. (2020). Statistical optimization of lipid production by the diatom Gyrosigma sp. grown in industrial wastewater. *Journal of Applied Phycology*, *32*(1), 375-387.
4. Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., & Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal*, *54*(4), 621-639.
5. d'Ippolito, G., Sardo, A., Paris, D., Vella, F. M., Adelfi, M. G., Botte, P., & Fontana, A. (2015). Potential of lipid metabolism in marine diatoms for biofuel production. *Biotechnology for Biofuels*, *8*(28), 1-10.
6. Chu, F., Cheng, J., Zhang, X., Ye, Q., & Zhou, J. (2019). Enhancing lipid production in microalgae Chlorella PY-ZU1 with phosphorus excess and nitrogen starvation under 15% CO_2 in a continuous two-step cultivation process. *Chemical Engineering Journal*, *375*, 121912.

7. Krishnamoorthy, N., Unpaprom, Y., Ramaraj, R., Maniam, G. P., Govindan, N., Arunachalam, T., & Paramasivan, B. (2021). Recent advances and future prospects of electrochemical processes for microalgae harvesting. *Journal of Environmental Chemical Engineering*, *9*(5), 105875.
8. Palanisamy, K. M., Paramasivam, P., Maniam, G. P., Rahim, M. H. A., Govindan, N., & Chisti, Y. (2021). Production of lipids by *Chaetoceros affinis* in media based on palm oil mill effluent. *Journal of Biotechnology*, *327*, 86-96.
9. Jayakumar, S., Bhuyar, P., Pugazhendhi, A., Rahim, M. H. A., Maniam, G. P., & Govindan, N. (2021). Effects of light intensity and nutrients on the lipid content of marine microalga (diatom) Amphiprora sp. for promising biodiesel production. *Science of the Total Environment*, *768*, 145471.
10. Mishra, M., Arukha, A. P., Bashir, T., Yadav, D., & Prasad, G. B. K. S. (2017). All new faces of diatoms: potential source of nanomaterials and beyond. *Frontiers in Microbiology*, *8*, 1239.
11. de Jesús-Campos, D., López-Elías, J. A., Medina-Juarez, L. Á., Carvallo-Ruiz, G., Fimbres-Olivarria, D., & Hayano-Kanashiro, C. (2020). Chemical composition, fatty acid profile and molecular changes derived from nitrogen stress in the diatom *Chaetoceros muelleri*. *Aquaculture Reports*, *16*, 100281
12. Bhuyar, P., Sundararaju, S., Rahim, M. H. A., Maniam, G. P., & Govindan, N. (2021). Enhanced productivity of lipid extraction by urea stress conditions on marine microalgae Coelastrum sp. for improved biodiesel production. *Bioresource Technology Reports*, *15*, 100696.
13. Lim, B. P., Maniam, G. P., & Hamid, S. A. (2009). Biodiesel from adsorbed waste oil on spent bleaching clay using CaO as a heterogeneous catalyst. *European Journal of Scientific Research*, *33*(2), 347-357.
14. Chtourou, H., Dahmen, I., Karray, F., Sayadi, S., & Dhouib, A. (2015). Biodiesel production of Amphora sp. and Navicula sp. by different cell disruption and lipid extraction methods. *Journal of Biobased Materials and Bioenergy*, *9*(6), 588-595.
15. Enamala, M. K., Enamala, S., Chavali, M., Donepudi, J., Yadavalli, R., Kolapalli, B., & Kuppam, C. (2018). Production of biofuels from microalgae-A review on cultivation, harvesting, lipid extraction, and numerous applications of microalgae. *Renewable and Sustainable Energy Reviews*, *94*, 49-68.

Flowering Performance of Two Pineapple Cultivars in Response to Flowering Hormones

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ABSTRACT

Pineapple (*Ananas comosus* L. Merr.) consists of many cultivars, and each of them was believed to have different physiological needs, thus requiring specific agronomic practices. One of the issues in pineapple management is that the flowering is poorly synchronized, eventually affecting pineapple fruit production. Thus, this study aims to evaluate the flowering performance of cv. Pada and cv. Sarawak in response to flowering hormones. A complete randomized design with three replications was implemented in the polybag study. Each replication comprises 15 plants. A 50 ml hormone solution was applied on a nine-month-old plant, consisting of nine treatments for cv. Pada (T1 – T9) and 10 treatments for cv. Sarawak (T1 – T10): calcium carbide (CaC_2) (T1: 0.5%, T2: 1.0%, T3: 1.5%), naphthaleneacetic acid (NAA) (T4: 5 ppm, T5: 10 ppm, T6: 15 ppm), and ethephon mixed with 2% urea and 0.04% calcium carbonate) (T7: 120 ppm, T8: 240 ppm, T9: 360 ppm, T10: 600 ppm). Results in cv. Pada showed that T3, T7, T8, and T9 could stimulate 100% flowering, while T1 and T2 were still sufficient to promote optimum flowering of at least 90%. However, the optimum flowering was unable to be achieved in cv. Sarawak, and the only best treatments were on ethephon applications, ranging from 55.5% (T8) to 66.7% (T9). Overall, we conclude that the cv. Pada responds well to hormones, particularly ethephon and CaC_2, but further study is needed for cv. Sarawak to obtain more synchronized flowering.

Keywords: Calcium carbide; cv. Pada; cv. Sarawak; ethephon; flowering synchronization; naphthaleneacetic acid

INTRODUCTION

A number of pineapples (*Ananas comosus* L. Merr.) cultivars have been introduced, while in Malaysia, currently, 12 cultivars have been registered. Besides, there is also another cultivar that has not been registered yet, for instance, cv. Pada was initially planted in some divisions of Sarawak (i.e., Mukah and Dalat), but now has been widely planted in that country [1]. As the physiological requirements vary between

cultivars [2], specific agronomical practices are needed to enable resources to be used efficiently. One of the issues in pineapple production was poor flowering synchronization even though the flowering hormone has been applied. This issue has become one of the factors that influence the type of cultivar to be planted [3]. This is because such a poor flowering synchronization causes a challenge in the fruit production schedule, hence affecting the production cost. An important criterion of hormone application in pineapple production was that at least 90% of plants should be flowered [4].

Several flowering hormones have been used in the pineapple industry and are mainly based on two main types of hormones, namely auxin and ethylene [2]. In Malaysia, ethephon, calcium carbide (CaC_2), and naphthaleneacetic acid (NAA) are commonly used due to market availability. The mechanism of these hormones was different, where the ethephon decomposes to produce ethylene, CaC_2 gives rise to acetylene, and NAA promotes 1-aminocyclopropane-1-carboxylic acid (an ethylene biosynthesis precursor) [5]. Also, the effectiveness of these hormones was less consistent [6, 7, 8]. Besides, determining hormone concentration was essential to stimulate flowering, and it can be affected by the type of cultivars, growing regions, and plant condition [9].

Therefore, this has led us to evaluate flowering performance on cv. Pada and cv. Sarawak, in Bintulu, Sarawak, Malaysia, using ethephon, CaC_2, and NAA, at different concentrations. The cv. Pada was selected because the record of agronomical practices was limited, and the cv. Sarawak was chosen because it is less sensitive to flowering hormones.

MATERIALS AND METHODS

The experiment was conducted at Universiti Putra Malaysia Bintulu Sarawak Campus, Malaysia, from January 2020 to December 2020. Two pineapple cultivars were assessed, namely, cv. Pada, and cv. Sarawak. The 16’ × 16’ polybag filled with 15 kg of Bekenu series soil was used. The sucker weight in the range of 300 to 500 g was selected and treated with fungicide and pesticide before planting. The planting arrangement was a double row system at 30 cm × 60 cm × 90 cm. The plants were maintained based on the Malaysian Pineapple Industry Board (MPIB) [10]. Irrigation was only performed when necessary, during the first three months to promote better plant growth.

A complete randomized design with three replications was implemented, and each replicate consists of 15 plants. The cv. Pada was provided with nine treatments (T1 – T9), while the cv. Sarawak consist of 10 treatments (T1 – T10); CaC_2 (T1: 0.5%, T2: 1.0%, T3: 1.5%), NAA (T4: 5 ppm, T5: 10 ppm, T6: 15 ppm), and ethephon (T7: 120 ppm, T8: 240 ppm, T9: 360 ppm, T10: 600 ppm). These ethephon treatments were mixed with 2% urea and 0.04% calcium carbonate to enhance their efficacy.

The treatments were applied on the nine-month-old plant during the early morning from 08:00 a.m. (26 °C) to 09:00 a.m. (32 °C), and approximately 50 mL of hormone solution were poured at the central plant rosette.

The number of flowerings was recorded after 60 days of treatment. Then, the flowering data were converted into percentages and analyzed descriptively using SAS JMP Pro 14.3.

RESULTS AND DISCUSSION

Table 1 Flowering performance of cv. Pada and cv. Sarawak after 60 days of treatments. The values are given as mean in percentage ± standard error.

Treatment	cv. Pada	cv. Sarawak
T1	91.1 ± 4.4	11.1 ± 5.9
T2	97.8 ± 2.2	0
T3	100.0 ± 0	0
T4	55.6 ± 4.4	24.4 ± 4.4
T5	44.4 ± 4.4	11.1 ± 4.4
T6	20.0 ± 6.7	0
T7	100.0 ± 0	60.0 ± 7.7
T8	100.0 ± 0	55.5 ± 11.8
T9	100.0 ± 0	66.7 ± 10.2
T10	-	57.8 ± 5.9

Based on cv. Pada (Table 1), the flowering percentage under ethephon and CaC_2 treatments at any concentrations were relatively higher than the NAA; also, both etephon and CaC_2 treatments were able to achieve at least 90% flowering. Furthermore, the T7 was sufficient to stimulate 100% flowering, although the concentration was approximately 33% lower than as recommended by MPIB on susceptible cultivars to flowering hormones such as Moris, Josapine, and Gandul [10]. There is a possibility that the ethephon concentration lower than T7 could stimulate flowering optimally, subsequently providing more cost efficiency to the farmers.

For CaC_2 treatments, only the highest concentration (T3) stimulated 100% flowering, and the trend of flowering percentage decreased as the concentration reduced. However, in NAA treatments, the trend was contradicted, presumably affected by the environmental condition. Previous studies demonstrated that the NAA was sensitive to light and temperature, which could destroy the NAA by decarboxylation [11, 12]. During NAA application, the T4 was firstly applied, followed

by T5, and T6, approximately 10 minutes apart; presumably, the magnitude of NAA decarboxylation on T6 was higher than on T4 because of rapid temperature increase.

Meanwhile, for cv. Sarawak (Table 1), only ethephon treatments (T7 – T10) showed a good response as compared with CaC_2 (T1 – T3) and NAA (T4 – T6). However, none of them was able to achieve 90% flowering. Also, unexpectedly the result of T10 was somewhat comparable to the lowest ethephon concentration (T7). A previous study showed that 400 ppm of ethephon was sufficient to stimulate 100% flowering (Smooth Cayenne - grown in China) [13]. Although the T10 was 200 ppm higher than the concentration used by Liu et al. [13], the flowering was almost 50% lower than that. Besides, 60% flowering was recorded when 1% CaC_2 was used (cv. Tainong 17 - grown in Taiwan) [14], but the similar concentration did not stimulate any plant in our study. These results may indicate that our growing condition was less susceptible to stimulate flowering on cv. Sarawak, probably because of frequent and high precipitation, where these conditions tend to keep promoting vegetative growth. In the case of NAA, the trend of flowering percentage was similar as in cv. Pada, and it is unsurprising as the condition during hormone application was almost the same.

CONCLUSION

Ethephon and CaC_2 treatments were sufficient to promote more than 90% flowering on cv. Pada. However, cv. Sarawak was poorly responded to any applied treatments, but ethephon were relatively better than CaC_2 and NAA. Further research can be conducted to reduce ethephon concentration on cv. Pada for more cost-efficient, while the cv. Sarawak needs further improvement, such as further increase of hormone concentration or multiple applications.

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REFERENCES

1. Department of Agriculture Sarawak (2009). *Nanas Pada a pineapple variety from Dalat*. Department of Agriculture Sarawak. Retrieved from https://doa.sarawak.gov.my/page-0-270-283-ARC-ARTICLES-Archives.html
2. Bartholomew, D. P. (2014). History and perspectives on the role of ethylene in pineapple flowering. *Acta Horticulturae, 1042*(1042), 269-284. https://doi.org/10.17660/ActaHortic.2014.1042.33
3. Rozlaily, Z., Nurul, S. A. G., Noorman, A. M., Mohd, A. H., Ahmad, H. B., Nor, A. R., … Mohamad, F. F. (2018). New pineapple for fresh market. *Buletin Teknologi MARDI*, *14*, 125-132.

4. Zuraida A. R., Hartinee, A., & Ayu, N. O. (2017). Technology production of MD2 pineapple seedlings through tissue culture and evaluation of field study. *Buletin Teknologi MARDI*, *12*, 9-17.
5. Cunha, G. A. P. (2005). Applied aspects of pineapple flowering. *Bragantia*, *64*(4), 499-516. https://doi.org/10.1590/S0006-87052005000400001
6. Dass, H. C., Randhawa, G. S., Singh, H. P., & Ganapathy, K. M. (1976). Effect of pH and urea on the efficacy of ethephon for induction of flowering in pineapple. *Scientia Horticulturae*, *5*(3), 265-268. https://doi.org/10.1016/0304-4238(76)90091-1
7. Py, C., Lacoeuilhe J. J., & Teisson, C. (1987). *The pineapple, cultivation and uses*. Paris: Maisonneuve G.P.
8. Raposo, R., Pereira, G. A. M., Matos, C. C., Barcellos, L. H., Paula, D. F., & Silva, A. A. (2019). Use of 2,4-D in mixture with ethephon and calcium carbide in pineapple crop. *Planta Daninha*, *37*, 1-10. https://doi.org/10.1590/S0100-83582019370100130
9. Sam Nureszuan, S. S., Noorasmah, S., & Shiamala, D. R. (2021). Flowering synchronization in pineapples (Ananas comosus L. Merr): A review. *Journal of Applied Horticulture*, *23*(2), 206-211.
10. MPIB (n.d.). *Manual tanaman nanas - Tanah mineral*. Retrieved from https://www.mpib.gov.my/en/publication/
11. Leeper, R. W., Gowing, D. P., & Stewart, W. S. (1962). Decarboxylation of alpha-naphthaleneacetic acid by pineapple leaves in sunlight. *International Journal of Applied Radiation and Isotopes*, *13*(7), 399-402. https://doi.org/10.1016/0020-708x(62)90012-1
12. Onaha, A., Higa, M., Nakasone, F., & Ikemiya, H. (2001). Effects of ambient temperature and defoliation on flower bud induction with chemicals in pineapple (Ananas comosus) plants. *Japanese Journal of Tropical Agriculture*, *45*(1), 1-7. https://doi.org/10.11248/jsta1957.45.1
13. Liu, S. H., Dou, M. A., Zang, X. P., & Sun, G. M. (2010). Flower forcing technique by etephon in pineapple. 7th International Pineapple Symposium, 13-15 July. Johor Bharu, Malaysia
14. Maruthasalam, S., Shiu, L. Y., Loganathan, M., Lien, W. C., Liu, Y. L., Sun, C. M., … Lin, C. H. (2010). Forced flowering of pineapple (*Ananas comosus* cv. Tainon 17) in response to cold stress, etephon and calcium carbide with or without activated charcoal. *Plant Growth Regulation*, *60*(2), 83-90. https://doi.org/10.1007/s10725-009-9421-9

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