Pertanika Journal of

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About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science is the official journal of Universiti Putra Malaysia. It is an open-access online scientific journal. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognised internationally as the leading peer-reviewed interdisciplinary journal devoted to the publication of original papers, it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields.

Pertanika Journal of Tropical Agricultural Science is a **quarterly** (*February*, *May*, *August*, and *November*) periodical that considers for publication original articles as per its scope. The journal publishes in **English** and it is open for submission by authors from all over the world.

The journal is available world-wide.

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Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance, and veterinary medicine.

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Pertanika was founded in 1978. A decision was made in 1992 to streamline *Pertanika* into 3 journals as Pertanika Journal of Tropical Agricultural Science, Pertanika Journal of Science & Technology, and Pertanika Journal of Social Sciences & Humanities to meet the need for specialised journals in areas of study aligned with the interdisciplinary strengths of the university.

Currently, as an interdisciplinary journal of agriculture, the revamped journal, a leading agricultural journal in Malaysia now focuses on tropical agricultural research and its related fields.

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To publish journals of international repute.

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Our goal is to bring the highest quality research to the widest possible audience.

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We aim for excellence, sustained by a responsible and professional approach to journal publishing. Submissions are guaranteed to receive a decision within 90 days. The elapsed time from submission to publication for the articles averages 180 days. We are working towards decreasing the processing time with the help of our editors and the reviewers.

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Pertanika is over 42 years old; this accumulated knowledge has resulted in Pertanika Journal of Tropical Agricultural Science being abstracted and indexed in SCOPUS (Elsevier), Clarivate Web of Science [ESCI], EBSCO, DOAJ, Agricola, ASEAN CITATION INDEX, ISC, Microsoft Academic, Google Scholar, National Agricultural Science (NAL), and MyCite.

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The abbreviation for Pertanika Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci*.

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Authors are not permitted to add or remove any names from the authorship provided at the time of initial submission without the consent of the journal's Chief Executive Editor.

Manuscript Preparation

Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words ***I**ntroduction*, ***M**aterials and Methods*, ***R**esults*, ***A**nd **D**iscussion*. IMRAD is simply a more 'defined' version of the "IBC" [*Introduction*, *Body*, *Conclusion*] format used for all academic writing. IMRAD indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: *Title*, *Authors*, *Keywords*, *Abstract*, *Conclusions*, *References*, and *Acknowledgement*. Additionally, some papers include *Appendices*.

The *Introduction* explains the scope and objective of the study in the light of current knowledge on the subject; the *Materials and Methods* describes how the study was conducted; the *Results* section reports what was found in the study; and the *Discussion* section explains meaning and significance of the results

Journal of Tropical Agricultural Science
Journal of Tropical Agricultural Science

and provides suggestions for future directions of research. The manuscript must be prepared according to the journal's **Instruction to Authors** (http://www.pertanika.upm.edu.my/Resources/regular_issues/Regular_Issues_Instructions_to_Authors.pdf).

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Authors who complete any submission are notified with an acknowledgement containing a manuscript ID on receipt of a manuscript, and upon the editorial decision regarding publication.

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Notification of the editorial decision is usually provided within 90 days from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

As articles are double-blind reviewed, material that may identify authorship of the paper should be placed only on page 2 as described in the first-4-page format in *Pertanika*'s **Instruction to Authors** (http://www.pertanika.upm.edu.my/Resources/regular_issues/Regular_Issues_Instructions_to_Authors.pdf).

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In the peer review process, 2 or 3 referees independently evaluate the scientific quality of the submitted manuscripts. At least 2 referee reports are required to help make a decision.

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What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are 7 steps to the editorial review process:

1. The journal's Chief Executive Editor and the Editor-in-Chief examine the paper to determine whether it is relevance to journal needs in terms of novelty, impact, design, procedure, language as well as presentation and allow it to proceed to the reviewing process. If not appropriate, the manuscript is rejected outright and the author is informed.

2. The Chief Executive Editor sends the article-identifying information having been removed, to 2 or 3 reviewers. They are specialists in the subject matter of the article. The Chief Executive Editor requests that they complete the review within 3 weeks.

   Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions for strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the research field.

3. The Editor-in-Chief examines the review reports and decides whether to accept or reject the manuscript, invite the authors to revise and resubmit the manuscript, or seek additional review reports. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the authors) are forwarded to the authors. If a revision is indicated, the editor provides guidelines to the authors for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors return a revised version of the paper to the Chief Executive Editor along with specific information describing how they have answered' the concerns of the reviewers and the editor, usually in a tabular form. The authors may also submit a rebuttal if there is a need especially when the authors disagree with certain comments provided by reviewers.

5. The Chief Executive Editor sends the revised manuscript out for re-review. Typically, at least 1 of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the Editor-in-Chief examines their comments and decides whether the manuscript is ready to be published, needs another round of revisions, or should be rejected. If the decision is to accept, the Chief Executive Editor is notified.

7. The Chief Executive Editor reserves the final right to accept or reject any material for publication, if the processing of a particular manuscript is deemed not to be in compliance with the S.O.P. of *Pertanika.* An acceptance notification is sent to all the authors.

   The editorial office ensures that the manuscript adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any minor queries by the editorial office. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, **only essential changes are accepted**. Finally, the manuscript appears in the pages of the journal and is posted on-line.

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ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS

Pertanika is over 40 years old; this accumulated knowledge has resulted in the journals being abstracted and indexed in SCOPUS (Elsevier), Clarivate-Emerging Sources Citation Index [ESCI (Web of Science)], BIOSIS, National Agricultural Science (NAL), Google Scholar, MyCite and ISC.

Pertanika Journal of Tropical Agricultural Science
Vol. 43 (4) Nov. 2020

Contents

Foreword

Welcome to the 4th 2020 issue of the Journal of Tropical Agricultural Science (JTAS)!

JTAS is an open-access journal for studies in Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university for the benefit of the world-wide science community.

This issue contains 19 articles; 1 is review article, 1 is short communication and the rest are regular articles. The authors of these articles come from different countries namely India, Indonesia, Japan, Malaysia, Nigeria, Taiwan, Thailand and Vietnam.

Articles submitted for this issue cover various scopes of Tropical Agricultural Science including: animal production; aquaculture; biotechnology; crop and pasture production; fisheries sciences; food and nutrition development; genetics and molecular biology; horticulture; plant physiology; soil and water sciences; and zoology.

A regular article entitled "Screening for Sarawak Paddy Landraces with Resistance to Yellow Rice Stem Borer, *Scirpophaga incertulas* (Walker) (Lepidoptera: Crambidae)" screened Sarawak paddy landraces for resistance to *S. incertulas*. Twelve Sarawak paddy landraces were hereby selected randomly for this study. Their levels of antixenosis resistance against *S. incertulas* were different. Kanowit is more preferred by *S. incertulas* for ovipositing on the abaxial leaf surface of rice plant. Whereas, the antibiosis resistance against the eggs and larvae of *S. incertulas* seems absent. The detailed information of this study is available on page 491.

A selected article entitled "Bali Bananas (*Musa* spp. L.) Genetic Relationship Based on Internal Transcribed Spacer 2 (ITS-2)" determined the genetic relationship of Bali banana cultivars using the internal transcribed spacer 2 (ITS-2) region as a molecular marker. The result showed that two clades were formed from thirty-nine banana samples, in which one clade was abundant in A genome (AA and AAA), and the other rich in the B genome (BB and ABB). It suggested that cultivars that had similar genomic compositions tended to be grouped within the same clade and separated with different genomic compositions. Further details of this study are found on page 583.

Mohammad Mijanur Rahman and his teammates from Universiti Malaysia Kelantan focused on the effect of *Asyastasia gangetica* and *Brachiaria decunbens* on intake, digestibility, and growth performance of rabbits. They concluded that the diet containing *A. gangetica* showed more benefits in terms of code protein (CP) and neutral detergent fibre (NDF) intakes, weight gain and feed conversion ratio (FCR). On the other hand, the diet containing *B. decumbens* gained lower rate of body weight (BW). Overall, a combination of concentrate and *A. gangetica* is recommended to be utilised for feeding the growing rabbits. Full information of this study is presented on page 609.

We anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

All the papers published in this edition underwent Pertanika's stringent peer-review process involving a minimum of two reviewers comprising internal as well as external referees. This is to ensure that the quality of the papers justifies the high ranking of the journal, which is renowned as a heavily-cited journal not only by authors and researchers in Malaysia but by those in other countries around the world as well.

In the last 12 months, of all the manuscripts processed, 37% were accepted. This seems to be the trend in Pertanika JTAS.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers, Editor-in-Chief and Editorial Board Members of JTAS, who have made this issue possible.

JTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

Chief Executive Editor
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Review Article

Effects of *Spirulina platensis* and *Chlorella vulgaris* on the Immune System and Reproduction of Fish

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ABSTRACT

This review briefly highlights previous studies on the effects of *Spirulina platensis* and *Chlorella vulgaris* on the health and reproduction of fish. These microalgae have diverse potentials. This study can be used as a stepping stone in advancing the aquafeed industry by formulating microalgae-based feeds. It can be made to specifically enhanced the health status of fish and its reproductive system through the supplementation and/or replacement of fishmeal or other plant proteins such as soybean meal. Hence, it could be more sustainable than depending on natural fish stocks. The usage of antibiotics and vaccines to solve the issue of disease outbreak in aquaculture, as well as the usage of hormones for the growth and reproduction of fish, can also be replaced by the usage of *S. platensis* and *C. vulgaris*. The inclusion of these microalgae in fish feed has affected hematological parameters and survival in fish as it boosts the numbers of white and red blood cells and thus affecting the immunity-stimulating capacity in fish. Besides, these microalgae also affect the fecundity and survival of fish eggs and thus directly affecting the reproduction performance of fish. *Spirulina platensis* affects eggs production and survival in fish whereas *C. vulgaris* enhances oxidative stress that affects the reproduction of White rabbits. This review aimed to deliver the results on the research of *S. platensis* and *C. vulgaris* on the immunity and reproduction of various fish species.

Keywords: *Chlorella vulgaris*, immunity, microalgae, reproduction, *Spirulina platensis*

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INTRODUCTION

The increasing human population worldwide has pressured the natural stocking of fish. Hence, aquaculture industries are blooming all over the world to support the growing demand for protein sources. This industry also provides food security and directly responsible in developing the livelihood of less-privileged communities. Aquaculture industry is being enforced and promoted as an important tool that drives economic growth in Malaysia and has been enlisted in the National Key Economic Area (NKEA), which highlights the 16 agro-food's entry point projects (EPP) (Yusoff, 2015). Statistics from the Department of Fisheries Malaysia (DOF) (2018) reported that the production of freshwater aquaculture was 102,500 tonnes in 2017 and increased to 105,700 tonnes in 2018, while the production of brackish water aquaculture was 324,300 tonnes in 2017 and 290,900 tonnes in 2018. The rapid growth of the aquaculture industry, particularly fish farming, has raised a number of issues in the health management of fish in terms of fish immunity against disease outbreak and the continuous supply of good quality fish seeds in terms of fish reproduction and developments.

This scenario is often related to the use of chemicals in aquaculture for a successful production (Subasinghe, 2004). However, such application is not widely encouraged as it introduces several risks to the production system, environment, and human health (Melba & Rohana, 2008). Thus, chemotherapeutics has been replaced with other alternative sources that are more acceptable in aquaculture practises, such as adding microalgae in fish feed as supplements. Various forms of algal meal applications have been studied. Generally, microalgae are used as larval feed by some fish farmers, and different levels of inclusion are added in fish feeds to boost their beneficial effects on fish conditions (Brown, 2002). Several microalgae possess high protein, lipid, and carbohydrate contents. Besides, the biomass of microalgae is rich in proteins, and it can strive and compete fairly for its quality and quantity compared with regular food proteins, such as fish, soybeans, and eggs (Ejike et al., 2017). A handful of researches and studies had been carried out in the past to analyse the potential effects of microalgae in fish, and such attempts are still in progress with various developments on specific parameters in fish, such as the immune and health system as well as the reproductive system. In this review, the effects of *S. platensis* and *C. vulgaris* on the immunity and reproduction of fish are briefly elaborated.

POTENTIALS OF MICROALGAE IN AQUACULTURE

The growth of the aquaculture industry has gone hand in hand with the growth of the population worldwide to ensure that ample fish supply as a part of protein sources can be fulfilled. The aquaculture industry is expected to solve food security and nutritional well-being, reduce poverty, and develop the economy (Melba & Rohana, 2008). However, the focus on developing

the aquaculture industry to fulfil such expectation has raised several issues in aquaculture. Among the most recognisable problem is the use of chemotherapeutants in aquaculture. Chemotherapeutics has benefits and is a key to a successful aquaculture production in activities, such as pond and tank constructions, feed formulation, growth promotion, health management, and booster of natural production (Melba & Rohana, 2008; Subasinghe, 2004). Nevertheless, the use of chemicals in aquaculture is not widely encouraged. Various researches and tests have been carried out to develop products that are friendlier to replace the use of chemicals in aquaculture. The organism that is known to be a more sustainable choice in replacing the use of chemotherapeutants in aquaculture is microalga. The growing interest in the multipurpose properties of microalgae has developed various microalgae applications in daily life. Microalgae are an alternative to sustainable aquaculture practices by playing roles in wastewater treatment and ingredient replacement in fish feed. The development of fish feed with the inclusion of microalgae is widely studied because of its encouraging effects on farmed fish. Microalgae can be used in aquaculture as feed, growth enhancers, and immunostimulants (Ahmad et al., 2018). However, microalgae must be easily cultured and nontoxic to be used in aquaculture (Spolaore et al., 2006). The criteria that are taken into consideration before incorporating microalgae as feed ingredients for fish are that they need to have the correct size, are easily ingested and digested by fish, and have high nutritional quality profiles (Brown et al., 1999; Renaud et al., 2002). Research on microalgae, such as *S. platensis* and *C. vulgaris*, their effects in enhancing the immunity and reproduction performance of fish and other organisms is continuous and evolving.

GENERAL INTRODUCTION

Spirulina platensis

Spirulina or *Arthrospira platensis* is a blue-green filamentous alga that inhabits freshwater bodies. Its name is acquired from its cylindrical shape with multicellular trichomes in an open left-handed helix (Figure 1) (Jung et al., 2019). Morphologically, *S. platensis* is a helicoid alga with a radial that is distinct from those of other species (Promya et al., 2008). This microalga has a protein content of up to 70% and is rich in vitamins, minerals, and essential fatty acids, such as linolenic and linoleic acids, and palmitic acid (Abdel-Tawwab & Ahmad, 2009). *Spirulina* has been used as a dietary supplement for a long time by the community that resides near the alkaline lakes where it is habitually found (Jung et al., 2019). Today, *Spirulina* is produced in numerous countries in Africa and America, such as Benin, Burkina Faso, Chad, Brazil, Chile, and Costa Rica, as well as Asian countries, such as Thailand, India, China, Vietnam, and Taiwan (Habib et al., 2008). However, the production of *Spirulina* is carried out in control conditions to avoid contaminations from other sources, such as blue-green algae, pesticides or heavy metal; thus, its general composition is affected by

the location and type of production (Table 1) (Jung et al., 2019). The rich contents of this microalgae species made it an interesting ingredient for testing as feed in fish. Various studies have analysed the immunology, disease resistance, oxidative stress, and growth performance of a number of aquatic animals based on *S. platensis*-supplemented feeds (Abdel-Latif & Khalil, 2014; El-Sheekh et al., 2014; Kim et al., 2013; Macias-Sancho et al., 2014; Promya & Chitmanat, 2011; Teimouri et al., 2013; Yeganeh et al., 2015).

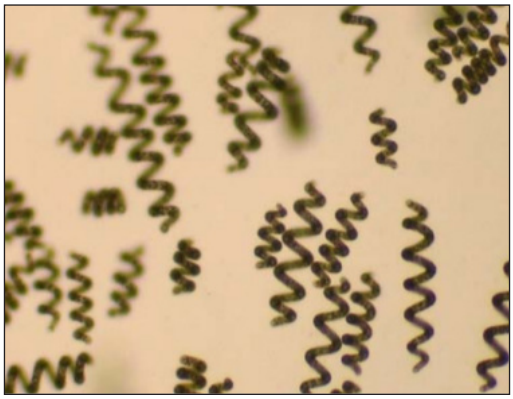

Figure 1. The microscopic view of *Spirulina*
Note. Adapted from "Earth food *Spirulina* (*Arthrospira*): Production and quality standarts", Retrieved June 01, 2020, from https://www.intechopen.com/books/food-additive/earth-food-spirulina-arthrospira-production-and-quality-standarts. Copyright 2012 by Koru. Adapted with permission

Table 1
The general composition of Spirulina*. Adapted from "*Spirulina platensis*, a super food?" (Jung et al., 2019)*

Components	Percentage (%)
Proteins	55-70
Carbohydrates	15-25
Lipids	6-8
Minerals	7-13
Humidity (dried algae)	3-7
Dietary fibers	8-10

Chlorella vulgaris

Chlorella vulgaris is a freshwater species and unicellular alga that contains a nutrient-dense super food, including various vitamins and minerals, 18 amino acids, and 60% protein (Khani et al., 2017). A report by Nick (2003) also stated that *Chlorella* possessed excess minerals, such as iron, calcium, potassium, magnesium, phosphorous, and 20 vitamins, such as pro-vitamin A, vitamins C, B1, B2, B2, B5, B6, B12, E, K; biotin, inositol, and folic acid. Morphologically, Beijerinck (as cited in Safi et al., 2014, p. 266) discovered *C. vulgaris* as the first microalga with a well-defined nucleus. *Chlorella* has a rigid cell wall that varies according to each growth phase and provides protection against invaders and harsh environment (Safi et al., 2014) (Figure 2). In addition,

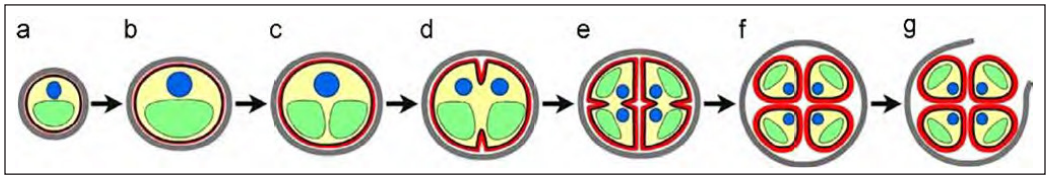

Figure 2. The growing phases of *Chlorella vulgaris*
Note. Adapted from "Morphology, composition, production, processing and applications of *Chlorella vulgaris:* A review" by C. Safi, B. Zebib, O. Merah, P. Y. Pontalier, and C. Vaca-Garcia, 2014, *Renewable and Sustainable Energy Reviews*, *35*, pp. 265–278. Copyright by Elsevier

this microalga has many fundamental components that are identical to actual plants, including cytoplasm, mitochondrion, and chloroplast. Besides, it is one of the microalgae that contain the highest quantity of chlorophyll compared with other plants (Raji et al., 2018). *Chlorella* has a unique phytonutrient property that is made up of vitamins, nucleic acid-related substances, amino acids, proteins, peptides, and sugars known as the *Chlorella* growth factor (CGF), which is found in abundance in the nuclei of the alga (Nick, 2003).

ENHANCEMENT OF FISH IMMUNE SYSTEM

Spirulina platensis

The properties of *S. platensis* that are rich in proteins and vitamins have a positive impact on the immunity of the animals that consumed it (Promya & Chitmanat, 2011). However, the effects of *S. platensis* on the immune system of fish may vary according to the levels of supplementation or inclusion. Promya and Chitmanat (2011) stated that a 5% supplementation of *Spirulina* in the feed resulted in higher red blood cell (RBC) and white blood cell (WBC) counts in African sharptooth catfish as well as advanced its immunity-stimulating capacity. The result is remarkedly different compared to a 3% supplementation of *Spirulina*, which gave a lower immunity in African sharptooth catfish. Besides, a feeding experiment on Nile tilapia with five levels of *Spirulina* inclusion (1.25, 2.5, 5.0, 7.5, and 10.0 g kg^{-1} diet) added to the basal diet showed that the RBC and WBC counts increased from 1.92×10^6 μL^{-1} to 2.54×10^6 μL^{-1} and 3.21×10^6 μL^{-1} to 4.02×10^6 μL^{-1}, respectively, as the *Spirulina* levels increased, although 5.0 g kg^{-1} *Spirulina* is the optimum amount that gave the highest specific growth rate, weight gain, and food conversion ratio with no substantial difference in the survival rate from one treatment to another (Abdel-Tawwab & Ahmad, 2009). These results showed that different levels of *Spirulina* inclusion in fish diet affect blood counts. Blood biochemical indexes can be an indicator that reflects the health status, physiological condition, metabolism, and immunity of fish (Zhou et al., 2001). In addition, alkaline phosphate (ALP) activity in fish is another parameter that is commonly used to study the immunity of fish. ALP is a non-specific phosphate hydrolase that is involved in fish metabolic regulation and plays a vital role in non-specific immune response in organisms (Huang et al., 2005). Lin et al. (2016) showed an increasing trend in the ALP activity of golden pomfret (*Trachinotus ovatus*) based on the 0%–6% supplementation of *S. platensis* in the fish diet and suggested that further experiment should be carried out for the ALP activity in fish. C-phycocyanin, a property of *Spirulina*, helps build immune capacity (Vonshak, 1997) and could be the reason for the enhancement of the immunity of fish based on the feeding trials with the inclusion or supplementation of *Spirulina*. Furthermore, a different approach of administering *Spirulina* through direct or oral method was

also investigated. A test on carp (*Cyprinus caprio*) intubated with 0 (control), 1, 10, and 25 mg doses of *Spirulina* suspended in sterilised physiological saline (0.85% NaCl) and subjected to *Aeromonas hydrophilia* (strain MU9901) infection showed that the number of bacterial cells in the liver and kidney of carp treated with *Spirulina* were lower compared with those of the control groups at the treatment intervals of 4, 8, and 12 hours and at 1 and 4 hours post-bacterial challenge (Watanuki et al., 2006). Another challenge test using sturgeon (*Huso huso*) that was intraperitoneally injected with *Streptococcus iniae* (strain ATCC29178) after 8 weeks of feeding period with 0%, 2.5%, 5%, and 10% of *Spirulina* added to the basal diet showed that the cumulative mortality of sturgeon decreased with the increasing supplementation of *Spirulina* (Adel et al., 2016). These challenge tests suggested that *Spirulina* could activate leucocyte activities in fish and thus increased the resistance against bacterial infections. Leucocytes are involved in superoxide production, cytokine release, and phagocytosis (Watanuki et al., 2006). The effects of *Spirulina* in enhancing the immunity of fish from disease infections and increasing the blood biochemical indexes in fish could create a specific market for *Spirulina*-based feed or supplements for aquaculture use. *Spirulina* could also be an alternative to replace the usage of antibiotics and vaccines, which are less environmentally friendly and have higher cost.

Chlorella vulgaris

Chlorella vulgaris is a green microalgae species with an engaging immunostimulant property that enhances the health and increases the life expectancy of fish (Gouveia et al., 2002). Formulated diet supplemented with 2%, 5%, 7%, and 10% of *C. vulgaris* dry powder gave higher values of C4, total immunoglobulin and lysozyme in koi carp than the control group (0% *Chlorella vulgaris* inclusion); thus, *C. vulgaris* could be involved in the modulation of the innate immunity of fish during the experimental period of 8 weeks (Khani et al., 2017). Khani et al. (2017) also found that the koi group fed with *C. vulgaris*-supplemented diets also had higher haemoglobin and haematocrit levels, and the highest values were obtained by 5% *Chlorella vulgaris* supplementation. These components are important for the survival of fish and linked to the oxygen-binding capacity of blood (Bielek & Strauss, 1993). The addition of *C. vulgaris* powder supplement to the formulated diet increased the levels of IgM, IgD, interleukin-22 (IL)-22, and chemokine (C–C motif) ligand 5 in Gibel carp (*Carassius auratus gibelio*) (Zhang et al., 2014). The expression of various cytokines, such as IL-8 and IL-1, in fish fed with microalga-supplemented diet is greatly affected (Díaz-Rosales et al., 2008). This report supports the immunostimulant potential of *C. vulgaris* in boosting the immunity of fish. Besides, *C. vulgaris* contains abundant carotenoid and thus subjected to various studies in yielding different types of carotenoids (Markou & Nerantzis, 2013). Fish with

high carotenoid content are more immune to fungal and bacterial infections (Gupta et al., 2007). Besides, carotenoids are soluble lipid pigments that play a role in the formation of skin colouring in ornamental fish [Kestemont et al. (as cited in Liang et al., 2012, p. 2); Paripatananont et al., 1999]. The biomass of *C. vulgaris* is more efficient in enhancing skin colouring and produces the highest deposition of carotenoid and red hue in three varieties of chromatic koi carps, namely, Kawari (red), Showa (black and red), and Bekko (black and white) (Gouveia et al., 2003). Colouring and pattern formation are important in ornamental fish as those traits will determine their quality (Li et al., 2008). The high bioavailability of carotenoids in *C. vulgaris* and the thin cellular membrane of this microalga are the possible characteristics that gave better efficiency for the skin colouring of fish, even for species that have relatively short digestive tracts (Gouveia et al., 1998). *Chlorella vulgaris* also remarkably enhances the growth performance and feed intake of koi carp at 5% inclusion in feed and other parameters, such as specific growth rate and protein and lipid efficiency rate, in contrast to the group without any *C. vulgaris* inclusion; thus, the growth enhancement is possibly caused by the high digestibility of this microalga (Khani et al., 2017). This enhancement may also be due to growth promoter properties, such as the adequate amounts of macronutrient and CGF in *C. vulgaris* (Badwy et al., 2008; Yamaguchi, 1996) and its high-quality protein content (Kang et al., 2013). However, further research on the growth effects of *Chlorella* should be carried out in various fish species and might not give the same positive effects. Its effect can be affected by the levels of supplementation, species-specific reactions to *Chlorella*, and the experimental period (Rahimnejad et al., 2016). Hence, the addition of *C. vulgaris* in feed for its carotenoid contents and property in stimulating the growth and immunity of fish is a promising idea as *C. vulgaris* is also known as a fast-growing microalga and can be a sustainable aquafeed ingredient in the future.

The addition or inclusion of *S. platensis* and *C. vulgaris* in fish diet and their effects on fish immune system are summarised in Table 2. Further trials on these microalga species on different fish stages, sizes, age, and environment should be investigated to improve the knowledge of suitable fish diet formulation with the addition of microalgae as natural supplements that could boost the immune system of fish. However, such approach requires a broad understanding on the abilities of different fish species to digest or utilise the target properties in microalgae, particularly the immunostimulant properties.

IMPROVEMENT OF FISH REPRODUCTION SYSTEM

Spirulina platensis

The effects of *S. platensis* on the reproduction and survival rates of fish were studied by several researchers throughout the years. The quality of fish brooders is the important key to a successful fish propagation. The features that define the reproductive capability and

Table 2
Summary of addition or inclusion of Spirulina platensis *and* Chlorella vulgaris *in fish diet and their effects on fish immunity*

Microalgae	Fish species	Diet / Test type(s)	Effects on immunity	Reference(s)
Spirulina platensis	*Clarias gariepinus* (African sharptooth catfish)	D1 (Control): Instant fish feed (obtained from animal feed supply) D2: Instant fish feed + 3% *Spirulina* algae D3: Instant fish feed + 5% *Spirulina* algae	Higher counts of WBC, RBC, and lysozyme activity assay in 3% and 5% *Spirulina* supplementation diets as compared to control	Promya and Chitmanat (2011)
	Oreochromis niloticus, L. (Nile tilapia)	D1 (Control): Basal diet (30.6% crude protein, 9.1% lipids, 4.72 kcal GE g^{-1}) D2: Basal diet + 1.25 g *Spirulina*/ kg diet D3: Basal diet + 2.5 g *Spirulina*/ kg diet D4: Basal diet + 5.0 g *Spirulina*/ kg diet D5: Basal diet + 7.5 g *Spirulina*/ kg diet D6: Basal diet + 10.0 g *Spirulina*/ kg diet	Increasing number of WBC, RBC, and lymphocytes as the amount of *Spirulina* added in diets increased	Abdel-Tawwab and Ahmad (2019)
	Trachinotus ovatus (Golden pomfret)	D1 (Control): Basal diet (50.27% crude protein, 8.35 crude lipid) D2: Basal diet + 1.0% *Spirulina* D3: Basal diet + 2.0% *Spirulina* D4: Basal diet + 3.0% *Spirulina* D5: Basal diet + 4.0% *Spirulina* D6: Basal diet + 5.0% *Spirulina*	Increasing trend in the alkaline phosphate (ALP) activity as the amount of *Spirulina* in the diet increased	Lin et al. (2016)
	Cyprinus caprio (Carp)	Direct intubation (0.1 ml suspension) Dose 1: 0 dose (0.85% NaCl) Dose 2: 0.85% NaCl + 1 mg *Spirulina* Dose 3: 0.85% NaCl + 10 mg *Spirulina* Dose 4: 0.85% NaCl + 25 mg *Spirulina*	Lower number of bacterial cell (challenged with *Aeromonas hydrophilia* - strain MU9901 infection) in liver and kidney as compared to control	Watanuki et al. (2006)
	Huso huso (Sturgeon)	D1: Basal diet (Control) D2: Basal diet + 2.5% pure dried *Spirulina* D3: Basal diet + 5.0% pure dried *Spirulina* D4: Basal diet + 10.0% pure dried *Spirulina*	Cumulative mortality of the sturgeon decreased with the increasing supplementation of the *Spirulina* in basal diet (challenged with *Streptococcus iniae* - strain ATCC29178) through intraperitoneal injection	Adel et al. (2016)

Table 2 (*continue*)

Microalgae	Fish species	Diet / Test type(s)	Effects on immunity	Reference(s)
Chlorella vulgaris	*Cyprinus caprio* (Carp)	D1: Formulated feed (Control) D2: Formulated feed + 2% dry powder *C. vulgaris* D3: Formulated feed + 5% dry powder *C. vulgaris* D4: Formulated feed + 7% dry powder *C. vulgaris* D5: Formulated feed + 10% dry powder *C. vulgaris*	Higher values of immune parameters: C4, total immunoglobulin, and lysozyme than the control Higher values of haemoglobin (Hb) and haematocrit (Ht) than control – 5% of *Chlorella vulgaris* supplementation gave highest values	Khani et al. (2017)
	Carassius auratus gibelio (Gibel carp)	D1: Formulated diet (Control) D2: Formulated diet + 0.4% *Chlorella* powder D3: Formulated diet + 0.8% *Chlorella* powder D4: Formulated diet + 1.2% *Chlorella* powder D5: Formulated diet + 1.6% *Chlorella* powder D6: Formulated diet + 2.0% *Chlorella* powder	Increasing immunoglobulin (Ig) M and D, interleukin-22 (IL)-22, and chemokine (C-C motif) ligand 5 (CCL-5) as the *Chlorella* supplementation in diet increased	Zhang et al. (2014)

performance of brooders rely on the eggs' fecundity, diameter, and hatchability rates (Chong et al., 2004; Kumaraguruvasagam et al., 2007; Izquierdo et al., 2001). *Spirulina platensis* provides a remarkably higher egg survival rate of 73%, shorter hatching and faster larval development compared with the commercial flake diet-fed group when given as sole food for zebrafish broodstock with the hypothesis that commercial flake does not contain as much Omega-6 fatty acid as *S. platensis* (Geffroy & Simon, 2013). In addition, *S. platensis* contains omega-6 fatty acid (which made up 41.2% of fatty acids), specifically gamma linolenic acid and linoleic acid (Qiang et al., 1997). These properties are the precursors of arachidonic acid, which is a remarkable constituent in the formation of prostaglandin that interferes in oocyte maturation, ovulation, and steroidogenesis (Pati & Habibi, 2002; Patiño & Sullivan, 2002). Hence, *Spirulina* is a promising diet for adult fish. *Spirulina platensis* also considerably increases the hatching percentage and total egg reproduced by yellow tail cichlid (*Pseudotropheus acei*) during a 12-week observation period (Güroy et al., 2012). This microalga species could possibly replace the administration of artificial hormones in enhancing reproduction performance by incorporating it in the diet of brooders. Besides, raw *Spirulina* was also tested on Nile tilapia as the primary feed and has increased

the egg production, hatching percentage, and survival rates of fish compared with conventional fish feed (Promya & Chitmanat, 2011). *Spirulina* as a replacement fish meal for the feeding of three-spot gourami (*Trichopodus trichopterus*) provides greater gonadosomatic indices (19.4%–21.85%) and affects the absolute fecundity of fish between 7,300 and 12,700 eggs per female at 2.5%–10% *Spirulina platensis* replacement levels than the group fed with fish meal only (Khanzadeh et al., 2016). The addition of this microalga can result in the enhancement of gonad maturation. Apart from that, *S. platensis* possesses fat soluble pigments (carotenoids), such as xanthophylls, B-carotene, echinenone, cryptoxanthin, and zeaxanthin (Nakagawa & Montgomery, 2007). Reproductive performance can be enhanced by dietary carotenoids (Watanabe & Vassalo-Agius, 2003). Increasing dietary carotenoid supplementation enhances the reproduction capability of different fish species, such as rainbow trout (*Oncorhynchus mykiss*) (Dabrowski et al., 1987), gilthead seabream (*Sparus aurata*) (Scabini et al., 2010), and yellow tail (*Seriola quinqueradiata*) (Vassallo-Agius et al., 2001, 2002). These discoveries show that the carotenoids in *S. platensis* play multiple roles, that is, it affects the colouration of fish and improves their reproductive performance. Thus, *S. platensis* can be one of the primary sources to be developed in ornamental fish industry by focusing on its capability to enhance the colouration and reproductive performance of the fish.

Chlorella vulgaris

Based on the knowledge obtained in this review, very scarce information is available on the effects of *C. vulgaris* on the reproduction of fish. Some of the studies found were only focused on mammals. For example, Sikiru et al. (2019) reported that *C. vulgaris* enhances oxidative stress, which was an exclusive biochemical complication that affected the reproduction in New Zealand White rabbits. Besides, the extract of *C. vulgaris* improves the histological adjustment of monosodium glutamate in ovarian tissue, the level of sex hormones, and increases the level of ovarian enzymatic antioxidants in adult female albino mice (Abdel-Aziem et al., 2018). Hence, its effects on fish should be further explored for potential use in aquaculture.

FUTURE PROSPECT AND CONCLUSION

The inclusion of *Spirulina platensis* and *Chlorella vulgaris* as feed supplement has substantial impacts on fish health and immunity. Planning better tests on a bigger scale of fish culture system is important to see the pattern of immunity and hematological parameters in natural culture conditions than in controlled laboratory environments. Besides, *S. platensis* has a potential in affecting the performance of fish brooder, which directly influences the quality and survival of eggs. Well-modified feed products based on *S. platensis* that focus more on the reproductive effects in fish could have a great future in aquaculture. Apart from that, more research should be

done to analyse the effects of *C. vulgaris* on fish to highlight the capability of carotenoids in evaluating the growth performance and immunity of fish. In conclusion, this review briefly presented the effects of *S. platensis* and *C. vulgaris* on the immunity and reproduction of fish. It is recommended that more studies should be conducted on the possible contributions of these microalgae in the aquafeed industry to create more environmentally effective antibiotics and vaccines to combat fish diseases in a culture system and enhance the reproduction of fish. Using renewable natural products, such as microalgae, will also benefit the aquaculture industry by having better practices that conserve the environment.

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Evaluation on Toxicity Level of *Terminalia catappa* Leaves Extract on Selected Cyprinids under Different Bath Concentrations

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ABSTRACT

This study aimed to determine the lethal concentration (LC_{50}) of *Terminalia catappa* leaves extract on three cyprinid species; carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and tiger barb (*Puntigrus tetrazona*) through the acute toxicity test. The leaves of *T. catappa* were extracted with methanol and prepared in various immersion concentrations (40, 80, 100, 150, 200, 250, 300, and 350 mg/L). These extracts were immersed in the aquarium and left for 24 h before performing the acute toxicity test. The water quality was also analyzed before and after adding the extract immersions into the aquarium. The acute toxicity test conducted for 96 h with 10 fishes of each cyprinid species (4.0-6.0 cm length) in 30 L water capacity aquarium. The mortality of each cyprinid species was recorded at 24 h time interval and LC_{50} of the extracts throughout 96 hours was determined through the probit analysis application. Specifically, the LC_{50} of *T. catappa* leaves extract were 349.89, 338.65 and 318.48 mg/L exhibited for carp, goldfish and tiger barb, respectively. A high concentration range of any plant-based extract has the potential to become toxic to particular fishes. Thus, it is an effort from this study to identify the safety margin of *T. catappa* leaves extract before its therapeutic values can be further manipulated and elucidated in aquaculture research.

Keywords: Leaves extract, lethal concentration, mortality, ornamental fish

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INTRODUCTION

The ornamental fish industry in Malaysia is expanding due to the increasing number of local fish exporters, even though there are still concerns over the economic viability and sustainability of this industry (Department

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of Fisheries [DOF], 2016). Cyprinidae considered among the popular fish families in Malaysia for the ornamental and food fish industries. Cyprinids were on the highest rank, which contributed about RM 103 million from a total of RM 341 million in ornamental fish production in 2015 (DOF, 2015). Among the cyprinid species, carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and tiger barb (*Puntigrus tetrazona*) are having a relatively high market demand due to its beautiful pattern and colors.

However, significant losses in many aquaculture industries from the uncontrollable disease outbreaks that emerge from various causes seemed to be unavoidable (Citarasu, 2012; Rodger, 2016). Progress in combating the diseases currently is time-consuming and has become slower through conventional protocols, since chemical drugs resistance problems are dramatically aroused (Liu et al., 2017; Song et al., 2015). To some extent, the over-dosage of antibiotics or antimicrobial treatments can spoil the captivating features and color of ornamental fishes, which ultimately affects its sale and market values (Marimuthu et al., 2012). As for issues related to the future of the ornamental fish industry, there are tremendous efforts and countless studies presently in search of alternative sources, especially from indigenous or herb plants that are convenient and harmless for disease treatments.

Terminalia catappa or locally known as 'ketapang' is a multi-purpose tree since almost all of its parts can utilize for human and animal benefits (Kadam et al., 2011). The dried leaves are said to have miraculous or therapeutic effects on the infected or injured fishes, according to locals in Southeast Asia (Chansue & Assawawongkasem, 2008). Therefore, it is common to see the leaves submerging in the culture tanks of fish breeders or even home aquariums as protection from diseases for their cultured fishes. *Terminalia catappa* leaves typically contain various phytoconstituents such as tannins, isovitexin, flavonoids and triterpenoids (Citarasu, 2010), which have proven efficient as fish anti-pathogenic through many *in vitro* tests (Allyn et al., 2018; Fakoya et al., 2019; Purwani et al., 2015; Walczak et al., 2017). The *in vivo* studies through bath treatment of these leaves on the infected fishes are still scarce and selective in certain species of ornamental fishes particularly on Siamese fighting fish (*Betta splendens*) and guppy fish (*Poecilia reticulata*) (Chansue & Assawawongkasem, 2008; Nugroho et al., 2017; Purivirojkul, 2012). Many researchers are likely more interested in the *in vitro* test compared to *in vivo* tests, as the preparations seem complicated with its toxicology and safety evaluations on live animals (Akinsanya et al., 2016; Manaharan et al., 2014). Although there are increasing numbers in plant remedy studies, their scientific works on its safety, toxicity and adverse effects for the specific host or target are not always discovered together at the same time (Kasthuri & Ramesh, 2018; Saad et al., 2006).

With the main focus on the immersion concentrations of *T. catappa* leaves extract, the present study aims to determine its toxicity level on the three selected cyprinid species, which are carp, goldfish and tiger barb. Information from this toxicity study would serve as an essential baseline for further studies in establishing *T. catappa* leaves extract as an alternative source of therapeutics against diseases in ornamental fishes.

MATERIALS AND METHODS

Preparation of *Terminalia catappa* Extract

The green leaves of wild-grown *T. catappa* were collected and dried under the sunlight for about one week with another 48 h drying with oven (Protech®-FSD 380) at 40°C. These dried leaves were pulverized to fine powders with a commercial blender (Butterfly® B-592) and freeze dried at -45°C (Labconco-7750030) as preparations for methanolic extraction. The extractions were performed for about 48 h using 100 g of *T. catappa* leaves powder with 1000 mL methanol according to the method by Yi et al. (2012). The excessive methanol was then evaporated using rotary evaporator (Eyela-N1100) and the obtained crude extract were kept in the freezer (-20°C) and used as a test solution.

Preparation of Fish Culture

The selected cyprinid species of carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and tiger barb (*Puntigrus tetrazona*) purchased from the local breeders; considerably similar in size (4.0-6.0 cm length). Prior to experiment, all fishes acclimatized under the laboratory conditions for seven days before conducting the acute toxicity test. The dissolved oxygen levels maintained within the standard concentration range for freshwater fishes between 3.0 to 7.0 mg/L (DOF, 2016) and the fishes were daily fed once with commercial fish pellet.

Acute Toxicity Test

Eight different concentrations of *Terminalia catappa* leave extract (40, 80, 100, 150, 200, 250, 300, and 350 mg/L), each with three replicates along with one control were set up in this test. The test conducted according to the method of Ekanem et al. (2004) within 96 h. The extracts initially immersed for 24 h before adding the 10 fishes of each cyprinid species into the 30-L tank aquarium. The water was continuously aerated without any change and fed not provided throughout the test. Any dead fish found under these circumstances during the observation at every 24 h was isolated from the aquarium. The total of death fishes throughout 96 h in the respective extract concentrations was finally recorded for determining the LC_{50} or lethal concentration of the extracts.

Water Quality Analysis

The quality of aquarium water with non-immersions and immersions of leaves extract was analyzed before performing the acute toxicity test. The pH, temperature,

dissolved oxygen and total ammonia-nitrogen of the water were measured using water quality probe (YSI Pro Plus). The final concentration of total ammonia-nitrogen was further determined using a Nessler-2458200 reagent set.

Statistical Analysis

Statistical analysis using one-way analysis of variance (ANOVA) was applied to the results of water quality analysis. The significant differences at 95% confidence level between the data samples distinguished using Duncan's multiple range test (Duncan-MRT). The lethal concentration or LC_{50} of *T. catappa* leaves extract on the tested cyprinid species was determined using probit analysis.

RESULTS

Acute Toxicity Test

No mortality was observed within the 96 h exposure for all cyprinid species in the concentrations ranging from 20 to 200 mg/L (Tables 1, 2, and 3). However, the mortality significantly increased when the tested concentrations distinctly increased from 250 to 350 mg/L. The highest bath concentration of 350 mg/L also demonstrated almost to 100% mortality in all cyprinid species, especially tiger barb.

Probit analysis was applied to determine the relative LC_{50} or lethal concentration of *T. catappa* that caused death in at least 50% from the tested cyprinid species in a specified period of 96 h. Through this analysis, different LC_{50} of *T. catappa* leaves

Table 1
Mortality rate (%) of carp (n=10) within 96 h bath immersion of Terminalia catappa *leaves extract at different concentrations*

Time (h)	Concentrations of extract (mg/L)								
	Control	40	80	100	150	200	250	300	350
24	0	0	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0	10
72	0	0	0	0	0	0	0	10	20
96	0	0	0	0	0	0	20	20	50
Total (%)	0	0	0	0	0	0	20	30	80

Table 2
Mortality rate (%) of goldfish (n=10) within 96 h bath immersion of Terminalia catappa *leaves extract at different concentrations*

Time (h)	Concentrations of extract (mg/L)								
	Control	40	80	100	150	200	250	300	350
24	0	0	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0	10
72	0	0	0	0	0	0	0	20	50
96	0	0	0	0	0	0	20	20	30
Total (%)	0	0	0	0	0	0	20	40	90

extracts were obtained on carp, goldfish and tiger barb with 349.89 mg/L (Figure 1), 338.65 mg/L (Figure 2), and 318.48 mg/L (Figure 3), respectively.

Table 3
Mortality rate (%) of tiger barb (n=10) within 96 h bath immersion of Terminalia catappa *leaves extract at different concentrations*

Time (h)	Concentrations of extract (mg/L)								
	Control	40	80	100	150	200	250	300	350
24	0	0	0	0	0	0	0	0	20
48	0	0	0	0	0	0	0	10	40
72	0	0	0	0	0	0	10	10	40
96	0	0	0	0	0	0	20	40	0
Total (%)	0	0	0	0	0	0	30	60	100

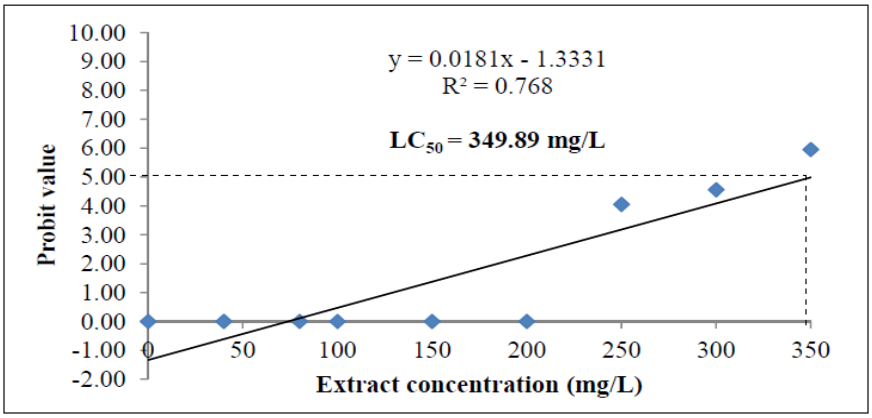

Figure 1. Probit analysis of determining the LC_{50} of *Terminalia catappa* leaves extract on carp (*Cyprinus carpio*) based on total mortality within 96 h

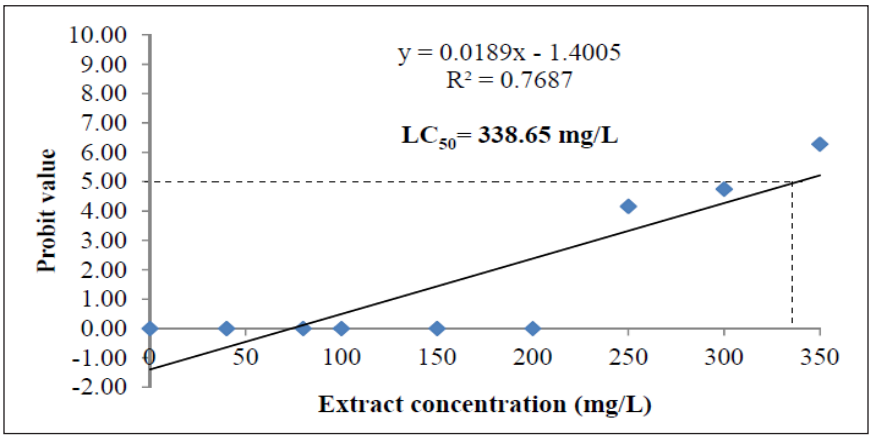

Figure 2. Probit analysis of determining the LC_{50} of *Terminalia catappa* leaves extract on goldfish (*Carassius auratus*) based on total mortality within 96 h

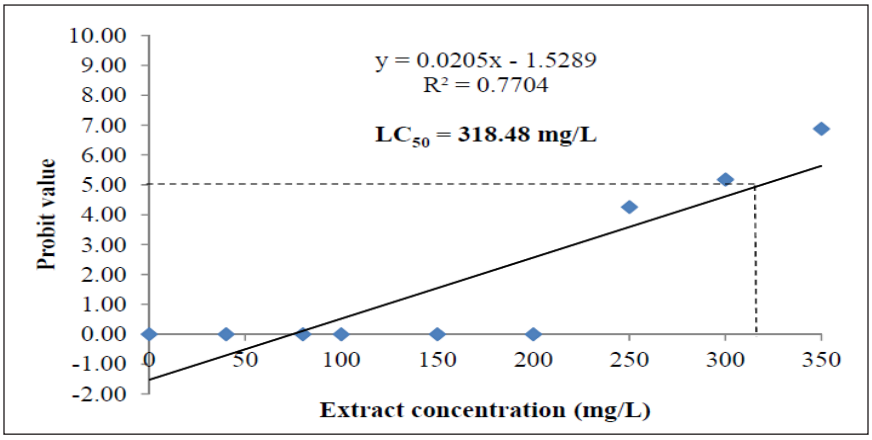

Figure 3. Probit analysis of determining the LC_{50} of *Terminalia catappa* leaves extract on tiger barb (*Puntigrus tetrazona*) based on total mortality within 96 h

Water Quality Analysis

The mean values for water quality analysis showed a paralleled increment between the extract concentrations and total ammonia-nitrogen concentrations but conversely different between the pH (Table 4).

DISCUSSION

The acute toxicity test of *T. catappa* leaves extract in this study revealed at a specific concentration range of its bath immersion could cause toxic and mortality on selected cyprinid species. Our findings were in agreement with Chansue and Assawawongkasem (2008)'s as well as Stratev et al. (2018)'s studies, which stated that any natural or herbal plant source could either be beneficial or detrimental to any fish since the level of its toxicity mainly depended on the applied extract

Table 4
Water quality parameters of different concentrations of Terminalia catappa *leaves extract for toxicology assessments on tested cyprinid species*

Concentration of extract (mg/L)	pH	Total ammonia-nitrogen (mg/L)
Control (no extract)	6.92 ± 0.05^{a}	0.43 ± 0.06^{a}
40	6.75 ± 0.04^{b}	0.82 ± 0.06^{b}
80	6.70 ± 0.02^{bc}	1.15 ± 0.06^{c}
100	6.64 ± 0.04^{cd}	1.45 ± 0.04^{d}
150	6.61 ± 0.04^{de}	1.91 ± 0.03^{e}
200	6.54 ± 0.04^{ef}	2.22 ± 0.04^{f}
250	6.51 ± 0.03^{f}	2.82 ± 0.04^{g}
300	6.42 ± 0.01^{g}	$>3.5^{h}$
350	6.39 ± 0.03^{g}	$>3.5^{h}$

Note. Data (mean) showed in two replicates with standard deviation. Values with the different letter ($^{a\text{-}h}$) in each column are significantly different at $p < 0.05$ based on Duncan-MRT

concentrations and targeted fish physical and species. Chansue and Assawawongkasem (2008) discovered that guppy (*Poecilia reticulata*) and Siamese fighting (*Betta splendens*) fishes (approximately 2.0 cm in length) had different toxicity effects by water extract of *T. catappa* leaves. Even if they are averagely similar in size, the guppy fish was found to be more sensitive than Siamese fighting fish with the LC_{50} for 96 h of 5.6 and 7.0 mg/L, respectively. Another study by Purivirojkul (2012) showed high LC_{50} (1,765.7 mg/L) of the *T. catappa* leaves extract on similar fish species utilized in Chansue and Assawawongkasem (2008)'s study; Siamese fighting fish but it is slightly bigger (about 4.0 cm in length) than the previous one. Furthermore, the concentrated plant extracts may also contribute to a severe cause to the non-target aquatic species in certain circumstances (Singh & Singh, 2002: Yunus et al., 2019). Therefore, a toxicological assessment of the plant extracts is indeed essential to conduct meticulously prior to usage on the targeted fish species or before other manipulation of the extract can further investigate.

The changes in pH value were observed where they were gradually reduced to acidic from the low to high concentrations of leaves extract in this study. These conditions were expected as *T. catappa* had the potential to lower the pH of water (Lee et al., 2016). However, *T. catappa* leaves extract at a low concentration ranging from 2.0 to 5.0 mg/L had proven to be non-effective on the acidity or alkalinity of the water, according to Bryan (2016). It showed acidic (pH 6.5-6.0) with 6.0 to 10.0 mg/L concentrations from the leaves extracted for three days using water in the study of Chansue and Assawawongkasem (2008). Most of the cultured fishes were well-tolerated with the pH ranges from 6.2 to 7.8 (Chansue, 2007; Chitmanat et al., 2005). Their growth and mortality might be affected and resulted from the rapid change of pH over 0.2 (Chansue & Assawawongkasem, 2008). Stone et al. (2013) also stated almost similar findings with Chansue and Assawawongkasem (2008), where the acceptable pH range for most fish species was within 6.5 to 9.0 and chronic pH level (below 6.5) may reduce the fish reproduction. Even our concentrations of the leaves extract varied, the acidic pH ranges and its effects on the tested fishes showed an agreement with the previous studies. No mortality was recorded in all tested cyprinid species between 40 and 200 mg/L of leaves extracts with acidic conditions within a pH of 6.75 to 6.54. The mortality of cyprinid species was only exhibited between 250 and 350 mg/L concentrations with lower pH than 6.54.

In general, many factors can contribute to the pH changes in the fish culture environment or condition. Ikhwanuddin et al. (2014) stated that the decreased pH or acidic conditions in any plant-based bath immersion could be reflected from the accumulation of its organic acids especially tannins. Tannin, a polyphenolic compound commonly found in most herbal plants may be employed as a source of alternative treatments in its adequate concentration as antibacterial, antiparasitic, antiviral and

antifungal for many fish species (Azrul et al., 2014; Chansue & Assawawongkasem, 2008; Chitmanat et al., 2005; Yunus et al., 2019). However, the excessive and high level of tannins in the water and feed formulation showed adverse effects on herbivorous and omnivorous fishes (Azrul et al., 2014; Mandal & Gosh, 2010). Chansue and Assawawongkasem (2008) found that heavy solid suspension adhered to the gills of guppy and Siamese fighting fishes through necropsy tests upon the completion of *T. catappa* leaves extract toxicity test. These findings could be evidence of irritation since the adhered gills were blocked for oxygen by a high concentration of tannins contained in the applied *T. catappa* leaves extracts. In the study of Borisutpeth et al. (2001), tannic acid at 97.5 mg/mL caused hyperplasia in epithelial cells of gill filaments, aneurysm of gill lamellae and disarray in tilapia; which ultimately died without histopathological changes in other organs within 96 h. Accumulation of significant level of tannin also found in the different fish species of Indian major and exotic carps and its tissues (liver and kidney) because of the tannin-like compounds presented in their feeding (Mandal & Gosh, 2010). Since no histopathological tests performed to support our findings, we stipulated that tannins at their high concentration levels and other active compounds (flavonoids, saponin, calcium oxalate and glycosides) contained in the *T. catappa* leaves (Tercas et al., 2017) have the potential to decrease the pH and ultimately caused mortality in the tested cyprinid species.

Total ammonia-nitrogen (TAN) is also another significant contributor to fish mortality other than tannins. Ammonia ionizes and deionizes the aquaculture system into NH_4+ and NH_3, respectively, which can be toxic depending on the levels present and fish species tolerance (Roberts & Palmeiro, 2008). Generally, any decomposing organic residue from the plant and fish waste has a great potential to raise the ammonia and pH levels in the water as protein break downs (Ip & Chew, 2010; Yavuzcan et al., 2017). Malaysia has set specific standards for acceptable and safety levels of TAN (ionized and unionized) for freshwater and marine fishes at 0.3 mg/L (DOF, 2016; White et al., 2008). At a low concentration of deionized ammonia of 0.05 mg/L, harmful effects such as poor growth rate, reduced fertility, increased stress, and susceptibility to disease could result in fish (da Silva et al., 2013). Meanwhile, at a high concentration that exceeds 2.0 mg/L, gills, and tissues can be damaged and ultimately causes mortality and death to the fish (Ip & Chew, 2010). TAN concentration in the present study found slightly higher in the control but extremely elevated (>3.5 mg/L) when the concentrations of leaves extract concurrently increased (Table 3). These evidences have enough to demonstrate that insignificant TAN concentration responsible to cause toxics to all tested cyprinid species in this study. However, other factors that influence the vulnerability of targeted fishes on TAN should consider as well. The toxicity level of TAN according to the standard is also dependent on the fish's

biological traits, sensitivity and adaptation levels to the affected environmental system (Wang et al., 2016). Remarkably, saltwater fish species found to be more sensitive to ammonia toxicity effects as compared to freshwater fish species according to Roberts and Palmeiro (2008). Therefore, we found that the tolerable capacity of our tested fishes varied towards the changes in their living environment even if they are from the same family. Goldfish managed to show higher tolerance than carp and barb in acidic pH condition and TAN concentration caused by the leaves extract immersion of *T. catappa*. Hence, overall results from this study can contribute significant knowledge to the aquaculture and local communities on the safety margin of these leaves to be utilized for their cultured fishes and provide promising insight into other potential use of *T. catappa* leaves extracts in fish ornamental industry.

CONCLUSION

The present study showed different effects of cyprinid species in various bath immersions of *T. catappa* leaves extract. The most tolerable threshold or safety concentration boundaries of the *T. catappa* leaves extract for all tested cyprinids were obtained up to 200 mg/L, where no mortality recorded among them. All tested cyprinids shared an almost similar LC_{50} between 300 and 350 mg/L. Further study based on these findings may be necessary to investigate the effectiveness of these leaves extract in improving the cyprinids' health and growth in a long term treatment.

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Botanical Extracts as Biofungicides against Fungal Pathogens of Rice

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ABSTRACT

Diseases such as blast, brown spot and sheath blight considerably affect the health and productivity of rice worldwide. Chemical fungicides have been routinely used in combating these diseases; however, a safe and environmental-friendly approach using bio-fungicides is desirable in disease management of food crop such as rice. Identification of botanical extracts with antifungal potentials would be instrumental in the development of bio-fungicides. In this study, the antifungal potentials of *Andrographis paniculata*, *Backhousia citriodora*, and *Phaleria macrocarpa* against selected rice fungal pathogens were analysed. Crude extracts obtained from leaves of these plants were diluted to 5, 10, 15, and 20% and tested against *Pyricularia oryzae*, *Exserohilum rostratum*, and *Rhizoctonia solani in vitro* using poisoned agar method. Percentage inhibition of diameter growth (PIDG) of each crude leaf extract against test pathogens was calculated. The aqueous extract of *A. paniculata* showed a significant mycelial inhibitory effect against *P. oryzae* at 20% concentration (PIDG 81.9%) as compared to other test concentrations and pathogens. On the contrary, the aqueous extract of *B. citriodora* at 15 and 20% concentrations had little influence on the mycelial growth inhibition on *P. oryzae* and *E. rostratum* with PIDG values less than 50%. In addition, *P. macrocarpa* methanol extracts at concentration of 10% and above significantly inhibited the mycelial growth of *P. oryzae*, *E. rostratum*, and *R. solani* (PIDG 100%). *Phaleria macrocarpa* leaf extract had been identified

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to give the highest efficacy against all three rice pathogens *in vitro* and therefore, has the potential to be developed into a bio-fungicide as a safe alternative to synthetic fungicides for disease management of rice.

Keywords: Biopesticide, botanical extract, green technology, percentage inhibition of diameter growth (PIDG), poisoned agar method

INTRODUCTION

Diseases occur in agriculture crops cause substantial losses to farmers around the world and are mostly caused by plant pathogenic fungi (Agrios, 2004). Plant fungal pathogens such as *Pyricularia oryzae* (teleomorph: *Magnaporthe oryzae*), *Exserohilum rostratum* (teleomorph: *Setosphaeria rostrata*), and *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*) cause important rice diseases such as blast, brown spot, and sheath blight, respectively. The estimated annual losses caused by rice blast range from nearly 10 to 30% globally (Skamnioti & Gurr, 2009). During rice blast epidemics, yield loss of rice can go up to 50% (Ashkani et al., 2015). Similarly, *R. solani* can cause reduction in yield by 50% when the environmental conditions are favourable (Richa et al., 2016). Meanwhile, *E. rostratum* has been identified as an emerging rice pathogen and its impact on rice productivity is still unknown (Toher et al., 2016).

Chemical fungicides have been considered as the most effective and commonly used in the management of most of the fungal diseases worldwide. For instance, probenazole, tricyclazole, azoxystrobin, isoprothiolane, and propiconazole are widely used in rice blast control (Gohel & Chauhan, 2015; Skamnioti & Gurr, 2009). Farmers are likely to choose chemical fungicides to protect their crops from fungal diseases because of its rapid effect, availability and cheaper price as compared to other methods of crop protection. However, synthetic fungicides possess detrimental attributes such as high and acute toxicity, long degradation period, accumulation in food chain and an extension of their power to destroy both useful organisms and harmful pests (Gatto et al., 2011). Moreover, long-term use of chemical fungicides may result in development of fungal resistant races and consequently, it will be more challenging to fight the disease. Therefore, it is vital to minimise the use of chemicals to maintain the sustainability of agriculture.

Use of naturally-occurring antifungal compounds in herbal plants has been regarded as one of the best alternatives to synthetic fungicides (Dissanayake & Jayasinghe, 2013; Kumar et al., 2017). Secondary metabolites such as phenols, flavonoids, and phenolic glycosides are produced abundantly in herbal plants and many of them possess antifungal activity. For many years, researchers have documented the antimicrobial properties and activities of plant oils and extracts. For instance, bioactivities of phytochemicals of *Andrographis paniculata*, a medicinal plant from the family Acanthaceae, including

andrographolide, isoandrographolide, neoandrographolide, 14-deoxy-11, 12-didehydroandrographolide, flavonoids, quinic acid derivatives, and xanthones have been well-reviewed by Ganapumane and Nagella (2020). Besides, four essential oils of *Backhousia citriodora*, an Australian shrub from the family Myrtaceae, appeared to be effective antimicrobial agents when tested against a wide range of pathogenic fungi and bacteria (Wilkinson et al., 2003). Likewise, the antimicrobial activity of essential oil of *B. citriodora* and citral, the major constituent of its essential oil against an array of bacteria, fungi and yeast have been also reported (Hayes & Markovic, 2002). On the other hand, the fruit extract containing flavonoid compounds such as kaempferol, myricetin, naringin, quercetin, and rutin of *P. macrocarpa*, a medicinal plant from the family Thymelaceae, exhibited varied antimicrobial activity against test bacteria and fungi (Hendra et al., 2011).

However, the above mentioned studies were conducted to evaluate the efficacy of these herbal extracts on human pathogens. It is hypothesized that these antimicrobial compounds would exert similar effects on plant pathogens. Percentage inhibition of diameter growth (PIDG) served as an indicator for antifungal activity of *Brucea javanica* extracts against several *Candida* species as compared to positive control (Nordin et al., 2013). Similarly, the antifungal effect of *Asteriscus imbricatus* extracts against *Botrytis cinerea* was compared using PIDG values (Senhaji et al., 2013). Therefore, in this study, we evaluated the antifungal potential of *A. paniculata*, *B. citriodora*, and *P. macrocarpa* against *P. oryzae*, *E. rostratum*, and *R. solani*, the three major fungal pathogens of rice *in vitro* using PIDG method.

MATERIALS AND METHODS

Collection of Plant Materials

The leaves of *A. paniculata* were collected from Herb Garden, University Agriculture Park, Universiti Putra Malaysia (UPM), Serdang, Selangor. Meanwhile, the leaves of *B. citriodora* and *P. macrocarpa* were collected from Department of Agriculture, Serdang, Selangor. The experiments were carried out in Mycology Laboratory, Department of Plant Protection, Faculty of Agriculture, UPM, Serdang, Selangor. *Phaleria macrocarpa* leaf samples were thoroughly washed and air-dried at room temperature (26 ± 2°C) according to Venkateswarlu et al. (2013). Meanwhile, *A. paniculata* and *B. citriodora* were dried at 50-60°C upon a thorough washing according to Buchaillot et al. (2009) with modifications. The samples were then separately ground to fine uniform texture using grinder (Retsch Model SK 100) and stored at room temperature (26 ± 2°C) until use.

Fungal Cultures

Fungal stock cultures were obtained from the Culture Collection Unit, Department of Plant Protection, Faculty of Agriculture, UPM, Serdang, Selangor. Fungal species

that were used in this study were *P. oryzae*, *E. rostratum*, and *R. solani*. The culture of each fungal species was sub-cultured and maintained on potato dextrose agar (PDA) and kept in the culture chamber at 26 ± 2°C.

Preparation of Plant Crude Extracts

Fifty (50) grams of ground leaves of *A. paniculata* and *B. citriodora* were separately soaked in 300 ml distilled water and stirred at 120 rpm for 24 h using an orbital shaker as described by Venkateswarlu et al. (2013). Then, each mixture was filtered using Whatman No-1 filter paper and the solvent was evaporated using a rotary evaporator, BUCHI Model R215W. The dried extract was collected in an air-tight container and stored at 4°C. The same method was used in preparation of crude extract of *P. macrocarpa*; however distilled water was replaced with 300 ml methanol as described by Aras et al. (2016).

Screening of Antifungal Activity

The antifungal test was carried out by testing four concentrations of extract (5%, 10%, 15%, and 20%) as compared to control (0%). The stock solutions of the crude extract of *A. paniculata* and *B. citriodora* were separately prepared by diluting the crude extract of each plant with distilled water at the ratio of 1:1 (w/v). Meanwhile, the stock solution of *P. macrocarpa* crude extract was prepared by diluting the crude extract with acetone at the ratio of 1:10 (w/v) as described by Mahlo et al. (2016). Further serial dilution was done to achieve test concentrations. Petri dishes containing 15 ml of poisoned medium were used. Then, a respective fungal plug (0.4 cm diameter) was placed at the centre of containing each plant extract at the defined concentrations. The antifungal activity of *A. paniculata*, *B. citriodora*, and *P. macrocarpa* extracts were separately tested against *P. oryzae*, *E. rostratum*, and *R. solani*. The plates were incubated at room temperature (26 ± 2°C) until the mycelial growth in control plates for certain fungal species had reached the edge of the plates. The colonial diameter was measured daily and PIDG values were calculated using Equation [1] as described by Lee et al. (2018):

$$PIDG(\%) = \frac{D1 - D2}{D1} \times 100 \qquad \text{Eq.1}$$

where;
D1 = Average increase in mycelial growth in control plates
D2 = Average increase in mycelial growth in treatment plates

Experimental Design and Statistical Analysis

The *in vitro* screenings of antifungal potential of all test plants were conducted in complete randomized design (CRD) with 5 treatments (0%, 5%, 10%, 15%, and 20%). There were 6 replicates for each treatment. Statistical analysis was conducted using SAS® software (SAS Institute, North Carolina State University, USA, Version 9.4, 2012) and comparison of means using least significant difference (LSD) at 5% probability level.

RESULTS AND DISCUSSION

Extract Yield and Antifungal Activity of *Andrographis paniculata* and *Backhousia citriodora*

About 5 g extract yield was obtained from 150 g of each plant. Each of the test fungal pathogen had different incubation period as follows: 12 days after inoculation (DAI) for *P. oryzae*, 5 DAI for *E. rostratum*, and *R. solani*, respectively. The effectiveness of antifungal activity of aqueous extract of *A. paniculata* differed among the test fungal pathogens (Figure 1). Aqueous extracts of *A. paniculata* exhibited different mycelial growth inhibitory activities on *P. oryzae* and *E. rostratum* as compared to *R. solani*. Figure 1 reveals that there had been a gradual rise in mycelial inhibitory activity on *P. oryzae* and *E. rostratum* as the concentration of aqueous extract of *A. paniculata* increased from 0% to 20%. This corroborates with findings of Olufolaji et al. (2015) on the increasing antifungal potency of several plant extracts with increasing concentration in the order against *P. oryzae*. Among all test concentrations, aqueous extracts of *A. paniculata* at 20% concentration significantly inhibited the mycelial growth of *P. oryzae* (PIDG 81.9%) while less inhibition was demonstrated on *E. rostratum* (PIDG 41.3%). It has been reported that both ethanol and aqueous extracts of *A. paniculata* were inactive against *P. oryzae* (Hu et al., 2001), which is contrary to the high inhibitory effect demonstrated in this study. Rajalakshmi et al. (2012) had reported different antifungal efficacy of *A. paniculata* crude extract when tested on five fungal species was attributable to phytochemical constituents of the plant. According to Singha et al. (2003), the significant antimicrobial activity of the *A. paniculata* aqueous extract may be due to the combined effect of the isolated arabinogalactan proteins and andrographolides. Besides, Nidiry et al.

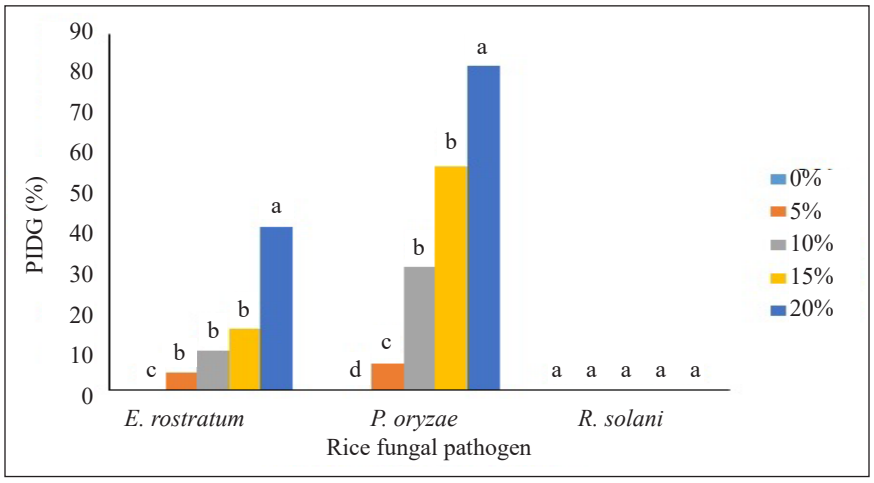

Figure 1. Percentage of inhibition of diameter growth (PIDG) of *Andrographis paniculata* against selected plant fungal pathogens. Measurement made at 5 days after inoculation (DAI) for *Exserohilum rostratum*, 12 DAI for *Pyricularia oryzae*, and 5 DAI for *Rhizoctonia solani*. Values are the means of 6 replicates. Means with the same letter are not significantly different at P = 0.05

(2015) had identified andrographolide as one of the antifungal compounds present in the methanol extract of *A. paniculata*, which resulted in spore germination inhibition of *Alternaria solani*. To date, the antifungal activities of *A. paniculata* leaf extract have been predominantly reported on phytopathogenic *Fusarium* species (Neela et al., 2014; Nidiry et al., 2015; Yasmin et al., 2008). This is the first report on evaluation of antifungal potential of *A. paniculata* aqueous leaf extract on *E. rostratum*. However, a further study is needed to identify the antifungal compounds present in *A. paniculata* leaves.

On the other hand, the PIDG values lower than 50% showed that the aqueous extract of *B. citriodora* did not exert any effective inhibitory activities on the mycelial growth of test organisms (Figure 2). Leaf paste of *B. citriodora* showed antibacterial activity against seven bacterial strains (Wilkinson et al., 2003). Ineffective mycelial inhibition of *P. oryzae* and *E. rostratum* by the aqueous extract of *B. citriodora* at the highest test concentration suggests low abundance of active compounds in the crude extract. Another possible reason is that the active compounds were not efficiently extracted using water suggesting organic solvents may perform better for *B. citriodora* extraction. Nevertheless, significant increase in PIDG values from extracts with concentrations of 15-20% indicating that the inhibitory activity of the aqueous extract of *B. citriodora* on *P. oryzae* and *E. rostratum* was concentration-dependent (Figure 2). Therefore, a further screening of the antifungal potential of crude leaf extract of *B. citriodora* at higher concentrations is needed to validate these plausible reasons. To date, no reports of antifungal activity of *B. citriodora* against plant pathogens were found in the literature. Present experiment suggests that *B. citriodora* may serve as good

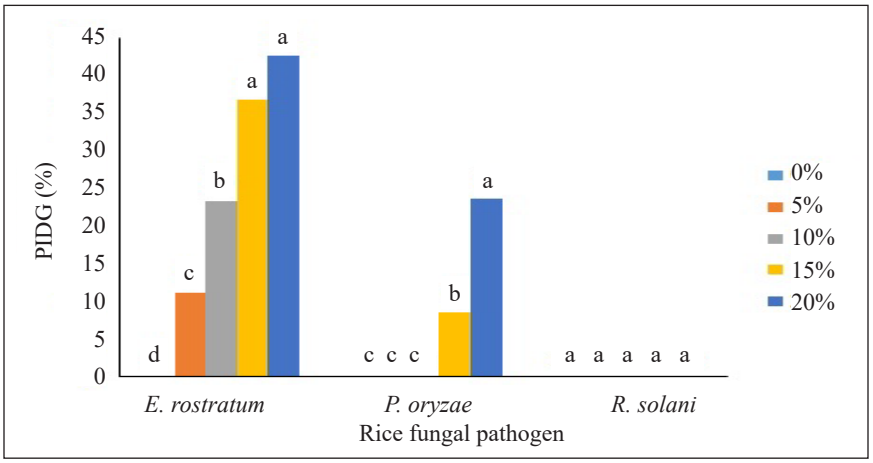

Figure 2. Percentage of inhibition of diameter growth (PIDG) of *Backhousia citriodora* against selected plant fungal pathogens. Measurement made at 5 days after inoculation (DAI) for *Exserohilum rostratum*, 12 DAI for *Pyricularia oryzae*, and 5 DAI for *Rhizoctonia solani*. Values are the means of 6 replicates. Means with the same letter are not significantly different at P = 0.05

natural resource of bioactive compounds for controlling *P. oryzae* and *E. rostratum*. Nevertheless, determination of suitable concentration of crude leaf extract of this herbal plant for inhibition of mycelial growth of *P. oryzae* and *E. rostratum* is crucial.

From Figures 1 and 2, it can be clearly seen that aqueous extracts of both *A. paniculata* and *B. citriodora* were ineffective in inhibiting the mycelial growth of *R. solani*. According to Kurucheve et al. (1997), the variation in the inhibitory effect of plant extracts is caused by qualitative and quantitative differences in antifungal properties.

Extract Yield and Antifungal Activity of *Phaleria macrocarpa*

Around 5 g of extract was obtained from 100 g of *P. macrocarpa* leaf powder. The incubation period of each test pathogen was as follows: 7 days after inoculation (DAI) for *E. rostratum*, 11 DAI for *P. oryzae*, and 5 DAI for *R. solani*. As presented in Figure 3, methanol extracts of *P. macrocarpa* were effective against *P. oryzae*, *E. rostratum*, and *R. solani* at test concentrations of 10% and above (PIDG 100%) as compared to control plates. The presence of an assortment of chemical compounds with antifungal and antibacterial properties in *P. macrocarpa* as reported by Altaf et al. (2013) might have contributed to mycelial inhibition of the test pathogens in this study. For instance, phorbolesters in *P. macrocarpa* seeds inhibited growth of certain fungi including *Aspergillus niger*, *Fusarium oxysporum*, *Ganoderma lucidum*, and *Mucor indicus* (Altaf et al., 2013). Furthermore, Cordell et al. (2001) had reported that flavanoids were the responsible compound for the antifungal activities in higher plants. However, it is important to identify the phytochemicals with antifungal properties of *P. macrocarpa* leaves in order to develop bio-fungicide

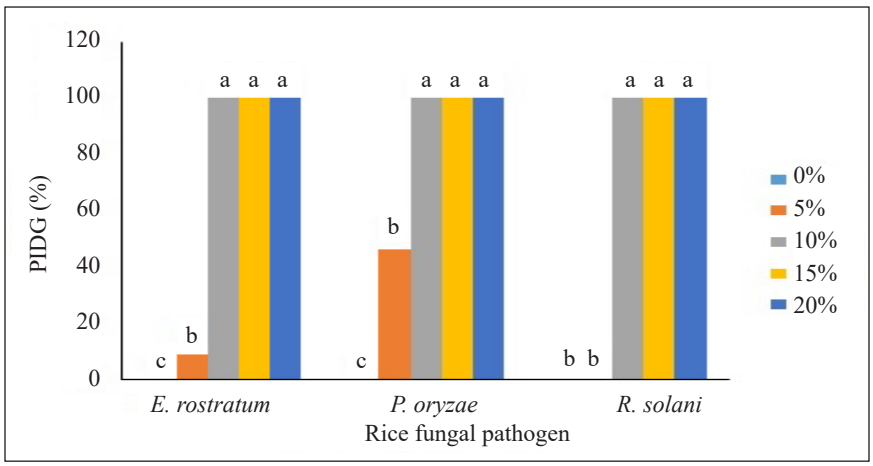

Figure 3. Percentage of inhibition of diameter growth (PIDG) of *Phaleria macrocarpa* against selected plant fungal pathogens. Measurement made at 11 days after inoculation (DAI) for *Pyricularia oryzae*, 7 DAI for *Exserohilum rostratum*, and 5 DAI for *Rhizoctonia solani*. Values are the means of 6 replicates. Means with the same letter are not significantly different at P = 0.05

as an alternative to chemical fungicides. On the other hand, 10% concentration and above had the highest inhibitory activity on the mycelial growth of all test pathogens and these treatments were significantly different from that of 5% concentrations. This suggests that 10% concentration of *P. macrocarpa* methanol extracts is sufficient to inhibit the mycelial growth of *P. oryzae*, *E. rostratum*, and *R. solani in vitro*. To our best knowledge, this is the first report on the antifungal potential of *P. macrocarpa* leaf extracts against fungal pathogens of rice.

CONCLUSION

The antifungal potential of *Andrographis paniculata*, *Backhousia citriodora*, and *Phaleria macrocarpa* against three selected rice fungal pathogens has been determined in this study. Among these herbal plants, *P. macrocarpa* leaf extract had the highest potential to inhibit the mycelial growth of *Pyricularia oryzae*, *Exserohilum rostratum*, and *Rhizoctonia solani* at concentration of 10%. Meanwhile, *A. paniculata* was proven effective against *P. oryzae* at the highest test concentration (20%). The results revealed that leaf extracts of *A. paniculata* and *P. macrocarpa* had high potential to be used as sources of biofungicides in management of rice diseases mainly rice blast. Further studies are required to identify the bioactive compounds of these two herbs and to determine their mechanism of antifungal activities.

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Status of Pepper Farming and Flower Composition of Different Pepper Varieties in Sarawak

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ABSTRACT

Pepper (*Piper nigrum* L.) is one of the most important cash crops in Sarawak. The productivity of pepper is consistently low due to the low yield of berry production. One of the major problems of pepper production is inconsistent flowering time. This is due to the morphology and inheritance of functional male, female and hermaphrodite (bisexual) flower in *P. nigrum* which affect the productivity of pepper. For the exploitation of pepper for its maximum production, the detailed of flower development and flower composition are important factors to be considered. A field survey was conducted to determine the status of farming practices and problems encountered by the farmers. The study was also done to determine the composition of flower which influenced the consistency of berry production in *P. nigrum* in Sarawak. Surveys were conducted at 18 pepper farms in Sarawak to determine the composition of flowers in different types of Sarawak pepper varieties which are Kuching, Semenggok Aman and Semenggok Emas. Nine spikes were harvested in each pepper vine. Three pepper vines were selected randomly for each variety. The spikes were then observed under 3D Keyence microscope to determine the number of flowers of each type of flower. The survey on the farming practices were also conducted. The composition of flower was found to be varied between varieties. 'Kuching' variety had less hermaphrodite flower when compared to 'Semenggok Aman' and 'Semenggok Emas' varieties. In addition, a proportion of 29% farmers had encountered root rot disease problem in their farm, while 21% stated that unsynchronisation of berries production in each harvesting time as a major problem.

Keywords: Flower composition, Sarawak pepper and berries production

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INTRODUCTION

Pepper mostly cultivated for its fruit, and widely known as the "king of spices" and "black gold" especially among the farmers (Ravindran, 2000). Pepper is known for their extensive culinary usages and has been used enormously in food seasoning as the flavour and its pungency blends well with most savoury dishes. The history of pepper cultivation in Malaysia was believed to have started in early 10th to 11th century when the south Indian kings started to expand their empire (Ravindran, 2000). In East Malaysia, the grow of pepper cultivation had been reported in 1840 where the Chinese settlers actively grew black pepper in Bau, Baram, Trusan and Limbang, in the state of Sarawak.

The cultivation of pepper area and the export value are increasing and becoming part of Malaysia's economic profit where pepper price is the significant determinant of the total revenue of Malaysia particularly for the state of Sarawak. Until then, pepper cultivating is the only source of livelihood for majority of the pepper farmers in the state (Kiong et al., 2010). Malaysia is still placed fifth among the world largest producers of peppers by representing 5.9% of total production in the world with 31,7073 tonnes during the year 2018 (Malaysian Pepper Board, 2018).

Malaysian Pepper Board (2013) reported that there were no wild varieties or land races of pepper but only cultivated varieties. Since it was originated from South Western of India, prior to year 1957, there were only two cultivated varieties of pepper first reported in Sarawak, which is 'Sarikei' and 'Kuching'. Until then, 'Kuching' became the cultivar of choice by farmers and known as the traditional cultivar. It is more vigorous in growth and produces much high yield as compare to 'Sarikei'. After several years, 'Semenggok Perak', 'Semenggok Emas' and 'Semenggok Aman' were released by Malaysian Pepper Board (MPB) in 1988, 1991 and 2006 respectively. However, currently there are three recommended cultivars and grow by most farmers in Sarawak which was 'Kuching', 'Semenggok Emas' and 'Semenggok Aman'. Each of these cultivars has different characteristic. Therefore, prior to its cultivar origin, Malaysia black pepper is known as 'Sarawak pepper' among the world traders over the decade.

Despite its popularity as a well known cultivar, the productivity of pepper is consistently low due to the low yield of berry production. The production of pepper for the past 10 years was inconsistent and farmers also encountered several problems in their farms. The yield was affected due to pests and diseases with the major problems such as 'Phytopthora foot root' caused by *Phytopthora capsici*, 'black berry' or anthracnose caused by *Colletotrichum capsici*, *C. gloeosporioide*, and *C. piperis*, and 'slow decline' a disease complex between the root-knot nematods (*Meloidogyne* spp) and the fungus *Fusarium* spp. (Malaysian Pepper Board, 2013).

Other than that, Khew (2019) also stated that the inconsistent flowering time in *P. nigrum* could be one of the

reasons for low yield of peppers. This is due to the morphology and inheritance of functional male, female and hermaphrodite (bisexual) flower in *P. nigrum* which affect the productivity where fruit ripening is not uniform even within a spike. For the maximum production of pepper, the detailed of flower development and composition should be considered due to the non-synchronous nature of flower development. Therefore, the objective of the study was to determine the composition of flower which influenced the consistency of berry production in *P. nigrum* in Sarawak.

MATERIALS AND METHODS

Survey on the Farming Practices

The survey was covered in the seven areas in Sarawak which were in Kuching, Serian, Sri Aman, Sarikei, Bintangor, Julau and Miri. Sampling respondents to represent each district and 18 selected pepper farmers surveyed in these areas were based on prior information provided by the MPB. Data were collected through face-to-face interviews based on questionnaires that include farmers ethnicity, education level, year of farm establishment, total of pepper vines, types of planted cultivar and the products that they produce. All of this information was used to conclude their farming status. Meanwhile, the other information about their monthly maintenance, the problems encountered the most and the action taken would be concluded as problems and management status from each farmer. The data were analysed by using Statistical Package for the Social Sciences (SPSS) software.

Spike Selection and Morphological Observation

The observations of flowers in pepper crop were carried out in 18 pepper growing farms around Sarawak, Malaysia (Table 1). Three Sarawak pepper varieties which are 'Kuching', 'Semenggok Aman' and 'Semenggok Emas' were selected to be used in this study. Nine spikes of each three vines were randomly selected. The plant canopy does not affect the formation of spikes and fruits (Satheeshan, 2000). The numbers of male flower, female flower and hermaphrodite in each spike were observed and calculated under 3D Keyence microscope to determine the flower composition. The mean of flower composition percentage of nine spikes of each farm was then plotted accordingly. The selection of vines was based on their height and yielding ability and free from pests and diseases. The vines were in the age group of one until five years old. They were grown under open conditions and were rain fed.

RESULTS AND DISCUSSION

Farming Practices and Problem Encountered by Farmers

As shown in Table 1 and Figure 1, there are four main varieties of pepper planted in Sarawak which are 'Kuching', 'Semenggok Aman', 'Semenggok Emas' and 'India' varieties where the most planted variety is 'Semenggok Aman'. This variety was released on 12 August 2006 in Sri Aman Sarawak by pepper research and development (R&D) team from Agriculture Research Centre, Semenggok, Sarawak.

'Semenggok Aman' produced a good fruit set, bigger berries and longer fruit spikes as compared to 'Kuching'. Moreover, this variety is proven to be tolerant to pepper weevil (*Lophobaris piperis* M.), known as stem borer. In terms of chemical quality, 'Semenggok Aman' contains high piperine, oleoresin, volatile and non-volatile oils compared to other cultivar varieties planted in Sarawak, followed by 'Semenggok Emas' and 'Kuching' (Malaysian Pepper Board, 2013).

Each cultivar has different characteristics (Table 2) and the major characteristics can be shown from their terminal shoot, where the tip is light purple in colour and leaves are ovate lanceolate in shape for 'Kuching' cultivar, with length: width ratio of 1.93 (Paulus & Sim, 2009 as cited in Paulus et al., 2011) and flower spike is pale yellow in colour (Figure 2a). 'Kuching' pericarp is the thinnest of all three cultivars. As for 'Semenggok Emas', the terminal shoot tip has intermediate anthocyanin coloration where the leaves ovate-lanceolate in shape with length: width ratio of 2.07 (Paulus & Sim, 2009 as cited in Paulus et al., 2011) and flower spike is yellow to golden yellow in colour (Figure 2c). Meanwhile for 'Semenggok Aman', the terminal shoot tip has intermediate anthocyanin coloration and the leaves are ovate in shape with length: width ratio of 1.52 (Paulus & Sim, 2009 as cited in Paulus et al., 2011), and flower spike is light green in colour (Figure 2b).

Table 1
Pepper farms location in Sarawak

Farms	Division	District	Location of farm	Cultivars
F1	Miri	Miri	Sg. Siam	Semenggok Aman, Semenggok Emas, Kuching
F2	Miri	Miri	Sg. Nakat	Semenggok Aman, Semenggok Emas, Kuching
F3	Miri	Miri	Sg, Merah	Semenggok Aman, Semenggok Emas, Kuching
F4	Sibu	Bintangor	Sg. Bakong	Semenggok Aman,Kuching
F5	Sibu	Julau	Nanga Bekiok	Semenggok Emas, Kuching
F6	Sibu	Julau	Nanga Bekiok	Semenggok Aman, Kuching
F7	Serian	Serian	Mapu Tragu	Semenggok Aman, Semenggok Emas, Kuching
F8	Serian	Serian	Bunan Punok	Semenggok Aman, Semenggok Emas, Kuching
F9	Serian	Serian	Bunan Gega	Semenggok Aman, Semenggok Emas, Kuching
F10	Sarikei	Sarikei	Sg. Rusa	Kuching
F11	Sarikei	Pakan	Lubuk Embawang	Semenggok Aman, Semenggok Emas, Kuching
F12	Sarikei	Pakan	Rh. Akun,Supi	Semenggok Aman, Semenggok Emas, Kuching
F13	Sri Aman	Sri Aman	Lubuk Ju	Semenggok Aman, Semenggok Emas, Kuching
F14	Sri Aman	Sri Aman	Lubuk Ju	Semenggok Aman, Semenggok Emas, Kuching
F15	Sri Aman	Sri Aman	Rh. Sherit,Undop	Semenggok Aman, Semenggok Emas, Kuching
F16	Kuching	Serikin	Jagoi	Semenggok Aman, Semenggok Emas, Kuching
F17	Kuching	Bau	Stass	Semenggok Aman
F18	Kuching	Bau	Stass	Semenggok Aman

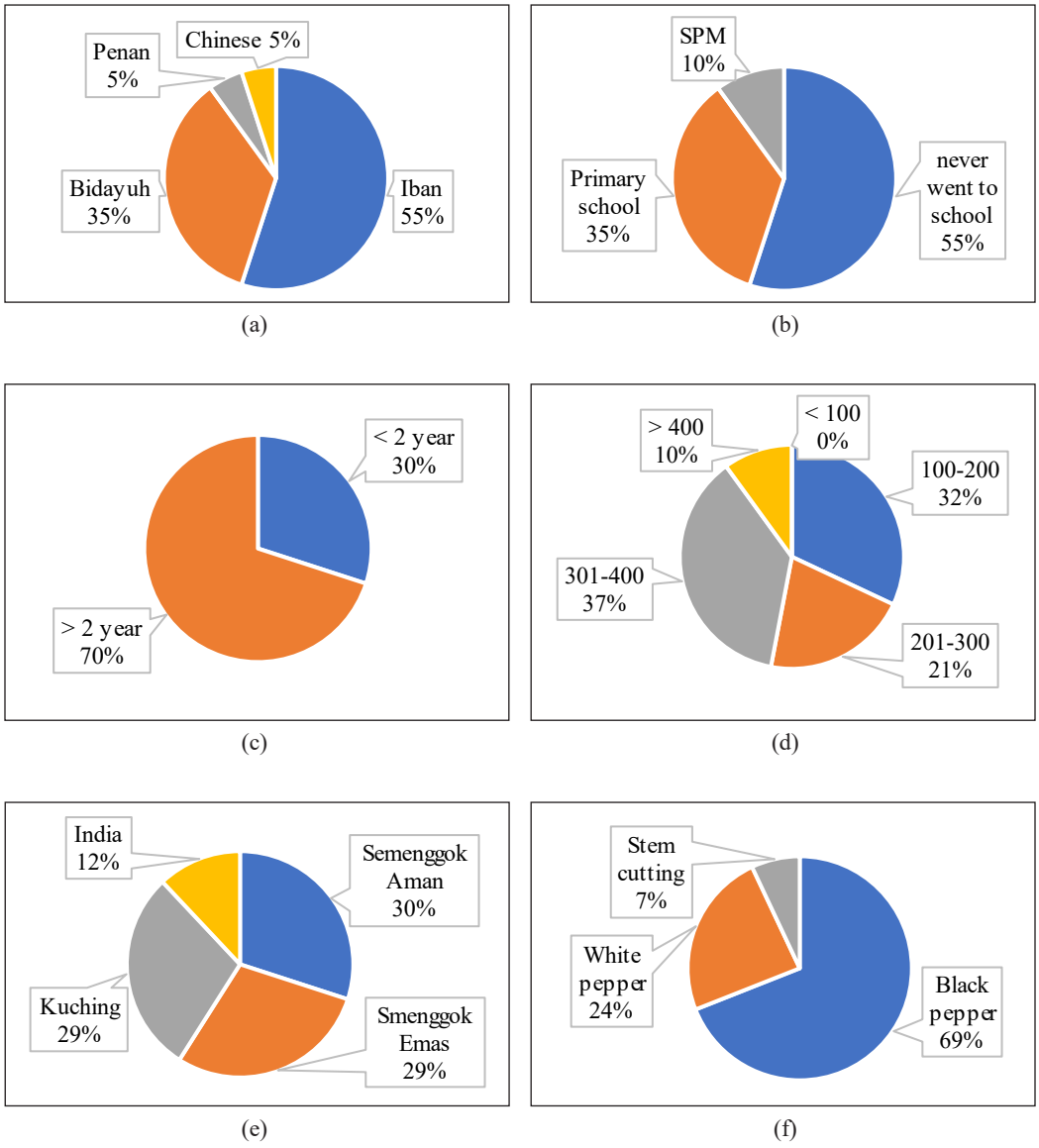


Figure 1. Pepper farming status in Sarawak. (a) ethnicity of pepper farmers; (b) education level of pepper farmers; (c) period establishment of pepper farm; (d) number of pepper vines planted in the farm; (e) type of pepper varieties planted in the farm; and (f) type of pepper products produced by pepper farmers

As for the farm management status, 90% of the farmers practice two times maintenance per month mostly for weeding, fertilization and pruning (Figure 3a-b). There are five main problems encountered by the farmers (Figure 3c). Up to 29% farmers counted diseases as their major problem in their farm, followed by 28% on inconsistent price of pepper commodity. Apart from the major problems, 21% of them

also agreed that the un-synchronization of berries formation also became a significant problem. Meanwhile, 12% of farmers agreed that they had a problem in applying fertilizer for their vine and 10% of them agreed there was un-synchronization of harvesting. Farmers also agreed that they were having spike shedding problems during flower blooming season. Basically, the farmers were seeking advice from MPB to address the problems (Figure 3d).

Table 2
Characteristics comparison between three Sarawak pepper varieties

Characteristics	**Variety**		
	Kuching	**Semenggok Emas**	**Semenggok Aman**
Green berry yield (kg/vine/year)	6-8	6-8	6-8
% Driage (conversion ration) Black pepper	33	31	33
% Driage (conversion ration) white pepper	24	22	22
Weight of 100 mature green berries (g)	13.8	15.6	15.7
Length of fruit spike (cm)	9.7	9.9	10.1
Chemical quality:			
% piperine	3.5	3.4	5.4
% oleoresin	11.0	11.0	15.5
% volatile oil	2.8	3.0	3.8
% non-volatile oil	7.9	8.0	11.5
Harvesting rounds per season	4-6	2-3 (more uniform ripening)	2-3 (more uniform ripening)
Susceptibility to *Phytophthora* foot rot disease	Highly susceptible	Susceptible	Less susceptible
Susceptibility to black berry disease	Highly susceptible	Tolerant	Tolerant
Susceptibility to pepper weevil	Susceptible	Less susceptible	Less susceptible

Source: Malaysian Pepper Board (2013)

(a) (b) (c)

Figure 2. Types of pepper spike in three different cultivar varieties. (a) Kuching, pale yellow in colour; (b) Semenggok Aman, light green in colour; and (c) Semenggok Emas, yellow to golden in colour

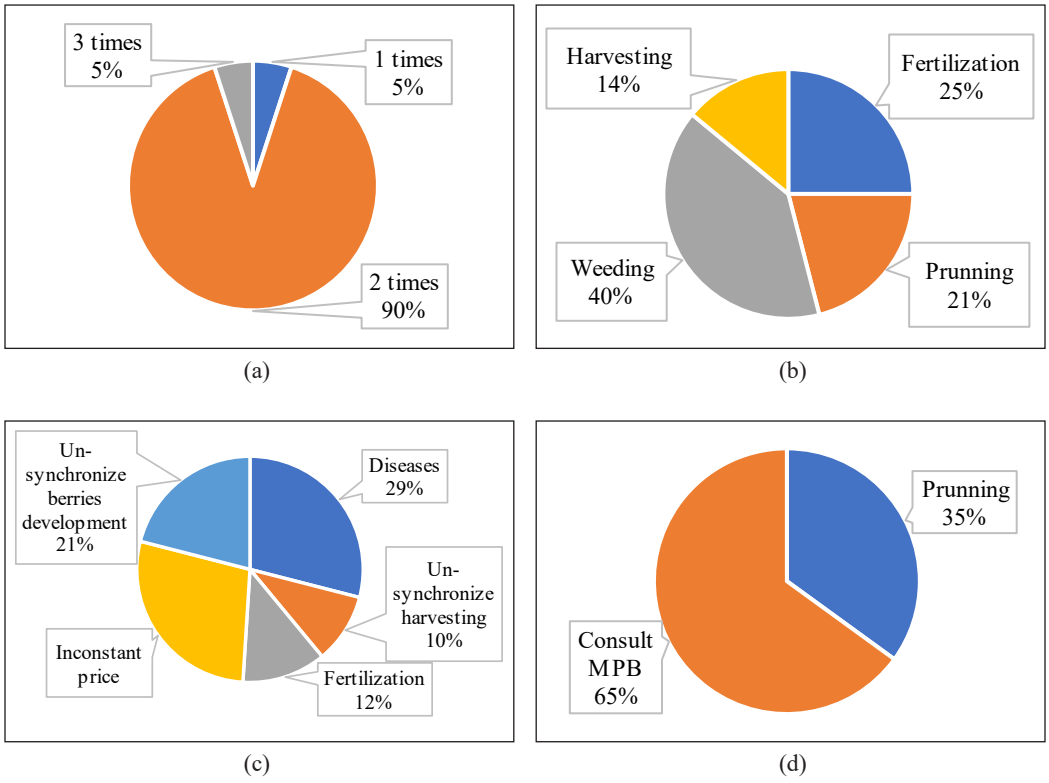


Figure 3. Pepper farm management status and problems encountered by pepper farmers in Sarawak. (a) frequency of vines maintenance in a month; (b) type of vines maintenance activity; (c) problems encountered by the pepper farmers; and (d) methods of solving problems encountered in pepper farms

Flower Composition between Three Different Varieties

Pepper flowers may be hermaphrodite or unisexual which is male and female (Figure 4). The results showed that the composition of flower varied between varieties, where the flower composition in each variety was different from dominantly female to purely bisexual (Parthasarathy et al., 2010). Flower compositions of different

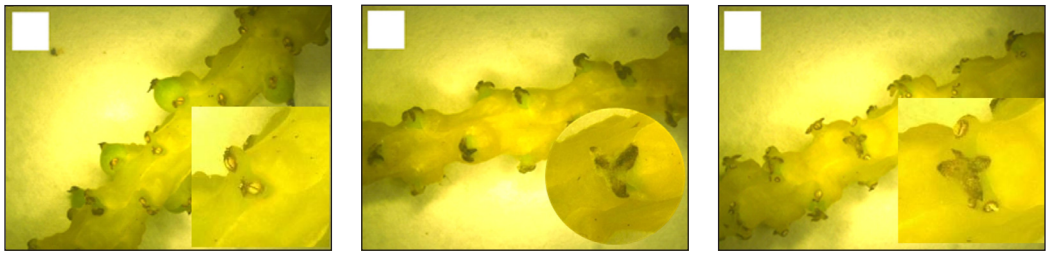
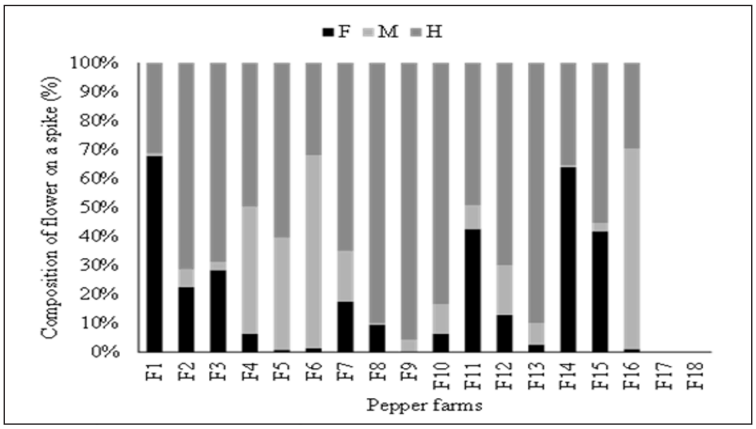
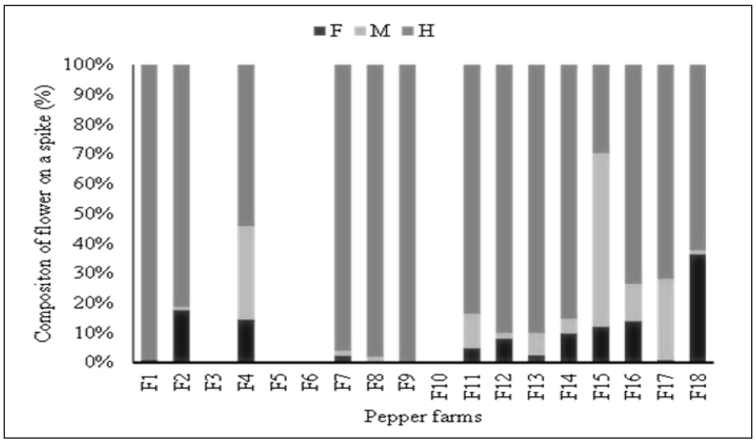


Figure 4. Type of flower on pepper spike. (a) male flower, only stamens are visible between the bracts; (b) female flower, only stigma is visible between the bracts; and (c) hermaphrodite flower, stamens are on both sides of the sigma



(a)



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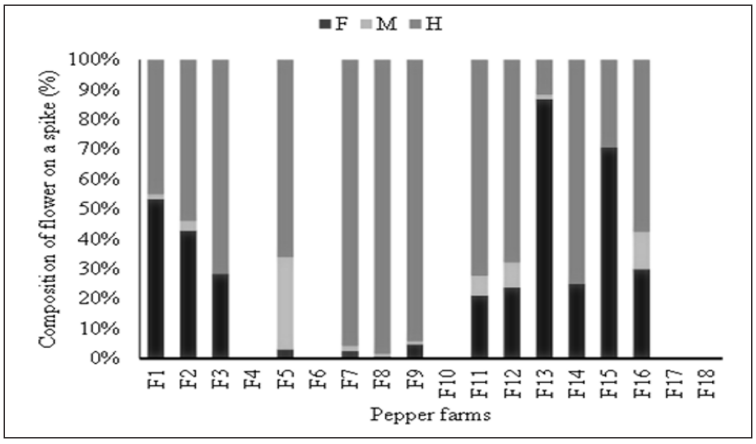

(c)

Figure 5. Composition of female (F), male (M) and hermaphrodite (H) flowers on different pepper varieties spikes. (a) Kuching pepper variety; (b) Semenggok Aman pepper variety; and (c) Semenggok Emas pepper variety

varieties were presented in Figure 5. The result showed that 'Semenggok Emas' variety at F13 had the highest percentage of female flowers (70.6%) followed by 'Kuching' variety with 69.5% of female flowers. Whereas, 'Semenggok Aman' variety had the lowest female percentage (36.5%). Meanwhile, 'Kuching' varieties had the highest percentage of male flower (69.3 %) located at F16 as compared to 'Semenggok Aman' and 'Semenggok Emas' but had the lowest percentage of the hermaphrodite flower. From the previous study, the percentage of hermaphrodite flowers in the plant varied according to the growth flush and regions (Hallad, 1991). The production of male flowers in spike resulted reduction of crop yield (Venugopal et. al., 2013). However, flower structure in pepper is likely to be influenced by the packaging of flowers in the inflorescence itself and therefore berries productivity is highly dependent on the growth and flowering behaviour of the vine (Satheeshan, 2000). In general, high percentage of bisexual flowers or hermaphrodite flowers is essential for effective pollination and fruit set (Ravindran et al., 2000) where the predominance of the female flowers in each spike is reported as the major cause of pollination failure and subsequent spike shedding instead of producing berries. As there are more than one hormone in the regulation pathway of black pepper, it is not easy to indicate the possible crosstalk between the plant hormones in the fruit development stages (Khew et al., 2019). Khew et al. (2019) also stated that salicylic acid played decisive roles in flowering and fruit set, whereas auxin, gibberellins and cytokinins played roles predominantly in the early fruit development stages during cell division and expansion. Abscisic acid (ABA) appears to play a role in fruit maturation and ripening in the fruit development process.

CONCLUSION

Based on the survey conducted, the major constrains faced by the farmers were unsynchronization of berries production at one time that can be related with flower compositions where a smaller number of hermaphrodite flowers were being observed at the surveyed farms. Thus, the initiation of hermaphrodite flowers through exogenous hormones application is very important to ensure the synchronisation of pepper vine, thus the production of berries could be increased.

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The Feasibility Study of Physicochemical Properties of Sarawak *Liberica* sp. Coffee Pulp

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ABSTRACT

Liberica coffee is a minor species that is planted all around the world. Therefore, there is little study conducted on this coffee species as only one percent is cultivated all around the world. In Malaysia, there is still no research focusing on coffee pulp from Sarawak *liberica* sp. and thus leading to this study. The wastes and by-product such as coffee pulps will become the residues as they were not needed in processing the coffee. This will create environmental pollution. Thus, this research aimed to evaluate the feasibility study on the physicochemical properties of coffee pulp from Sarawak *liberica* sp. including determination by colorimetric assays for phenolic and flavonoid content, antioxidant activity, and reducing sugar analysis. The antibacterial activities of coffee pulp were evaluated against Gram-positive, *Staphylococcus aureus*, and Gram-negative *Salmonella typhimurium* using a disc diffusion method. As a result, Sarawak *liberica* sp. coffee pulp extract contained total phenolic content of 24.24 mg GAE/g of coffee pulp, a total flavonoid content of 39.39 mg QE/g of coffee pulp, DPPH scavenging activity of 92.24 ± 0.03%, reducing sugar analysis of 13.13 mg GE/g of coffee pulp, and there was no significant effect of antibacterial activities. Therefore, the physicochemical study determination in this study would add values toward Sarawak

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liberica sp. coffee pulp by-product and thus reducing the disposal of Liberica coffee wastes in the future.

Keywords: Antibacterial activity, antioxidant properties, coffee pulp, flavonoid compound, phenolic compound, reducing sugar analysis

INTRODUCTION

Coffee is a common beverage which is an important product throughout the world. Some products from coffee beans can be obtained such as essence, coffee caramel, and coffee syrup. There are three main coffee species planted throughout the world which are Arabica (80%) and Robusta (20%), while Liberica is the minimal species that is less than 1%. Unlike in Malaysia, Arabica is the minor species that were grown due to its climatic condition, while Liberica (73%) and Robusta (27%) are the major coffee species that are grown in this country (Ismail et al., 2014). Liberica and Robusta are suitable to be cultivated in Malaysia because the optimum growth temperature is 18 to 28°C, but the maximum temperature for these two species is at 34°C as stated by Ismail et al. (2014). In a developing country, over 10 million of small producers make their living growing coffee. Coffee comes from the genus *Coffea* which originated in tropical Africa (Maurin et al., 2007). It is important to know about the chemical and physical properties of coffee beans because the coffee that is freshly harvested will undergo various processes such as grinding, roasting, packing, and transporting process. The processing of coffee berries comprises two different methods which are dry and wet (Berecha et al., 2011). The skin and pulp of the coffee beans will be removed and become the residues as they are not needed to process the coffee. As there a wide world production of coffee, it will associate with ecological damages as the residues of coffee can lead to environmental pollution if it is not handled well (Cubero-Abarca et al., 2014). Most of the coffee pulp is dumped into a landfill or river stream directly during wet processing without proper handling of waste (Geremu et al., 2016). The absence of a method that can utilize the use of coffee by-product is one of the main problems to use the coffee pulp for biomass productions due to the high moisture content (Cubero-Abarca et al., 2014). However, due to the myriad problems such as disposal problems of this by-product and cause environmental pollution, this material has received greater attention compared in the past. Many researchers have developed some ideas to reduce the environmental pollution by using the waste by-product in fermentation of edible fungi, animal feeds, extraction of active ingredients, and compost (Marcel et al., 2011; Murthy et al., 2012). The coffee pulp is rich with minerals, nutrients, amino acids, polyphenols, and caffeine (Arellano-González et al., 2011; Ploypradub et al., 2010). In addition, the coffee pulp has also been found to have antimicrobial properties against bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Escherichia coli*, and *Bacillus*

subtilis as stated by Runti et al. (2015). In Malaysia, there was a previous study that had been done on this coffee species, but it was only focused on the coffee berries and beans physical properties (Ismail et al., 2014). However, there is still no study that focuses on the physicochemical properties of coffee pulp from *liberica* sp. particularly Sarawak adapted *liberica* coffee sp. and thus leading to this study. Therefore, this research aimed to evaluate the physicochemical properties of coffee pulp from *liberica* sp., to study the antioxidant potential of coffee pulp extract using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and to investigates the antibacterial potential of coffee pulp extract against Foodborne bacteria, Gram-positive, *Staphylococcus aureus* and a Gram-negative *Salmonella typhimurium*.

MATERIALS AND METHODS

Collection and Preparation of Plant Material

The Sarawak *liberica* coffee sp. pulp was used as the main substrate in this study. The Sarawak *liberic*a sp., the coffee sample was supplied by RekaJaya Plantation Sdn. Bhd. in Kuching, Sarawak. The pulp of the coffee beans was separated from the beans and crushed to obtain a greater surface area. Consequently, the crushed coffee pulp sample was oven-dried at 105°C for 5 days. The dried coffee pulp sample was analyzed for its moisture content as well as dry matter.

Dry Matter and Moisture Content

A drying dish was oven-dried at 60°C for 10 min and subsequently was cooled to room temperature. The dried drying dish was weighed using an analytical balance. Then, the coffee pulp sample was dried at 105°C in an oven for 5 days, cooled to ambient temperature, and was weighed again. The drying, cooling, and weighing processes were repeated until a persistent weight was obtained. The percentage of moisture content of coffee pulp was computed using the formula:

$$\text{Moisture content (\%)} = \frac{(w1-w2)}{w1} \times 100\%$$

According to this formula, the weight of an amount of coffee pulp was determined as w1 and the weight of dried coffee pulp sample was determined as w2. The percentage of dry matter was determined using the formula below:

Dry matter (%) = 100% - moisture content (%)

Extraction of Sarawak *Liberica* sp. Coffee Pulp Sample

The extraction of coffee pulp using methanol as a solvent was done by referring to the previous procedure by Redfern et al. (2014). The coffee pulp sample was dried and ground using mortar and pestle to fine powder form. The powdered coffee pulp was collected and placed inside a thimble of the Soxhlet extractor. An amount of 200 mL of solvent which was methanol was added into the Soxhlet apparatus. Next, the solvent was heated using the isomantle and evaporated.

This process was run for 8 hours. After the process was finished, the methanol was removed, leaving about 2 to 3 mL yield of the essential oil.

Total Phenolic Content Determination

The total phenolic content (TPC) was investigated using the Folin-Ciocalteu method as exhibited by Rahayu et al. (2013). Gallic acid (GA) was used as a standard thus the TPC was indicated as mg/g gallic acid equivalence (GAE) from the calibration curve (R^2=0.9995). To form the stock solution, 10 mg of GA was dissolved in 100 mL of distilled water as the solvent (0.1 mg/mL). For the standard curve, different concentration of 0, 0.02, 0.04, 0.06, 0.08, and 0.10 mg/mL of (GA) were made from the stock solution. Then, 100 µL of the extract was added with 0.75 mL of the stock Folin-Ciocalteu's reagent. The mixture was let to settle down at room temperature for 5 min before adding 0.75 mL of 6% sodium carbonate solution. The mixture was then shaken thoroughly before allowing to mix at room temperature for 90 min. UV-Vis spectrometer was then used to measure the absorbance at 725 nm. Each sample then was repeated thrice for this procedure.

Total Flavonoid Content Determination

The total flavonoid content (TFC) of the pulp extracts was determined referring to the procedure of colorimetric assay done by Ahlem et al. (2014). First, 75 µL of 5% sodium nitrite ($NaNO_2$) solution was added to 125 µL of the extract. Then, the mixture left for 6 min, before inserting 150 µL of 10% aluminum trichloride ($AlCl_3$). After leaving for another 5 min, 750 µL of NaOH (1M) was added into the combination. The mixture then was added with distilled water to reach 2500 µL mark. After 15 min of incubation, the mixture was observed to turn pink in coloration. The absorbance of the blend then was measured at 510 nm. Next, the calibration curve was made from multiple concentrations of quercetin. In this study, quercetin was used as a standard flavonoid and conveyed as Quercetin equivalence (mg QE/mL extract). Quercetin stock solution was made by mixing 20 mg quercetin with 20 mL of methanol thus obtaining a concentration of 1.0 mg/mL. Next, it was diluted to 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL of quercetin. Absorbance was measured by using the spectrophotometer at 510 nm.

Antioxidant Activity of Sarawak *Liberica* sp. Coffee Pulp

The antioxidant properties of the extract were determined by the procedure previously done by Daniel and Workneh (2017) with slight modifications. By using 2,2-diphenyl-1-picrylhydrazyl (DPPH), the free radical scavenging activity of the extract was formulated below:

$$\% \text{ RSA} = \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \times 100\ \%$$

Firstly, a concentration of 1 mg/mL of coffee pulp extracts was used as a working standard. It was diluted to obtain

concentration 1000, 500, 250, 125, 62.5, 31.25, 15.63, and 7.8 $\mu g/mL^{-1}$. An amount of 0.5 mL of diluted solvent extracts was inserted into a respective test tube and 1 mL of (0.006% in methanol) of DPPH was added. The solutions were left for half an hour in darkroom temperature. After 30 min, the absorbance was determined using a UV-Visible spectrophotometer at 520 nm wavelength. Ascorbic acid was set as a positive control. The mixture without any extracts, but with the DPPH and methanol would be used as control. The DPPH free radical-scavenging activity of the coffee pulp extract would be calculated as the following equation and the IC_{50} value was determined from the graph of the percentage DPPH free radical scavenging activity against concentrations of the samples.

Reducing Sugar Analysis Determination

The DNS (3,5-dinitrosalicylic acid) assay was the way used to analyze reducing sugars such as glucose referred to the previous research done by Marisa et al. (2017) with some modifications. Solution A and solution B were prepared as DNS reagent. For solution A, 1.0 g of DNS was diffused in 20 mL of 2M NaOH. For solution B, 30.0 g of potassium sodium tartrate tetrahydrate (Rochelle salt) was dissolved in 50 mL of distilled water. Both solutions were stirred until complete dissolution. Then, solutions A and B were mixed and heated to homogenize. By using distilled water, the volume was fixed to 100 mL and stored in an amber bottle at 4°C. After that, 1 mL of coffee pulp extracts and 1 mL of DNS reagent were placed in tubes of 10 mL. Then, the tubes were taken to a bath thermostatized at 100°C for 5 min. Then, it was cooled down to room temperature. After that, the volume was completed with 8 mL of distilled water and homogenized. Then, the absorbance was read at 540 nm in a spectrophotometer and subsequently was analyzed to determine glucose concentration. The concentration of 1.0 mg/mL of glucose solution was prepared as a working standard stock solution. It was diluted to obtain 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL glucose working standard. A standard was plotted by using glucose to estimate glucose equivalent values. The reading was compared against a reagent blank solution.

Antibacterial Activity of Coffee Pulp Extract

Bacteria Inoculum Preparation. In this study, a Gram-positive, *Staphylococcus aureus,* and a Gram-negative *Salmonella typhimurium* were selected for testing. These bacteria were acquired from the Microbiology Laboratory of Faculty of Resource Science and Technology (FRST), UNIMAS culture collection. These bacteria were pre-cultured in Mueller-Hinton Broth and incubated at 37°C for 24 hours. The inoculum of each bacteria was standardized to 0.5 McFarland standard and measured at 620 nm via UV-vis spectrophotometer.

Antibacterial Susceptibility Test. The antimicrobial properties of the extract were tested using the disc diffusion method referring to the procedure done by Mohamed

et al. (2018) with few adjustments. First, the coffee pulp extract was redissolved in 1% dimethyl sulphoxide (DMSO) to obtain the final concentration of 100 mg/mL The solution was then diluted further with 1% DMSO to the concentration of 10, 50 and 100 mg/mL. An amount of 100 µL of bacterial inoculums prepared was spread onto Mueller-Hinton Agar. Then the disc (6 mm in diameter) was dripped with 10 µL of different concentrations of coffee pulp extracts were placed on the agar. Gentamicin (10 µg) was used as positive control while 1% of DMSO as a negative control. The agar plate was incubated at 37°C for 24 hours and the experiment was done thrice. The diameters of inhibition zones were measured.

RESULT AND DISCUSSION

Total Phenolic Content

Polyphenols play a crucial role in human health to protect from diseases caused by oxidative stress and free radical (Geremu et al., 2016). In this assay, GA was used as standard because it can inhibit the oxidation and hydrolysis of oils and fats because of their free radical scavenging and antioxidant nature (Kahkeshani et al., 2019). This colorimetric assay is easy to perform, low cost, and rapid making it extensively used in the UV/Vis spectrophotometric method. In this colorimetric reaction, a blue complex was formed because polyphenol in the coffee extract was reacted with redox reagents which are a Folin-Ciocalteau reagent that can be quantified with UV/Vis spectrophotometry (Blainski et al., 2013).

As a result, an amount of 24.24 mg GAE/g was found in the *Coffee liberica*. In this test, the TPC in the coffee pulp extract was estimated in terms of GA equivalent (mg GAE/g of coffee pulp). Based on a previous study by Geremu et al. (2016), coffee pulp from *Coffee arabica* exhibited the highest amount of phenolic content when it was extracted with methanol compared to ethanol and acetone. In this study, methanol was used as a solvent in the extraction of coffee pulp samples. Methanol is also known to be a competent solvent and is usually used to extract natural oxidative components from plants or natural materials. This is because methanol mixture has greater efficiency and high polarity towards the extraction of polar phytochemicals such as phenolic and flavonoids. Nevertheless, methanol is the recommended solvent when extracting polyphenols due to its capability to obstruct the activity of polyphenol (Geremu et al., 2016). Other previous studies also indicate that the capacity of phenolic content in the coffee pulp depends on a few reasons including the variety of extraction methods. Samar et al. (2018) had stated that the total polyphenols were notably differed in the roasted, raw, and spent coffee that could be influenced due to the heat treatment. Based on the studied, roasted coffee bean showed a very low amount of total polyphenol content in the coffee bean. This factor might also influence the amount of phenolic content in the coffee pulp extract from *Coffee liberica*

as the coffee pulp sample was oven-dried until the sample was completely dried.

Total Flavonoid Content

Flavonoid is the most familiar, major, and extensively dispersed sole group of phenols. It is available in plants with tremendous efficacious antioxidants and by producing combination with metal ions, it is also obstructed metal-initiated lipid oxidation (Samar et al., 2018). In this study, quercetin was used as standard because it is a plant pigment that is a vigorous antioxidant flavonoid and more specifically a flavonol that is found in most plants (David et al., 2016). The TFC was determined using a colorimetric assay. The yellow coloration produced is due to the pattern of a compound between the aluminum ion, the carbonyl groups, and the hydroxyl groups of flavonoids (Al-Farsi et al., 2018).

The color changes indicate that the coffee pulp sample from *Coffee liberica* has an amount of flavonoid content. The TFC in coffee pulp extract was estimated as quercetin equivalent (mg QE/g of coffee pulp). A total of 39.39 mg QE/g of coffee pulp was found in the coffee pulp sample extract that determined *Coffee liberica* has a slight amount of flavonoid. In the previous study that was conducted by Geremu et al. (2016), roasted coffee beans had the lowest amount of flavonoid content compared to raw and spent coffee bean which was 30.65 mg catechin/g while raw and spent coffee beans were 52.07 and 34.32 mg catechin/g respectively. In general, the number of total flavonoid contents in the coffee pulp varied because it was influenced by the extraction method. It was described that the flavonoid content was influenced by the temperature in the extraction method which was above 40℃ (Geremu et al., 2016). The solvent polarity in the extraction method plays an important part in increasing phenolic solubility and it is proven that the regaining of flavonoid is influenced by the solvent and the polarity (Nadiah & Uthumporn, 2015).

Antioxidant Activity of Sarawak *Liberica* sp. Coffee Pulp

The antioxidant is a molecule that is ready to give an electron to a charged free radical and counteracts it, hence decreasing its magnitude for cell corruption. The radical scavenging activity is one of the various mechanisms to measure the antioxidant activity of plant extract. 2,2-diphenyl-1-picrylhydrazyl (DPPH) is one of the compounds that have a proton free radical and shows maximum absorption at 520 nm. This study used the DPPH method because it is low cost and easy to evaluate the radical scavenging activity. In agreement with Sagar and Singh (2011), this method was able to react with the varieties of DPPH concentration regarding the sample type and allowed DPPH to react gradually even with weak antioxidants and with sufficient time given. Besides, the colorimetric assay was conducted to measure the antioxidant activity of coffee pulp sample extract. The purple color dims rapidly when DPPH confronts proton radical scavengers (Daniel & Workneh, 2017).

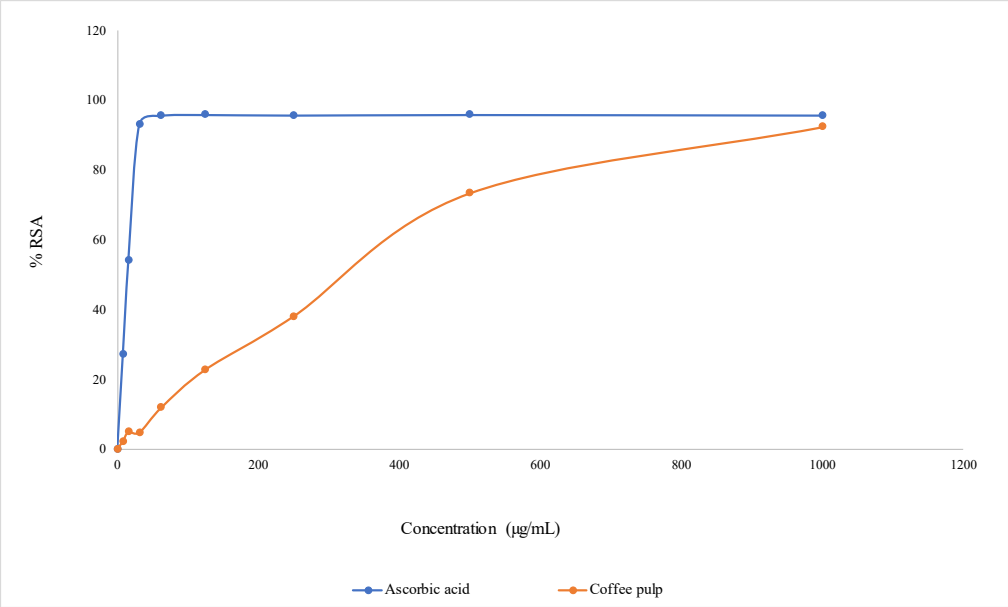

Figure 1. Radical scavenging activity of ascorbic acid and coffee pulp extract

The antioxidant activity of coffee pulp sample extract was calculated as a percent of radical scavenging activity. Based on the result (Figure 1), Liberica coffee pulp sample extract had a high level of radical scavenging activity which was 92.24 ± 0.003% RSA. This is in agreement with Geremu et al. (2016) stated that the interaction of solvents in the extraction played a crucial part in the effects of DPPH scavenging activity. Based on the study, methanolic extract gives a higher value of radical scavenging activity compared to ethanol and acetone. The study indicates that polyphenols play an important role due to their rummage capability on free radicals which they have hydroxyl groups and the extracts performed the highest antioxidant activity have the highest concentration of polyphenols. Sultana and Anwar (2008) also described that the increase in the magnitude of DPPH free radical scavenging capacity and the increased antioxidant activity were due to the increase in the accumulation of polyphenol compounds also. Therefore, polyphenol also plays an important role that may contribute to the antioxidant action.

The inhibition concentration (IC_{50}) value is defined as the concentration of antioxidant required for 50 % scavenging of DPPH radicals. The IC_{50} was calculated for the extraction of coffee pulp sample extract. It is a parameter that is extensively used to compute the antioxidant activity of organic and non-organic compounds. In this step, the standard used was ascorbic acid and it had a

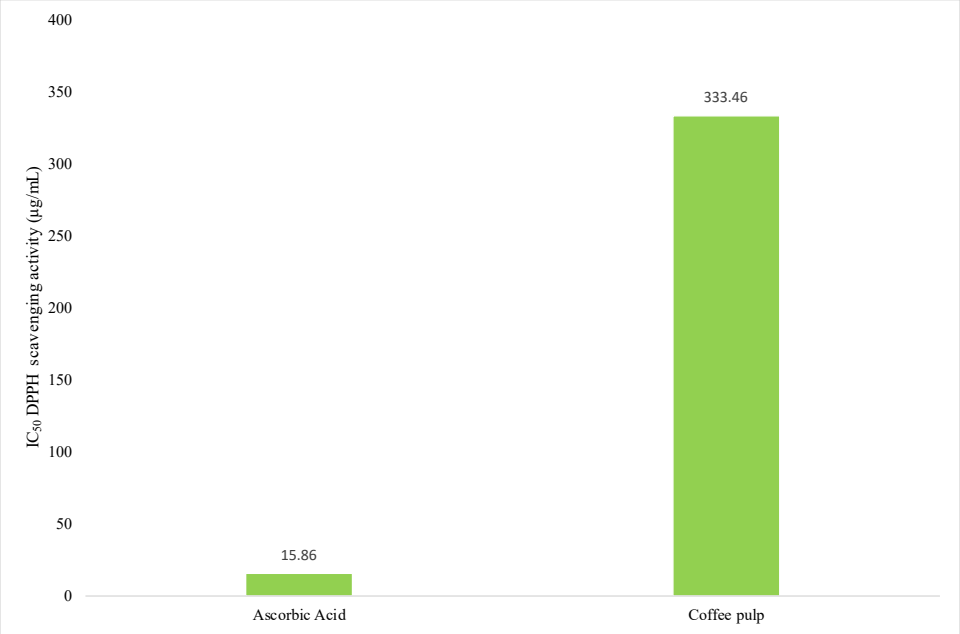

Figure 2. IC_{50} of DPPH scavenging activities in ascorbic acid and coffee pulp extract of Liberica coffee

lower value of IC_{50} which was 15.86 µg/mL. Based on Figure 2, the coffee pulp sample extract showed a high-value IC_{50} which was 333.46 µg/mL and it might correspond to a low antioxidant activity compared to ascorbic acid. This was supported by Maisuthisakul et al. (2007) that a smaller value of IC_{50} correlated to higher antioxidant activity and ascorbic acid was an example of a substance that had high antioxidant activity. Referring to the study conducted by Geremu et al. (2016), the solvent also influenced the IC_{50} value because ethanol extract in the studied gave the highest value of IC_{50} which indicates that it had least potent antioxidant activity. The lowest IC_{50} value was also estimated when extracting using methanolic solvent due to the higher antioxidant activity of methanolic extracts and the total amount of polyphenols. The differences IC_{50} values between solvent properties might be because of their reaction with the solvent used and internal properties of plant compounds

Reducing Sugar Analysis of Sarawak *Liberica* sp. Coffee Pulp

Reducing sugar is any sugar that is capable of becoming as a reducing agent because of its free ketone group or aldehyde group. It is most commonly determined by the reaction with a stabilized alkaline solution of a copper salt. In this study, the DNS assay was conducted to determine the reducing sugar activity in the coffee pulp sample extract. The DNS method is a colorimetric method

that comprises a redox reaction between the reducing sugar and 3,5-dinitro salicylic acid available in the sample. The carbonyl group of sugars has a reducing power which can be oxidized to the carboxyl group by mild oxidizing agents. The DNS reagent is yellow colored and is reduced to 3-amino-5-nitro salicylic acid which is red-brown color. Marisa et al. (2017) had stated that the brightness of the color was in correlation to the sugar concentration.

Based on this study, an amount of 13.13 mg GE/g of the coffee pulp of reducing sugar analysis was identified in the coffee pulp sample extract from *Coffee liberica* that indicates that coffee pulp sample has a slight amount of sugars. The reducing sugar analysis was estimated in the terms of glucose equivalent (mg GE/g of coffee pulp). The color changes during the DNS assay of coffee pulp sample extract showed a change from yellow to red-brown color that indicates that the coffee pulp sample has an amount of sugar. Glucose was used as a standard in this study and the color changes were correlational to the glucose concentration. The changes from yellow coloration to red-brown coloration was increasing with the increasing of the concentration of glucose. In this assay, the 3,5-dinitrosalicylic acid acted as an antioxidant; Rochelles salt that avoids the dissolution of oxygen in the reagent, and lastly, sodium hydroxide were to give the medium needed for the redox reaction to happen (Marisa et al., 2017).

Antibacterial Activity of Sarawak *Liberica* sp. Coffee Pulp

The antibacterial properties of coffee pulp were conducted using the disc diffusion method and evaluating the susceptibility of *Coffee liberica* against foodborne bacteria. The results revealed that there was no antibacterial activity of the coffee pulp extract against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Salmonella typhimurium*) bacteria as no inhibition zone was observed against these bacteria (Figures 3 and 4). Duangjai et al. (2016) stated that coffee pulp extracts showed powerful nutrients and antioxidant activities. Different concentration of 10, 50, and 100 mg/mL of coffee pulp was used in this test. The coffee pulp sample extract was redissolved in Dimethyl Sulfoxide (DMSO) because Awoufack et al. (2013) stated that DMSO was suitable to dissolve the compounds because in the previous studied, acetone was used as the solvents but it did not dissolve all the compounds well.

The coffee pulp sample extract showed no inhibition zone and it might due to the concentration variation. Some reports suggested the antibacterial activity of plant extracts was influenced by phytochemicals such as phenolic acids, malic acids, tannin, caffeine and hydroxycinnamic acid that are the reason for the antibacterial activity (Kabir et al., 2014). The coffee pulp sample extract might have low phytochemical properties that cause no formation of an inhibition zone. It is difficult to predict

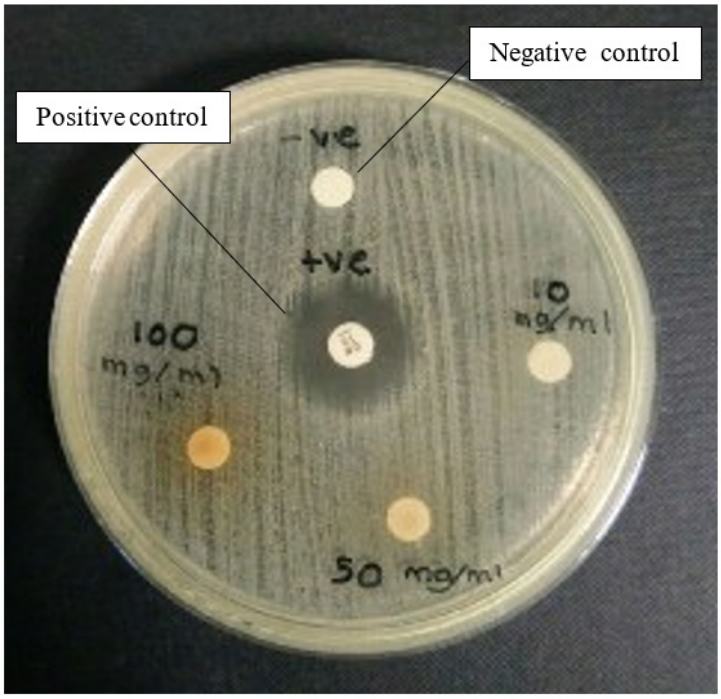

Figure 3. Disk diffusion test of Sarawak *liberica* sp. coffee with different concentration against *Staphylococcus aureus*

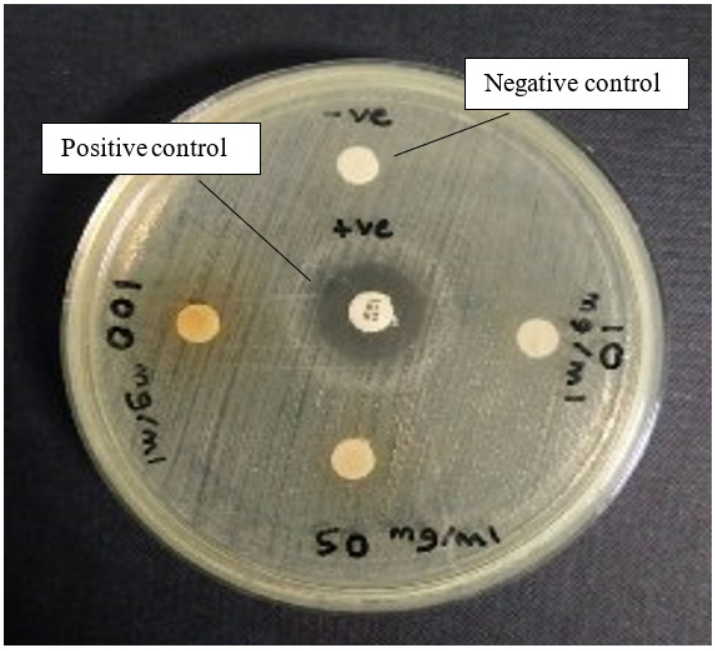

Figure 4. Disk diffusion test of Sarawak *liberica* sp. coffee with different concentration against *Salmonella typhimurium*

the susceptibility to antibacterial agents because results were reported in the previous studies using different types and parts of the plants. It is necessary to isolate the bioactive compounds associated with the antibacterial activity to properly identify the mode of action of antibacterial agents and test against numerous different strains and species of bacteria.

CONCLUSION

This research has successfully determined the physicochemical properties, antioxidant and antibacterial activities of Sarawak *liberica* sp. coffee pulp. The Sarawak *liberica* sp. coffee pulp showed the potential of values added product in conjunction with the amount of phenolic, flavonoid contents, reducing sugar analysis, and a higher level of radical scavenging activity that could have great importance as therapeutic for beneficial use. Comprehensive improvement can be made by estimating the physicochemical properties of coffee pulp using different processing methods and solvents for extraction.

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Screening for Sarawak Paddy Landraces with Resistance to Yellow Rice Stem Borer, *Scirpophaga incertulas* (Walker) (Lepidoptera: Crambidae)

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ABSTRACT

The yellow rice stem borer, *Scirpophaga incertulas* (Walker) is a prevalent pest in paddy fields worldwide. In Sarawak, a survey on pest of paddy carried out from 2009 to 2011 covering 166 paddy fields revealed that rice stem borers caused 11.4% of total paddy damage. In order to reduce the damage, identifying resistance paddy variety is crucial. The objective of this study was to screen Sarawak paddy landraces with resistance to *S. incertulas*. Twelve Sarawak paddy landraces were selected randomly for this study. Antixenosis resistance screening was performed in aquariums (60 x 28 x 33 cm). Three replications of one-month-old seedlings were randomly arranged in aquarium and exposed to adult *S. incertulas*. The number and position of egg mass on each plant were recorded. Egg mass abnormalities were also observed. For antibiosis, rice culms of two-month old seedlings from each landrace were infested with larvae. The length of surviving larvae from five rice culms was measured. The experiment revealed variations in landrace

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of preference for ovipositing. Among the twelve Sarawak paddy landraces, Kanowit was more preferred by *S. incertulas* for ovipositing in comparison to other paddy landraces suggesting susceptibility towards the pest. Abaxial leaf surface was the preferred oviposition site. There was no clear antibiosis response of the paddy landraces towards *S. incertulas* larvae in this study.

Keywords: Antibiosis, antixenosis, rice insect pest

INTRODUCTION

Cultivated rice, *Oryza sativa* is one of the dominant staple foods in many countries, especially in the Asian region. However, insect pests remain to be one of the major threats to the quantity and quality of rice production (Bhadauria & Singh, 2009). Generally, among many insect rice pests, the stem borer stands out as one of the most serious pests due to the wide-scale damage on crop yield (Khan et al., 2003). A state-wide rice pest survey in 166 rice fields in Sarawak, rice stem borer infestation has been found responsible for 11.4% of rice damage in the fields of Sarawak (Gumbek & Hamsein, 2011). Yellow rice stem borer, *Scirpophaga incertulas* (Walker) is one of the stem borer species found commonly throughout the fields of South East Asia and accounts to the highest overall damage (Chakraborty & Deb, 2008; Hugar et al., 2010). *S. incertulas* is also found throughout the rice fields in Sarawak.

Adults of *S. incertulas* are harmless to rice plants. The larvae, however, can cause significant damage via burrowing and feeding on the rice stem (Satpathi et al., 2012). Damages by yellow rice stem borer has been detected in different growth stages ranging from seedlings to adult plants (Dale, 1994; Hamsein et al., 2020). Symptoms of infestation in the early stages are concealed and will only become apparent in later stages. A common symptom of infestation during the vegetative phase of rice is known as dead heart, a condition where the leaf base is destroyed, causing the leaves to dry out. Another symptom during the generative phase, white head, occurs when the stem is attacked, causing the panicles to be cut off from water and nutrient, leading to desiccation (Bashir et al., 2004).

Usage of insecticide has been found to aggravate damage done by rice stem borer due to the elimination of the predators and parasites of rice stem borer. Rice stem borer infestation has been found to be significantly higher in areas practicing chemical control, compared to areas in Sarawak where insecticide is seldom used (Rothschild, 1970). As a result, a more effective and sustainable control method has to be introduced. One of the alternatives to control insect pest is by planting resistant variety. Resistance is determined by the presence of antibiosis and antixenosis properties in plant. These mechanisms allow a plant to appear unfavourable as a target of infestation by insect pest. Resistance towards insect pest is an efficient and durable built-in control measure, convenient and does not cause harm to human health or the environment (Chaudhary et al., 1984).

Different paddy variety/landraces may have different resistance level (Devasena et al., 2018). Varieties/landraces known to have resistance are valuable candidates for resistance breeding. The objective of this study was to screen Sarawak paddy landraces with resistance to *S. incertulas*.

METHODS

Seedlings Preparation

A total of twelve locally collected wetland paddy landraces were used in this experiment (Table 1). The seeds were germinated by soaking in distilled water and sowed in planting trays. For antixenosis experiment, healthy seedlings were selected and transplanted randomly (three seedlings per landrace) into an enclosed aquarium (60 x 28 x 33 cm) filled with planting media consisting of topsoil and sand (2:1). There were six landraces per aquarium. One-month-old rice plants were used.

For antibiosis experiment, healthy seedlings were transplanted into pots (⌀ = 30 cm; 16L) filled with planting media as mentioned earlier (five seedlings per pot). Culms of 20 cm were harvested from two-month-old rice plants for experimental usage. Compound fertilizer 12N: $12P_2O_5$: $17K_2O$: 2MgO+TE was applied at the rate of ≈ 9.7 g per aquarium and ≈ 2.4 g per pot biweekly until the plants were ready for use.

Antixenosis Experiment – Choice Experiment

One-month-old plants (in aquarium) were subjected to *S. incertulas* infestation.

Table 1

Twelve wetland paddy landraces subjected to Scirpophaga incertulas *infestation*

Landrace	Code	Seed Source
Padi Pulut	UNISRA-11	Kg. Paun Gahat
Padi Hitam	UNISMT-21	Kg. Pueh
Padi Segangging	UNISRA-25	Melugu Skim
Padi Kanowit	UNISRA-26	Melugu Skim
Padi Merawi	UNIPDW-51	Padawan
Padi Adan	UNIBKN-61	Long Semadoh, Ba'kelalan
Padi Rutan	UNISRA-33	Sg. Tenggang
Padi Upah	UNISRA-35	Sg. Tenggang
Padi Merah	UNIPDW-18	Kg. Pesak
Padi Selasih	UNISRA-40	Melugu Skim
Padi Labat	UNISMT-23	Kg. Pueh
Padi Pulut Hitam	UNISMT-22	Kg. Pueh

Female *S. incertulas* were collected from the paddy field of Kampung Skuduk-Chupak (1°15'7.12"N, 110°25'59.83"E) using white screen light trap. A total of 50 adult females were released directly into each aquarium after captured in the field. Three days after infestation, the number and position of egg masses found on the leaves were recorded. Egg mass abnormalities as described by Hilker and Meiners (2006) were recorded. Three repetition were carried out for this experiment. Relative value for number of egg masses (Equation 1) was used for analysis:

$$\frac{\text{Total egg masses on leaf of a plant}}{\text{Total egg masses found on leaf of all plants}}$$

[Equation 1]

Oviposition site of preference, adaxial *vs.* abaxial, was analysed using two-sample *t*-test (α =0.05). Landrace of preference for oviposition was analysed using one-way analysis of variance (ANOVA) (α = 0.05; using IBM SPSS software version 24).

Antibiosis Experiment – Non-choice Experiment

The culm (20 cm) of the paddy landraces were harvested and placed in separate transparent containers (10 culms per container). A moist cotton piece was used to cover the lower end of the culms. One newly hatched *S. incertulas* larva was placed at the top end of each culm. The containers holding the culms were left on lab bench at room temperature (25 - 27°C). Seven days after the infestation, the length of surviving larvae from five rice culm was measured using a ruler. Two repetitions were carried out for this experiment and the data was analysed using one-way ANOVA (α = 0.05; using IBM SPSS software version 24).

RESULTS

On each plant, 0 to 16 egg masses were found. Size of egg masses ranged from 2 mm^2 to 32 mm^2. Egg masses were found mostly on the surface of leaf blades, while only 1 - 4 egg masses were found on the stems of plants from each aquarium (Table 2).

Table 2

Egg masses recorded across six aquariums on different surfaces

Aquarium	Egg mass on plant		Egg mass on non-plant surface	Total egg mass
	Leaf	Stem		
1	42	3	14	59
2	92	3	21	116
3	54	1	9	64
4	46	1	11	58
5	71	2	9	82
6	75	4	3	82

Leaf Surface of Preference by *Scirpophaga incertulas* for Oviposition

Between abaxial and adaxial leaf surface, there was a significant different in the number of egg masses found on the abaxial and adaxial surfaces (p-value = 0.0001). The mean relative numbers of egg masses laid on the abaxial and adaxial surface of paddy landraces were 0.04 ± 0.003 and 0.02 ± 0.002, respectively, where more egg masses were found on abaxial surface.

Antixenosis Resistance of Sarawak Paddy Landraces against *Scirpophaga incertulas*

Among the twelve paddy landraces, there was a significant difference in the relative number of egg masses found on the leaf of seedlings (p-value = 0.001). In general, the twelve paddy landraces can be grouped into landraces with low (7 landraces), intermediate (4 landraces), and high (1 landrace) number of egg masses (Table 3). The classification is based on the homogenous subsets created by Tukey HSD. The one landrace in the high egg mass number category, Kanowit, was more preferred by the female *S. incertulas* for oviposition (mean relative number of egg masses, 0.12 ± 0.018) when compared to the landraces in the low egg mass number category. Egg mass abnormalities described by Hilker and Meiners (2006) were not observed (Figure 1). All egg masses were intact, and no drop-off egg masses were seen. There were also no signs of neoplasm formation or necrosis under or around egg masses attached to the plant surface.

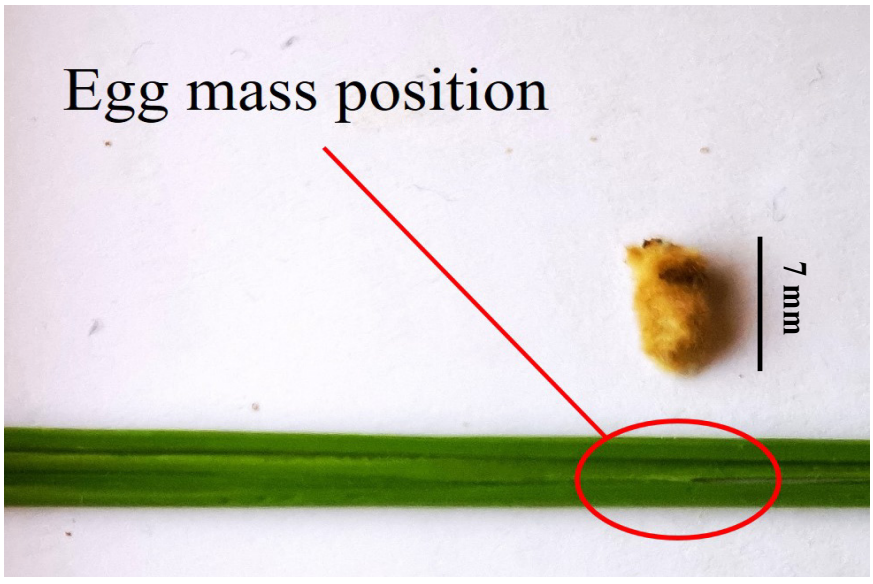

Figure 1. Egg mass of *Scirpophaga incertulas* artificially removed from its deposited position on paddy leaf blade. Neoplasm formation or necrosis under or around egg masses was absent

Antibiosis Resistance of Sarawak Paddy Landraces against *Scirpophaga incertulas*

All twelve landraces from both repetitions had at least five culms with surviving larvae. The length of larvae removed from the culms measured between 1.5 mm to 5.2 mm. The average larvae length was measured at 2.9 mm. There was no significant difference in the length of survived larvae (p-value = 0.333) (Table 3).

Table 3

Mean relative number of egg masses oviposited and the larvae length of Scirpophaga incertulas *on each paddy landrace*

Landraces	Mean relative number of egg mass ± SE*	Grouping based on mean relative number of egg mass[!]	Mean larvae length(mm) ± SE*
Padi Merawi	0.03 ± 0.009 [a]	Low number	2.82 ± 0.312 [a]
Padi Pulut	0.03 ± 0.009 [a]	Low number	2.97 ± 0.208[a]
Padi Upah	0.04 ± 0.012 [a]	Low number	3.25 ± 0.214 [a]
Padi Hitam	0.05 ± 0.011 [a]	Low number	2.51 ± 0.137 [a]
Padi Adan	0.05 ± 0.011 [a]	Low number	3.07 ± 0.246 [a]
Padi Pulut Hitam	0.05 ± 0.013 [a]	Low number	2.66 ± 0.273 [a]
Padi Merah	0.05 ± 0.009 [a]	Low number	2.74 ± 0.240 [a]
Padi Selasih	0.06 ± 0.013 [ab]	Intermediate	2.73 ± 0.169 [a]
Padi Segangging	0.06 ± 0.014 [ab]	Intermediate	2.99 ± 0.158 [a]
Padi Labat	0.07 ± 0.014 [ab]	Intermediate	2.66 ± 0.193 [a]
Padi Rutan	0.07 ± 0.018 [ab]	Intermediate	3.14 ± 0.163 [a]
Padi Kanowit	0.12 ± 0.018 [b]	High number	2.73 ± 0.116 [a]

Note. * Means within the same column followed by the same letter are non-significantly different (p<0.05) based on Tukey HSD; [!] Grouping is based on homogeneous subsets generated by Tukey HSD

DISCUSSIONS

Decision of female insects in choosing oviposition site (leaf surface) is crucial for the survival of their larvae. A recent study by Hamsein et al. (2020) had screened six Sarawak paddy landraces for resistance towards *S. incertulas* in a net house environment. The free choice experiment showed that *S. incertulas* preferred to oviposit on the abaxial of leaf surface, and on the leaf of older and taller plants. Adult females of *S. incertulas* showed no specific preference between the six Sarawak paddy landraces for ovipositing. In a non-choice experiment by Cheok et al. (2019), under uniform age, *S. incertulas* showed no specific preference for ovipositing on adaxial and abaxial leaf surface, as well as

on the Sarawak paddy landraces tested. In this study, *S. incertulas* showed a preference to oviposit on the abaxial leaf surface in agreement to Shahjahan (2002) and Hamsein et al. (2020). A possible reason for the oviposition choice could be that abaxial side decreases the chance of the egg masses to be exposed to predation and parasitism (Renwick & Chew, 1994).

It was also observed in this study, under uniform age and plant height, *S. incertulas* showed variations in landrace of preference for ovipositing. The Kanowit paddy landrace seemed to be more preferred for ovipositing by *S. incertulas* as compared to certain landraces. A variation in preference for oviposition by *S. incertulas* was also observed by Rustamani et al. (2002) in a field experiment in Pakistan. The variations of preference may have occurred due to the presence and absence of oviposition deterrent chemicals (antixenosis) between the 12 Sarawak paddy landraces. Evidence of oviposition deterrent chemicals forming after egg mass deposition on plants had been reported for cabbage against *Pieris brassicae*, the cabbage butterfly (Blaakmeer et al., 1994). According to Bertea et al. (2019), insects produce substances during ovipositing which act as elicitors that triggers plant defence response. The elicitors were able to trigger electrical signals and change Ca^{2+} homeostasis resulting in defensive responses from the plant through hormone signalling pathways (salicylic acid pathway) (Bertea et al., 2019). The condition may be similar for rice in response to ovipositing of *S. incertulas*. Landrace which is more preferred for ovipositing may not have or have a low level of oviposition deterrent chemicals. However, there is no study yet on such rice response.

To date, 18 Sarawak paddy landraces were used to study the landrace of preference for ovipositing by *S. incertulas* collected from Kampung Skuduk-Chupak (1°15'7.12"N, 110°25'59.83"E). Only the Kanowit landrace was more preferred by S. *incertulas* (Table 4). Additional screening and combination of different landraces will allow the identification of susceptible and resistance landrace.

Egg deposition is the precursor to future insect (larvae) infestation (Lortzing et al., 2019). To counteract, hypersensitive response in the form of necrosis and neoplasm formation (antibiosis resistance) has been observed in plants as a lethal direct defence against insect eggs (Petzold-Maxwell et al., 2011). Formation of neoplasm and necrotic tissue may cause attached egg masses to drop off, therefore removing the possibility of larvae hatching and infesting the plant. The egg masses observed in this study were healthy and were attached firmly to the plant surface. Thus, it could be assumed that the 12 paddy landraces lacked antibiosis resistance. Signs of neoplasm and necrotic tissue formation were not observed in areas of egg mass deposition otherwise (Hilker & Meiners, 2006).

An alternative method of observing antibiosis resistance is by studying larvae growth. Visible effects of antibiosis can be observed on the larvae in the form of high mortality, longer development period

Table 4

List of Sarawak paddy landraces subjected to ovipositing preference test by Scirpophaga incertulas*. All insect samples were collected from the same location, Kampung Skuduk-Chupak (1°15'7.12"N, 110°25'59.83"E)*

Landrace	Seed source	Result reported	Plant age (month)	Test	Citation
Padi Bajong	ARC, Semongok	No preference	1, 2, & 4	Free choice	Hamsein et al. (2020)
Padi Selasih	ARC, Semongok	No preference	1, 2, & 4	Free choice	Hamsein et al. (2020)
Padi Bario	ARC, Semongok	No preference	1, 2, & 4	Free choice	Hamsein et al. (2020)
Padi Bubok	ARC, Semongok	No preference	1, 2, & 4	Free choice	Hamsein et al. (2020)
Padi Biris	ARC, Semongok	No preference	1, 2, & 4	Free choice	Hamsein et al. (2020)
Padi Nyamuk	ARC, Semongok	No preference	1, 2, & 4	Free choice	Hamsein et al. (2020)
Padi Bajong	ARC, Semongok	No preference	2	Non-choice	Cheok et al. (2019)
Padi Bario	ARC, Semongok	No preference	2	Non-choice	Cheok et al. (2019)
Padi Bubok	ARC, Semongok	No preference	2	Non-choice	Cheok et al. (2019)
Padi Merawi	Padawan	No preference	1	Free choice	Current study
Padi Pulut	Kg. Paun Gahat	No preference	1	Free choice	Current study
Padi Merah	Kg. Pesak	No preference	1	Free choice	Current study
Padi Adan	Long Semadoh, Ba'kelalan	No preference	1	Free choice	Current study
Padi Pulut Hitam	Kg. Pueh	No preference	1	Free choice	Current study
Padi Hitam	Kg. Pueh	No preference	1	Free choice	Current study
Padi Labat	Kg. Pueh	No preference	1	Free choice	Current study
Padi Upah	Sg. Tenggang	No preference	1	Free choice	Current study
Padi Rutan	Sg. Tenggang	No preference	1	Free choice	Current study
Padi Selasih	Melugu Skim	No preference	1	Free choice	Current study
Padi Segangging	Melugu Skim	No preference	1	Free choice	Current study
Padi Kanowit	Melugu Skim	Preferred	1	Free choice	Current study

Note. ARC = Agriculture Research Centre

or decreased size (Padmaja, 2016). In the current study, there was no difference in the length of *S. incertulas* larvae infested in culm cuttings of different landraces. This may suggest that the 12 paddy landraces have no antibiosis resistance towards the larvae or among the paddy landraces, there are landraces having weak level of antibiosis resistance which result in insignificant different between them. Presence of antibiosis resistance in rice plants has been proven to exist in rice against *Chilo suppressalis* (Hosseini et al., 2011; Tabari et al., 2017). According to Hosseini et al. (2011), smaller stem diameter may be one form of resistance associated morphological characteristics that disrupt the *S. incertulas* larvae infestation. A smaller stem diameter provides less feeding space as well as limited food sources for the development of larvae (Hosseini et al., 2010, 2011; Sarwar, 2013).

CONCLUSION

In conclusion, *Scirpophaga incertulas* prefers to deposit their egg masses on the abaxial leaf surface of rice plant. The 12 Sarawak paddy landraces vary in the level of antixenosis resistance against *S. incertulas*. Among the landraces, Kanowit is more preferred by *S. incertulas* for ovipositing in comparison to seven other landraces. Antibiosis resistance against the eggs and larvae of *S. incertulas* seems absent.

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Diversity, Abundance, and Foraging Behavior of Ants (Hymenoptera: Formicidae) Scavenging on American Cockroach in Various Habitats of Nasarawa State, Nigeria

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ABSTRACT

Ants play a vital role in removing dead arthropods from the environment. Complex foraging patterns are used by ants to locate food items and overwhelm even larger insects such as cockroach. Consequently, the biotic interaction between the ants and the American cockroach, *Periplaneta americana*, another home infesting and a vector of major food-borne diseases, may lead to microbial handover and ease the spread of mechanically transmitted human pathogenic microbes. This study was done to determine the diversity and abundance of cockroach-foraging ants in Nasarawa State, Nigeria. Ten households were randomly selected from 14 locations: 5 residential communities from three most urbanized areas (Lafia, Akwanga, and Keffi) and 5 rural communities. Four remaining locations were nonresidential from Lafia and Akwanga, respectively. A total of 1,364 ants belonging to three subfamilies (Myrmicinae, Formicinae, and Ponerinae) were collected from 140 households. *Pheidole rugaticeps* Emery recorded the highest relative abundance (52%) followed by *Pheidole decarinata* Santschi (16%), *Pheidole* sp. (17%), *Camponotus maculatus* (7%), *Paratrechina longicornis* (7%), while both *Crematogaster* sp. and *Brachyponera sennaarensis* recorded the lowest relative abundance (1%). There is a significant difference in the species diversity between the urban and the rural communities. *Pheidole rugaticeps*, *P. decarinata*, *Pheidole* sp., and *P. longicornis* were more organized in foraging and operating in group by recruiting nest-mate

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for collecting fragments of dead cockroach. Studies on the epidemiology, conservation implications, and biocontrol potentials of these *Pheidole* species are recommended.

Keywords: Abundance, diversity, *Periplaneta americana*, *Pheidole*, scavenging ants, rural, urban

INTRODUCTION

Ants are vital arthropod pests associating with human habitation and have a diverse ecological significance in several ecosystems (Hölldobler & Wilson, 1990). About fifty ant species are reported to have adapted to the urban setting due to its heterogeneity that offers them food, nesting sites and biotic interactions (Benson & Harada, 1988; Hölldobler & Wilson, 1990; Reyes-Lopez et al., 2003). Some ants can live outdoors and forage indoors while others nest indoors and previous studies have noted that foraging activities of ants may be detrimental or beneficial (Gathalkar & Sen, 2018). Many ants invade human dwellings and cause serious direct and indirect harm, including human health issues, building damage, and interruption of other wildlife. As scavengers, they play a massive role in emptying the environment of arthropods. Their high rate of scavenging habit suggests how considerably successful they are at locating invertebrates carcasses than other scavengers like flies, cockroaches, and vertebrates (Tan & Corlett, 2012). They remove the whole or fragments of the dead invertebrates by using complex foraging patterns (Moffett, 1988), such as recruiting other nest-mates (Tan & Corlett, 2012) and/or mass foraging density (Beckers et al., 1989). This group operation as well as aggression assist them to overpower even relatively larger insects (Tan & Corlett, 2012), and foraging abilities of many ant species may have a devastating effect on the biodiversity of local taxa.

Moreover, the gathering and the storage of corpses and feces by ants have been shown to have tremendous effects on other lives and chemical components around them (Dauber et al., 2001; Dauber & Wolters, 2000; Lavelle et al., 1997; Petal, 1998). Previous studies have revealed that bacteria can remain viable and persist within the nest of ant becoming a reservoir for infection of other members of the colony (Beatson, 1972). Their presence in the ecosystem directly enables the spread of human pathogens (Boursaux-Eude & Gross, 2000; Fonseca et al., 2010). Contamination of hospital apparatuses by ants leads to the mechanical transmitting of diseases (Eichler, 1990; Lima et al., 2013), allergies, stings, and bites (Goddard, 1993; Syukriah Sabtu & Ab Majid, 2020; Williams et al., 2001), and food contamination (Lee, 2001). In the tropics, many ant species have already been incriminated as vectors of pathogens (Sarwar, 2015). Food-borne disease pathogens such as *Serratia*, *Citrobacter*, *Klebsiella*, *Enterobacter*, *Proteus*, *Staphylococcus*, and *Yersinia pestis* were isolated from ants (De Zarzuela et al., 2005; Simothy et al., 2018). *Vibrio cholerae* has previously been recovered from both ants and cockroaches (Sarwar, 2015). Yet, data on the ant's role in mechanical transmission

of human pathogens is scanty compared to flies or cockroaches (Sarwar, 2015).

American cockroaches (*Periplaneta americana*) are one of the house-infesting invertebrates (Graczyk et al, 2005; Mpuchane et al., 2005) that are frequently seen in bathrooms, toilets and other parts of the houses (Dehghani et al., 2014). They are ideal carriers of various pathogens due to the filthy nature of their breeding and feeding habits (Chaichanawongsaroj et al., 2004; Graczyk et al., 2005). They play a vital role in spreading major food-borne diseases like diarrhea, dysentery, cholera, tuberculosis, and typhoid fever (Fotedar et al., 1992; Graczyk et al., 2005; Saitou et al., 2009). More species of pathogens are harbored by *P. americana* than any other cockroach species studied (Pai et al., 2003; Prado et al., 2002). Recently, the infestation trend of cockroaches has increased in household environments (Nasirian, 2017). Dead *P. americana* can frequently be observed around houses due to high infestation which attracts foraging ants into the house. The foraging activities may lead to microbial interchange and can be of epidemiological concerns as ants presence can ease the propagation and spread of human pathogens (Boursaux-Eude & Gross, 2000; Fonseca et al., 2010). Therefore, this study was to determine the diversity, abundance, and foraging behavior of ants scavenging on American cockroach in various habitats of Nasarawa State, Nigeria. The data from the study can be useful in managing household pests, especially mechanical vectors that spread diseases.

MATERIALS AND METHODS

Study Area and Sampling

This study was done to determine the diversity, abundance, and foraging behavior of ants scavenging on American cockroach in various habitats of Nasarawa State located in a middle belt of Nigeria with an estimated land area of 2,733km^2 and a population size of 330,720 according to the 2006 census by the National Population Commission. Ten households were randomly selected based on the consent of the households and relevant authorities from 14 locations, 5 residential communities from three most urbanized areas, which includes Lafia (Lafia East, Shinge, and Shabu), Keffi (GRA), Akwanga (low-cost housing estate) as well as 5 rural communities (Akunza, Gandu, Akunzan Sama, Gwandare, and Kurikyo). Four remaining locations were nonresidential areas including Federal University Lafia, Nasarawa State Polytechnic, Dalhatu Araf Specialist Hospital, and a primary healthcare center (PHC) from Lafia and Akwanga, respectively (Figure 1). Ethical clearance was obtained from the Ethics Committee of the State Ministry of Health, Nasarawa State, as the survey involves the use of insecticides (knock down, Guangzhou Konnor Daily Necessities Co., Ltd, Guangdong, China). In each community eight to ten households or sampling points were selected, where cockroaches and ants scavenging on American cockroach were collected in and around toilets, rooms, and kitchens after spraying insecticides. However, toilets and bathrooms were the most infested parts of residents by *P. americana* (Dehghani et

al., 2014). The sampling took place from November 2018 to February 2019.

Observation of Foraging Behavior, Collection, and Identification of the Insects

The sample collection was done during night hours (20:00h to 22:00h) as American cockroaches are nocturnal insects. Flushing agents (knock down, Guangzhou Konnor Daily Necessities Co., Ltd.) were sprayed around cockroach observing spots such as toilets with the consent of the household (Alias et al., 2018). Thirty minutes after spraying the insecticide, we returned to the location and made the observations. The cockroaches were observed and recorded. Ants foraging on the live and dead cockroaches were monitored in and around the sprayed toilets. Dead American cockroaches were also placed along the ant trail routes in the selected houses. Where ants were observed collecting or foraging on live or dead cockroaches, both the ants and the host cockroaches were collected and preserved in 70% ethanol solutions for further analysis in the laboratory. Ants scavenging both dead and life cockroaches were gently scooped into a 20 ml vial container and the cockroaches that have not hosted ants were collected into a jar containing 70% alcohol solution for preservation for later analysis. Several ant species foraging around each cockroach were recorded to ascertain their diversity and abundance in the area. The sprayed spots were checked the following morning. The identification of the collected cockroach was done with the aid of morphological identification keys (Bell, 1981). Identification of the collected ants

Figure 1. Map of the collection sites in Nasarawa State, Nigeria. Refer to Table 1 for abbreviation of the numbers 1-14

was done using taxonomic keys (Fischer et al., 2012) and the colored images at the websites (antweb.org and antsofafrica.org, respectively). The behavior of the ants found foraging on the cockroaches was also monitored during collection. How they collect the cockroach, whether they collect live or dead cockroach, and how they transport the cockroach (whole or fragments) were all recorded. In addition, other insects that were collected by these ants and the nature of their caste and trail were listed down as well.

Data Analysis

The data collected were tabulated and statistically analyzed using SPSS Statistics 20.0. Kruskal-Wallis test was conducted to determine if there was a relationship between the distribution of *P. americana* and ants scavenging on this cockroach species in the study area. A post hoc test was conducted to compare the means of the various locations. The diversity index of cockroach-foraging ants in the urban and the rural communities was determined using the biodiversity calculator excel sheet (Zar, 1996). Diversity *t*-test was used to analyze the statistical difference between the communities and overall relative abundance of ants was determined using the formula (Alias et al., 2018) below:

$$\text{Relative abundance of a species} = \frac{\text{Number of individuals of the same species}}{\text{Number of individuals of all the species}} \times 100$$

RESULTS

Distribution of American Cockroach and The Ants Scavenging on American Cockroach

A total of 36-pit latrine (rural communities) and 89 water system toilets (rural communities) from 136 residential and nonresidential premises in the rural and the urban communities were sampled by spraying insecticide, respectively. Dead cockroaches after spraying insecticides in the water system toilets and the pit latrine toilets are presented in Figure 2.1 and Figure 2.2, respectively. A total of 3,298 American cockroaches (78.71% and 21.29% from the rural and the urban, respectively) were recorded. The result of the post hoc test is presented in Table 1 and showed that Akunza, one of the rural communities, recorded the highest mean of *P. americana* (92.50±23.64), while primary healthcare center (PHC) Akwanga, one of the urban communities, recorded the lowest mean (2.00±0.98). Moreover, the overall result from the Kruskal-Wallis test showed a significant difference in the overall distribution of the American cockroach across the fourteen sampled locations, ($X^2(13) = 40.048$, $p<0.001$). The independent samples *t*-test ($t(136) = 6.13$, $p<0.001$) also showed a significant difference in the distribution of the American cockroach between the rural and the urban areas.

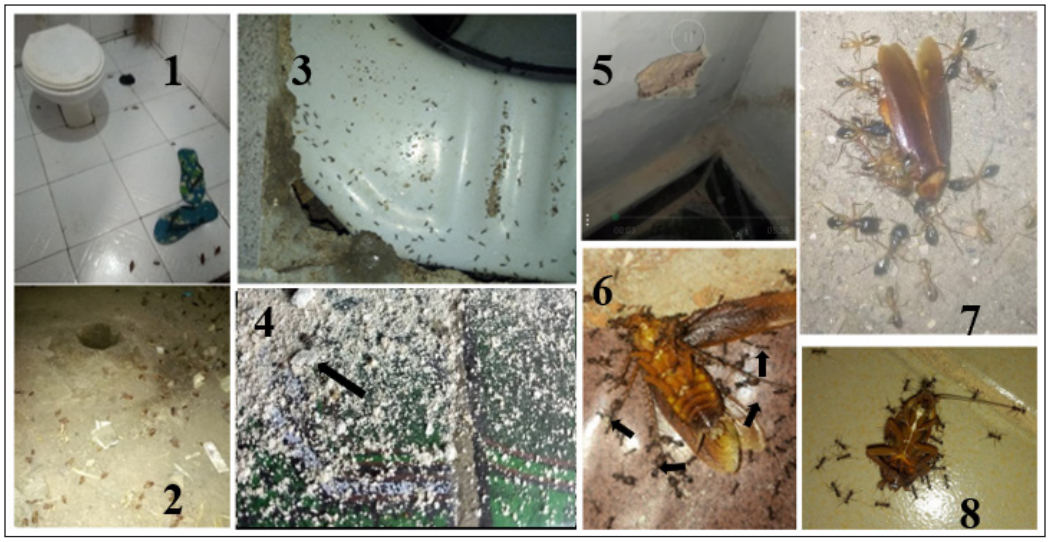

Figure 2. Dead cockroaches in water system (1) and pit latrine (2) after spraying insecticides; nest of *Paratrechina longicornis* in a toilet (3); sands and carcasses of *Brachyponera sennaarensis* just below the nest of a *Pheidole decarinata* (4); nest of *P. decarinata* on the wall (5); *Pheidole rugaticeps* spread-eagling a live cockroach (6); *Camponotus maculatus* foraging on a dead cockroach (7); and a swarm *P. longicornis* carrying a dead cockroach to their nest (8)

A total of 1,364 ants (31% rural and 69% urban) were collected from the 136 sampling points. Unlike the distribution of the cockroach, the overall distribution ants scavenging on American cockroach obtained from Kruskal-Wallis test showed no statistically significant difference across all the locations ($X^2(13) = 9.455$, $p = 0.738$) and between the rural and the urban areas (*t*-test, $t(25) = 0.15$, $p<0.876$). The highest mean occurrence of ants scavenging on American cockroach was recorded in Akunza (20.80±15.72) and the lowest was recorded in Gandu Sarki (0.00±0.00) as shown in Table 1. The percentage of each ant species collected around *P. americana* in each location is shown in Table 2. *Pheidole rugaticeps* had been recorded in all except 4 locations (Table 2). Other species such as *Crematogaster* sp., *B. sennaarensis*, and *P. megacephala* were only recorded in one location. Table 2 shows the total number and the percentages of each ant species collected foraging on *P. americana* in each location. Lafia East had the highest number of ant species (Table 2).

Species Diversity and Abundance of the Ants Scavenging on American Cockroach

The 1,364 ants collected from 136 sampling spots belong to three subfamilies: Myrmicinae (85.27%), Formicinae (13.41%), and Ponerinae (1.32%). *Pheidole* species were the most dominant ants in all communities and forms the most abundance subfamily, Myrmicinae (together with *Crematogaster* sp.). The overall result of the

Table 1
Locations, coordinates, range, and mean abundance of Periplaneta americana *and ants scavenging on American cockroach in the sampling locations*

Serial no.		Locations	Habitat*	Coordinates	N	Range		Mean	
						American cockroach	Ants	American cockroach	Ants
1	Urban	Lafia East (LE)	UB/RA	08°29'33.57" N 08°32'27.10" E	10	1-40	18-38	11.40(±3.67)	11.50(±5.37)
2		GRA, Keffi	UB/RA	08°50'53.25" N 07°53'08.48" E	10	0-34	22-69	6.56(±3.60)	16.89(±9.43)
3		Fulafia (FUL)	NRA	08°28'25.70" N 08°33'22.42" E	9	0-21	0-34	4.89(±2.39)	3.78(±3.78)
4		Shinge (SHG)	UB/RA	08°30'37.92" N 08°29'34.34" E	9	0-58	31-67	12.44(±6.20)	10.89(±7.80)
5		Shabu (SHB)	UB/RA	08°34'45.06" N 08°33'32.73" E	9	0.60	0-21	13.80(6.77)	2.10(±2.10)
6		Nasarawa State Polytechnic (POLY)	NRA	08°32.47.55" N 08°32'09.81" E	10	0-14	0-72	2.78(±1.48)	8.00(±8.00)
7		Dalhatu Specialist Hospital (DASH)	NRA	08°30'08.95" N 08°31'21.95" E	9	0-44	20-103	8.60(±4.60)	18.70(±11.36)
8		Low-cost Housing, Akwanga (LHE)	UB/RA	08°55'38.29" N 08°24'46.91" E	10	0-7	44-115	2.80(±0.88)	15.90(±11.85)
9		Primary Healthcare Center (PHC), Akwanga	NRA	08°54'50.41" N 08°24'51.86" E	10	0-10	0-87	2.00(±0.98)	8.70(±8.70)
10	Rural	Akunza (AKZ)	RR/RA	08°28'11.69" N 08°35'24.03" E	10	15-206	5-156	92.50(±23.64)	20.80(±15.72)
11		Gandun Sarki (GND)	RR/RA	08°46'59.37" N 08°00'57.68" E	10	1-121	-	47.70(±11.95)	0.00(±0.00)
12		Akunzan Sama (AKZS)	RR/RA	08°28'07.87" N 08°36'04.02" E	10	10-134	20-42	48.90(±17.44)	6.20(±4.45)
13		Gwandara (GDR)	RR/RA	08°34.11.95" N 08°29'50.59" E	10	50-105	0-50	35.20(±11.37)	5.00(±5.00)
14		Kurikyo (KRK)	RR/RA	08°31'32.09" N 08°35'51.59" E	10	5-113	25-76	35.30(±10.68)	10.10(±7.73)
		Total			136			23.18(±3.329)	9.90(±7.24)

Note. UB = Urban areas; RR = Rural areas; RA = Residential areas; NRA = Non-residential areas. Kruskal Wallis test shows a significant difference in the overall distribution of the American cockroach, $X^2(13) = 40.048$, $p<0.001$ across all locations and between the rural and the urban ($t(136) = 6.13$, $p<0.001$) according to the independent samples *t*-test. As for ants the test shows no significant difference in the overall distribution $X^2(13) = 9.455$, $p = 0.738$ and between the rural and the urban areas ($t(25) = 0.15$, $p<0.876$)

Table 2
Total number of ant species collected foraging on Periplaneta americana *and their percentages (%) occurrence in each location*

	Myrmicinae				Formicinae		Ponerinae			
Communities	*Pheidole rugaticeps*	*Pheidole decarinata*	*Pheidole* sp.	*Crematogaster* sp.	*Camponotus maculatus*	*Paratrechina longicornis*	*Brachyponera sennaarensis*	Total	No. of ant spp.	%
LE	-	36	-	20	14	45	18	133	5	9.75
GRA	61	69	-	-	22	-	-	152	3	11.14
FUL	-	-	34	-	-	-	-	34	1	2.49
SHG	67	-	-	-	31	-	-	98	2	7.18
SHB	-	-	-	-	-	21	-	21	1	1.54
POLY	72	-	-	-	-	-	-	72	1	5.28
DASH	103	64	-	-	20	-	-	187	3	13.71
LHE	115	44	-	-	-	-	-	159	2	11.66
PHC	87	-	-	-	-	-	-	87	2	6.38
AKZ	47	-	156	-	5	-	-	208	3	15.25
GND	-	-	-	-	-	-	-	0	0	0.00
AKZS	20	-	42	-	-	-	-	62	2	4.55
GDR	50	-	-	-	-	-	-	50	1	3.67
KRK	76	-	-	-	-	25	-	101	2	7.40
Total	698	213	232	20	92	91	18	1,364		100
%	51.17	15.62	17.01	1.47	6.74	6.67	1.32			

relative abundance showed that *P. rugaticeps* (51.2%) recorded the highest abundance, together with *P. decarinata* (15.6%) and other *Pheidole* species (17.1%) made up 83.8% of all the ants collected. This explains the fact that *Pheidole* was the most abundant and diverse formicids in the area. However, *B. sennaarensis* (subfamily: Ponerinae) and *Crematogaster* sp. (subfamily: Myrmicinae) were the least collected with (1.3% and 1.5%, respectively) as shown in Table 2. The community-based distribution of these ants showed a difference in the diversity of ants scavenging on American cockroach between the urban communities (H' = 1.35, E = 0.7) and the rural communities (H' = 0.93, E = 0.67). Based on the result of the diversity t-test ($t(800) = 28.26$, $p>0.05$), it indicated that they were more diverse in the urban communities. The genus, *Pheidole* was the dominant ant species in all the communities and *P. rugaticeps* was the most abundant species in almost all the communities. Surprisingly, the result of the diversity reveals that the richness of species in the urban communities was increased (Figure 3). The highest percentage of the ants was recorded in Akunza (15.25%) while Gandun Sarki (0.00%) had the lowest percentage and both are the rural communities. However, the highest number of ant species (5 ant species) was recorded in Lafia East, which is an urban community (Table 2). The relative abundance of the ants scavenging on *P. americana* based on the communities studied is presented in Figure 4.

Foraging Behavior of the Ants Scavenging on American Cockroach

Table 3 shows the total number of each species of ants and the percentage of American cockroach infestation by these ants. *Pheidole rugaticeps* had the highest number and percentage of infestation with up to 54% as shown in Table 3. Among the ant species collected, *P. rugaticeps*, *P. decarinata*, *Pheidole* sp., and *P. longicornis* were more organized in their foraging activity by operating in the group and recruiting nest-mate. All the ants were

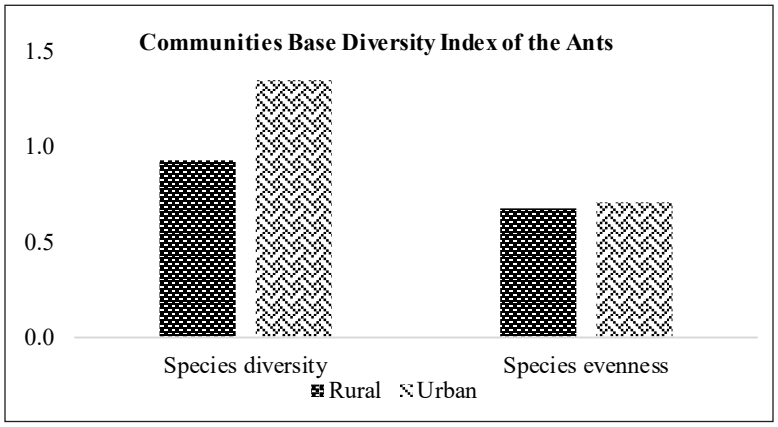

Figure 3. Species diversity and species evenness of the ants in the rural and the urban communities

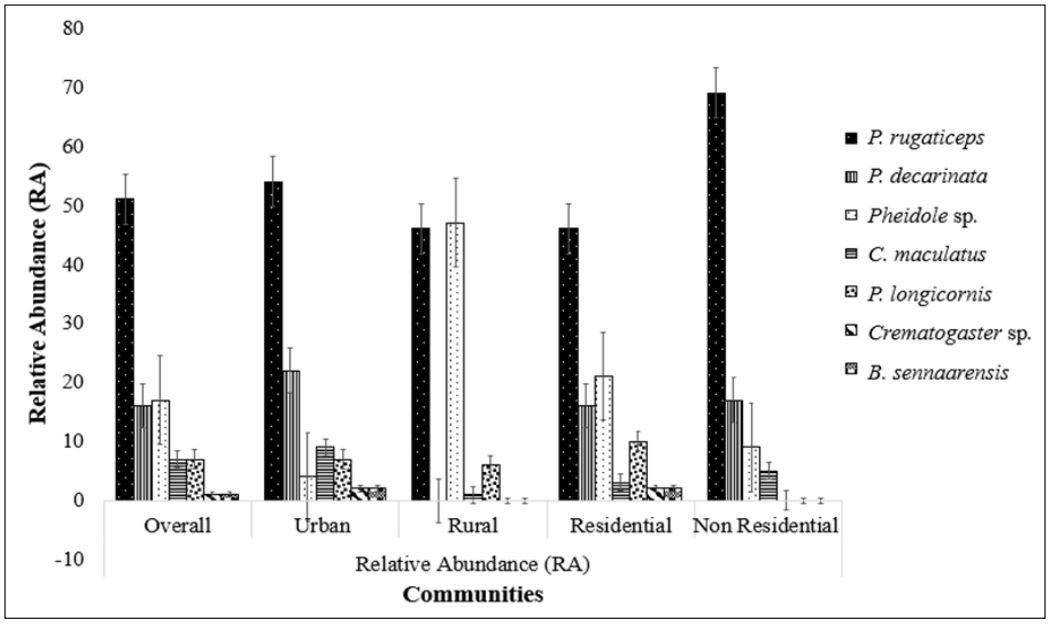

Figure 4. Relative abundance of the scavenging on *Periplaneta americana* according to communities (overall, urban, rural, residential, and non-residential communities)

observed collecting fragments of the dead cockroach after fragmenting it. Furthermore, *P. longicornis* and *Pheidole* species, except for *P. decarinata*, were also observed dragging the whole cockroach into their nest and sometimes, they overwhelm live struggling American cockroaches by spread-eagling (unfolding the legs and the wings of the cockroach) as shown in Figure 2.6. *Pheidole decarinata*, the tiniest of all the collected ants were also observed recruiting nest-mates. However, major workers of *P. decarinata* broke the dead cockroach into fragments and the minor workers carried them back to their nest it to their nest. *Pheidole rugaticeps*, *P. decarinata*, *Pheidole* sp., *P. longicornis*, and *Crematogaster* sp. were all collected indoors mainly near and inside the toilets. Figure 2.3 shows a nest of *P. longicornis* in a void at the edge of Turkish toilet while Figure 2.5 shows an indoors nest of *P. decarinata* in a cavity on the wall. Moreover, Figure 2.8 shows *P. longicornis* dragging a cockroach to their nest. Surprisingly, the study observed that most of the *Pheidole* ants came out of the nest only when the size of the prey was relatively large and proximate to their nest. The majors (soldiers) of this group of ants aid the workers in transporting the prey especially when the prey was relatively bigger. However, when the distance of the prey was far from the nest, only the minors were observed collecting the prey to their nest. *Camponotus maculatus* was also collecting only fragments of the dead cockroach and moved the fragments to their nest. Figure 2.7 shows *C. maculatus* foraging on dead cockroaches.

Table 3
The frequency, density, infestation level (%), and other foraging activities of the of the ant species collected around Periplaneta americana *between November 2018-Febroury 2019*

Ant species	N	No. of *Periplaneta americana* infested	Average no. per cockroach	Infestation (%)	Mode of infestation	Collects	Indoors/ Outdoors
Pheidole rugaticeps	698	26	26.8	54	Dead and alive	Whole/ Fragment	Indoors and Outdoors
Pheidole decarinata	213	7	30.4	15	Dead	Fragment	Indoors and Outdoors
Pheidole sp.	232	4	58	8	Dead and alive	Whole/ Fragment	Outdoors
Camponotus maculatus	92	5	9.2	10	Dead	Fragment	Outdoors
Paratrechina longicornis	91	4	11.4	8	Dead and alive	Whole/ Fragment	Indoors and Outdoors
Crematogaster sp.	20	1	20	2	Dead	Fragment	Outdoors
Brachyponera sennaarensis	18	1	20	2	Dead	Fragment	Outdoors
Total	1,364	48	28.4	100			

DISCUSSION

Insects are by large underrepresented in studies related to biodiversity and conservation despite the importance they play globally in the overall ecosystems functioning, stability, and monitoring (Fox, 2013; McKinney, 1999; Thomas et al., 2004; Wilson, 1976). Apparently, few studies exist on ant populations and trends in most ecosystems, except for few invasive species (Cooling & Hoffmann, 2015; Vogel et al., 2010). This, however, reveals uncertainty in the future of many ants (Sánchez-Bayo & Wyckhuys, 2019). Fast-growing human activities because of the trade and development are major promoters of the establishment of exotic species that can have a tremendous impact on biodiversity around human settlements. Widespread litter and trash accumulations, particularly in cities, are suitable habitats for the establishment of ant species (Sharaf et al., 2017). Dead cockroaches are one of such liters around homes that attract foraging ants into the house. Most importantly, this finding reveals that *Pheidole* species are the dominant ant species collecting dead *P. americana*. Consequently, these ants have previously been reported to have successfully spread their range globally and regularly found in kitchens, restaurants, greenhouses, and gardens. Other ants, such as *P. longicornis* and *Brachyponera* species, collected in this study were also shown to be attracted to human settlement by the accumulation of litter and trash (Sharaf et al., 2017).

Recent research has revealed that the mean infestation trend of *P. americana* in household environments has an increasing range of 50.0–75.0% (Nasirian, 2017). It has also been established that there was a high abundance of American cockroaches in most Nigerian households (Bala & Sule, 2012; Iwuala & Onyeka, 1997). Recently, a study has shown that bathrooms and toilets are the most infested by this cockroach species in houses (Dehghani et al., 2014). The present study compared the infestation of an American cockroach in toilets between the rural and the urban communities as well as residential and non-residential areas and found that the infestation was higher in the rural communities than in the urban communities. This may not be unconnected to the level of hygiene of these two sets of communities as cockroach infestation has been revealed to have a significant correlation with poor sanitation (Nasirian, 2017) and are the commonest indoor pest in low-income housing (Brenner, 1995; Wang et al., 2008). Little or no application of insecticides and poor toilet facilities (particularly use of pit latrine) are other problems associated with these rural communities and low-income housing in the urban communities. Similarly, studies have also revealed that an increasing percentage of Nigerians live in poverty and it is higher in the rural areas.

Nevertheless, the Kruskal-Wallis test showed that the distribution of the ants scavenging on cockroach at all locations did not differ statistically. This finding further supports the idea that most ants are generalist feeders with the ability to thrive them almost everywhere (Cerdá & Dejean, 2011). Nevertheless, it is interesting to note from the richness of the species that more species have been recorded in the urban communities than in the rural communities. This must be due to the adaptation of several species of ants to the urban environment because of its heterogeneity that offers them food, nesting sites and biotic interactions (Benson & Harada, 1988; Hölldobler & Wilson, 1990; Reyes-Lopez et al., 2003). Although these results slightly differ from some published studies (Mckinney, 2002; Ab Majid et al., 2016) that there are fewer species diversities of ants and many taxa in the urban core than in the rural areas, they are consistent with the fact that suburban has a high ant species richness. This is because the urban communities sampled in this study are, to a certain extent, suburban. Study has also shown that widespread litter and trash deposits are suitable habitats for the formation of abundant species, especially in the city centers (Sharaf et al., 2017). Dead arthropods are one of those deposits around human settlements with a high concentration that attracts other insects to the house, especially ants. Dead *P. americana* is one of these deposits, where the level of sanitation is poor, which makes human settlement suitable for many ant species.

The present study determined the diversity and abundance of the ants that forage on American cockroach and 1,364 ants were collected belonging to three subfamilies of the Formicidae: Myrmicinae, Formicinae, and Ponerinae. *Pheidole* species

(Myrmicinae) were the most dominant ants in terms of diversity and abundance. The overall result of the relative abundance showed that *P. rugaticeps* recorded the highest abundance followed by *P. decarinata* and other *Pheidole* species. Generally, *Pheidole* species are revealed to have discriminating capability of both collecting corpses and effectively invading colonies of other competing ants (Dejean et al., 2007). This study also observed that *P. rugaticeps* not only foraged on dead cockroaches, but was also able to spread-eagle struggling *P. americana* shortly after insecticide application and drag the cockroach into their nest. Highly organized foraging activity of teamwork and nest-mate recruitment observed from the *Pheidole* species provide them with the prospect of excluding other competitors in the habitat. This study also observed *P. rugaticeps* catching other live competitors, such as *C. maculatus* and collecting other dead insects. This clearly agrees with the fact that *Pheidole* is the most abundant and diverse formicids within tropical areas (Ward, 2000; Wilson, 2003) with the highest species richness globally (Longino, 2009) and worldwide distribution (Wilson, 1976). Other ant species that were previously observed scavenging on dead cockroaches and corpses of other arthropods, such as *P. longicornis* and *Brachyponera* sp. (Sharaf et al., 2017), were recorded in this study (2% and 8%, respectively). *Paratrechina longicornis* has also been reported as the most widely distributed ant species (Wetterer, 2008) feeding on a host of live and dead arthropods, and transmit pathogenic microorganisms (Roxo et al., 2010). Likewise, a pr*evious study have also* observed *Brachyponera spp*. scavenging on dead cockroaches and other organisms (Rice & Waldvogel, 2017). Our studies have not recorded *B. sennaarensis* and *Crematogaster* spp. in the rural communities, although they are among the most abundant insect species in human settlements, and *B. sennaarensis has* previously been *reported* in Nigeria (Al-khalifa et al., 2015). Whereas, *C. maculatus* recorded 10% of the ants observed around dead *P. americana* in this study. Previous study have also pointed out that media workers of *C. maculatus* and other ants can recruit nestmates and capture preys dead or alive of 4-18 mm in size (Dejean, 1988).

Most ants are generalist feeders, even the predators collect corpses of insects and other arthropods (Cerdá & Dejean, 2011). They are the most successful scavengers that feed on even corpses of invertebrates (Tan & Corlett, 2012). Nest-mates recruitment in large numbers who troop out in mass, helps them in transporting the cockroach to their nest by spread-eagling the live cockroach (Figure 2.6). These mass density foraging activity has been described as a factor for the success of most ground dwelling ants (Beckers et al., 1989). Mass recruitment and collective foraging along well-defined trail system perhaps employed when bait is larger than their size are features exhibited by all the *Pheidole* species and *P. longicornis* collected during this study (Moffett, 1988). Surprisingly, the study also observed that major workers of the *P. rugaticeps* came out of their nest only when the size of

the prey was relatively large. However, *P. decarinata* and *C. maculatus* were found only collecting fragments of dead cockroach and this has previously been reported by Yamamoto et al. (2009). Moreover, the majors of *P. decarinata* only fragment the corpses of the cockroach while the minors transport the fragments to their nest. This ability is a possibility that these ant species may be a serious source of ecological and conservation concern for other native urban ecosystem taxa (Kouakou et al., 2018). The aggressive behavior of the *Pheidole* species can also be used to control them (Lim & Ab Majid, 2019).

Though not much has been reported of ant's medical and veterinary importance like flies or cockroaches, it has been confirmed that *Vibrio cholerae* could be obtained from ants and cockroaches (Sarwar, 2015). In the tropics, ants have already been incriminated as vectors of pathogens (Sarwar, 2015). Gathering and storing corpses and feces by ants have been disclosed to have huge effects on other lives and chemical components around them (Dauber et al., 2001; Dauber & Wolters, 2000; Lavelle et al., 1997; Petal, 1998). Studies have revealed that bacteria can remain viable and persist within the nest of ant and become a reservoir for other workers of the colony to be infected (Beatson, 1972). Interestingly, most of the ant species recorded in this study nested inside toilets (see Figure 2.3) or a few meters away from the toilets. The foraging pattern and activities, as well as nest sites of these ants, can have health implications on the host community, especially *Pheidole* species, *C. maculatus*, and *P. longicornis* that were frequently collected in the aforementioned places. Their foraging activity on *P. americana*, an ideal carrier of several pathogenic microorganisms due to the filthy nature of its breeding habits and feeding mechanism (Chaichanawongsaroj et al., 2004; Graczyk et al., 2005) can be a source of great concern. *Periplaneta americana* harbored more species of pathogens than other cockroach species (Pai et al., 2003; Prado et al., 2002) and the foraging activity of ants on them can be an unnoticed medium of dissemination of diseases causing pathogens in human societies, particularly during an outbreak of diseases such as cholera.

CONCLUSIONS

Pheidole rugaticeps, *P. decarinata*, *Pheidole* sp., *C. maculatus*, *P. longicornis*, *Crematogaster* sp., and *B. sennaarensis* were the ant species that foraged on *P. americana* around human habitat and these scavenging ants had higher species richness in the urban than the rural communities. *Pheidole* species were the most abundant group of ants. The organized foraging patterns such as nest-mate recruitment, teamwork, and raiding in mass exhibited by *Pheidole* species must be the reason for their success. The rural communities had a higher abundance of *P. americana* than the urban communities because of poor toilet facilities and household's inability to afford insecticides, which are serious problems in most tropical rural households. Studies

on the epidemiological and conservation implications of the *Pheidole* species in the urban communities are recommended. The foraging patterns of the *P. rugaticeps* may also be an important area of exploration for biological control of insect pests.

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Species Richness of Leaf Roller and Stem Borers (Lepidoptera) Associated with Different Paddy Growth and First Documentation of Its DNA Barcode

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ABSTRACT

Leaf folder and stem borer are pest moths (Lepidoptera) of paddy crop and caused serious damage and significant rice yield loss. The richness, abundance, and diversity of the pest moths were calculated in one paddy planting season and sampled from a model conventional paddy field, located on the west coast of Peninsular Malaysia (Sabak Bernam, Selangor). The adult and immature stages of moths associated with paddy plants have been sampled using active sampling namely sweep net and stem cross-cutting. A total of 189 individuals belonging to five species under two families (Crambidae and Noctuidae) were recorded. Overall, the richness (R'), diversity (H'), and evenness (E') index of lepidopteran species were 0.76, 1.51, and 0.90, respectively. The richness and species abundance throughout the paddy stages were discussed. The DNA barcode of five collected species using cytochrome oxidase subunit 1 (*CO1*) viz. *Cnaphalocrocis medinalis* (Guenée) (leaf folder), *Scirpophaga incertulas* (Walker), *Chilo auricilius* Dudgeon, *Sesamia inferens* (Walker), and *Parapoynx stagnalis* (Zeller) (stem borers) were presented. This study's outcomes are very important as the initial

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stage for conservation purposes, especially in managing the strategy in handling the pest species populations in the paddy field.

Keywords: Agricultural ecosystem, *CO1*, genetic, grain insect pests, infestation, Lepidoptera, Malaysia

INTRODUCTION

Lepidoptera is the second-largest insect group comprising moths and butterflies. It plays a pivotal role as a food source for birds and small mammals, apart from being potential dispersal agents (Lee at al., 2002), and nutrient recyclers (Mercks et al., 2013). Lepidopteran is also very diverse in many agricultural ecosystems such as in paddy fields, oil palm plantations, orchards, and secondary forest ecosystems. This group of species mostly acts as pests during their larval stages such as the bagworm species, *Metisa plana* (Halim et al., 2017), fruit bunch moth, *Tirathaba* sp. (Yaakop & Abdul Manaf, 2015), and other lepidopteran pests (Ghazali et al., 2015).

Paddy fields are considered temporary aquatic and terrestrial habitats for these species. The paddy ecosystem or area is flooded with water throughout the planting season and is dried after the harvesting season. Thus conditions become an ideal environment for lepidopteran species (Bahaar & Bhat, 2011). Paddy grains are one of the most economically important products because it is a food source for human beings (Sie et al., 2008), but the grains have also been recorded to be infested by lepidopteran species (Syarifah Zulaikha et al., 2018).

DNA barcoding is a novel approach used to precisely identify any species using the short and standard gene region, the cytochrome oxidase subunit 1 (*CO1*). This approach enables taxonomists to precisely identify species and helps stakeholders to combat pests and the invasive species so as to ensure food safety and security (Hebert & Gregory, 2005). The latest findings reveal that identification using DNA barcoding helps to resolve problems related to species diversity (Hebert et al., 2004a, 2004b). This approach is found to be the most effective technique to support and reconfirm morphological identification (Halim et al., 2018; Nor Atikah et al., 2019). Moth species are very difficult and complicated to accurately identify because of damaged scale structures in the adult stage, as well as its very dull colouration. As leaf rollers and stem borers become pests during the larval stage, this approach enables species identification during their immature stages.

Few researchers work on moths (Scoble, 1992), hence many moths are still not yet identified. In addition, distributional and host studies are also lacking (Janzen, 1988). Several studies in Malaysia and other Southeast Asian countries have been indirectly conducted on leaf folder and stem borers in terms of diversity and abundance and pesticide applications, i.e. by Bhatnagar (2004), Faleiro et al. (2006), and Ooi et al. (2015). However, information is scarce and limited; none of the studies focussed

on molecular work such as DNA barcoding analysis for Malaysian species. Hence, the abundance and richness of moth pests are still unclear, making conservation efforts more difficult. This study is thus carried out to provide information on paddy-associated moth species and to measure the abundance and richness of paddy field moths, as well as to provide the DNA barcode of obtained moth species.

METHODS

Sampling Locations

This study was conducted at a conventionally cultivated paddy field in Parit 4 Timur, Sungai Panjang, Sabak Bernam, Selangor, Malaysia (3°42' 763" N 101°46'317"E), as a model sampling site. The plot size was approximately 10250 hectares (102.5 m x 100 m).

Insect Sampling

The sampling area was divided into four plots and then each plot was divided into four subplots. Two-line horizontal transects of 50 m each were selected randomly in the subplot and the adult stage sampled by using the sweep net, then larval stage specimens of leaf rollers were handpicked from the subplots. Sampling was carried out from November 2017 till March 2018 (1 season) according to paddy growth stages; vegetative, reproductive and mature stages at three time-zone replicates i.e. morning (1000-1100 hr), afternoon (1200-1300 hr), and evening (1400-1500hr) for the netting methodology. A total of 20 stems/ plants were also collected from a total of five paddy plants that were selected randomly from each subplot. Paddy stems were later cut longitudinally to examine the presence of larvae and pupae.

Laboratory Work

Field specimens were brought back to the Entomology Laboratory for the sorting and identification process. The adult and larval stages were preserved in 100% alcohol for molecular work.

Species Identification

The identification of species was based on external morphological characters (adult only) and conducted up to species level using a stereo microscope Zeiss Stemi DV5 by referring to a species key by Hampson (1896) and Khan et al. (1988, 1991). Seven (7) individual representatives (different morphology) were labelled with RC, LFC, LFM, LFG, KC, KK, and KH then identified using the molecular approach or DNA barcoding (Hebert et al., 2003). The DNA barcoding procedure involves DNA extraction, polymerase chain reaction (PCR), DNA purification, DNA sequencing, and DNA sequence analysis.

DNA Extraction and Polymerase Chain Reaction (PCR)

The DNA of the moth specimens was extracted using appropriate protocols by QIAGEN DNeasy® Blood and Tissue Kit (Germany). Extraction procedures were conducted using the manual provided by the

manufacturer. An Eppendorf machine was used to perform polymerase chain reaction (PCR) while cytochrome oxidase subunit 1 (*CO1*) was amplified using a set of primers, forward: LCO1490 5' GGT CAA CAA ATC ATA AAG ATA TTG G 3' reverse: HCO22198 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3' (Hebert et al., 2003). The PCR condition is as follows; initial denaturation at 95°C for 3 min followed by denaturation at 95°C for 30 s, annealing at 52.2°C for 1 min and extension at 72°C for 30 s, and the final extension at 72°C for 10 min in a total of 30 cycles. For PCR reagents, 25 µL consisted of 12.5 µL of 1× GoTaq® Green Master Mix, 7.5 µL of ddH2O, 1-3 µL of 10 ng DNA template, 1.0 µL of 200 nm forward and reverse primers. The PCR product was later purified using the GF-1 PCR Clean-Up kit (Vivantis) to remove dNTP, primers, and buffer. The end product was then used to perform electrophoresis gel at 90V and 30 min using agarose gel 1.5%.

Sequencing Analysis, Editing, and Alignment of DNA

The purified PCR product was sent to First Base Sdn. Bhd. Shah Alam, Selangor, Malaysia for sequencing analysis to determine the variation and identity of the base sequence. DNA sequence editing was done using Sequencher software. The alignment was done on both forward and reverse alignments to ensure the validity of the base.

Alignment, Basic Local Alignment Search Tool Analysis (BLAST) and Phylogenetic Analysis

The online software, Basic Local Alignment Search Tool (BLAST) was used to ensure that the amplified sequence is the sequence of choice. BLAST showed a maximum hit for the respective species only, as available in GenBank (http://www.ncbi.nlm.nih.gov/genbank). PAUP* 4.0 was implemented to construct the neighbour joining (NJ) tree using Kimura's two parameter algorithm model with bootstrap analysis (1,000 replications).

Data Analysis

Ecological indexes such as the Shannon diversity index (H'), evenness index (E'), and Margalef's richness index (R') were evaluated using paleontological statistical software (PAST) version 2.17c.

RESULTS

Overall Composition of Lepidoptera

A total of 189 individuals belonging to five species under two families were collected from Sabak Bernam, Selangor. Species *Chilo auricilius* Dudgeon were recorded with the highest abundance with 33% (62 individuals) (Figure 3b), followed by 29% of *Parapoynx stagnalis* (Zeller) (54 individuals) (Figure 3a), 15% (29 individuals) of *Scirpophaga incertulas* (Walker) (Figure 3e), 14% (27 individuals) *Cnaphaphalocrocris medinalis*

(Guenée) (Figure 3c), and the lowest abundance *Sesamia inferens* (Walker) with 9% abundance (17 individuals) (Figure 3d). All species were stem borers except for *Cnaphalocrocis medinalis* which is a leaf roller. The stem borers belonged to family Crambidae with one species (*Sesamia inferens*) from Noctuidae.

Richness and Diversity of Moths

Shannon index (H'), evenness index (E'), and Margalef's richness index (R') counted for moths obtained in Sabak Bernam, Selangor were 1.51, 0.90, and 0.76, respectively. Pielou (1975) stated that the Shannon index was at the lowest if its range is between 0.0-2.5. Moth diversity in the paddy field is therefore low as H'= 1.518, in addition to evenness and richness at 0.90 and 0.76, respectively.

The Abundance of Lepidoptera Species at Different Paddy Growth Stages

Lepidoptera sampling in the Sabak Bernam, Selangor paddy field was carried out at three different stages i.e. vegetative, reproductive, and mature stages. Based on Figure 1, 5 moth species were recorded in all paddy growth stages with 189 individuals and the mature stages had the highest number of moths with 84 individuals, followed by 71 and 34 for the reproductive and vegetative stages, respectively. At the vegetative stage, *Parapoynx stagnalis* (Walker) had the highest number with 32 individuals followed by *Chilo auricilius* and *Cnaphalocrocis medinalis* with one individual each. At the vegetative stage, there were no records of *Sesamia inferens*. At the reproductive stages, four moth species were recorded i.e. *Chilo auricilius* (46 individuals), followed by

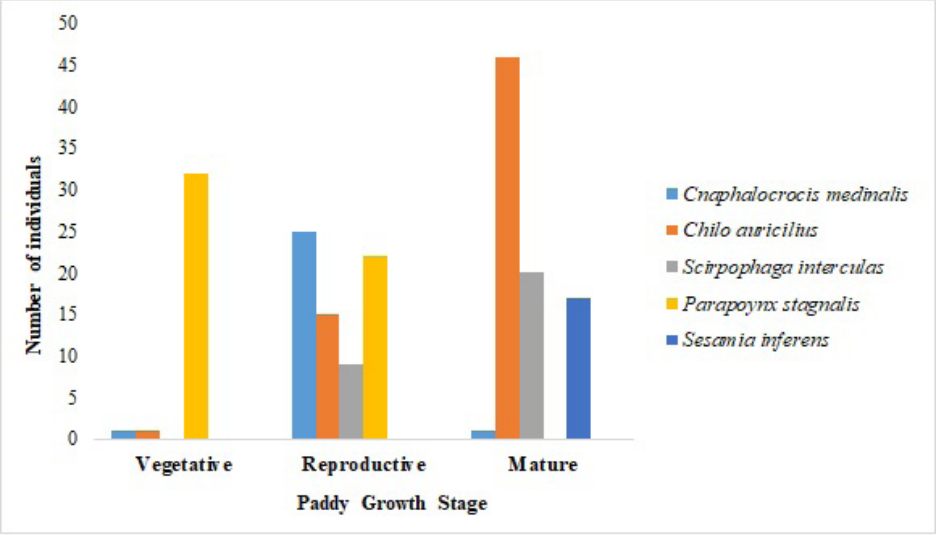

Figure 1. Lepidoptera species richness at different stages of paddy growth

Scirpophaga incertulas (20 individuals), *Sesamia inferens* (17 individuals), and *Cnaphalocrocis medinalis* (1 individual).

DNA Barcode and Genetic Distance

A total of five species were sequenced and matched with online BLAST to detect the percentage of similarities with available data in the GenBank. Samples of LFM and LFG were referred to a different species, *Cnaphalocrocis medinalis* morphologically but molecular work determined that they were the same species. All the other species showed a high percentage of similarity with the GenBank data (between 97-99%) (Table 1) and all species were located at specific lineages, showing that they belonged to different species (Figure 2). The genetic distance analysis (also presented in Table 2) shows the distance between genera between 0.112-0.166 (Table 2).

Table 1

Percentage similarity of the sequences deposited in the GenBank using BLAST analysis

Sample code	Species	Percentage of similarity (%) (compared to the GenBank data)	Accession no. (sequences submitted to GenBank)
LFM	Cnaphalocrocis medinalis	99 (*Cnaphalocrocis medinalis)*	MT357089
LFC	Chilo auricilius	98 (*Chilo auricilius*)	MT357091
LFG	Cnaphalocrocis medinalis	99 (*Cnaphalocrocis medinalis)*	MT357090
KH	Chilo auricilius	98 (*Chilo auricilius)*	MT357092
KK	Scirpophaga incertulas	98 (*Scirpophaga incertulas*)	MT357093
KC	Sesamia inferens	99 (*Sesamia inferens*)	MT357094
RC	*Parapoynx stagnalis* (= *Nymphula depunctalis*	94 (*Parapoynx stagnalis*)	MT357095

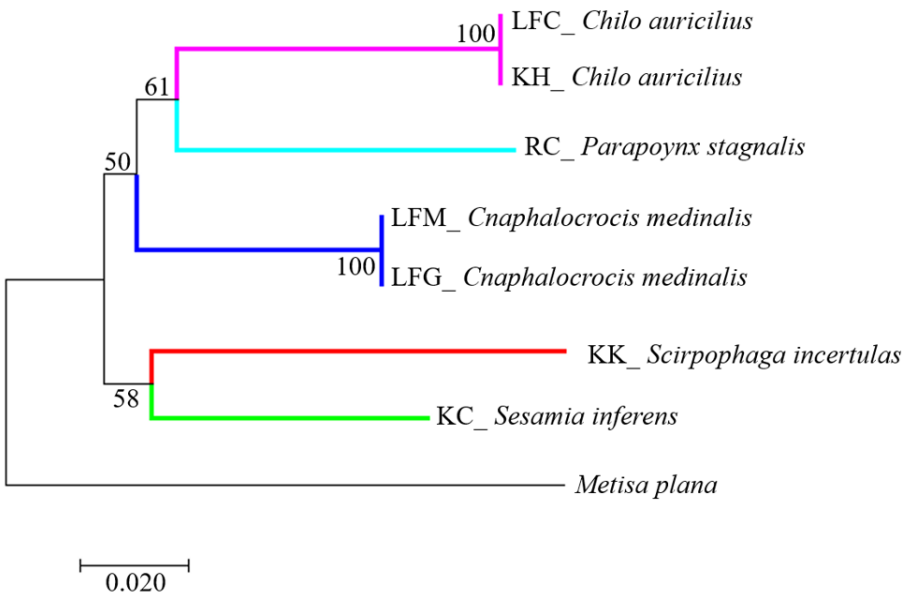

Figure 2. Neighbour joining tree resulting from the *CO1* sequences on five species

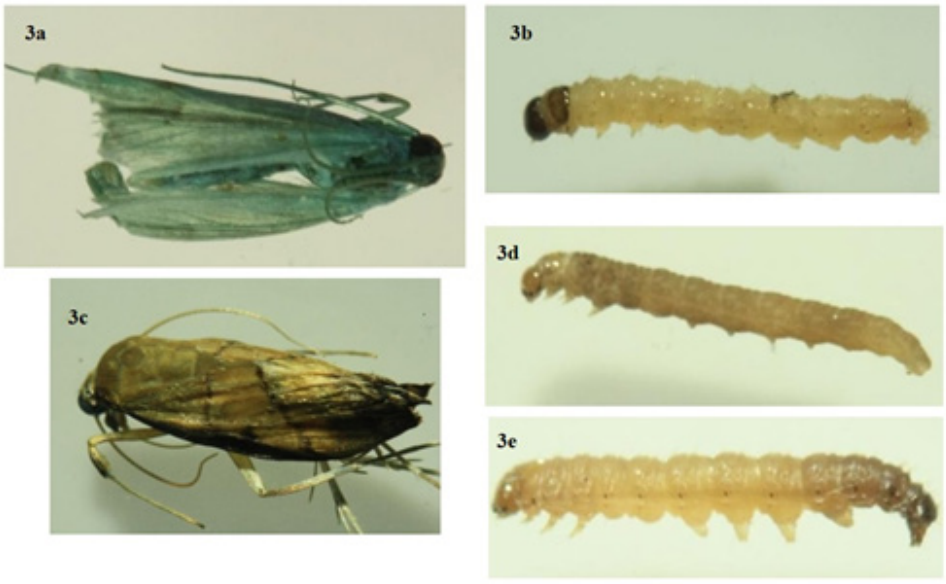

Figure 3. Photos of larval and adult stages of lepidopteran species: (a) *Parapoynx stagnalis*; (b) *Chilo auricilius*; (c) *Cnaphalocrocis medinalis*; (d) *Sesamia inferens*; (e) *Scirpophaga incertulas*

Table 2

Genetic distance between species implemented in the phylogenetic analysis

	[1]	[2]	[3]	[4]	[5]	[6]
[1] *Cnaphalocrocis medinalis*						
[2] *Chilo auricilius*	0.112					
[3] *Scirpophaga incertulas*	0.142	0.164				
[4] *Sesamia inferens*	0.116	0.120	0.135			
[5] *Parapoynx stagnalis*	0.121	0.119	0.166	0.131		
[6] *Metisa plana*_Outgroup	0.914	0.909	0.942	0.925	0.909	

DISCUSSION

This is an extensive study on pest moth species in a conventional paddy ecosystem. To date, no study has been specifically conducted on pest moth species in Malaysia and her neighbouring countries. Studies by Babendreier et al. (2020), Norela et al. (2013), Ooi (2015), Razali et al. (2015), and Siregar et al. (2017), were conducted with myriad aims and different parameters. In addition, stem borer and leaf folder species are difficult to study due to their behaviour of living inside the stem and leaf roll during their immature and pest stages (Razali et al., 2015). The model sampling site in Sabak Bernam was thus chosen to understand the richness and abundance of pest moth species in conventional rice ecosystems.

According to Norela et al. (2013), the System of Rice Intensification (SRI) supports a high diversity of insects especially on Lepidoptera, rather than conventional planting. In this study, five species were successfully collected from the conventional paddy ecosystem, consisting of two families (189 individuals) of pest moths. However, in Norela et al. (2013), there were only eight species (140 individuals) of pests and non-pests of Lepidoptera. Bahaar and Bhat (2011) stated that the paddy field was an example of an appropriate ecosystem for insect species, especially Lepidoptera due to adaptation to the disturbed habitat. According to Norela et al. (2013), the pest species collected in that study are not sampled from the SRI ecosystem. It is possible that natural enemies played a part in pest control as nitrogenous fertilizers had not been applied extensively to SRI-cultivated paddy. A paddy field is highly influenced by constant physical, chemical, and biological changes, therefore organisms that inhabit paddy fields are regarded as opportunists that are able to undergo extreme physiological and behavioural adaptations

A study by Praveen (2017) also recorded moth diversity in paddy fields in Palakkad, India, reporting results that were more or less similar to the Shannon diversity index, in terms of the number of family and species with 0.995, 0.90; 4, 2; 9, 5 respectively. In

their study, specimens from the families Noctuidae, Pyralidae, Saturniidae, and Sphingidae were collected, of which *Cnaphalacrocis medinalis* and *Spodoptera mauritia* were common species. However, in this study, only two families (Noctuidae and Crambidae) as well as *Chilo auricilius* were found to be the most abundant species. All species, both leaf folder and stem borers are widely recorded in Malaysia (Cuong & Cohen, 2002).

Referring to Nasiruddin and Roy (2012), *Cnaphalocrocis medinalis* and *Chilo auricilius* showed the highest infestation rate at the reproductive and mature stages, while *Scirpophaga incertulas* existed in almost all growth stages of paddy, but were not collected during the vegetative stage. Their findings were similar the results of this study. At reproductive stage, *Cnaphalocrocis medinalis* showed the most abundance, with 25 individuals. According to De Kraker et al. (1999), this species of leaf roller starts to appear in the fourth week of plant shift without consistent population density. The number of eggs is highest at the late vegetative stage which is the seventh week after plant shift while the number of larvae is highest one to two weeks after the late vegetative stage. Leaf damage caused by leaf roller larvae can be seen in the fourth week of plant shift and damage on the leaves is highest during the reproductive stage then decreases during the mature stages. Destruction to paddy plants occurs because larvae fold leaves longitudinally using silky thread and eventually feed on them, resulting in white, dry leaves (Goco, 1921).

Kakde and Patel (2014) showed that the percentage of infestation of *Scirpophaga incertulas* was 5.79% and 4.93% at the mature stages in conventional and in SRI paddy fields, respectively. The level of infestation by stem borers was lower in SRI-cultivated paddy fields compared to that in conventional-cultivated paddy fields. However, in this study, the infestation of *S. interculas* was high during the maturation and vegetative stages; almost 23% and 11%. In addition, *Parapoynx stagnalis*, a winged moth, was the most dominant species at the vegetative stage. It caused maximum damage during this stage and a high level of destruction was reported during the first 4 weeks of plant shift (Pulin et al., 1998). Ramasubbaiah et al. (1978) also recorded 35-40 days as the optimum duration needed by *P. stagnalis* to cause maximum damages. Litsinger et al. (1994) documented 2-6 weeks as the duration in which maximum infestation by species larvae could occur for their survival. This thus proves that the winged moth has a high adaptability rate at the vegetative stage because of a high oviposition rate, fast larvae development, high survival rate, as well as mature and bigger larvae size.

The morphological identification of insect species is first based on adult specimens. However, a lack of taxonomic keys and availability for a particular life stage and sex causes difficulties in the identification process (Ball & Armstrong, 2006). Identification using morphology is time-consuming and tedious especially for the adult stags, but almost impossible at

the larval stage. Therefore, DNA barcoding is an effective approach to overcome time constraints and to avoid misidentification at the larval stage. Molecular identification has also been popularly applied for precise and fast identification especially in the agricultural field (Ghazali et al., 2014a, 2014b, 2015).

All sequences were blasted and were highly supported with 98-99% similarity with the data available in the GenBank. The species *Parapoynx stagnalis* was identified based on its sequenced data; however, the species presented as *P. stagnalis*, was found to be taxonomically synonymous with *Nymphula depunctalis*. The phylogenetic analysis has also proven that all five species (seven individuals) were clearly separated and supported as a single species located at specific lineages on the tree. This approach is the most popular in published barcoding papers, especially for insect species (Halim et al., 2017; Nor Atikah et al., 2019; Razali et al. 2015).

CONCLUSION

A total of 189 individuals of moth pests belonging to two families and five species were collected and barcoded in this study, namely *Cnaphalocrocis medinalis* (Guenée) (leaf folders), *Scirpophaga incertulas* (Walker), *Chilo auricilius* Dudgeon, *Sesamia inferens* (Walker), and *Parapoynx stagnalis* (Zeller). The identification was confirmed using *CO1* sequencing data with the application of BLAST analysis and the sequences were successfully deposited in the GenBank.

Even though the richness, abundance and diversity of species were quite high compared to earlier studies, they were previously measured with different parameters and aims, and are thus significantly incomparable. No final conclusion can hence be made as to whether the conventional paddy ecosystem in Sabak Bernam, Selangor has an unusually high record of pest insect species. However, it does denote that an appropriate method of pest control should be implemented.

More studies should therefore be carried out in the near future to ensure updated and credible data on richness and abundance of Lepidoptera in cultivated and conventional paddy ecosystems based on different growth stages, longer sampling durations (several seasons), and with similar sampling methods. The outcomes from this study are very important for the initial stages of conservation, especially for paddy field pest management strategies.

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The Effect of Nanozeolite Concentration in a Delivery System of *Ha*NPV$_1$ to the Lethal Time against *Crocidolomia pavonana*

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ABSTRACT

The constraints on the effectiveness of nuclear polyhedrosis virus (NPV) as biocontrol are usually due to environmental factors such as temperature and ultraviolet (UV) exposure. Zeolite has been commonly used as a carrier or delivery system for nuclear polyhedrosis viruses. In this study, zeolite powder was reduced into nanosized particles by beads milling method and was investigated for the effect of its concentration in the delivery system of *Helicoverpa armigera* nuclear polyhedrosis virus (*Ha*NPV$_1$) on the lethal time against the larvae *Crocidolomia pavonana*. The formulation used three concentrations of nanozeolite suspension, 0.5, 1, 1.5, and 2 wt.% applied for each 4×10^7 of *Ha*NPV$_1$. A randomized block design (RBD) method was applied with 3 replications. The results showed that the scanning electron microscope (SEM) from nanozeolite was seen coating the entire surface of the *Ha*NPV$_1$ polyhedra and an increase of zeolite concentration caused acceleration of the lethal time of *C. pavonana* instar III. Thus, the fastest lethal time was 1.2 days receiving a concentration of 2 wt.%, which was significantly higher compared to without delivery (2.9 days). The increase of the zeolite concentration up to 2 wt.% in the delivery system for *Ha*NPV$_1$ improved their performance on lethal time

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and mortality against *C. pavonana*. It was concluded that nanozeolite as a delivery system enhanced and created a synergy in infecting *C. pavonana*.

Keywords: *Crocidolomia pavonana*, *Ha*NPV$_1$, nanoparticle, nanozeolite, pest control

INTRODUCTION

Crop caterpillars (*Crocidolomia pavonana*) is one of the highly harmful pests to cabbage (*Brassica oleracea* var. Capitata L). *Crocidolomia pavonana* frequently attacks cabbage plants at the early stage of crop growth, leaves holes, and may attack primordial tissues causing plants to stop growing. If there is no effort to control the pest, particularly in the dry season, it can cause harvesting failure (Yuliadhi et al., 2016). Therefore, it is important to apply integrated pest control (IPC) to deal with this insect. However, farmers usually use synthetic insecticides to control insects, which is harmful to the environment (Razak et al., 2014). Thus, it is important to have an alternative biocontrol to avoid the use of synthetic insecticides, i.e. microbial agents such as bacteria, fungi, and viruses.

One of the potential viruses usually used as a biocontrol is baculovirus which belongs to a family of entomopathogenic. This virus attacks arthropods, especially insects from the Lepidoptera order. Baculovirus is used as a microbial agent because it is safe, easily mass-produced, highly pathogenic to insects, and easily formulated and applied. One of the developed baculoviruses is nuclear polyhedrosis viruses (NPV), which is packaged in a protein matrix called "polyhedra" (Ompusungu et al., 2015). One strain of NPV is *Ha*NPV which is isolated from larvae *Helicoverpa armigera*. This virus is known to have infected the Lepidoptera order when polyhedra ingested by target pest insects (Govindaraju et al., 2011). Furthermore, to enhance the number of produced virus, the *Ha*NPV was sub-cultured and isolated in an alternate host of *Spodoptera litura,* named as *Ha*NPV$_1$ (Miranti et al., 2015).

Despite the capability of this virus to infect the targeted pest insect, the environmental factors influence the viability of this virus in the field application. To maintain the effectiveness and viability of this virus, zeolites were used as a carrier or delivery system to protect the virus from environmental constraint (Melanie et al., 2017). It was reported that the use of zeolite as a drug carrier enhanced solubility and effectively modulates drug (Karavasili et al., 2017). The zeolites were also reported applied in controlling the insect pest *Chironomus riparius*. It was found that zeolite concentration determined the effectiveness of their control (Lorenz et al., 2017). In addition, it is also expected that the delivery system (zeolite) is in synergy to support the virus-infected insect target. Some researchers tried to enhance the delivery system by reducing the size of the powder aims to obtain higher toxic effects due to the greater surface area (Wibowo & Putra, 2013). Therefore, this study

aimed to investigate the effectiveness of nanozeolite as a delivery system of *Ha*NPV$_1$ and investigated the effect of nanozeolite concentration on the lethal time against *C. pavonana*.

MATERIALS AND METHOD

Preparation *Ha*NPV$_1$ Suspension

The *Ha*NPV$_1$ was produced by infecting the *Ha*NPV$_1$ in *Spodoptera litura* as an alternate host. The virus was isolated after only 1 passage in *S. litura* larvae. *Spodoptera litura* third instar larval was infected by 4×10^5 OBs/mL of virus suspension. The infected larval was collected in a glass container and stored at 4°C. Then, the cadavers (40 larval) were crushed by mortar and mixed with 20 mL Tris buffer (1 mM, pH 7.6) solution and 20 mL 0.1% sodium dodecyl sulfate (SDS) solution. This mixture was stored at 4°C for 24 h (Miranti et al., 2015).

After storage, the mixture of the virus was filtered with two layers of the filter. Filtering using 2 layers of cotton cloth. The suspension of the virus was centrifuged at relative centrifuge force (RCF) 1,931 x *g* for 15 min at 4°C. The supernatant was suspended in 5 mL Tris buffer (1 mM, pH 7.6) solution and 5 mL 0.1% SDS solution and subsequently centrifuged at RCF 1, 931 x *g* for 15 min at 4°C. The centrifugation was conducted just for separating viruses from other debris. The last supernatant was suspended with mixed Tris buffer (1 mM, pH 7.6) solution and 0.1% SDS solution by adding 0.2% sodium azide to prevent the virus suspension from contaminant (Miranti et al., 2015).

To count the OBs numbers of a virus, 0.1 mL virus suspension was mixed by adding 0.9 mL of Tris buffer (1 mM, pH 7.6) and 0.1% SDS with a 1: 1 ratio. The suspension of the virus with concentration 4×10^7 OBs/mL in the liquid medium was used for bioassay.

Zeolite Beads Milling

Firstly, received zeolite was ball milled into -400 mesh. One hundred and fifty grams (150 g) of zeolite suspended into 2 liters of water and mixed using a stirrer at a speed of 2,000 rpm for 2 h. The suspension was then milled with a bead milling method for 3 h. This was a wet bead milling process utilized zirconia with 30 μm in size and detailed explain elsewhere (Joni et al., 2010; Rochima et al., 2018). The size and size distribution of the zeolite particles were performed using particle size analysis (PSA, HORIBA Scientifica SZ-100, HORIBA, Ltd. Japan) and their morphology observed with a scanning electron microscope (SEM, HITACHI SU3500. HITACHI High-tech GLOBAL, Japan).

*Ha*NPV$_1$ and Nanozeolite Formulation

The *Ha*NPV$_1$ formulation with nanozeolite carrier was obtained by mixing 1 mL of *Ha*NPV$_1$ suspension with a concentration of 4×10^9 OBs/mL with 99 mL of nanozeolite suspension at various concentration according to the treatment (i.e. 0.5, 1, 1.5, and 2 wt.%). This formula was used for bioassay testing against *C. pavonana*.

Bioassay Test

The vegetable as a host plant of *C. pavonana* was obtained from the Vegetable Research Institute (BALITSA) Lembang, Bandung, West Java, Indonesia. Test insects of *C. pavonana* were used at instar III. The observation of lethal time was conducted at various treatments including their control using only cabbage and in comparison, to the only *Ha*NPV$_1$. Thus, there were 8 levels of various treatment as follows:

C0 : Control (only cabbage)
C1 : *Ha*NPV$_1$
C2 : 0.5 wt.% Nanozeolite
C3 : 1 wt.% Nanozeolite
C4 : 1.5 wt.% Nanozeolite
C5 : 2 wt.% Nanozeolite
C6 : *Ha*NPV$_1$ + 0.5 wt.% Nanozeolite
C7 : *Ha*NPV$_1$ + 1 wt.% Nanozeolite
C8 : *Ha*NPV$_1$ + 1.5 wt.% Nanozeolite
C9 : *Ha*NPV$_1$ + 2 wt.% Nanozeolite

The *C. pavonana* larvae were placed 10 individual larvae in a plastic container for each treatment and subjected to acclimatization to ensure the health of larvae. Acclimation by way of the *C. pavonana* larvae was placed in condition without feed for 3 h before the applications of the formulation. The application means that the cabbage coated with the formulated biocontrol and was infected by means of oral ingestion. The application of formulations to larvae was carried out for 7 days of observation.

Data Analysis

This research is a biological test using descriptive exploratory methods in the laboratory. The research design used was a single factor randomized complete block design (RCBD). Each treatment was repeated three times based on the results of calculations using Federer's formula, namely (t-1) (n-1) ≥ 15. In this study, 30 experimental plots were obtained. The obtained data were analyzed using analysis of variance (ANOVA) with their significance were determined by Duncan's multiple distance test (DMRT) at a level of 5%. The average of lethal time was calculated using the formula in Equation (1) for 7 days observation (Tamimi et al., 2016).

$$W = \frac{\sum W_i \bullet Z_i}{Y} \quad (1)$$

where,

W = Average lethal time

W_i = Lethal time of test insects on the day i of infection

Z_i = Number of dead insects on the first day of infection

Y = Number of test insect death

RESULTS AND DISCUSSION

Particle Size Analysis (PSA)

Figure 1 shows the size distribution of zeolite before and after beads milling. The results showed that the initial size distribution of the zeolite particles with an average size of 499 nm with a high polydispersity index (1.626) means that zeolites were partially agglomerated. After beads milling, the

average size of zeolite particles was 175 nm with a very low polydispersity index (0.776) means that particles were relatively homogenous in size. The obtained zeolite suspension with homogenous in size ensures their affectivity on coating the virus.

Figure 2 shows the SEM images of zeolite before and after beads milling with magnification 20, 000. The result indicated that the morphology of zeolite was changed after beads milling. Before beads milling, the morphology of the zeolite particles was flaky and agglomerated as highlighted in a red circle as shown in Figure 2a. In contrast, the morphology of zeolite particles after beads milling was changed into smaller sized as highlighted in a red circle as shown in Figure 2b. Figure 2c shows the *Ha*NPV$_1$ with spherical in morphology highlighted in a dotted red circle. This is consistent with research conducted by Sudhakar and Mathavan (1999), in which the PIB *Ha*NPV$_1$ was spherical in morphology and some of them were irregular in shape. The nanozeolite has encapsulated the surface of the *Ha*NPV$_1$ as indicated in Figure 2d, which is highlighted with a dotted red circle. Nanozeolite can be used as a carrier material of *Ha*NPV$_1$ with visible nanozeolite covering the entire surface of the *Ha*NPV$_1$ polyhedra.

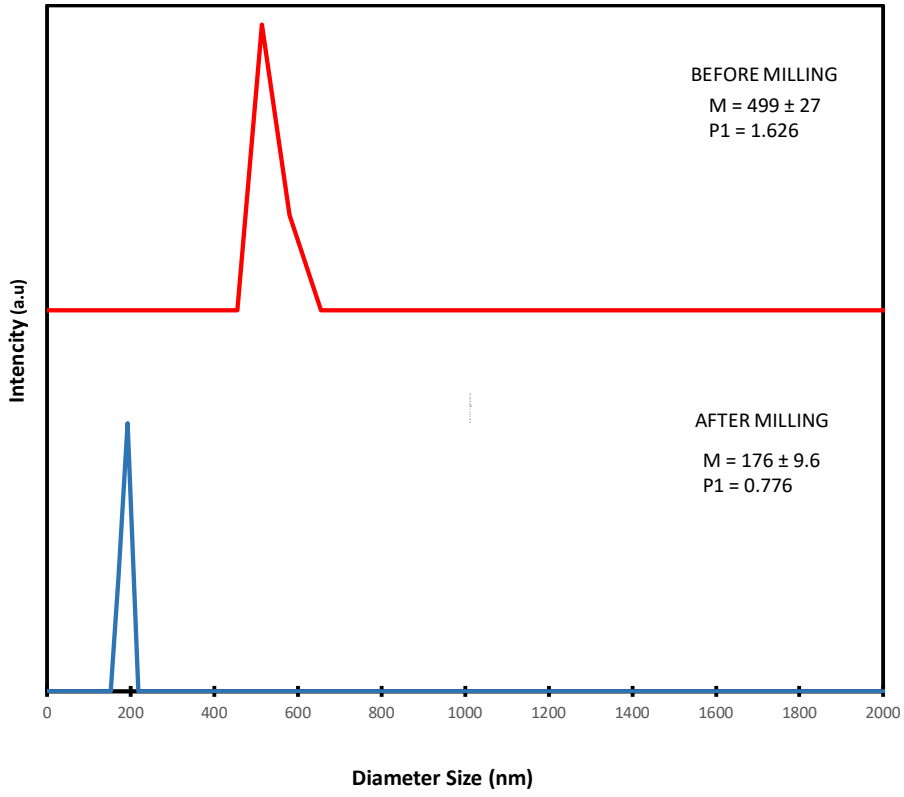

Figure 1. The size distribution of zeolite before and after beads milling

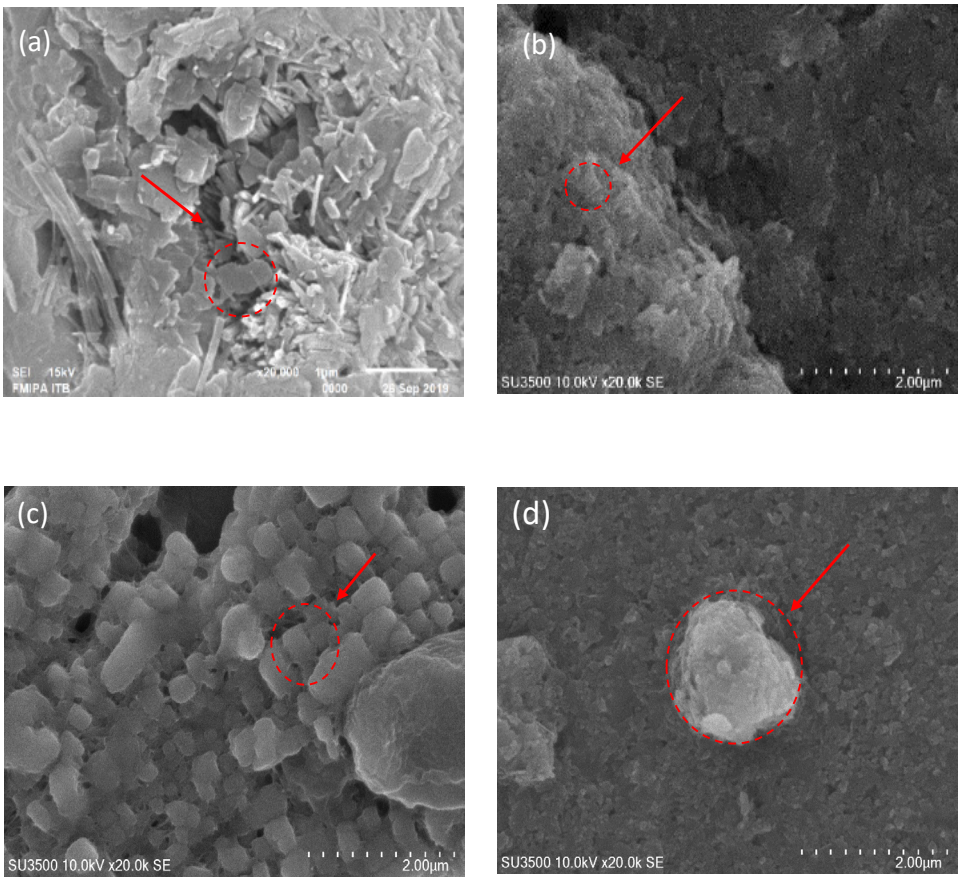

Figure 2. The SEM images of zeolite with magnification 20, 000 (a) before beads milling, (b) after beads milling, (c) *Ha*NPV$_1$, and (d) *Ha*NPV$_1$ with nanozeolite

Effect of Nanozeolite Concentration on Lethal Time and Mortality against *Crocidolomia pavonana*

The lethal time of the *C. pavonana* larvae in all treatments showed significant differences (Table 1). The use of *Ha*NPV$_1$ as control only produced a lethal time of 2.9 days. While the treatment with the only nanozeolite with a concentration of 0.5 wt.% decreased the lethal time up to 2.6 days and the significant difference compared to the treatment of only *Ha*NPV$_1$. However, the increase of nanozeolite concentration to 1 wt.% did not significantly improved the lethal time. The lethal time significantly improved when the concentration of zeolite 1.5 and 2 wt.% were used with a lethal time correspondingly 1.7 and 1.3 days. There were significant differences in the use of nanozeolites with concentrations of 0.5, 1, 1.5, and 2 wt.% with *Ha*NPV$_1$ compared with the use of nanozeolites alone. The results of the

analysis showed the fastest lethal time in the use of nanozeolite 2 wt.% with the fastest time of death, which was 1.2 days. These results are supported in Figure 3a showing *C. pavonana* larvae infected with *Ha*NPV$_1$ undergoing regurgitation within 2.9 days. whereas *C. pavonana* larvae infected with nanozeolite and *Ha*NPV$_1$ underwent regurgitation within 1.2 days. The fastest lethal time of larvae (1.2 days) was obtained from the application of nanozeolite 2 wt.% as a delivery system for *Ha*NPV$_1$. All treatment showed a significant deferent in the mortality of the larvae compared to the control. It was highlighted that the treatment with only *Ha*NPV$_1$ received quite lower mortality (86%) compared to other treatments (100%), however, it was not significantly different.

Figure 3 shows the photo images of larvae *C. pavonana* infected with *Ha*NPV$_1$, infected with nanozeolite, infected with *Ha*NPV$_1$ with the delivery system of nanozeolite. The larvae of *C. pavonana* was infected with the virus *Ha*NPV$_1$ appeared to be settled in the corners and on the sidelines of the cabbage crop leaves (Figure 3a). It was also observed that the larvae slowed their movements and tended to be settled with the body of the larvae becoming flabby and emitted a brown liquid. This phenomenon is in accordance with research reported by Rao et al. (2015), which stated that some of the common symptoms of attacked by a virus caused lethargy, skin discoloration, wet or very moist stools, and liquid regurgitation. The infected larva is generally characterized by reducing the ability to eat, slow motion,

Table 1

The lethal time and mortality of Crocidolomia pavonana *larvae*

Treatment	Average lethal time (day)	Average mortality (wt.%)
Control	0 ± 0^{a}	0 ± 0^{b}
*Ha*NPV$_1$	2.9 ± 0.65^{e}	86 ± 23^{a}
0.5 wt.% Nanozeolite	2.6 ± 0.30^{de}	100 ± 0^{a}
1 wt.% Nanozeolite	2.4 ± 0.47^{de}	100 ± 0^{a}
1.5 wt.% Nanozeolite	1.7 ± 0.20^{bc}	100 ± 0^{a}
2 wt.% Nanozeolite	1.3 ± 0.05^{b}	100 ± 0^{a}
0.5 wt.% Nanozeolite + *Ha*NPV$_1$	2.4 ± 0.02^{cd}	100 ± 0^{a}
1 wt.% Nanozeolite + *Ha*NPV$_1$	2.0 ± 0.25^{cd}	100 ± 0^{a}
1.5 wt.% Nanozeolite + *Ha*NPV$_1$	1.7 ± 0.55^{bc}	100 ± 0^{a}
2 wt.% Nanozeolite + *Ha*NPV$_1$	1.2 ± 0.10^{b}	100 ± 0^{a}

Note.
- Numbers in columns are average ± SD
- Numbers followed by a different letter in a column were significantly different according to Duncan's multiple range test $p < 0.05$

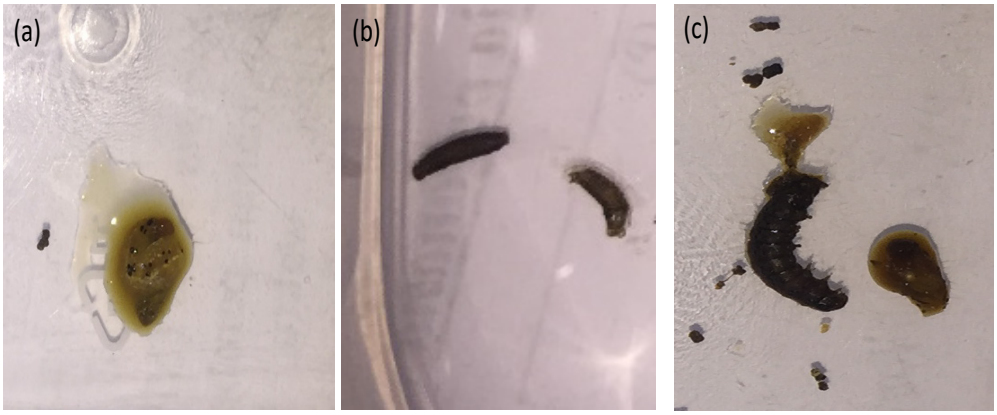

Figure 3. The photo images: (a) larvae *Crocidolomia pavonana* infected with *Ha*NPV$_1$, (b) larvae *C. pavonana* infected with only nanozeolite, and (c) larvae of *C. pavonana* infected *Ha*NPV$_1$ with nanozeolite

swollen body, due to the replication of the virus in the body (Bedjo, 2017). It is also the infected larvae that showed their body color turn pale, not actively moving, a larval body is flabby and secreted milk-brown liquid which contained polyhedra and reduced food activity (Arlita et al., 2014). This condition usually occurs 24 h after the larvae are infected. In line with the research reported by Sanjaya et al. (2011), which stated that the results of histological incision of the middle intestine of larvae *S. litura* within 24 h after treatment the damage occured to the outermost layer and the peritrophic membrane. Virion replicates or self-propagate in the cells of the insect's body so that eventually the insect dies because the whole body undergoes lysis. The infection is polyorganotrophic, which means the virus at the same time infects multiple tissues such as the epidermis, tracheal matrix, fat bodies, hemocytes, central nervous system cells, and pericardial (Das et al., 2019).

Figure 3b shows the death larvae with their bodies were dried out. This might be due to the absorption of liquid in the body of the larvae by nanozeolite. The absorption process by zeolites occurred because of its structure and also a high polarity of nanozeolite (Ginting et al., 2007). Figure 3c shows the death larvae infected with *Ha*NPV$_1$ with the delivery system of nanozeolite caused emitted a brown liquid and their bodies were dried out. This was an indicators for the synergy between *Ha*NPV$_1$ and nanozeolite infected the larvae.

Figure 4 shows the effect of treatment on the behavior of larvae consumption of cabbage. This behavior was investigated to know the important effect of additional nanozeolite 2 wt.% in the delivery system and also its role in the delivery system of *Ha*NPV$_1$. The *C. pavonana* larvae infected by only *Ha*NPV$_1$ were still able to consume cabbage before the death occurred and indicated by the existence of faces in the container (Figure 4a). This indicated that

Figure 4. The effect of treatment on the behavior of larvae consumption of cabbage: (a) cabbage leaves consumed by *Crocidolomia pavonana* infected with *Ha*NPV$_1$, (b) cabbage leaves consumed by *C. pavonana* infected with nanozeolite, (c) cabbage leaves consumed by *C. pavonana* infected with *Ha*NPV$_1$ with nanozeolite as a delivery system

the virus needs time to infected larvae (Sanjaya et al., 2011). In contrast, the cabbage leaves were partially consumed by the larvae when infected with only nanozeolite 2 wt.%, significantly less consumed compared to those infected only with *Ha*NPV$_1$ (Figure 4b). This might due to the larvae having suffered in the digestive tract because zeolite absorption leads to dry out the body of the larvae. It is also reported the similar phenomena that zeolite kills insects mainly by abrasive action or by absorption of epicuticular lipids from the insect exoskeleton causing excessive dehydration (Lu et al., 2017). Also, zeolites work by creating a barrier film by covering the leaves with a white powdery film, which adheres and irritates insects (De Smedt et al., 2016).

The cabbage leaves remained unconsumed by larvae infected by *Ha*NPV$_1$ with delivery system 2 wt.% of nanozeolite and no feces were found (Figure 4c). This is an indication of the synergetic effect of viruses and zeolite as a delivery system. The biocontrol formulated with *Ha*NPV$_1$ and nanozeolites as the delivery system was consumed from the leaves cabbage by the larvae. The consumed cabbage with the formulation was ingested by the larvae and firstly, the nanozeolite particles were absorbed by larval midgut and then the polyhedra directly entered the larval midgut lead to infection of the larval body cells. This phenomenon was also reported that the use of zeolites accelerated the lethal time in *Tuta absoluta* and as a result absorbed the liquid in the insect's body (De Smedt et al., 2016). In this study, the use of *Ha*NPV$_1$ tailored with a delivery system of nanozeolite was effective in accelerating the lethal time and significantly enhanced the mortality against *C. pavonana* larvae.

CONCLUSION

The increase of the zeolite concentration up to 2 wt.% in the delivery system for *Ha*NPV$_1$ improved their performance on lethal time and mortality against *Crocidolomia pavonana*. It was also found that nanozeolite as a delivery system enhanced and created a synergy in infecting *C. pavonana*. The virus

encapsulation with nanozeolite allowed the application of the formulation in the field since nanozeolite possible to protect the virus from UV exposure and other environmental factors. We also found that only nanozeolite received high performance as pest control.

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Extraction of High-quality RNA from Metabolite and Pectin Rich Recalcitrant Calyx Tissue of *Hibiscus sabdariffa* L.

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ABSTRACT

Hibiscus sabdariffa L. is no stranger to the field of pharmacology, as its calyx extract is highly rich in beneficial compounds and has been demonstrated to possess antihyperglycemic, antihypertension, anticancer and antioxidant properties. Thus, it is labelled as a functional food with great health benefits and therapeutic potentials. The medicinal and nutritional components of the calyx are well reported. On the contrary, not much is known about the molecular machineries governing the biosynthesis of beneficial compounds in this plant. Obtaining good yields of high-quality RNA is crucial for the success of downstream research pertaining to molecular biology. However, the presence of high quantities of phenolic compounds, polysaccharides, mucilage and pectin in the fibrous calyx tissue poses major challenges for RNA extraction in *H. sabdariffa*. Here, we modified a CTAB-based method for efficient extraction of high-quality RNA from the calyx tissue. High quality RNA samples having RNA integrity number of more than eight were successfully extracted. The purities of RNA samples were also confirmed by the A260/280 and A260/230 values. Subsequent successful preparation of a sequencing library using one of the RNA samples extracted via the modified CTAB method further emphasized the efficiency of this extraction protocol and quality of the RNA samples. The results showed that the modified CTAB method was effective in extracting good quality RNA from the challenging calyx tissue of

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Hibiscus sabdariffa L. suitable for sensitive downstream application.

Keywords: CTAB, high viscosity, mucilage, polysaccharide, RNA integrity, roselle, transcriptome profiling

INTRODUCTION

Ribonucleic acid (RNA) of high quality is the key to success for any downstream molecular work associated with transcriptomics such as cDNA library preparation, RNA-sequencing and real time quantitative reverse transcription polymerase chain reaction (RT-qPCR). RNA quality and quantity which emphasize on the yield, purity and reliable integrity require fundamental attention. However, with the high content of polyphenols, polysaccharides, and other secondary metabolites in flavonoid rich plants such as *Hibiscus sabdariffa* L. (Olaleye, 2007), it is difficult or perhaps impossible to extract pure RNA without contaminations. A major issue is the oxidation of phenols abundantly present in such tissues that forms quinone, an aromatic compound that binds RNA and inevitably affects downstream applications (Loomis, 1974). These polyphenolic compounds together with polysaccharides are able to bind and potentially co-precipitate with the nucleic acids (Gasic et al., 2004) leading to RNA extract of poor yield and low purity.

Many efficient and rapid extraction kits are developed and introduced to ease the challenges faced in RNA isolation from difficult plant samples. One example of such commercial kits is the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) that incorporates chemicals such as guanidine thiocyanate and guanidine hydrochloride in its extraction buffers, which enhance disruptive ability during the lysis step. In addition, the washing steps in the protocol that include two different types of wash buffers, one of which (Buffer RW1) contains the chemical guanidine thiocyanate further aids in the purification of the RNA samples. The potent binding capacity of the silica membrane is exceptionally sensitive and does not bind to 5S/5.8S rRNA, tRNA and other low-molecular-weight (LMW) RNAs. This alleviates the chances of high-quality RNA being extracted from tissue samples with profuse phenolic and other interrupting compounds. However, the cost incurred for RNA extraction using commercial kits are normally more expensive than conventional methods.

Thus, efforts to develop or modify conventional method for RNA extractions are continuously being carried out. CTAB extraction methods have consistently been used in extracting RNA in previous studies done on an array of troublesome plants including those that are rich in polysaccharides and phenolic compounds (Chang et al., 1993; Gambino et al., 2008; White et al., 2008; Wong et al., 2014). The extraction buffer that is based on cetyl trimethylammonium bromide (CTAB), a cationic surfactant that acts as a robust detergent, aids in breaking cell walls and in separating nucleic acids

from polysaccharides (Jaakola et al., 2001). Like many other conventional extraction methods, the CTAB method too includes four basic steps with the initiation of a nucleic acid extraction step, phase segregation step utilizing chloroform, and a precipitation step to separate the RNA from DNA, followed by a final washing step. Nonetheless, customized modifications to the basic CTAB protocol are almost always required in order to be suitable for use in each of the designated plant species.

The calyx tissue of *H. sabdariffa* is highly abundant in secondary metabolites and pectin. Pectin is a high-molecular-weight carbohydrate polymer, which poses additional challenges to RNA extraction from plant tissue. Hence, in this study, a CTAB-based protocol (Zeng & Yang, 2002) was improved with customized modifications (hereafter referred to as MCM) to suit our tissue sample. We had also extracted RNA from the same tissue type using Qiagen RNeasy Plant Mini Kit (hereafter referred to as RNeasy Kit), a guanidine-based protocol, for comparison. Comprehensive comparisons were made on the quantity and quality of the RNA samples extracted. One of the RNA samples extracted using the MCM was further tested for construction of RNA sequencing library. Based on the results obtained, we strongly recommend MCM protocol as a promising method to isolate high quality and quantity RNA from recalcitrant tissue such as the calyx of *Hibiscus sabdariffa* L.

MATERIALS AND METHODS

Materials

Calyx Tissue Sample. Calyx tissues were harvested at >25 days after blossoming, flash-frozen in liquid nitrogen, segregated into individually labeled bags and stored in a -80˚C freezer.

Chemicals, Reagents and Extraction Kit. All chemicals and reagents used were of molecular grade. Cetyltrimethyl ammonium bromide (Bio Basic Inc, Canada); disodium salt, dihydrate (Bio Basic Inc, Canada); chloroform (VWR Chemicals, USA); ethanol absolute (VWR Chemicals, USA); Tris hydrochloride (Amresco, USA); polyvinylpyrrolidone (Bio Basic Inc, USA); β-mercaptoethanol (Bio Basic, USA); isoamyl alcohol (Merck Schuchardt ohg, Germany); lithium chloride (Sigma-Aldrich, USA); sodium chloride (Sigma-Aldrich, USA); RQ1 RNase-free DNase (Promega, USA); diethyl pyrocarbonate (Bio Basic Inc, Canada); RNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

DEPC-treated and RNase-free Apparatus. All the apparatus used for the RNA extraction were pre-treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) to remove any presence of RNase. All apparatus was immersed completely in DEPC-treatment solution overnight and autoclaved for 45 minutes at 121˚C. Subsequently, the apparatus was dried in an oven for 3 days and cooled down before use. The benches and working area were sprayed, wiped and

cleaned with 70% ethanol and RNaseOut™ solution (G-Biosciences, India), a chemical that acts as an inhibitor towards RNases.

Methods

Sample Grinding with Liquid Nitrogen. Preceding the addition of samples, the mortar and pestle were pre-chilled with liquid nitrogen. A total amount of 100mg calyx sample was pulverized into fine powder in liquid nitrogen using the pre-chilled mortar and pestle. The resulting talcum powder-like sample was then scraped quickly into a pre-chilled 1.5ml micro-centrifuge tube, sealed off with its cap and kept back into liquid nitrogen to maintain its frozen state before being extracted using the two methods discussed in the sections below. RNA extractions were conducted in triplicate for each method.

MCM Protocol.

Pre-preparation of Chemicals. The CTAB extraction buffer used had a composition of 2% (w/v) CTAB, 2% (w/v) NaCl, 100mM Tris-HCL, 25mM EDTA and 2% (v/v) dH_2O. Final addition of 2% (w/v) of polyvinylpyrrolidone (PVP) and 2% (v/v) β-mercaptoethanol were done separately and the buffer was warmed up in a water bath at 65˚C for 10 minutes prior to being used for extraction.

CTAB Protocol (Zeng & Yang, 2002) with Modifications. A total of 100mg of ground powder-like calyx sample was used for RNA extraction. The sample was allowed to slightly thaw right before the addition of 800µl pre-warmed CTAB buffer. The sample and CTAB buffer were mixed using a vortex and incubated in a water bath for 10 minutes at 65˚C. An equal volume of chloroform: isoamyl alcohol solution was then added into the mixture and mixed vigorously using a vortex to form a homogenized mixture. The homogenized mixture was centrifuged at 10,000 × *g* for 15 minutes at 4˚C to separate the mixture into different phases according to density. The upper aqueous phase containing nucleic acid was transferred into a new sterile 1.5ml microcentrifuge tube. A second addition of an equal volume of chloroform: isoamyl alcohol solution was done but mixed thoroughly with gentle inversions of the tube. Once completely mixed, the mixture was then centrifuged again at 10,000 × *g* for 10 minutes at 4˚C. The upper aqueous phase was transferred into a new 1.5ml microcentrifuge tube, and 0.33 volumes of 8M LiCl was added and mixed thoroughly via gentle pipetting. The sample was precipitated for 24 hours at -20˚C. After overnight precipitation, the sample was centrifuged for 35 minutes at 10,000 × *g* at 4˚C. The supernatant was carefully removed without disturbing the RNA pellet. The washing steps were commenced with subsequent additions of 75% ethanol, 80% ethanol, and 95% ethanol with repeated centrifugation at 10,000 × *g* at 4˚C and removal of supernatant, after each addition of ethanol. The pellet was air dried. A final volume of 20µl of RNase-free water was added onto

the pellet and mixed via gentle pipetting. The RNA sample was kept in -80˚C freezer immediately.

RNeasy Kit Protocol. Similarly, a starting material of 100mg of ground calyx sample was used and the RNeasy Kit protocol (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany) was conducted based on the manufacturer's handbook.

DNase-treatment. The DNase-treatment of RNA samples extracted using MCM and RNeasy Kit were performed post extraction and on-column respectively using RQ1 RNase-Free DNase (Promega Corporation, Madison, USA) according to the manufacturer's instructions.

Quality and Quantity Check (QC) of Samples. The isolated RNA samples were electrophoresed on a 1% agarose gel in 1× TAE buffer and stained with 4% (v/v) Midori Green staining dye (Genetics Nippon, Genetics Europe GmBH). A 2-Log DNA Ladder was used (BioLab, New England) and the gel was visualized using the ENDURO™ gel imaging system (Labnet International, Inc, Edison, NJ, USA). Sample purity and yield, and RNA integrity number (RIN) were further evaluated by means of a Qubit Fluorometer 2.0 (Life Technologies Corporation, Carlsbad, USA), NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) respectively.

Preparation of Sequencing Library using Sample 3(b). A sequencing library was prepared using sample 3(b) extracted via MCM. Terminator™ 5'-phosphate-dependant exonuclease (Epicentre, Madison, USA) and ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicentre, Madison, USA) were used for rRNA-depletion and library construction respectively following the manufacturer's protocol. The ScriptSeq cDNA libraries were quantified using Qubit® 2.0 Fluorometer (Life Technologies Corporation, Carlsbad, USA) and the size distribution was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) via a high sensitivity DNA chip. In order to achieve optimum sequencing of reads, at least more than 60% of the libraries should fall between the size fragment range of 200-1000 bp stipulated on the electropherogram profile. Sequencing was performed by Illumina NextSeq 500 Sequencer Platform (Illumina, Inc, California, USA). Raw sequencing reads were further subjected to quality trimming and filtering, in which good quality reads were acquired and quantified using the Bowtie 2 (Langmead et al., 2009; Langmead & Salzberg, 2012) and BBDuk (Bushnell, 2014).

RESULTS

The RNA samples extracted using the MCM denoted as 3(a), 3(b), and 3(c), and those extracted using the RNeasy Kit denoted as 3(i), 3(ii), and 3(iii) as seen in the gel image presented in Figure 1 clearly

showed two apparent double bands in each sample. For all the six samples, the upper band conspicuously appeared brighter and much thicker than their respective lower bands. Moreover, minimal background and smearing were seen for all samples indicating minor to no occurrence of RNA degradation. Table 1 documents the yield, absorbance ratios of A260/230 and A260/280, and RNA integrity numbers (RIN) of these samples. On average, RNA samples extracted via MCM recorded an average yield of 6803.33ng; whilst RNA samples extracted through RNeasy Kit recorded an average yield of 4721.67ng, from 100mg of starting material each. Thus, this indicated that the MCM had exceeded the RNeasy Kit in terms of yield.

In the context of sample purity, the MCM isolated RNA samples have average A260/230 and A260/280 absorbance ratios of 1.70 and 1.78, respectively; whereas the RNeasy Kit had isolated RNA samples with an average absorbance value of 0.81 for A260/230 and 1.22 for A260/280. These absorbance values clearly showed that the purity of RNA samples obtained using MCM are notably closer to the ideal range 1.8-2.0 of standard values accepted for both A260/230 and A260/280 compared to those extracted using RNeasy Kit. Figures 2 and 3 show the bioanalyzer graph results recording the RIN values measured based on the development of two prominent peaks representing the 18S and 25S bands for all RNA samples alongside a digital gel image for samples 3(a), 3(b), and 3(c), and samples 3(i), 3(ii), and 3(iii) respectively. RNA samples obtained using MCM documented RIN values ranging from 8.00 to 8.20; whereas samples extracted using RNeasy Kit documented RIN values ranging from 7.60 to 8.50.

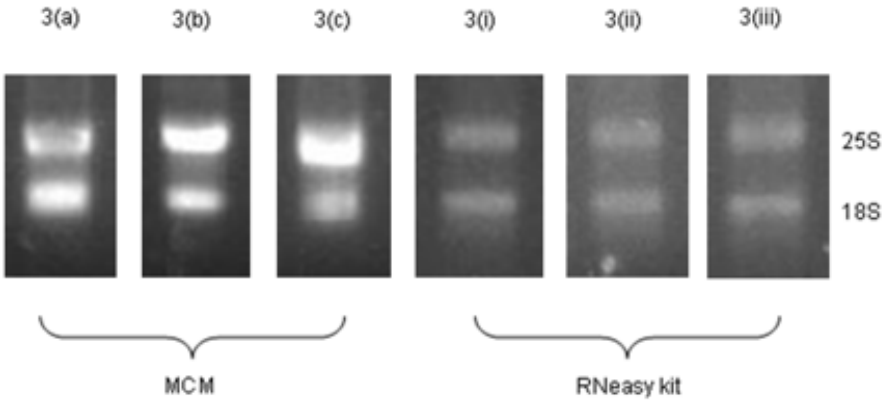

Figure 1. RNA samples of *Hibiscus sabdariffa,* samples 3(a), 3(b), and 3(c) extracted using MCM, and samples 3(i), 3(ii) and 3(iii) extracted using the RNeasy Kit electrophoresed on 1% agarose in 1× TAE buffer. The double bands represent an upper 25S and a lower 18S rRNA bands

Table 1

Yields, absorbance ratios and RNA integrity numbers (RIN) of RNA samples extracted from the calyx tissue of Hibiscus sabdariffa

Extraction Method	Sample	Yield (nanogram)	A260/230 ratio	A260/280 ratio	RIN
CTAB	3(a)	7150	1.58	1.63	8.10
	3(b)	4180	1.74	1.84	8.20
	3(c)	9080	1.80	1.88	8.00
RNeasy Plant Mini Kit	3(i)	4660	0.66	1.24	8.50
	3(ii)	4690	0.92	1.25	7.60
	3(iii)	4815	0.84	1.18	7.80

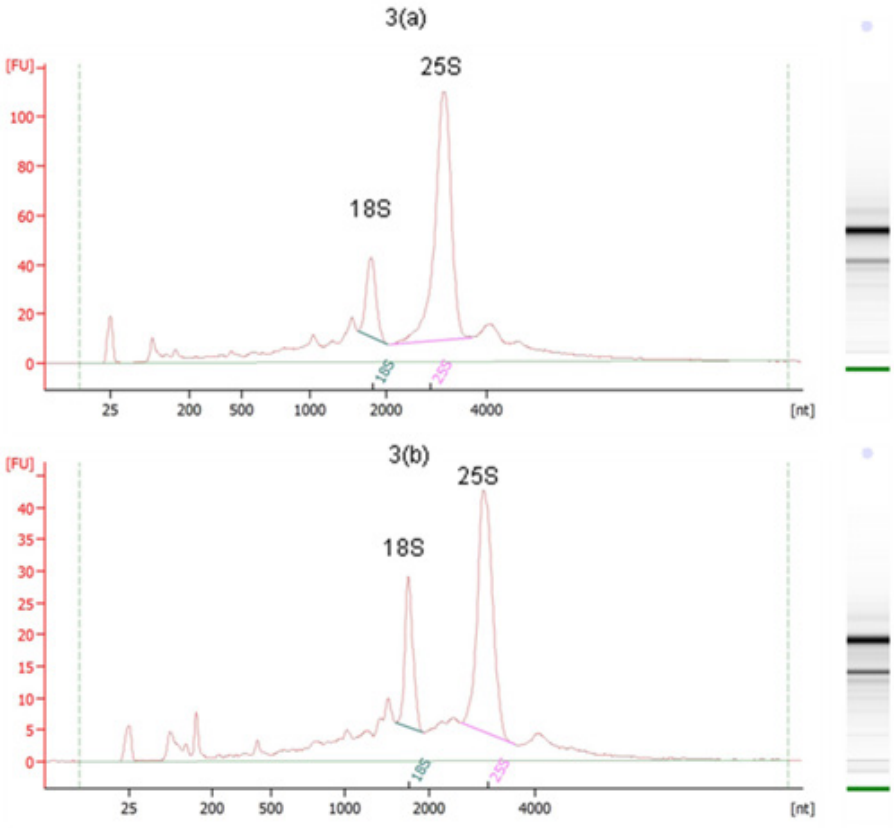

Figure 2. Digital image representations of the RNA bands and graphic representation of RNA integrity based on the two rRNA peaks (18S and 25S) for samples 3(a), 3(b), and 3(c) extracted from *Hibiscus sabdariffa* using the modified CTAB method (MCM)

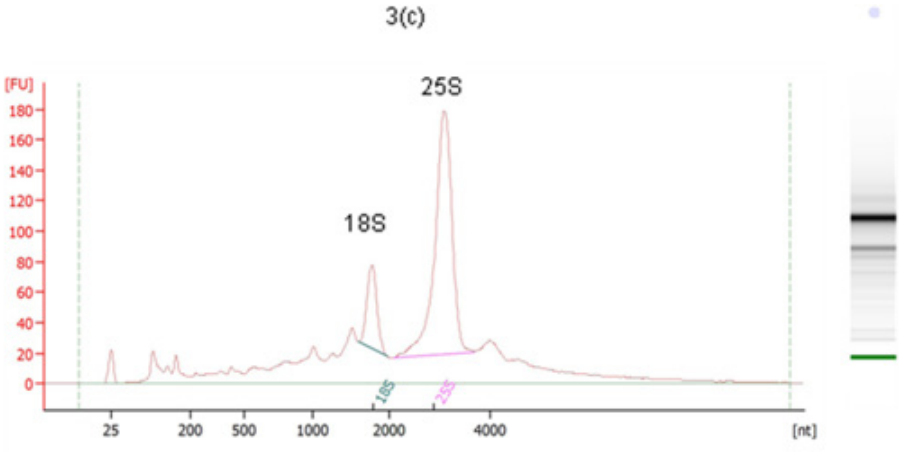

Figure 2. (Continued)

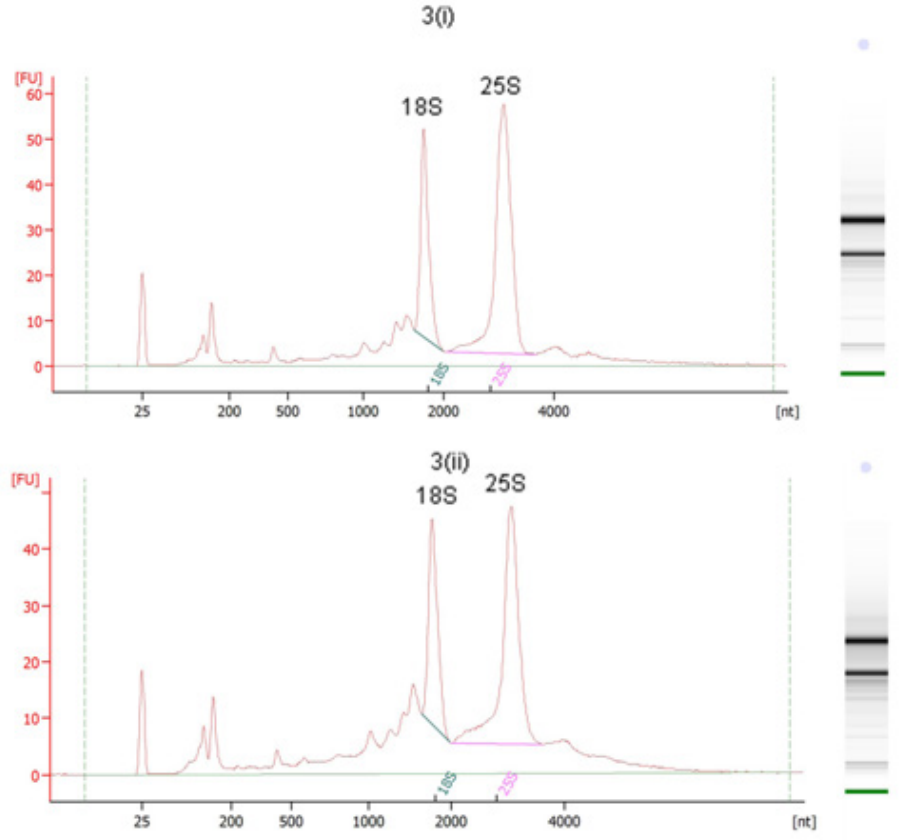

Figure 3. Digital image representations of the RNA bands and graphic representation of RNA integrity based on the two rRNA peaks (18S and 25S) for sample 3(i), 3(ii), and 3(iii) extracted from *Hibiscus sabdariffa* using RNeasy Kit

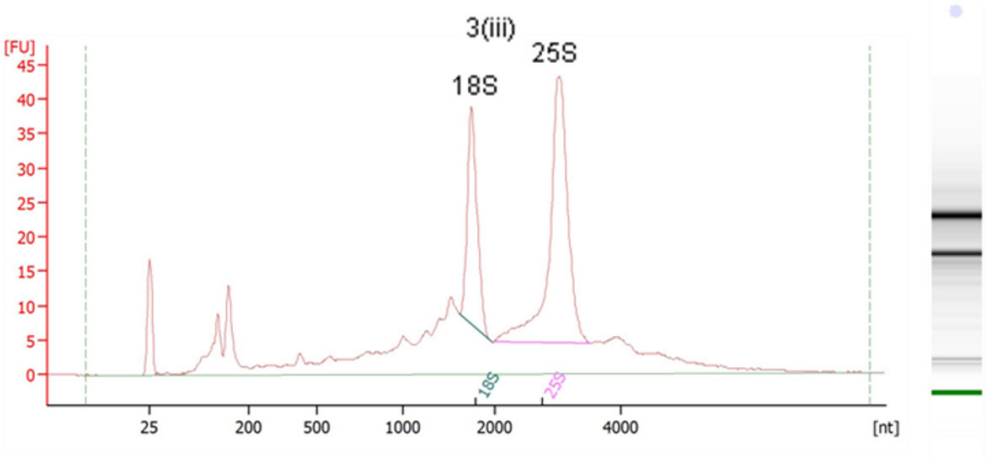

Figure 3. (Continued)

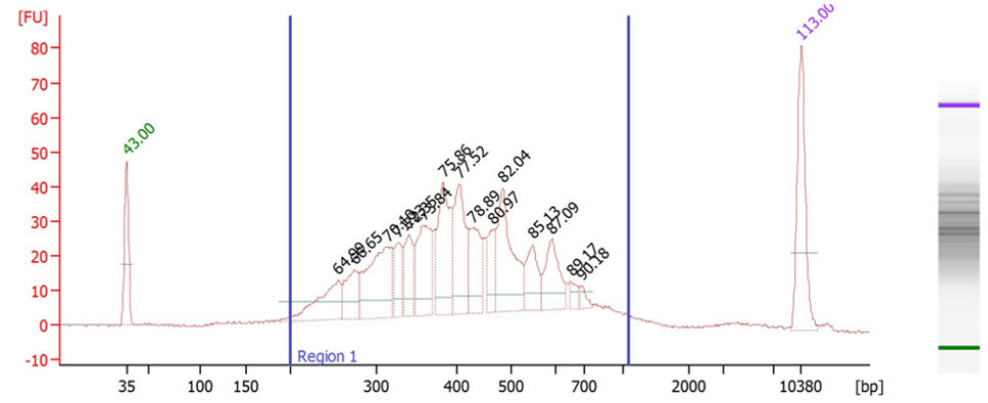

Figure 4. Electropherogram of the sequencing library prepared from sample 3(b)

One of the RNA samples 3(b) extracted using MCM was further tested for the preparation of RNA sequencing library. Quality of the RNA sample is a critical and fundamental factor in determining the quality of sequencing library and output data. The quality of the library was assessed using high sensitivity DNA assay on 2100 Bioanalyzer (Agilent Technologies, Germany). Figure 4 shows the electropherogram result of

sequencing library prepared from sample 3(b). The library had a concentration of 1295.95pg/µl and average fragment size of 433bp. Ninety-five percent of the fragment were distributed between 200-1079bp and size distribution (co-efficient variation) was 32.6%. Sixty percent of the sequencing output attained PHRED score quality ≥30 (reads with probability of error = 0.001), generating a total of 71,210,684 high-quality reads (unpublished data). Thus, the number of high-quality reads demonstrate a good and successful sequencing library preparation, which meets the requirements for subsequent *de novo* assembly alongside further downstream analysis.

DISCUSSION

One of the main problems faced in studying plant genetics is the difficulties in isolating high-quality nucleic acid (i.e. DNA and RNA) from plant tissue. RNA is important when gene expression profiling and functional genomics are the subjects of the study. The presence of phenolic compounds, polysaccharides, and various secondary metabolites in the fibrous calyx tissue of *Hibiscus sabdariffa* has made RNA isolation very challenging. The presences of mucilage and pectin in particular, makes complete homogenization of the calyx tissues impossible during isolation of RNA.

RNA extract with high yield or concentration is always desirable for downstream application such as preparation of sequencing library. In the context of RNA concentration, both methods yielded good amounts of RNA. However, on average, a difference of more than 2,000ng in yield between samples extracted via MCM and RNeasy Kit were recorded, which indicated the greater capacity of the MCM in extracting higher yields of RNA. Eukaryotic RNA is theoretically characterized as having double rRNA bands that are made up of an upper 25S band and a lower 18S band in plants. The presence of these two bands with the 25S band showing higher intensity is used as an indicator to reflect RNA intactness. In this study, the RNA samples extracted using MCM (Figure 1) clearly showed a thicker and more intense 25S band in relative to the lower 18S band, similar to those were extracted using the RNeasy Kit.

The RNA samples extracted from the calyx tissue using the two methods were intact in structure reflected by the reasonably superior RNA integrity numbers ranging from 7.60 to 8.50 as shown in Table 1, Figure 2, and Figure 3. The RIN values ranged from 1 to 10 with 10 representing the most intact RNA (Schroeder et al., 2006). RIN values were determined through the 25S and 18S peaks formed on the bioanalyzer graphs with the integration of algorithms. These peaks symbolize the quantity of rRNA molecules present in the samples which are mostly composed of cytosolic, chloroplastic and mitochondrial rRNAs (Kim & Haj-Ahmad, 2014). An ideal graph would show two prominent peaks with the 18S peak being lower in height in comparison to the 25S peak as demonstrated by samples extracted in this study. A noticeable difference in peak heights acts

as a strong indicator of intact RNA having minor/no degradation that consequently affects the RIN value (Schroeder et al., 2006). It had been shown in studies that RIN values can affect gene expression profile leading to misinterpretation of the result particularly in qPCR and RNA sequencing (Fleige & Pfaffl, 2006; Wang et al., 2016). Recommended RIN values of more than 5 were considered good; whereas RIN values of more than 8 were considered excellent in regards to total RNA most efficient for downstream work (Fleige & Pfaffl, 2006).

RNA purity as indicated by the A260/230 and the A260/280 ratios showed comparative deviations for samples extracted using both methods, from the ideal range of 1.8 to 2.0 ratio value that is generally considered as "pure" RNA sample (Skrypina et al., 2003). The three replicates of RNA extracts isolated using MCM recorded absorbance values ranged from 1.58 to 1.88, with an average value of 1.70 for A260/230 and an average value of 1.78 for A260/280. In contrast, for the RNA samples extracted using RNeasy Kit, the absorbance values deviated further from the range of acceptable purity with A260/230 and A260/280 ratios obtaining average values of 0.81 and 1.22, respectively. Deviation of the A260/230 and A260/280 absorbance ratios from the acceptable range of 1.8-2.0 may indicate either the contamination of phenol, guanidine or other organic salts, and the contamination of proteins in the DNA samples, respectively (Wang & Stegemann, 2010). Based on these values, it is worth noting that the MCM produced RNA extracts with less contaminants comparatively.

The customized modifications made to the CTAB protocol that was used initially in an oil palm study conducted by Zeng and Yang (2002) had led to effective results in enhancing RNA quality and quantity. In our initial attempt, adoption of the Zeng and Yang (2002) CTAB method with no modification had resulted in consistent failures with the persistent formation of either a brown pellet, absence of pellet or the inability of the pellet to dissolve completely during the elution step. Hence, either non-intact RNA was continually being produced or no RNA was extracted. Other conventional method that integrated sodium chloride precipitation and isopropanol precipitation in order to extract RNA from samples having high starch levels (Li & Trick, 2005) was also tested but without any success. Under the modified conditions, the starting material used was decreased to 100mg from the 500-700mg suggested by Zeng and Yang (2002). Nevertheless, the rest of the procedure was not scaled down despite the reduced amount of plant starting material but was maintained the same as that used for 500-700mg of oil palm material. This was due to the consistency of the calyx tissue sample that turned thick and viscous upon homogenization with the extraction buffer because of the presence of pectin and mucilage. Initial trials using the minimum amount of 500mg suggested in the CTAB protocol by Zeng and Yang (2002) consistently led to incomplete breakage of cell walls and separation of nucleic acid due

to the challenging nature of the calyx tissue. Moreover, the tough and fibrous nature of the calyx tissue lengthened the time required to completely mix the sample in the extraction buffer after grinding, to the extent of allowing oxidation to occur rapidly. This unavoidably had led to the degradation of the nucleic acid. Thus, a reasonable amount of 100mg of sample was found to work well with the protocol due to the nature of the tissue sample used after several attempts. The extraction buffer was able to work well at homogenizing the sample entirely with the reduced sample input amount.

The ingredients used for the extraction buffer were like those of the Zeng and Yang's (2002) method, with only two changes made. Firstly, the addition of PVP was done separately just before warming up the CTAB buffer. PVP helps to prevent the oxidation of polyphenols in cell walls (Loomis & Battaile, 1966) and the addition of PVP into the CTAB buffer just prior to extraction prevented the formation of colloids and ensured no alteration was done towards its main function. Secondly, the inclusion of 0.05% spermidine trihydrochloride in Zeng and Yang's (2002) protocol was omitted since it binds and precipitates DNA rather than RNA. In our protocol, the separation between these two nucleic acids (i.e. RNA and DNA) was done solely using LiCl precipitation which had aided in reduction of steps. Precipitation was optimally effective at a molarity of 2.67M rather than 2.5M used in the original procedure of Zeng and Yang (2002). The increase in molarity had resulted in a better precipitation of RNA.

As for the protocol, the RNA samples were precipitated at -20˚C during the 24 hours incubation period rather than at 4˚C as was done by Zeng and Yang (2002). Lastly, the washing steps were also improved to suit the calyx RNA sample whereby three consecutive washing steps were performed using three different concentrations of ethanol in order to gradually wash off any unwanted residual compounds meticulously. As such, 75% and 80% ethanol were used to separate and dissolve residual compounds such as salts present in the sample and 95% ethanol was used to bind any leftover water clinging to the RNA pellet to allow swift drying of the pellet before elution. The combination of all these modifications had yielded high quality RNA from recalcitrant calyx tissue of the *Hibiscus sabdariffa* L. reproducibly.

CONCLUSION

The overall results of the RNA quality for the samples extracted using the CTAB method adapted from Zeng and Yang (2002) with modifications (MCM) and the RNeasy Plant Mini Kit (RNeasy Kit) produced comparatively similar qualities in terms of the RIN values. Both extraction protocols produced credible RNA qualities with RIN values exceeding 7.0. However, the absorbance values and yields of the RNA obtained via these two methods differed substantially with MCM scoring slightly higher points above the RNeasy Kit. Hence, in this study it is evident that MCM is efficient to extract RNA of excellent quality and quantity from the calyx tissue of roselle,

which allows downstream molecular studies on this important crop. We are optimistic that the modified CTAB method (MCM) developed in this study will be useful for RNA extraction from other difficult plant tissues of similar nature.

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Chitosan-based Edible Coating Prolongs *Musa troglodytarum* L. ('Pisang Tongkat Langit') Fruit Shelf-life and Changes the *ACS1* and *ACO1* Gene Expression Profile

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ABSTRACT

Musa troglodytarum L. ('Pisang Tongkat Langit'), a banana cultivar which originated from Eastern Indonesia, has an economic potential due to the high β-carotene content on its pulp. Being a climacteric fruit, *M. troglodytarum* has a short shelf-life that can reduce fruit quality. In this study, the effect of 1.25% (w/v) chitosan coating on *M. troglodytarum* fruit shelf-life and *ACS1* and *ACO1* gene expression analysis using quantitative PCR were evaluated. Results showed that the application of chitosan coating delayed the fruit ripening process for two days by delaying several fruit physical and chemical changes. *ACS1* and *ACO1* gene expression analysis showed a different expression pattern, the expression level was lower on chitosan-coated fruits on the first day compared to control. In conclusion, chitosan-based edible coating delayed *M. troglodytarum* fruit ripening and changed the *ACS1* and *ACO1* gene expression pattern, compared with the chitosan coating effect on Cavendish banana which also prolonged fruit ripening and suppressed *ACS1* and *ACO1* expression in a previous research.

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INTRODUCTION

Banana has been considered as the world's number four important food crop (Instituto de Promocion de Exportaciones e Inversiones, 2016). This fruit is widely consumed in the world due to its high nutrition. The ripe fruit has 89 kcal per 100 g fresh weight for energy source, carbohydrates, fibers, proteins, fats, calcium, iron, vitamins, and potassium (Pareek, 2015). One of the local banana cultivar from Eastern Indonesia that has an economic potential is *Musa troglodytarum* L. ('Pisang Tongkat Langit'), belongs to the *Australimusa* section (Ploetz et al., 2007). This banana pulp has a high β-carotene content (520-2,780 $\mu g \cdot 100\ g^{-1}$), a pro-vitamin A precursor (Englberger et al., 2003b). Thus, this banana could be a good source of vitamin A. Vitamin A is an essential nutrient in the human diet and it has been reported that vitamin A deficiency is a world health problem and is related to mortality rate especially in developing countries (Englberger et al., 2003a).

Banana is a climacteric fruit where ripening is associated with a sharp increase followed by a rapid decline of ethylene production in the early climacteric period (Liu et al., 1999). This condition could speed up the fruit ripening process and shorten the fruit's shelf-life. Postharvest technologies have been developed to solve this problem, such as using controlled atmosphere (CA) (Ahmad et al., 2001) and modified atmosphere packaging (MAP) (Kudachikar et al., 2011), but these technologies are relatively expensive.

An alternative postharvest technology which is relatively low cost is edible coating. Edible coating has received much attention because it is capable of forming a thin film that could prevent moisture loss and oxygen diffusion into the plant tissues, thus maintaining its postharvest quality (Jianglian & Shaoying, 2013; Xing et al., 2016). One promising edible coating biopolymer is chitosan, which is a chitin derivative that is known to be non-toxic, biodegradable, biocompatible, and biofunctional. Furthermore, chitosan is considered to be Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) (Luo & Wang, 2013).

Chitosan-based edible coating has been applied on some banana cultivars and could extend their shelf-life, such as on 'Berangan' (*Musa sapientum*, AAA group) (Malmiri et al., 2011; Maqbool et al., 2011) and Cavendish (*Musa acuminata*, AAA group) (Lustriane et al., 2018; Pratiwi et al., 2015). Though the study of chitosan effect on banana ripening had been confirmed through physical and chemical analysis, only limited study had been conducted on the molecular mechanism, such as analysis of the expression of *ACS* and *ACO* genes on chitosan-coated Cavendish banana (Dwivany et al., 2018; Lustriane et al., 2018; Yamamoto et al., 2018). *ACS* is a gene encoding 1-aminocyclopropane-1-carboxylic acid synthase (ACS), while *ACO* encodes 1-aminocyclopropane-1-carboxylic acid oxidase (ACO). These two key enzymes play an important role in ethylene biosynthesis (Xu & Zhang, 2015).

Moreover, *ACS1* and *ACO1* expression were associated with ripening process of banana fruit (Karmawan et al., 2009; Liu et al., 1999; López-Gómez et al., 1997), and these genes had been used as markers for the ripening process of chitosan-coated Cavendish banana (Dwivany et al., 2018; Lustriane et al., 2018). It was reported that these genes had been succesfully isolated and characterized from *M. troglodytarum* fruit pulp using *ACS1* and *ACO1* primers of *Musa acuminata* (AAA group) (Dwivany et al., 2020). But, there had been no report on the effect of chitosan coating to extend the shelf-life and *ACS1* and *ACO1* gene expression of *M. troglodytarum* fruit. Therefore, this study aimed to investigate the effect of chitosan coating on the shelf-life and *ACS1* and *ACO1* expression of *M. troglodytarum* fruit.

In this study, the chitosan coating concentration was 1.25% according to the optimized concentration to prolong the shelf-life of Cavendish banana at room temperature by Lustriane et al. (2018). Besides, 1% acetic acid solution, the solvent of chitosan solution, used as a coating to analyze its effect on the fruit postharvest storage, since Du et al. (1997) found that acetic acid coating affected pear fruit physical characteristics during storage.

MATERIALS AND METHODS

Materials

'Pisang Tongkat Langit' (*Musa troglodytarum* L.) mature green fruits were harvested from Ullath Village, Saparua Island, Maluku Province (Moluccas Islands), Indonesia (Figures 1a and 1b). Eight hands, which consisted of 5 until 13 fingers, were chosen randomly from eight bunches. Each bunches were derived from different herbs (Figure 1c). The fingers then were separated from each hand (Figure 1d) and selected visually for similarity in relative peel color, size, and without physical damage and infection of fungi on the fruit surface. The fingers, which weight ranged from 186.60 g to 256.32 g, were washed with commercial soap. Then the fruits were air-dried and the tips were wiped with 70% ethanol. One finger was used for each replicate, with three replicates per treatment sets per evaluation date. Meanwhile, three fingers were used for control samples. Chitosan (food graded) was purchased from Biotech Surindo, Indonesia, with high molecular weight and 85% degree of acetylation. All chemicals used in this research were analytical grade.

Preparation and Application of Chitosan Coating

Fruits were grouped into two experimental sets, i.e. 1.25% chitosan-coated and 1% acetic acid-coated fruits, and a control (uncoated fruits) set. There were three replicates per treatment sets per evaluation date. 1.25% chitosan solution was chosen according to a study conducted by Lustriane et al. (2018). Meanwhile, 1% acetic acid solution was used to examine its effect on the postharvest storage of *M. troglodytarum* fruits, since it was the main solvent for chitosan.

The 1.25% (w/v) chitosan solution and 1% (v/v) acetic acid solution were prepared

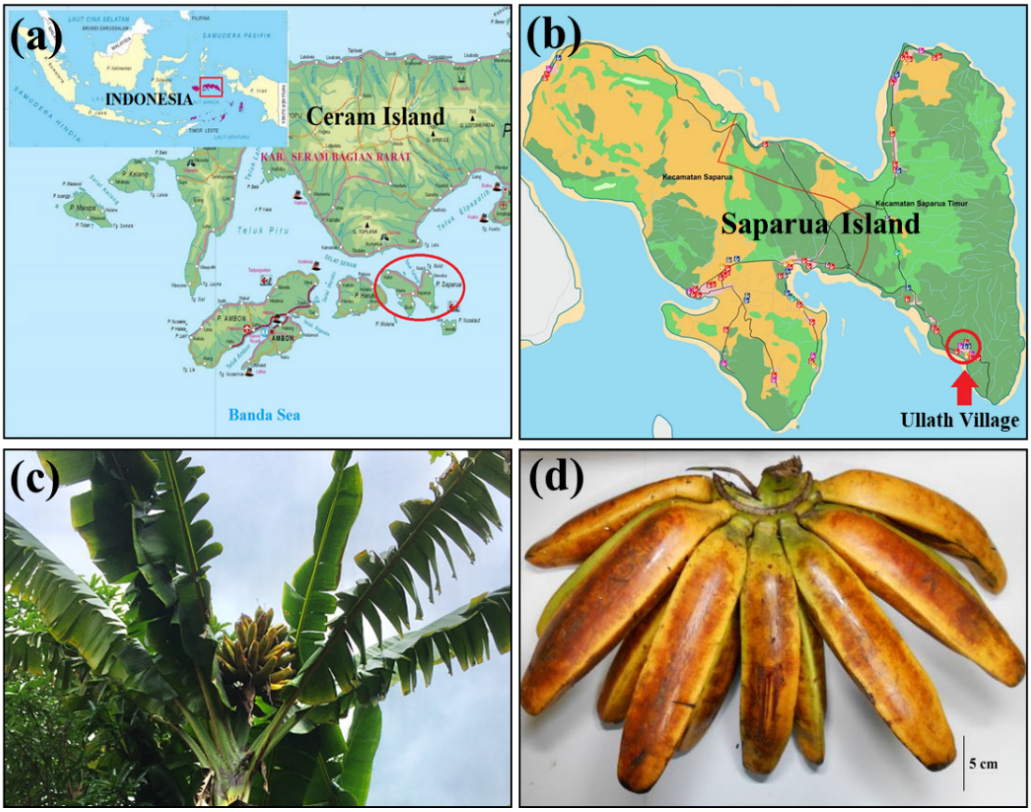


Figure 1. Sampling location of *Musa troglodytarum* L. ('Pisang Tongkat Langit'). (a) Saparua Island in Maluku Province is circled in red. The inset shows the position of the map in Indonesia [Ministry of Education and Culture of Indonesia (Kemdikbud), 2018], (b) Location of Ullath Village (red arrow) in Saparua Island (Kemdikbud, 2015), (c) *Musa troglodytarum* herb and bunches, (d) Hand of *M. troglodytarum* fruits

according to Malmiri et al. (2011) method with modification.

Briefly, 1% (v/v) acetic acid solution was prepared by dissolving glacial acetic acid in distilled water. The 1.25% (w/v) chitosan solution was prepared by dissolving the chitosan powder in 1% (v/v) acetic acid solution. Both of the solutions were agitated with a magnetic stirrer until homogenous. The pH of solution was adjusted to 5.5-5.6 by adding 3 N NaOH. Coating application was done by dipping the fruits into chitosan or acetic acid solution for 2 min and air-dried at room temperature for 2 hours. Then the fruits were stored at ambient temperature of 27±0.7°C and relative humidity of 82.57±3.62% for 9 days. The postharvest analysis was carried out on days 0, 1, 3, 5, 7, and 9 of storage.

Fruit Physical Analysis

Observation of the Peel and Pulp of the Fruit. Changes on the peel and pulp of the fruit were noted and photographed at each

observation days of storage, which was described by Dadzie and Orchard (1997).

Weight Loss Determination. Weight loss was measured, which was described by Dadzie and Orchard (1997) as well as Lustriane et al. (2018), by comparing the initial fruit weight (day 0) and the fruit weight on each observation days during storage. The weight loss percentage was obtained by calculating the difference between the initial weight and final weight, then dividing the result with the initial weight and multiplying with 100%.

Pulp to Peel Ratio. The middle part of the fruit was cut transversely 1.0 cm thick, then the peel and pulp were weighed separately. The pulp to peel ratio was calculated by dividing the pulp weight with the peel weight (Dadzie & Orchard, 1997).

Fruit Chemical Analysis

Starch into Sugar Coversion. Starch into sugar conversion analysis was conducted using iodine starch staining techniques developed by Dadzie and Orchard (1997). The iodine staining solution was made by dissolving 1% potassium iodide and 0.25% iodine in warm distilled water. The middle part of the fruit was cut transversely 1.0 cm thick, then the peel was separated from the pulp. Then the pulp was immersed at a depth of 5 mm for 20-30 seconds in the staining solution. The iodine starch pattern of each fruit was compared with the chart of banana pulp stained surface with iodine staining (Blankenship et al., 1993).

Total Soluble Solids (TSS). Total soluble solids (TSS) content of fruit pulp was measured by using a refractometer (ATAGO) which was described by Dadzie and Orchard (1997). Briefly, 15 g fruit pulp was homogenized with 45 mL of distilled water using a blender to give three times dilution. The homogenate was centrifuged at (18,600 x *g*) for 5 min using microcentrifuge (Thermo Fisher Scientific™ Heraeus Pico™ 21). Then a few drops of the supernatant were dripped on the refractometer prism before reading. The results were multiplied by three and expressed as degree Brix (^{o}Brix). Calibration of the refractometer was done before measurement by placing a few drops of distilled water to give a 0 ^{o}Brix reading.

Fruit Surface Microstructure Analysis

The 1.25% chitosan-coated and uncoated fruits on day 1 after storage (unripe fruit) were used for fruit surface microstructure analysis, as described by Lustriane et al. (2018) with slight modification. Square pieces (0.5 cm x 0.5 cm) of 2 mm thick peel from the fruits were cut transversely from the middle part of the fruit. The samples were freeze dried (freeze dryer VD-550R, Taitec Corporation) for 25 hours, then were coated with gold (Au) using ion sputter coater (MC1000, Hitachi). The microstructure of peel outer surface was analyzed by using scanning electron microscopy (JSM-6510A, JEOL Ltd.) using an accelerating voltage of 10 kV and viewed in 1,000x zoom.

Sensory Quality Evaluation

Sensory quality evaluation was conducted using hedonic test which was described by Amerine et al. (1965). Sixteen semi-trained panelists were chosen to evaluate untreated (control) and treated fruits (days 5, 6, and 7 of storage at 27±0.7°C). Fruit samples were presented randomly to the panelists and rated on a seven-point hedonic scale (1 = extremely dislike, 7 = extremely like) for aroma, taste, and fruit overall acceptability.

Isolation of Total RNA and cDNA Synthesis

RNA isolation was carried out on uncoated (control) and 1.25% chitosan-coated fruits on observation days of 0, 1, 3, 5, and 7. Total RNA was isolated from fruit pulp using Cordeiro's method (Corderio et al., 2008). cDNA synthesis was done by using the isolated RNA as template and iScript™ cDNA Synthesis (Bio-Rad Laboratories) as mix reagent.

***ACS1* and *ACO1* Gene Expression Analysis**

Total cDNA from the pulp of uncoated and 1.25% chitosan-coated fruits were used for *ACS1* and *ACO1* gene expression analysis by quantitative PCR (qPCR). Specific primers were used to amplify the cDNA fragments of *MaACS1* (primer of Cavendish banana *ACS1* gene), *MaACO1* (primer of Cavendish banana *ACO1* gene), and a reference gene, *MaGAPDH* (primer of Cavendish banana *GAPDH* gene). The primer pairs used to amplify *ACS1*, *ACO1*, and *GAPDH* are presented in Table 1. The qPCR was performed by MyGo Pro® real time PCR instrument connected with MyGo Pro PCR software, using Thunderbird® SYBR® qPCR mix reagent (Toyobo). In each qPCR analysis, three samples were used for

Table 1

Primer sequences of analyzed genes

Gene Name	Primer Name	Primer Sequences	Reference
MaGAPDH (Reference Gene)	*MaGAPDH* Forward	5'-TCAACGACCCCTTCATCAC-3'	Karmawan et al. (2009)
	MaGAPDH Reverse	5'-AGCAGCCTTGTCCTTGTCA-3'	
MaACS1	*MaACS1* Forward	5'-CCGAGACTGGATGAAGAAGAA-3'	Karmawan et al. (2009)
	MaACS1 Reverse	5'-GTCTGGGTCAAATCTGGCTC-3'	
MaACO1	*MaACO1* Forward	5'-CGAGATGCTTGCGAGAAATGG-3'	Dwivany et al. (2018)
	MaACO1 Reverse	5'-TGCAGCAAATTCCTTCATCGC-3'	

triplicate. The qPCR cycle condition was set at 95°C initial hold for 60 s, followed by 40 cycles of denaturation (95°C, 15 s), annealing (60°C, 30 s), and extension (72°C, 30 s). This was followed by a melting stage from 60°C to 97°C (4°C increments for 30 s each at the initial stage and 0.5°C increments for 1 s each at the final stage).

Gene expression levels were normalized using the reference gene. Then the relative gene expression levels were calculated using obtained Ct (cycle threshold) value by $2^{-\Delta\Delta Ct}$ method as formulated by Livak and Schmittgen (2001).

Statistical Analysis

Statistical analysis was conducted using IBM SPSS Statistics ver. 20. Analysis of variance (ANOVA) was used to measure the treatment effect and followed by Tukey's HSD as *post hoc* test. Differences were considered to be significant when the *P*-values ≤ 0.05.

RESULTS AND DISCUSSION

Fruit Physical Analysis

Changes on Peel and Pulp Color. Changes on peel and pulp color are often used to estimate fruit ripening stages. The effects of coatings on peel and pulp color changes are presented in Figure 2a. As observed, peel color of uncoated (control) and 1% acetic acid-coated fruit were yellowish orange on day 3 and orange on day 7, while 1.25% chitosan-coated peel color just changed to yellowish orange on day 5 and orange on day 9. Moreover, control and 1% acetic acid-coated fruit showed a faster deterioration compared with 1.25% chitosan-coated fruit on day 9. Observation of the pulp color showed that the pulp color of the control fruit changed from yellow to orange on day 3, this color was obtained on day 5 for the 1% acetic acid-coated fruit. On 1.25% chitosan-coated fruit, the pulp reached the orange color on day 9. These results indicated that chitosan coating delayed the changes in the peel and pulp color.

Chitosan coating formed a thin film on fruit surface which can be seen on microstructure observation (Figure 4). This thin film provides a physical barrier against water and gas, which causes a reduction in the diffusion of O_2 into and CO_2 out from the plant tissue (Xing et al., 2016). If internal O_2 decreases, ethylene biosynthesis is inhibited and ripening process is slowed down, including chlorophyll degradation on peel and pulp (Knee, 1980). Chitosan coating may also inhibit carotenoid pigment production in the fruit. This may happen since the carotenoid production is affected by ethylene (Rodrigo & Zacarias, 2007). Delay on peel color changes are also observed on other chitosan-coated banana fruits, such as 'Berangan' (Maqbool et al., 2011) and Cavendish (Lustriane et al., 2018). Chitosan coating also delays fruit decay because of its antimicrobial properties (Jianglian & Shaoying, 2013; Xing et al., 2016). Delay on fruit decay was also observed on chitosan-coated raspberry (Tezotto-Uliana et al., 2014) and Cavendish banana (Lustriane et al., 2018). On Cavendish banana, it was reported that chitosan coating (concentration

1.25%) delayed the fruit deterioration until 2-3 days (Lustriane et al., 2018).

Weight Loss. The effect of coatings on weight loss is shown in Figure 2b. Fruit weight loss is one of important parameter to determine fruit postharvest quality (Maqbool et al., 2011). Generally weight loss increases during ripening. However, according to statistical analysis, all of the treatments did not show any significant differences ($P \leq 0.05$). This result showed that chitosan coating did not affect weight loss of *M. troglodytarum* fruit. Fruit weight loss is related with moisture loss from respiration and transpiration during ripening. Chitosan coating is supposed to prevent moisture loss and weight loss of fruits (Eshghi et al., 2014). Therefore, chitosan coating can be added with a plasticizer such as glycerol to increase its permeability to water so it can restrain transpiration and decrease fruit weight loss (Malmiri et al., 2011).

Pulp to Peel Ratio. The effect of coatings on pulp to peel ratio can be seen on Figure 2c. Generally, pulp to peel ratio increased until it reached a peak, then it decreased at the end of the ripening process. The peak values of each treatment were significantly different ($P \leq 0.05$) from one another. Each treatment reached its peak value at different storage time, 1% acetic acid-coated fruit on day 5, control on day 7, and 1.25% chitosan-coated fruit on day 9. This suggested that chitosan coating slowed down the changes in the pulp to peel ratio.

During fruit ripening, pulp and peel tissues undergo changes in water and soluble sugar content, which lead to osmotic pressure differences between pulp and peel. Therefore, the pulp and peel weight ratio is increased (Dadzie & Orchard, 1997). Chitosan coating slows down starch conversion into soluble sugar (Maqbool et al., 2011). Hence, osmosis from pulp to peel is inhibited and pulp to peel ratio changes is delayed. Lustriane et al. (2018) and Pratiwi et al. (2015) reported that pulp to peel ratio of chitosan-coated Cavendish banana was lower than the uncoated banana.

Fruit Chemical Analysis

Starch into Sugar Conversion Analysis. The effects of coatings on changes of starch degradation pattern are visualized in Figure 3a. Starch degradation in 1.25% chitosan-coated fruit pulp was the slowest among all treatments. This could be seen from the presence of starch staining on the middle part (placenta) and the edge part of the 1.25% chitosan-coated fruit pulp on day 5, while control and 1% acetic acid-coated fruit only showed starch staining on the edge of the pulp.

During fruit ripening, starch is degraded into soluble sugar by amylase activity, hence this decreases the starch content and increases the soluble sugar content of the pulp (do Nascimento et al., 2006). In chitosan-coated fruit, ethylene production is inhibited (Maqbool et al., 2011). As a result, starch degradation rate decreases, since amylase activity is affected by

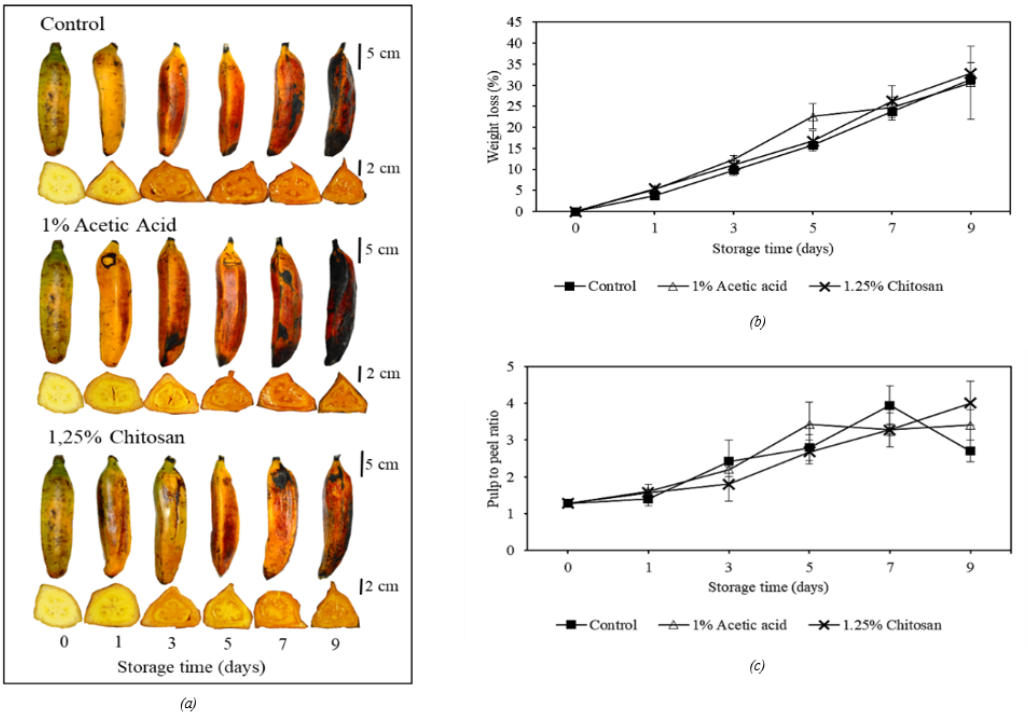
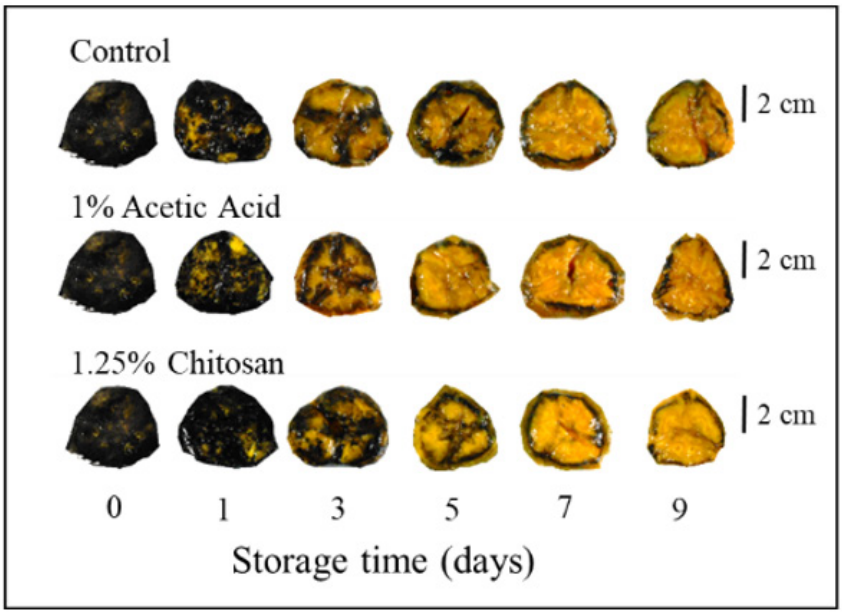


Figure 2. Changes of the physical characteristics of uncoated (control), 1% acetic acid-coated, and 1.25% chitosan-coated *Musa troglodytarum* fruits during ripening. (a) Changes on the peel and pulp colors, (b) Weight loss (significant at $P \leq 0.05$), (c) Pulp to peel ratio (significant at $P \leq 0.05$). Error bars indicate standard deviation (SD) (n = 3)

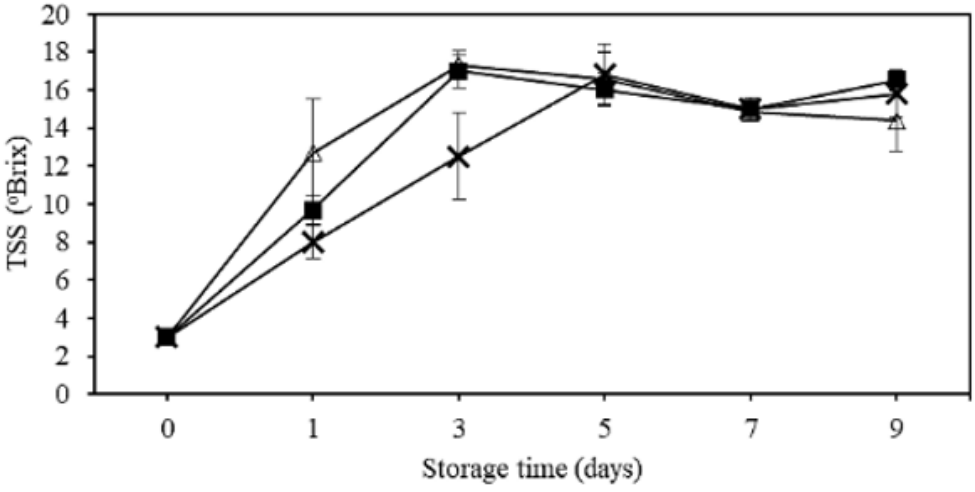
ethylene (do Nascimento et al., 2006). The effect of chitosan coating on delay of starch degradation were also reported on 'Berangan' (Maqbool et al., 2011) and Cavendish bananas (Lustriane et al., 2018).

Total Soluble Solids (TSS). The effects of coatings on total soluble solids (TSS) content during ripening are shown in Figure 3b. Generally, all treatments showed an increase of TSS until day 3 or day 5, and then followed by constant values until day 9. TSS on 1.25% chitosan-coated fruit was lower ($P \leq 0.05$) than control and 1% acetic acid-coated fruit from day 0 until day 3. However, TSS was not significantly different ($P \leq 0.05$) between all treatments on day 5 until day 9. This result showed that chitosan coating decreased TSS values of *M. troglodytarum* fruit until day 3.

Total Soluble Solids, which consists of soluble sugar and organic acids, increases in fruit pulp during ripening (Dadzie & Orchard, 1997). It is caused by starch degradation into soluble sugar (do Nascimento et al., 2006) and production of organic acids (McGlasson & Wills, 1972). In chitosan-coated fruit, there is a decrease in



(a)



(b)

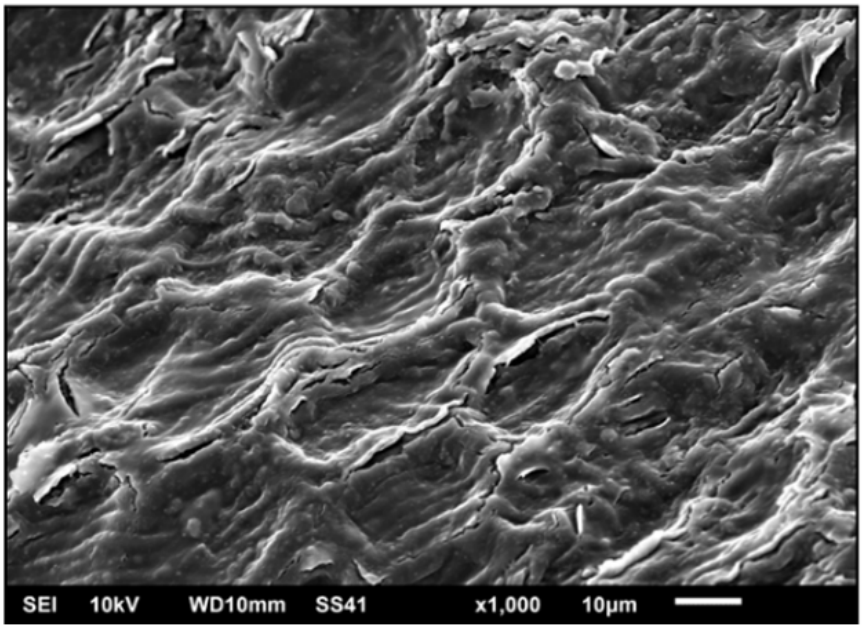
Figure 3. Changes on the chemical characteristics of uncoated (control), 1% acetic acid-coated, and 1.25% chitosan-coated *Musa troglodytarum* fruits during ripening. (a) Changes of the starch pattern on the pulp, (b) Total soluble solids of the pulp (significant at $P \leq 0.05$). Error bars indicate standard deviation (SD) (n = 3)

internal O_2 (Ali et al., 2011). Low O_2 content decreases soluble sugar concentrations (Ali et al., 2011) and Krebs cycle related organic acids content (citrate, malate, glutamate, and aspartate acid) (McGlasson & Wills, 1972). The effect of chitosan coating on the decrease of TSS had also been reported on papaya (Ali et al., 2011) and 'Berangan' banana (Malmiri et al., 2011).

According to physical and chemical analysis, chitosan-coated fruit shows delay on its ripening compared with control and acetic acid-coated fruits which show no delay. Meanwhile, acetic acid-coated fruit shows no significant differences with control, except for the pulp to peel ratio, which may be caused by the damaging effect on the peel by acetic acid as described by Du et al. (1997). Therefore, chitosan coating and control coating are used for fruit surface microstructure and quantitative PCR analysis.

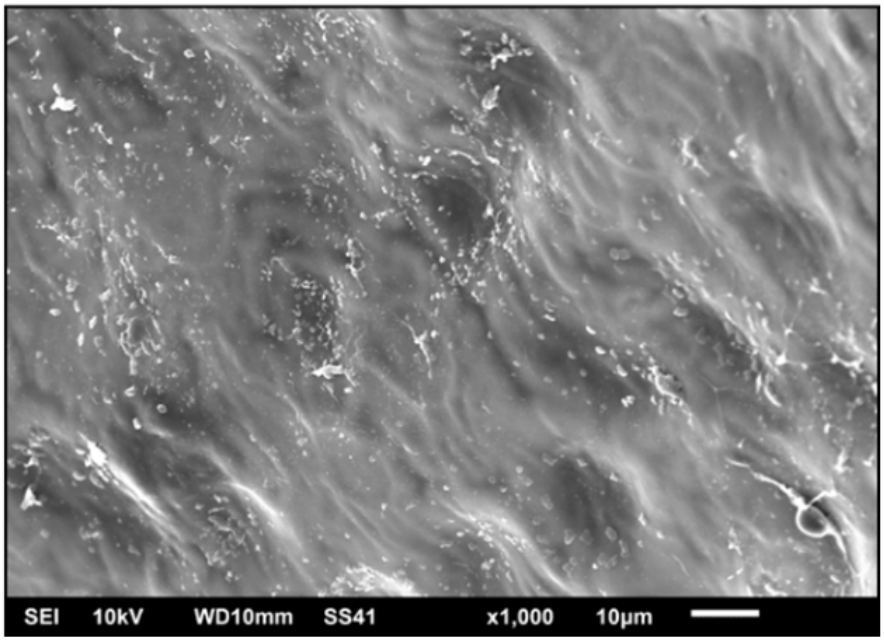
Fruit Surface Microstructure Analysis

Surface microstructure of uncoated (control) and 1.25% chitosan-coated *M. troglodytarum* fruits at day 1 of storage are shown in Figure 4. We used this stage to examine the thin film formation on the chitosan-coated fruit peel surface after one day of the coating process and compared it with the uncoated fruit (control). The microstructure of uncoated fruit showed porous surface and epidermal cells clearly. Meanwhile, chitosan-coated fruit surface was coated with a film; hence the pores and the epidermal cells were covered. Chitosan



(a)

Figure 4. SEM micrographs of *Musa troglodytarum* peel surface at day 1 of fruit storage (1,000x zoom) (a) Uncoated (control) fruit peel surface,



(b)

Figure 4. SEM micrographs of *Musa troglodytarum* peel surface at day 1 of fruit storage (1,000x zoom). (b) The 1.25% chitosan-coated fruit peel surface, which shows a thin film formation

coating formed a thin film which provided a barrier against gas and water, thus modifying internal O_2 and CO_2 in the fruit tissues (Xing et al., 2016). This condition decreased respiration and ethylene biosynthesis rate, hence decreasing the decay rate, peel color change, pulp to peel ratio, TSS, and delayed starch degradation of chitosan-coated *M. troglodytarum* fruits, which were also observed on chitosan-coated Cavendish bananas (Lustriane et al., 2018).

Sensory Quality Evaluation

The effects of coatings on sensory quality are shown in Table 2. According to panelists, the chitosan-coated fruit had lower ($P \leq 0.05$) aroma and taste quality compared with control on day 7. Meanwhile, uncoated and coated fruits showed no significant differences ($P \leq 0.05$) on overall acceptability on days 5, 6, and 7. These results indicated that chitosan coating could only keep the overall fruit quality, but not the aroma and taste quality on the last day of storage. This might have happened since the chitosan film was too thick and resulted in CO_2 accumulation in the fruit tissues, hence inducing anaerobic respiration and ethanol production which might have affected the fruit aroma and taste on the last day of storage (Xing et al., 2016).

Table 2

Sensory quality evaluation of coated and uncoated Musa troglodytarum *L. fruits at days 5, 6, and 7 of storage at 27±0.7°C*

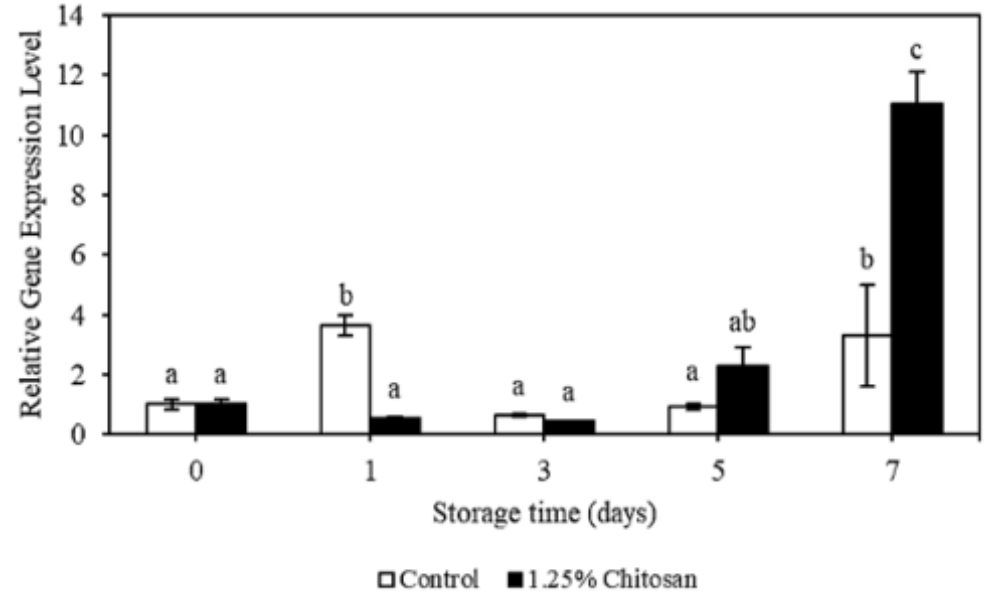
Parameter	Storage Time (Days)	Treatment		
		Control	1% Acetic Acid	1.25% Chitosan
Aroma	5	4.48 ± 0.60 a	4.50 ± 0.53 a	4.23 ± 0.75 a
	6	3.98 ± 1.07 a	4.06 ± 1.22 ab	4.15 ± 1.17 a
	7	4.13 ± 0.87 a	3.88 ± 0.65 ab	3.29 ± 0.76 b
Taste	5	4.42 ± 1.00 a	4.35 ± 0.83 a	4.44 ± 0.76 a
	6	4.46 ± 1.03 a	4.81 ± 0.92 a	4.50 ± 1.33 a
	7	4.40 ± 0.96 a	4.02 ± 1.04 ab	3.44 ± 1.11 b
Overall Acceptability	5	3.71 ± 1.77 a	3.44 ± 1.41 a	3.77 ± 1.11 a
	6	3.50 ± 1.45 a	3.00 ± 1.53 a	3.81 ± 1.25 a
	7	3.58 ± 1.15 a	3.29 ± 0.86 a	3.69 ± 1.26 a

Note. Scales 1 to 7 (1 = extremely dislike, 7 = extremely like). Mean values ± standard deviation in the same column with a same letter are not significantly different ($P \leq 0.05$) (n = 3)

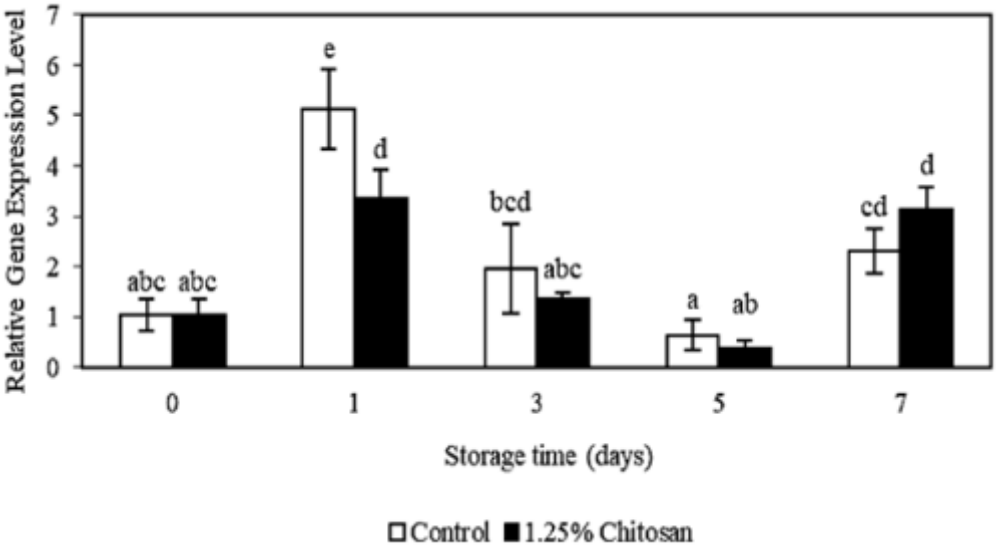
Expression Analysis of *ACS1* and *ACO1*

The effect of chitosan coating on *ACS1* and *ACO1* expression of *M. troglodytarum* fruits are presented in Figure 5. In this analysis, only uncoated (control) and chitosan-coated samples were used, since the physical and chemical analysis result of control and acetic acid-coated samples showed no significant differences. On uncoated banana (control), *ACS1* and *ACO1* expression level increased until day 1, decreased on day 3 and day 5, then increased again on day 7 although it was smaller than day 1. This expression pattern is also found on the other banana as biphasic peaks and considered as a unique pattern among banana (Lustriane et al., 2018; Pathak et al., 2003). Furthermore, the biphasic expression level pattern on *ACS1* is concomitant with biphasic ethylene production and respiration rate (Pathak et al., 2003). However, Pathak et al. (2003) explained that the mechanism on this biphasic pattern was still not understood and needed to be explored.

ACS1 and *ACO1* expression level was lower ($P \leq 0.05$) on chitosan-coated fruit compared with control on day 1. However, there were no significant differences ($P \leq 0.05$) between control and chitosan-coated fruit on day 3 and day 5. On day 7, *ACS1* expression level on chitosan-coated fruit was higher ($P \leq 0.05$) than control, while *ACO1* expression level showed no significant differences ($P \leq 0.05$) compared with control. This suggested that 1.25% chitosan coating suppressed the expression



(a)



(b)

Figure 5. Chitosan coating effect on (a) *ACS1* and (b) *ACO1* expression level of *Musa troglodytarum* fruit pulp during storage at 27±0.7°C for 7 days. Bars marked with a same letter are not significantly different ($P \leq 0.05$). Error bars indicate standard deviation (SD) (n = 3)

of *ACS1* and *ACO1* in the early ripening stage (day 1) and increased *ACS1* expression in the late ripening stage (day 7).

Chitosan coating caused the decline of ethylene production rate on banana (Maqbool et al., 2011). This condition may affect the suppression of *ACS1* and *ACO1* expression level on the early ripening stage since ethylene influenced *ACS* and *ACO* expression (Liu et al., 1999; López-Gómez et al., 1997). The same expression pattern was also observed in Cavendish banana (Lustriane et al., 2018). Low *ACS1* and *ACO1* expression level from day 1 to day 3 might decrease ethylene production, hence slowing the changes in peel and pulp color, pulp to peel ratio, starch content, and TSS on chitosan-coated fruits in the early ripening stage (day 1 until day 3).

The rise of *ACS1* and *ACO1* expression level on day 7 (Figures 5a and 5b) might be caused by the physical barrier effect of chitosan film against ethylene diffusion (Xing et al., 2016). Consequently, internal ethylene might be trapped and accumulated inside chitosan-coated fruits at the late ripening stage. Ethylene was autocatalytic at climacteric period, which means the additions of ethylene would trigger ethylene biosynthesis (McMurchie et al., 1972). Therefore, ethylene accumulation on the last ripening stage (day 7) may trigger *ACS1* and *ACO1* expression and more ethylene production, causing the peel and pulp color, pulp to peel ratio, starch content, and TSS on chitosan-coated fruit to show no differences compared to control on the last day of storage. The increase of *ACS1* expression level at the late ripening stage was also observed on chitosan-coated Cavendish banana (Dwivany et al., 2018).

On day 7, *ACS1* expression level on chitosan-coated fruits increased dramatically compared with control. However, *ACO1* expression level also raised but showed no significant differences compared with control. This might be due to low O_2 level caused by the physical barrier from chitosan coating. *ACO* is a gene encoding ACO enzyme that converts ACC to ethylene with the presence of O_2 (Xu & Zhang, 2015). Therefore, low O_2 might cause the ACO activity and *ACO1* expression level to be lowered and showed no sharp increase compared with *ACS1* expression level. Moreover, *ACS1* showed higher mRNA accumulation in the ripening process and ethylene presence than *ACO1* as reported by Liu et al. (1999). Measurement of ethylene production should be conducted to clear this mechanism.

CONCLUSION

Chitosan coating could be an alternative postharvest technology with a potential to prolong *M. troglodytarum* ('Pisang Tongkat Langit') shelf-life, because it maintained several postharvest qualities, i.e. peel and pulp condition, pulp to peel ratio, starch content, and TSS in the early ripening stage. Chitosan coating also affected fruit ripening at the molecular level, i.e. suppressed *ACS1* and *ACO1* gene expression in the early ripening stage. As compared with Cavendish banana, chitosan coating (concentration

1.25%) also prolonged the banana shelf-life until the eleventh day of storage and suppressed *ACS1* and *ACO1* gene expression (Lustriane et al., 2018).

Further gene expression analysis related with *M. troglodytarum* ethylene biosynthesis, signaling, and perception, and fruit ripening is required to reveal the mechanism clearly. In the future, chitosan coating can be developed and modified with the addition of plasticizer and natural antimicrobial compounds to get more optimal result in delaying *M. troglodytarum* fruit ripening.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Bali Bananas (*Musa* spp. L.) Genetic Relationship Based on Internal Transcribed Spacer 2 (ITS-2)

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ABSTRACT

Banana is one of the most essential commodities in Bali island. It is not only for nutrition sources but also for cultural and religious aspects. However, Bali banana genetic diversity has not been explored; therefore, in this study, we focused on its genetic relationship using a molecular approach. This research aimed to determine the genetic relationship of Bali banana cultivars using the internal transcribed spacer 2 (ITS-2) region as a molecular marker. A total of 39 banana samples (*Musa* spp. L.) were collected from Bali island. The ITS-2 DNA regions were then amplified and sequenced from both ends. ITS-2 sequences were predicted using the ITS2 Database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/). The multiple sequences alignment was performed using ClustalX for nucleotide-based tree and LocARNA to

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provide the secondary structure information. Phylogenetic trees were constructed using neighbor-joining (Kimura-2-parameter model, 1,000 bootstrap). The result showed that two clades were formed, one clade was abundant in A genome (AA and AAA), and the other rich in the B genome (BB and ABB). This result suggested that cultivars that had similar genomic compositions tended to be grouped within the same clade and separated with different genomic compositions. This study gives perspectives that ITS-2 sequences in bananas are quite similar and differ much compared to other families. Secondary structure has been described to provide more robust resolving power in phylogenetic analysis.

Keywords: Genetic diversity, ITS-2, phylogenetic tree, secondary structure

INTRODUCTION

Banana is one of the most important crops, with 100 million metric tons being produced annually in 130 tropical and subtropical countries (Pillay & Tenkouano, 2011). It was reported that bananas were initially derived from naturally crossing of bananas in Papua New Guinea and Eastern Indonesia, then differentiated and spread to Southeast Asia and finally throughout the world (Nelson et al., 2006). In Indonesia, banana is a valuable commodity with the highest biodiversity throughout the country (Hapsari et al., 2017). Particularly on the island of Bali, in which most of its inhabitants are Hindus, bananas have pivotal roles in the procession aspects of the customs, culture, and religious ceremonies. Not only in quantity, but the diversity of bananas is also crucial for the people of Bali, especially for custom processions and offerings (Rai et al., 2018).

DNA barcoding is a method used in species identification using standard areas of DNA in an organism. This method uses short molecular markers in organism DNA and is considered promising to identify species of an organism and determine their relationship with one another. The molecular markers used should be variable enough and enable closest species identification but need to be conservative to simplify PCR amplification and alignment of sequences among distant species. One type of molecular marker used universally is the internal transcribed spacer (ITS) (Zhang et al., 2015).

Most of the banana accessions are polyploid (diploid or triploid) and believed that various cultivars originated from intra- and inter-specific hybridizations between wild bananas of *Musa acuminata* (A genome) and *Musa balbisiana* (B genome) (De Langhe et al., 2009). To date, the immense diversity of banana cultivars on the island of Bali has not been fully explored, especially in the aspect of genetics and molecular biology. To the best of our knowledge, there are some molecular studies on banana in Indonesia (Hapsari et al., 2018; Meitha et al., 2020). However, there was no report on banana molecular relationships in Bali. As one of the most popular markers in phylogenetics, ITS-2 sequences are considered accurate owing to their conserved nucleotides. As a means of

identification by using the DNA barcoding method, ITS-2 can supplement the use of morphological characteristics analysis (Guzow-Krzemińska & Węgrzyn, 2000).

ITS is a region located between the ribosomal rDNA locus. This region is often used by researchers to determine biodiversity and molecular relationship, especially in plants. The molecular marker of the ITS region is chosen because it has several advantages. The ITS area is relatively short (at around 200 bp) and has a high-copy-number in the genome, making it easier to be isolated, amplified, and analyzed (Poczai & Hyvönen, 2010). ITS-2 region, the primary source of variations in the ITS sequence, is shorter and easier to be sequenced. Besides, the ITS-2 region exhibits significant sequence variability at the species level or lower (Yao et al., 2010). Therefore, the ITS-2 region has been considered as an excellent phylogenetic marker and a promising standard region for barcode DNA (Zhang et al., 2015). The purpose of this research is to determine the genetic variability of banana cultivars in Bali Island using the ITS-2 region as a molecular marker.

METHODS

Tissue Collection and Preservation

We collected thirty-nine cultivars of bananas from nine regencies in Bali, as presented in Table 1 and Figure 1 was visualized according to area coordinates, as shown in Table 2. Cultivars were sampled one replicate by cutting approximately 10 cm of the cigar leaves with a sterile blade and stored in a 50 mL conical tube filled with 2 g silica gel (SiO_2) to reduce the humidity. Once arrived in Bandung, liquid nitrogen was used to preserve the cigar leaves and leaves were stored in -80^{o}C before used.

Table 1
Banana cultivars collected from Bali island

No.	Cultivar	Locality	No.	Cultivar	Locality
1.	Kayu	Gianyar	11.	Sobo	Klungkung
2.	Sabe Macan	Klungkung	12.	Buah	Karangasem
3.	Mas Bali	Gianyar	13.	Ketip Kerta	Gianyar
4.	Sangket	Klungkung	14.	Poh	Gianyar
5.	Keladi	Klungkung	15.	Bali	Gianyar
6.	Tulang	Gianyar	16.	Lumut	Gianyar
7.	Kepok Tanjung	Klungkung	17.	Ambon Kuning	Gianyar
8.	Rojo Molo	Klungkung	18.	Mas Marlin	Gianyar
9.	Tembaga	Gianyar	19.	Nangka	Gianyar
10.	Gancan	Klungkung	20.	Ketip Tulang	Gianyar

Table 1 *(Continued)*

No	Cultivar	Locality	No	No Cultivar	Locality
21.	Klutuk	Denpasar	31.	Bile	Buleleng
22.	Padi	Gianyar	32.	Ketip Sari	Jembrana
23.	Muli Kuning	Gianyar	33.	Raja Bulu	Gianyar
24.	Kaiki	Karangasem	34.	Susu Hijau	Denpasar
25.	Siam	Gianyar	35.	Panjang	Gianyar
26.	Kapal	Gianyar	36.	Tanduk	Jembrana
27.	Bunga	Karangasem	37.	Seribu	Klungkung
28.	Cavendish	Gianyar	38.	*Musa velutina*	Gianyar
29.	Kepok	Gianyar	39.	*Musa ornata*	Gianyar
30.	Kole	Gianyar			

Table 2

Locations of sample collection with the village of each regency and city

Regency/City	Village	Coordinate of Global Positioning System
Denpasar	Penatih	8°36'28.79"S, 115°14'14.87"E
Gianyar	Guwang	8°36'39.23"S, 115°17'23.82"E
	Celuk	8°36'5.59"S, 115°15'53.85"E
	Kerta	8°19'49.64"S, 115°17'32.69"E
Klungkung	Besan	8°31'5.73"S, 115°27'5.85"E
	Pesinggahan	8°32'58.88"S, 115°27'27.02"E
Karangasem	Rendang	8°25'41.02"S, 115°25'7.79"E
Bangli	Jehem	8°24'54.90"S, 115°22'15.16"E
Buleleng	Anturan	8°9'12.42"S, 115°3'4.93"E
Badung	Abiansemal	8°32'15.60"S, 115°12'51.66"E
Tabanan	Marga	8°25'51.67"S, 115°10'34.55"E
Jembrana	Bading Kayu	8°25'24.13"S, 114°54'59.59"E

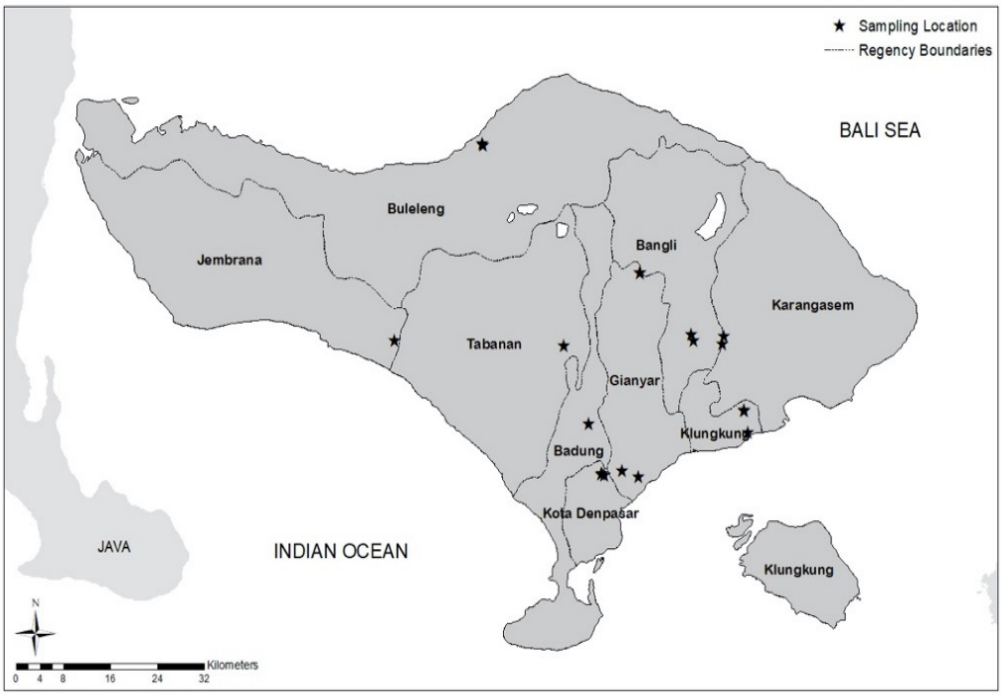

Figure 1. Sampling locations of banana cultivars in Bali island with stars showing the geographical position of Penatih, Guwang, Celuk, Kerta, Besan, Pesinggahan, Rendang, Jehem, Anturan, Abiansemal, Marga, and Bading Kayu area

DNA Isolation and Amplification of ITS-2 Region

Thirty-nine (39) samples had been collected and each sample had one replicate. All samples were ground using mortar and pestle with the help of liquid nitrogen, which was poured periodically until the sample became powdery fine. The DNA was isolated using the modified Doyle and Doyle method, by multiple washing and cleaning process using Isopropanol (Doyle & Doyle, 1991). The quality and quantity of DNA isolates were measured using NanoDrop™ spectrophotometer. The 260/280 ratio was measured to check the quality and quantity of the DNA in which TE buffer was used as a solvent. Gene amplification was performed by polymerase chain reaction (PCR) method (initial denaturation temperature 94°C for 5 minutes, annealing temperature of 55°C for 30 seconds, and elongation temperature of 72°C for 10 minutes; with 40 denaturation cycle) (Meitha et al., 2020). The 50 µL of PCR reaction comprised 5 µL of DNA samples and mixed with 25 µL of GoTaq® Green Master Mix (serial number: AF9PIM712 1016M712, Promega Co., USA), which consisted of DNA Polymerase, Reaction Buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400

μM dCTP, 400 μM dTTP, and 3mM $MgCl_2$ (Promega Co., USA); 15 μL of nuclease-free water; and 2.5 μL of each of the primary ITS-S2F and ITS-S3R. The primer sequences used in the reaction were ITS-S2F: 5'-ATGCGATACTTGGTGTGAAT-3' and ITS-S3R: 5'-GACGCTTCTCCAGACTACAAT-3' (Gu et al., 2013). The PCR results were confirmed using electrophoresis (agarose 1%, TAE 1x buffer, 70V-30 minutes). The PCR amplified products were sequenced using Sanger sequencing platform via Macrogen Inc., South Korea. Samples that have been successfully sequenced then used to make contig using the CodonCode Aligner application (http://www.codoncode.com/aligner/).

Prediction of ITS-2 Secondary Structure and Phylogram Construction

The secondary structure ITS-2 region was predicted using ITS2 Database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) with E-value cut off less than 1e-16 and model possessing the highest transfer helices while as for motif prediction, E-value less than 0.01 and Viridiplantae model were opted (Keller et al., 2009; Koetschan et al., 2009). Multiple ITS2 sequences were aligned with ClustalX algorithm (Sievers & Higgins, 2014), within an integrated program, namely SeaView 4 (Gouy et al., 2010). Consensus representation of multiple sequence alignment was illustrated using WebLogo 3 (http://weblogo.threeplusone.com/), and the probability of the bases are shown in bits unit (Crooks et al., 2004). Logo consensus was manually juxtaposed to its motif prediction to depict the conserved nucleotides better.

We added other closely related plants of *Musa* (*Ensete ventricosum*, *Ensete glaucum*, and *Musella lasiocarpa*) to inquire further whether the ITS-2 sequences among them are conserved but adequately robust to different group species within Musaceae family. To provide a broader illustration of plant evolution and systematics through phylogeny, different ITS-2 sequences of various plants from 4 families were designated as operational taxonomical units (OTUs). Those group of plants collected from ITS2 Database were Strelitziaceae (*Strelitzia reginae* and *Strelitzia alba*), Poaceae (*Hordeum vulgare*, *Zea mays*, and *Orzya sativa*), Brassicaceae (*Brassica rapa*, *Arabidopsis thaliana*, and *Arabidopsis lyrata*), and Fabaceae (*Medicago cretace* and *Medicago polyceratea*) (Koetschan et al., 2009). ITS-2 rRNA sequences of Bali bananas have been uploaded to GenBank and can be freely accessed, whose accession numbers are MN718989-MN719027.

Phylogram construction comprised multiple sequence alignment, tree inference, and visualization. Firstly, ITS-2 (51 OTUs) sequences were aligned with ClustalX software (Sievers & Higgins, 2014). The tree was inferred using neighbor joining (NJ) with Kimura-2-parameter (K2P) model and 1,000 bootstrap disturbances using SeaView program (Gouy et al., 2010). To compare, we managed to construct a phylogram using the same tree inference method enriched with secondary structure

information of ITS-2 sequence using Clustal algorithm coupled with LocARNA program (Will et al., 2012). Last, trees were visualized and annotated using FigTree 1.4.4 (Rambaut, 2018).

RESULTS AND DISCUSSION

Amplification of ITS-2 Area

The ITS-2 region amplification produced fragments with a size of ~500 bp. The amplified products did not only consist of the ITS-2 region, which based on the literature had a length of 205 bp to 227 bp (Hřibová et al., 2011), but also the 5.8S rDNA region and the 28S rDNA region which were the flanking sequences of the ITS-2 region (Yao et al., 2010). This was not a problem in constructing the phylogeny of ITS-2 due to a trimming process that was conducted following ITS-2 annotation. The visualization of the amplification results can be seen in Figure 2.

ITS-2 Secondary Structure

We evaluated the secondary structure of ITS of *M. acuminata* (A genome donor) and *M. balbisiana* (B genome donor), as shown in Figure 3. A variety of molecular and evolution mechanisms have resulted in ITS-2 that can be distinguished based on their secondary structure. The most striking differences observed were the internal loop size and the extra loop in helix I in B genome (Figure 3B) compared to the A genome (Figure 3A). We also found that from the alignment of Bali bananas represented in logo consensus, the first motif of U-U mismatch (II left) was the least conserved motif among the other three motifs.

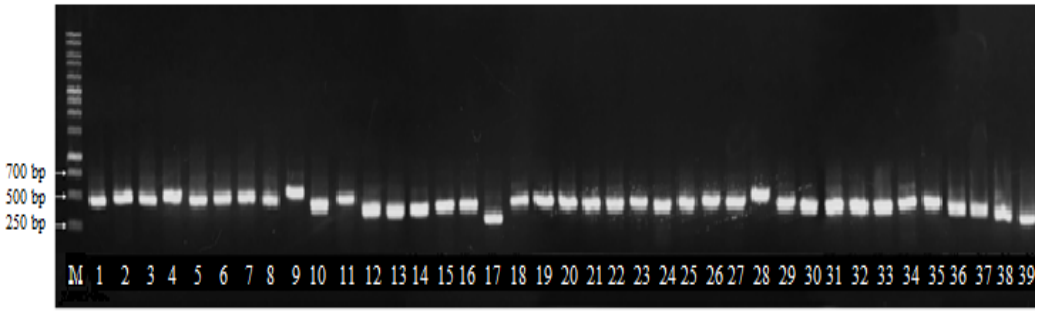

Figure 2. Electrophoregram of DNA amplicons of 5.8S rDNA, ITS-2, and 28S rDNA of 37 banana cultivated varieties, *Musa ornata*, and *Musa velutina* with no band shearing: M = 1 kb DNA marker ladder, 1 = Kayu, 2 = Sabe Macan, 3 = Mas, 4 = Sangket, 5 = Keladi, 6 = Tulang, 7 = Raja Molo, 8 = Tembaga, 9 = Gancan, 10 = Sobo, 11 = Buah, 12 = Ketip Kerta, 13 = Poh, 14 = Bali, 15 = Lumut, 16 = Ambon Kuning, 17 = Mas Marlin, 18 = Nangka, 19 = Ketip Tulang, 20 = Klutuk, 21 = Padi, 22 = Muli Kuning, 23 = Kaiki, 24 = Siam, 25 = Kapal, 26 = Bunga, 27 = Cavendish, 28 = Kepok, 39 = Kole, 30 = Bile, 31 = Ketip Sari, 32 = Raja Bulu, 33 = Susu Hijau, 34 = Panjang, 35 = Tanduk, 36 = Seribu, 37 = Kepok Tanjung, 38 = *Musa ornata*, and 39 = *Musa velutina*. The bands are shown to have lengths of 500 bp, hence the flanking sequences were trimmed to produce the ITS-2 sequence only

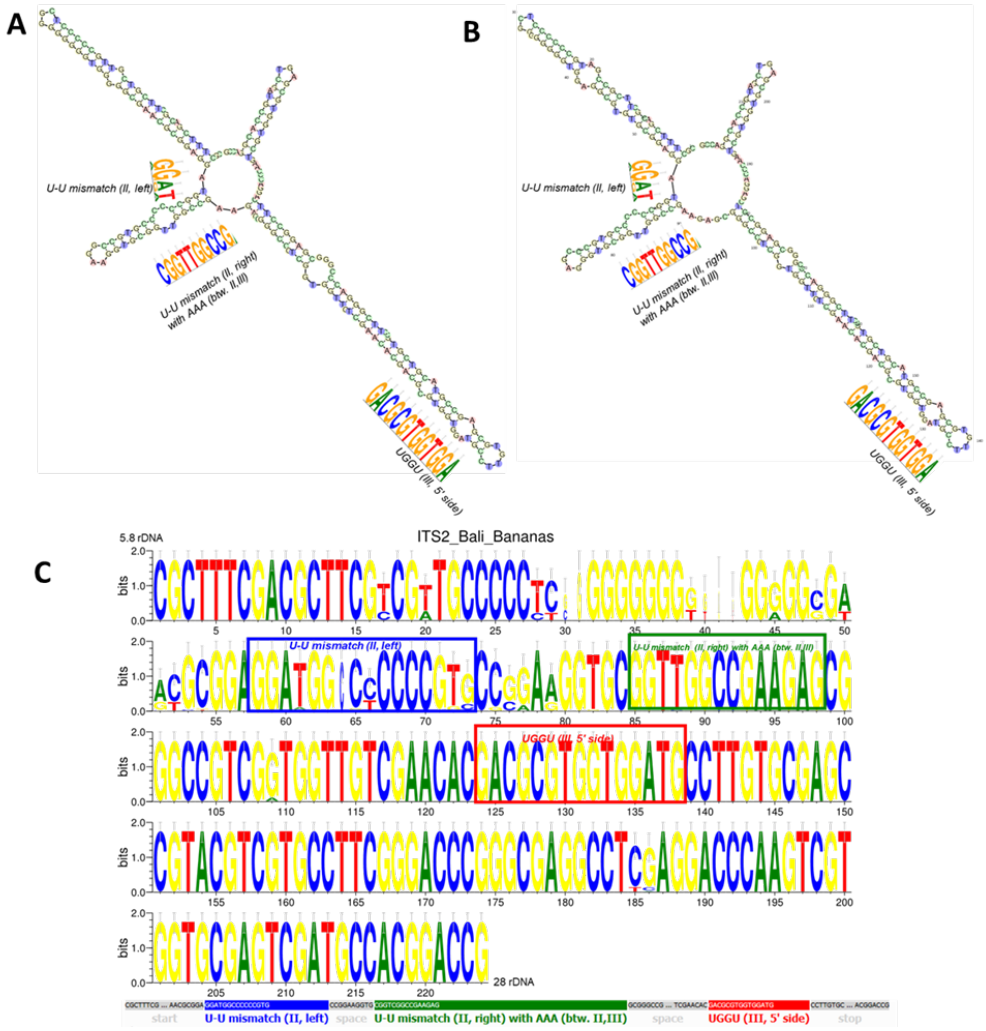

Figure 3. Secondary structure and motif prediction of ITS-2: A) secondary structure of ITS-2 predicted from A genome banana, B) secondary structure of ITS-2 predicted from B genome banana, and C) consensus of ITS-2 sequences of 32 banana cultivars represented in nucleotides logo (C = blue, G = yellow, T = red, A = green). The probability of each nucleotide is represented in bits unit. The 224 bp ITS-2 is paired with the motif prediction comprises start, two spacers, three motifs (represented in boxes outlined with blue, green, and red colors), and stop sequence

ITS-2 functional secondary structure is proven to be conserved in plants DNA (Hershkovitz & Zimmer, 1996; Jobes & Thien, 1997). ITS-2 spacer had four unique helices structure, as shown in Figure 4, and the analysis of the two-dimensional structure proved that the structure was highly conserved throughout the eukaryotes (Schultz et al., 2005). Helix II is characterized by the content of pyrimidine-pyrimidine nodules. Helix III characterized as the longest and containing the most conserved primer on 5' includes TGGT (UGGU sequences for RNA) sequence (Schultz et al., 2005). As for helix, I and IV are more diverse, and only helix II and III that could be identified in general to all eukaryote (Coleman, 2007). The functions of ITS-2 as spacer are largely unknown, and ITS-2 sequences are not incorporated into the mature rRNA. Nevertheless, they encode signals for the rRNA transcripts processing properly (Hillis & Dixon, 1991). This inferred function is itself dependent on secondary structure of the ITS RNA.

Phylogenetic Tree Analysis

Standard multiple sequence alignment programs, such as MAFFT (Katoh & Standley, 2013) or T-Coffee (Notredame et al., 2000), cannot be used to construct a phylogenetic tree by including the secondary structure information. Rather, these tools use virtually similar approaches based on nucleotides information such as similarity. Despite significant variations in nucleotides, the ITS-2 secondary structure has been described to be highly conserved, possessing four helices and motifs (Schultz et al., 2005). As an alternative, combining multiple loci of gene markers with the ITS-2 will provide a better substitute to infer a phylogenetic tree. The demerit of using additional loci is that it is time-consuming and can be costly; furthermore, adding nucleotides information does not guarantee the truest evolutionary history (Huelsenbeck et al., 1996).

To provide a comparison, we inferred trees using two alignments: nucleotides information-based and one with additional secondary structure information, as shown in Figure 4.

To analyze the genetic variability among *Musa* species and the family of Musaceae, we also studied other families of Strelitziaceae, Poaceae, Brassicaceae, and Fabaceae. Coupled with additional OTUs of *Ensete* and *Musella* species, this strategy hopefully can shed light in terms of ITS-2 usefulness. Particularly, it may provide a depiction of major and minor classification in plants. Based on the same taxa of families, both phylograms, as shown in Figure 4, showed a consistent clade clustering. Our analyses showed the monophyletic origin of Musaceae comprised *Musa*, *Ensete*, and *Musella* species, this result is concordant to previous analysis (Hřibová et al., 2011). Musaceae is shown to closely relate to Strelitziaceae as they are the group in order of Zingiberales. Given two types of strategies in constructing the phylogenetic tree, the secondary structure is far more conserved in comparison with the nucleotide sequences. The genera of *Ensete* and *Musella* are not shown as outgroups

Figure 4. Phylograms constructed from the ITS-2 region from banana cultivars and other plants from other families (Strelitziaceae, Poaceae, Brassicaceae, and Fabaceae): A) tree inferred using NJ (neighbor-joining) with no information of secondary structure, B) NJ tree constructed from the information of nucleotide sequences and secondary structure. Numbers on the branches show bootstrap value, and the terminal nodes represent species name in which genome composition for banana cultivars are shown. The numbers following non-*Musa* spp. are the GI accession of ITS-2 that were retrieved from the database

within *Musa* clade in the tree constructed from nucleotide and secondary structure information (Figure 3B).

The monophyletic group of subclass of commelinids, known as the core of monocots, has been described as a closely related group based on molecular analysis (Barrett et al., 2013). The group of comelinids, such as corn (*Zea mays*), barley (*Hordeum vulgare*), and paddy (*Oryza sativa*) are shown topologically close to bananas. Not only they have extant similar phenotypic characteristics, but they also have a close molecular relationship based on ITS-2 rDNA analysis. Liliopsida (monocots) groups are shown as distant relatives meaning a greater variance above taxa of families was observed.

The drawback of using the ITS-2 rDNA marker in phylogenetic analysis is that ITS-2 may be heterogeneous. This characteristic can be understood by its presence in plant species and polyploid ones as multiple divergent ITS sequences in several loci. ITS-2 sequences have been used in a wide range of taxonomy analysis ranging from order to subspecies while other barcoding markers such as *rbcL* (gene encoding ribulose biphosphate carboxylase large chain) and (mitochondrial DNA) mtDNA have narrower ranges (Coleman, 2003). Although their versatility of usefulness, ITS-2 sequences appeared to be less conserved (differ too much) in major groups or higher taxa (Baldwin et al., 1995). We found that this claim could be well-conceived as we evaluated the alignment of different families, particularly in the subdivision of dicots. Thus, the strategy used in choosing an alignment approach remained a challenging task.

One of the intricate comprehensions in phylogenetics is polytomy, a clade of OTUs that are not formed a series of two way-splits. Commonly, this phenomenon appears as a result of unresolved phylogeny owing to the limitedness of phylogenetic software (Slowinski, 2001). This is not the case when it comes to several ITS-2 region clusters. Our data showed that some banana cultivars have 100% identity, such as in the B genome group, and appeared as a polytomy. Subsequently, we reconstructed the trees by excluding non-banana plants as well as ornamental bananas (*M. velutina* and *M. ornata*) (Figure 5).

The phylogenetic trees in Figure 5 show that the OTUs based on ITS-2 were clustered according to their families in which banana cultivars were grouped into two clades indicating their dominant genome composition: A genome and B genome. This clade described the genome composition of the banana cultivar. The first clade contained banana cultivars that had the genome AA, AAA, and AAB. In the second clade, banana cultivars had the genomic composition of ABB and BB. In conclusion, ITS-2 region can be used to classify banana species rather than banana cultivars. However, the closely related *M. velutina* and *M. ornata* made the clustering analysis seemed complicated (Figure 4). Constructed trees of ingroup edible bananas (nucleotide information only and one added with secondary structure information) seem to have similar topology

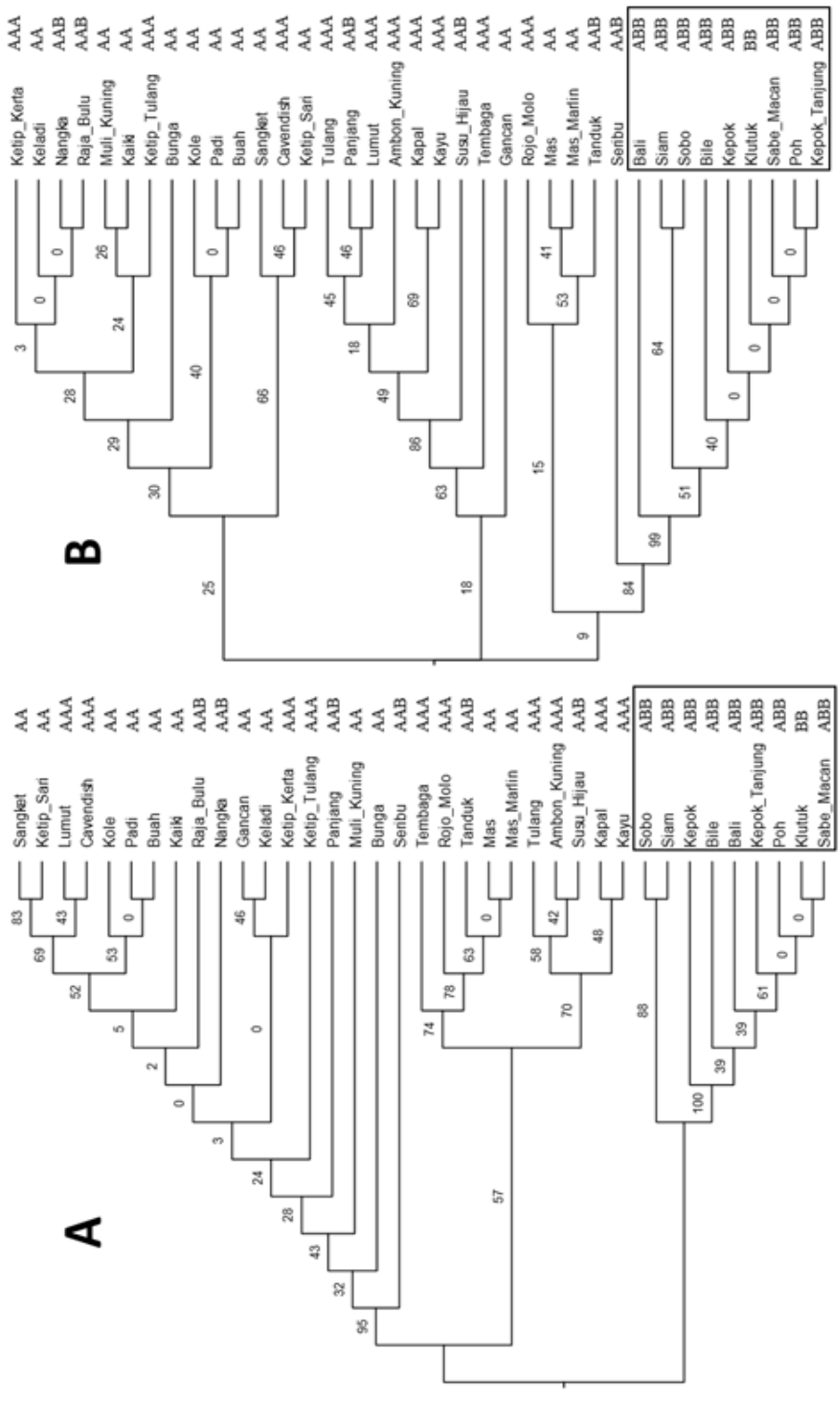

Figure 5. Dendograms of edible banana cultivars inferred from ITS-2 sequences: A) tree constructed using NJ and B) tree constructed using NJ with secondary structure information attained from LocARNA software

and cluster (Figure 5). Both strategies were able to group bananas based on their genome composition even though the B genome group was clearly separated in the former strategy.

In terms of Bali bananas diversity, it is also imperative to be concerned about the cultivation and conservation of useful cultivars such as 'Kayu AAA' and 'Bile ABB' cultivar (also known as 'Haji' cultivar in Nusa Tenggara). 'Kayu' cultivar possesses distinctive value since the variety is only cultivated in Bali island, generally harnessed for offering in ceremonies (Rai et al., 2018).

Being one of the most diverse cultivars resulting from several wild species crossing, bananas have more than thousand of cultivars. Molecular barcoding such as this is beneficial to evaluate the diversity of bananas regarding their chromosomal genome character. Further analysis of multiple ITS-2 sequence types origin in terms of evolutionary mechanisms, and their utilization can shed some light on genome relationship and polyploid ancestry (Bailey et al., 2003; Dadejová et al., 2007). Banana cultivars that have a relative proportion of similar genome compositions are placed in the same clade. Thus the molecular relationship of them is closer than that of a banana cultivar with a different relative proportion of genomic compositions. By juxtaposing two phylograms, we observed that additional information from a secondary structure could provide a stronger resolving power in phylogenetic analysis rather than just solely relying on nucleotide information.

CONCLUSION

This study showed that from thirty-nine banana samples from Bali island, two clades were formed, one clade was abundant in A genome (AA and AAA), and the other rich in the B genome (BB and ABB). This ITS-2 study also suggested that cultivars with similar genomic compositions would tend to be grouped within the same clade.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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BRI1 Signaling in the Root is Mediated through the SERK1 and SERK3 Co-receptors

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ABSTRACT

Brassinosteroid (BR) is a class of polyhydroxysteroids plant hormones known to regulate shoot and root growth. Genetic and molecular analyses demonstrate that receptor kinase BRI1 protein acts as a perceiver for BR. One of the characteristics of *bri1* mutant's phenotypes is the complete BR insensitivity in the root. Biochemical evidences of the BRI1 protein complex indicates that somatic embryogenesis receptor kinase 1 (SERK1) and SERK3 participate in the BR pathway in *Arabidopsis* root. While only *serk3* mutants show partial reduction to BR sensitivity, *serk1* presents a normal BR penetration phenotype compared to the wild type. Interestingly, the double mutant *serk1serk3* displays more, but not full resistance to BR in root length assay. In this study, we aimed to enhance the BR insensitivity of the double mutant *serk1serk3* by crossing *serk1* mutant allele with a strong *serk3* and *bri1* mutant alleles. In our study, by generating *serk1-3serk3-2* double mutants, a complete insensitivity to BR that phenocopied *bri1-301* mutant was recorded. However, we were unable to increase BR resistance in the root of *serk1-3serk3-2* double mutant by crossing with *bri1* mutant allele in the triple mutant *serk1-3serk3-2bri1*. As a result, all the BRI1 signaling in the root was mediated through the SERK1 and SERK3 co-receptors. Additionally, we established that based on conventional BR assays, the At1g27190 protein was also involved in BR signaling. Preliminary data indicated that the triple mutant *serk1serk3-2At1g27190* showed a dwarfed phenotype. Whether or not this dwarfed phenotype is linked to BRI1 signaling impairment needs to be further investigated.

Keywords: At1g27190, brassinosteroid, brassinosteroid insensitive, somatic embryogenesis receptor kinase

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INTRODUCTION

Brassinosteroids (BRs) are essential regulators of plant growth and development affecting a broad spectrum of processes at the molecular, cellular, and physiological levels (Fan et al., 2016). The importance of BR in plant development is illustrated by the dramatic phenotypes of *bri1* (brassinosteroid insensitive) mutant plants failing to produce or perceive BR (Bücherl et al., 2013). These *bri1* mutants are characterized by short roots, dwarfed stature, male sterility, rounded leaves, and photo-morphogenetic defects (Kang et al., 2017; Sun et al., 2017). Major progresses in understanding the BR signaling cascade have been performed (Belkhadir & Chory, 2006; Belkhadir & Jaillais, 2015). However, considering the highly pleiotropic defects of the *bri1* mutants and the ubiquitous expression of *bri1* gene, it remains to be established how BR specificity can be achieved in this signaling system (Hutten et al., 2017).

Some models suggest the involvement of interacting partner proteins for BRI1. A gain-of-function screen identified a leucine rich repeat-receptor like kinase (LRR-RLK) protein, somatic embryogenesis receptor-like kinase 3 (SERK3) (Brandt & Hothorn, 2016; Santiago et al., 2013; Sun et al., 2013), which hetero-dimerizes with BRI1 (Russinova et al., 2004). Since the formation of the SERK3-BRI1 complex is BR-dependent (Kinoshita et al., 2005; Wang et al., 2005), SERK3 is suggested to play a key role in downstream signaling events in BR-dependent manner. Another line of biochemical evidences also indicates that SERK1 contributes to the BR pathway (Albrecht et al., 2012; Karlova et al., 2006; van Esse et al., 2012). SERK1 and SERK3 are parts of a large family of 13 SERK-like receptors with 5 closely related members (SERK1 to SERK5) (Liu et al., 2020). The mild phenotypes of the single mutants *serk3* and *serk1* indicate that other SERK members could participate in the BRs signaling as well (Zheng et al., 2018). These findings have shed lights that spatial and temporal expression of SERK-proteins might be responsible for the specificity of the BRs signaling (van Esse et al., 2016).

Due to the redundancy in the SERK protein family, it remains debatable whether all responses of the BRI1 signaling are mediated through SERKs receptors (van Esse et al., 2013). Using a set of double, triple and quadruple mutants within the subfamily SERK1 to SERK5, the results exhibit that only *serk1* enhances the BR insensitivity of *serk3* mutant roots. However, data also indicates that SERK1 and SERK3 partially account for the BR insensitivity root phenotype, i.e. the double *serk1serk3* mutant does not show complete insensitivity as *bri1* mutants do (Albrecht et al., 2008).

There have been several hypotheses to explain the various penetrations of BR sensitivity phenotype in the root. Firstly, *serk3* mutant allele used in the previous studies is not the strongest allele (Albrecht et al., 2008, 2012; Kemmerling et al., 2007). Secondly, there could be other co-receptors than the SERK are involved in BR signaling cascade. We identified in the SERK3 and SERK1 protein complex, another LRR-

RLK, At1g27190 (unpublished data). Finally, an alternative BRI1 pathway that does not utilize of the SERK co-receptors is also proposed.

In our study, we established that the level of BR insensitivity was dependent on which *serk3* mutant allele used in the generation of double mutant *serk1serk3*. Our report also depicted that based on conventional brassinolide (BL) assays, the At1g27190 protein was involved in BR signaling pathway. We also showed that the triple mutant *serk1-1serk3-2at1g27190* revealed a dwarf phenotype. Determining this dwarf phenotype linked to BRI1 signaling impairment needs to be further investigated.

MATERIALS AND METHODS

Plant Growth Conditions

Unless otherwise specified, plant materials used in this study were *Arabidopsis thaliana* ecotype Columbia as the wild type control. Freshly harvested seeds sterilization were carried out as previously described (Lindsey III et al., 2017). Once the sterilized seeds were collected, they were germinated on 0.5x Murashige and Skoog medium (Duchefa) contained with 1% sucrose. After vernalized for 2 days at 4^{o}C, seeds were transferred to growth chamber with the following conditions: 22^{o}C, light intensity of 130-150 $Em^{-2}s^{-1}$, 16:8h, light: dark photoperiod and relative humidity of 80% as previously described conditions (Junker et al., 2015). Subsequently, 10 day-old seedlings were transplanted to soil and grown under the same environment conditions. For the selection of transgenic seedlings, 0.5x Murashige and Skoog medium (Duchefa) supplemented with 1% sucrose and 15mg L^{-1} phosphinothricin (PPT; Duchefa) were used.

For the hypocotyl length and root length experiment with or without BR, after vernalization for 2 days, the seeds were vertically kept in the growth chamber in the darkness for 5 days, or in the light for 7 days, respectively. Every experiment was biologically repeated three times. Images were analyzed by ImageJ software.

PCR-based Genotyping

The *serk1-1*, *serk3-1* (Kemmerling et al., 2007; Russinova et al., 2004), *bri1-301*, and *bri1-19* were previously described (Alonso et al., 2003; Zhang et al., 2018). PCR genotyping for single, double, and triple mutants was performed with published primers combinations (Albrecht et al., 2005, 2008).

Gene Cloning and Plant Transformation

For the production of BRI1-GFP construct, the full length *BRI1* gene cDNA were cloned with iProof™ high-fidelity DNA polymerase and then fused to *green fluorescent protein* (*GFP*) gene by primers engineered with *Nco*I restriction sites.

To produce the *BRI1* promoter construct, a 2-kb region upstream of the start codons of the *BRI1* gene was cloned in the pGEM®-T vector (Promega Corporation) with iProof™ high-fidelity DNA polymerase. The pGEM-T cloned promoters were inserted via *Sal*I-*Nco*I in a modified pBluescript

vector containing the *GFP* gene inserted as *Nco*I-*Bam*HI fragment in front of the T*nos* terminator. The entire open reading frames of *BRI1* gene as described above were then inserted as *Nco*I fragments. The resulting full cassettes were then sub-cloned into a modified pFluar vector via *Sal*I-*Sma*I (Stuitje et al., 2003). These constructs were further referred to as PBR1: BRI1-GFP.

These constructs were verified by sequencing and were electroporated in *Agrobacterium tumefaciens* strain C58C1 containing a disarmed C58 Ti plasmid (Koncz et al., 1989). The constructs were transformed into the *serk1-3serk3-2* mutant backgrounds by the floral-dip method (Clough & Bent, 1998). All the primers were designed and provided by Catherine Albrecht, Laboratory of Biochemistry, Wageningen University.

RESULTS AND DISCUSSION

Generation of the Double Mutant *serk1-3serk3-2* and *serk1-3serk3-2bri1-301*

Based on root length assay, it was reported that the *serk1-1* was weaker allele than the *serk1-3*, while both alleles showed a similar male sterile phenotype (Albrecht et al., 2008). Similarly, the *serk3-1* allele was weaker than the *serk3-2* allele in innate immunity assays but not in BR-related assays (Chinchilla et al., 2007; Kemmerling et al., 2007). Furthermore, although the double mutant *serk3-1serk4* displayed a viable dwarf stature, the double mutant *serk3-2 serk4* was lethal. These data clearly indicated that the different *serk1* and *serk3* mutant alleles showed various level of penetrance of BR sensitivity. Additionally, we also theorized that the level of BR resistance could be increased by incorporating *bri1* mutant allele in the double mutant *serk1-3serk3-2*. As a result, the double mutant *serk1-3serk3-2* and triple mutant *serk1-3serk3-2bri1-301* were generated. We further analyzed the BR-related phenotypes of these two mutant lines in the root inhibition assay.

BR-related Phenotypes of the Double Mutant *serk1-3serk3-2* and *serk1-3serk3-2bri1-301*

Root length assay was performed to test whether the BR sensitivity of the double mutant *serk1-3serk3-2* and triple mutant *serk1-3serk3-2bri1-301*. Seedlings were grown vertically with different BR concentrations under the light. The root lengths were subsequently photographed and analyzed. Although the *serk1-3* single mutant did not show a reduced BR sensitivity (Albrecht et al., 2008), an increase in BR sensitivity even at high BR concentrations (100nM) could be observed in the double mutant *serk1-3serk3-2*, which phenocopied the BL response of the *bri1-301* mutant (Figure 1) (Greene et al., 2003). It was also noteworthy the triple mutant *serk1-3serk3-2bri1-301* did not enhance the BR insensitivity. Although we could observe a strong BR-related phenotype in the root, we did not record any increased dwarfism in the *serk1serk3* double mutant as compared to the *serk3-2* single mutant. Taken together, these data clearly showed that the double mutant *serk1-3 serk3-2* enhanced the BR insensitivity.

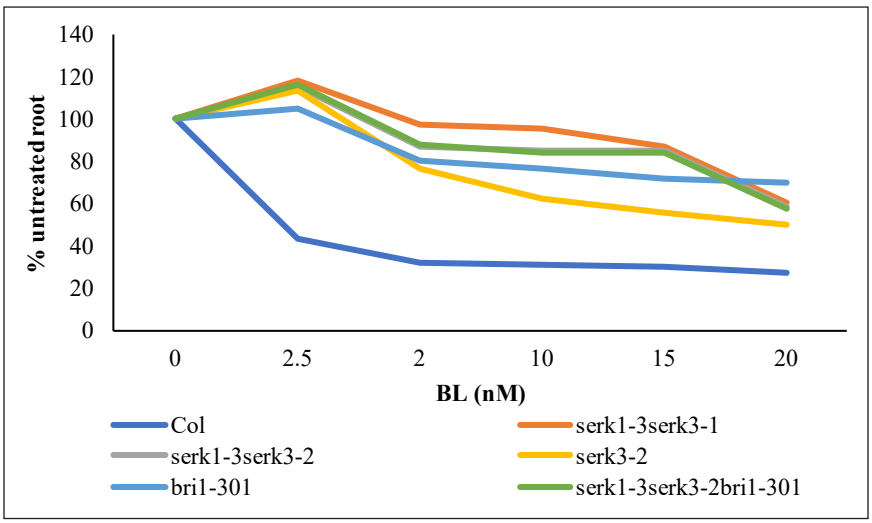

Figure 1. Root length assay of *bri1-301* and *serk3-2* single mutants, *serk1-3* in combinations with *serk3-1*, *serk3-2*, and *serk3-2bri1-301* backgrounds. Wild type *Col* serves as control treatment (*Note*. BL = Brassinolide)

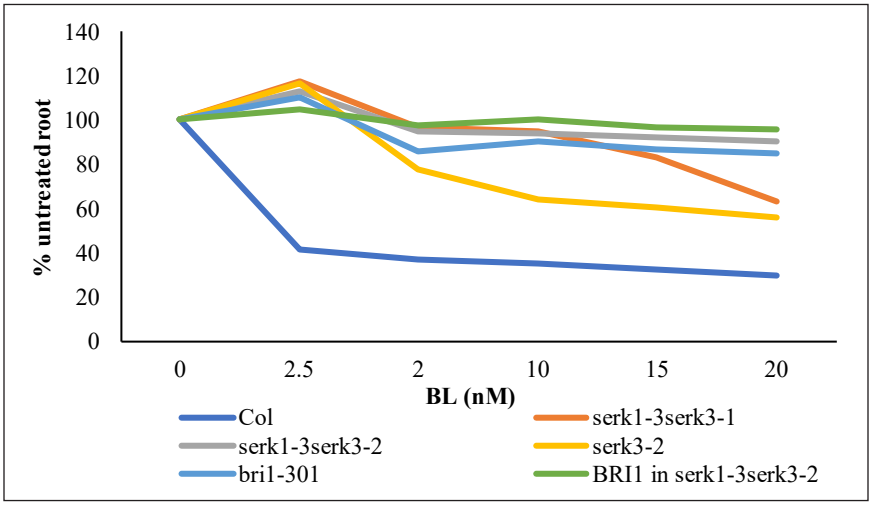

Figure 2. Root length assay of *bri1-301* and *serk3-2* single mutants, transgenic *pBRI1::BRI1* in *serk1-3serk3-2* background, and *serk1-3* in combinations with *serk3-1* and *serk3-2* backgrounds. Wild type *Col* serves as control treatment (*Note*. BL = Brassinolide)

An Alternative SERK-independent BRI1 Pathway

It has been shown that BRI1 protein can operate in the homo-dimeric state, thus suggesting an alternative BRI1 pathway that would operate without the SERK co-receptors. This would imply that it is possible to partially or fully rescue a *serk1serk3* mutant phenotype by supplementing a transgene *BRI1* in a *serk1serk3* mutant background. As a result, the *serk1serk3* strong double mutant was crossed with *BRI1*

overexpressing line. The *BRI1/serk1serk3* lines were subsequently analyzed in the root inhibition assay. We used the overexpressing *BRI-GFP* line and the *bes1-D* mutant line, which showed an overexpression BR phenotype due the dominant positive mutation in the BES1 transcription factor, as controls. Our data presented that *BRI1/ serk1serk3* lines displayed BR insensitivity (Figure 2). This indicates that in the root the BRI1 signaling is mediated through the SERK1 and SERK3 co-receptors.

Characterization of the *At1g27190* Mutants

Our protein mass spectrometry experiments demonstrated that *At1g27190* participated in the same complex with BR1, SERK1, and SERK3 (data not shown). Consequently, mutant line of *At1g27190* gene was obtained for characterization. The mutant *At1g27190* lines illustrated indistinguishable phenotypes compared to the wild type. Since PCR genotyping indicated that the T-DNA inserted in the *At1g27190* gene was located in kinase region, the *At1g27190* mutant gene would encode a non-functional protein lacking approximately 200 amino acids of the kinase domain (unpublished data).

We then proceeded to cross *At1g27190* mutant line with the *serk* mutants to observe any enhancement in reported *serk* mutant phenotypes. Double, triple mutants were developed by crossing the *At1g27190* mutants with *serk1-1serk2-1serk3-1* and *serk1-1serk2-1serk3-2* triple mutants. Based on the genotyping and phenotyping results, double mutants *serk1-1 at1g27190*, *serk3-2 at1g27190*, *serk3-1at1g27190*, triple mutants *serk1-1serk3-1at1g27190* demonstrated an indistinguishable phenotype from the wild type. However, triple mutants *serk1-1 1serk3-2 at1g27190* exhibited dwarf phenotype (Figure 3). Whether *At1g27190* acts in synergy with the SERK co-receptors and enhances the phenotype of the *serk1serk3* double mutant needs further confirmation.

Figure 3. Phenotypic analysis of 12-week-old *Arabidopsis thaliana* used in this study. (A) wild type; (B) *serk1-1*; (C) *serk3-2*; (D) *serk1-1 at1g27910*; (E) *serk3-1 at1g27910*; (F) *serk3-2 at1g27910*; (G) *serk1-1 serk3-1at1g27910*; (H) *serk1-1 serk3- 2at1g27910*, respectively.

BR-related Phenotypes of the *At1g27190* Mutants

As a first step to evaluate the involvement of *At1g27190* in BR signaling pathway, two different alleles of single mutant *At1g27190 BIL3-N616632* and *BIL3-632078*, were tested in the root length assay and the hypocotyl assay. In the root length assay, the *At1g27190* mutants showed the same level of BL insensitivity as the *serk3-1* (Figure 4) at low (2.5 to 10nM) concentrations of BL. In the hypocotyl length assay, it was previously reported that the *serk3* mutant alleles, *serk3-1* and *serk3-2*, had shorter hypocotyls than the wild type when grown in dark (Li et al., 2002; Nam & Li, 2002). In our study, the dark-grown hypocotyls of the *At1g27190* mutants were decreased compared to the control wild-type plants (Figure 5).

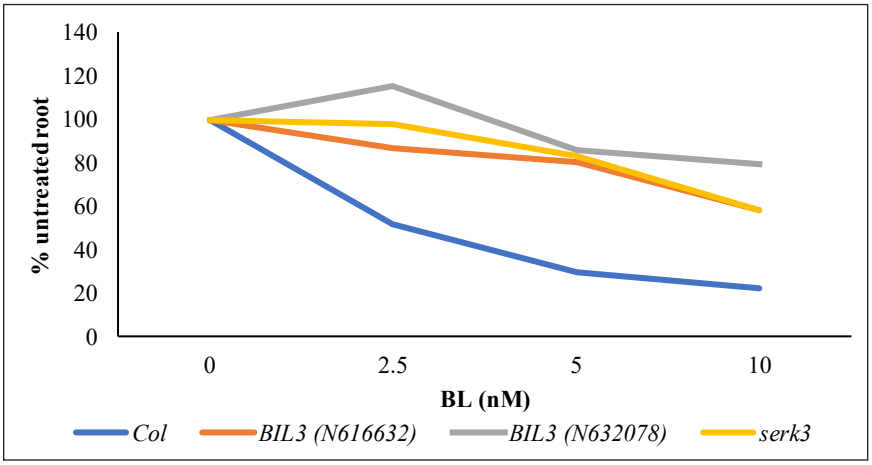

Figure 4. Root length assay of *At1g27190* T-DNA tags. Wild type *Col* serves as control treatment (*Note*. BL =Brassinolide)

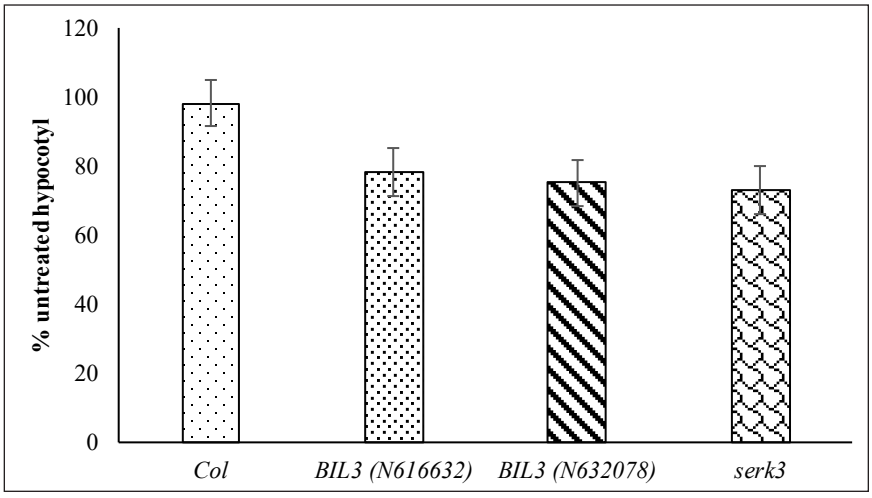

Figure 5. Hypocotyl length assay of *At1g27190* T-DNA tags. Wild type *Col* serves as control treatment

CONCLUSION

Our data indicated that the double mutants *serk1-3serk3-2* and *serk1-3serk3-2-bri1-301* depicted a complete BR insensitive phenotype in the root comparable to that of a *bri1* mutant. As a result, we propose that all the BRI1 signaling in the root is mediated through the SERK1 and SERK3 co-receptors. Our report also depicted that based on conventional BL assays, the At1g27190 protein was involved in brassinosteroid signaling and the triple mutant *serk1-1serk3-2at1g27190* revealed a dwarf phenotype. Determining this dwarf phenotype linked to BRI1 signaling impairment needs to be further investigated.

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Effects of Diets Containing *Asyastasia gangetica* and *Brachiaria decumbens* on Intake, Digestibility and Growth Performance of Growing Rabbits

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ABSTRACT

The beneficial effects of feeding rabbits with forages have been recommended. However, limited study has been made on feeding values of locally available forages in rabbits. Therefore, an experiment was conducted to evaluate the effect of *Asyastasia gangetica* and *Brachiaria decunbens* on intake, digestibility, and growth performance of rabbits. Twelve rabbits were distributed into three diets: (i) 100% commercial pellet as control (T1), (ii) 50% pellet plus *Asyastasia gangetica ad libitum* (T2), and (iii) 50% pellet plus *Brachiaria decumbens ad libitum* (T3). Daily feed intake, nutrient digestibility, weekly body weight, and feed conversion ratio (FCR) were measured. Intakes of total dry matter (DM) (121.2-134.3 g/d) and organic matter (OM) (115.1-132.5 g/d) were similar (p>0.05) for all the groups. The crude protein (CP) intake of rabbits fed with T1 (10.1 g/d) and T3 (9.6 g/d) diets was similar (p>0.05), but lower (p<0.05) than T2 (14.1 g/d) diet. Ether extract intake of rabbits fed with T2 (2.9 g/d) and T3 (3.9 g/d) diets was similar (p>0.05), but lower (p<0.05) than T1 (5.1 g/d) diet. Neutral detergent fibre (NDF) intake was higher (p<0.05) for rabbits fed with T3 (55.8 g/d) diet followed by T2 (41.7 g/d) and T1

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(31.7 g/d) diets. There were no differences (p>0.05) on the digestibilities of DM, OM, and CP among treatments. Total weight gain and daily weight gain were higher (p<0.05) for rabbits fed with T1 (568 and 11.0 g) and T2 (468 and 9.0 g) diets than T3 (155 and 3.3 g) diet, respectively. The lowest FCR was obtained with T1 (12.3) and T2 (13.9) diets, whereas the highest was obtained with T3 (30.3) diet. In conclusion, diet containing *Asyastasia gangetica* showed more benefits in terms of CP and NDF intakes, weight gain and FCR than the diet containing *Brachiaria decumbens*. A combination of concentrate and *Asyastasia gangetica* is recommended as a partial replacement for concentrate in rabbit production.

Keywords: *Asyastasia gangetica*, *Brachiaria decumbens*, digestibility, growth performance, intake, rabbit

INTRODUCTION

Rabbit farming is gaining popularity in smallholder farmers as an alternative source of animal protein. Rabbit is considered as one of the suitable high quality animal proteins in developing countries (Owoleke et al., 2016). Smallholder farmers get faster benefits from rabbit farming compared to other farming systems (e.g., cattle farming), because rabbits require low investment, show short generation interval and have the ability to consume forage grasses. For profitable rabbit production, feed is considered as one of the most important inputs. When rabbits receive feeds containing required amount of energy and protein, they can provide better quality meat as a healthy diet for human consumption. Production of high quality meat at lower prices is achievable when locally available forage plants are included in rabbit's diet.

Safwat et al. (2014) stated that many forages could be used in ration formulation by replacing the costly protein sources. It depends on the forages' chemical composition, viability, palatability, and anti-nutritional factors. Rabbits fed leaves of browse plants and concentrate showed better performance than those fed tropical grasses with concentrate as reported by Amata and Okorodudu (2016). Adigun et al. (2014) also reported that 66% of wheat offal could be replaced with *Asyastasia gangetica* leaf meal as a fibre source in the diet of rabbit.

Asyastasia gangetica could be used as a substitute for legumes as reported by Adetula (2004). It is considered as suitable protein and mineral sources for goats (Khalil, 2016). Because of its ability to fast-grow naturally, resulting in low production cost, and high nutritive value, *A. gangetica* is utilised as forage for ruminants in South-East Asia; it is either grazed or cut for stall feeding (Gopal et al., 2013). Besides having these attributes, this plant can tolerate shade well. It grows prolifically in Malaysia especially at shaded and plantation areas (Chuku et al., 2018). Most of the farmers also feed their livestock with forages like *Brachiaria decumbens*. In Malaysia, *B. decumbens* is being used as a main plant in farming pasture and it is the most favored species for grazing ruminants (Low, 2015).

However, level of concentrate offered to rabbits should not be below 50% (50g) when fed with bracharia hay as recommended by Iyeghe-Erakpotobor et al. (2006), and poor utilisation of bracharia hay in their study was observed especially when offered low concentrate level.

However, there is little information about the effect of these locally available forage grasses on rabbit performance. It is important to know about the nutrient status of above mentioned forages as it can support the production and growth of rabbits. Therefore, this study was conducted to compare the feeding effect of two local tropical forage grasses (*Brachiaria decumbens* and *Asyastasia gangetica*) with supplementation of concentrate on intake, nutrient digestibility, growth performance, and feed conversion ratio of weaned rabbits.

MATERIALS AND METHODS

Study Site and Experimental Design

An experiment with rabbits was carried out in rabbit house located at Agro Techno Park, Universiti Malaysia Kelantan (UMK), Jeli campus, Kelantan. All animal handling and procedures were approved by the UMK Animal Care and Use Ethics Committee (UMK/FIAT/ACUE/UG1/2018). The average daily temperature and monthly rainfall were 28^{o}C and 260 mm during the experimental period (July – October 2019).

The experiment was conducted for 69 days (10 days as an adaptation period, 52 days as a growth trial and 7 days as a collection period) using twelve unsexed weaned mixed breed rabbits, about 2 months of age, which were obtained from local supplier. Before data collection, rabbits with an initial average body weight (BW) of 1080.8±275.4 g were adapted to the new enviroment for 10 days, when only commercial pelleted compound feed were offered them on *ad libitum* basis (140 g/head/d). During adaptation period, the average daily pellet intake was 116.7±11.4 g/head, which was about 11.0% of initial their BW. Based on these data, all rabbits with an average BW of 1175.3±287.8 g were divided into three dietary groups consisting of 4 rabbits of each: (i) commercial pelleted compound feed on *ad libitum* (11.0% of their BW) basis which was served as control (T1), (ii) half of the control (5.5% of their BW) plus *Asyastasia gangetica* on *ad libitum* (75 g dry matter/head/d) basis (T2), and (iii) half of the control (5.5% of their BW) plus *Brachiaria decumbens* on *ad libitum* (83 g dry matter/head/d) basis (T3). Throughut the experimental period, the quantities of offered pellet (for T1), *Asyastasia gangetica* (for T2) and *Brachiaria decumbens* (for T3) were adjusted weekly to ensure at least 20% refusals. The quantities of offered pellet for T2 and T3 treatments were determined for individual rabbit on the basis of their BW, and adjusted weekly to account for BW changes. The pellet and grasses were offered in separate feeders. Water was supplied *ad libitum* in automatic pipe drinkers.

Both types of grass were harvested at pre-flowering stage daily in the morning from the experimental field of Agro Techno Park, UMK, and fed to rabbits as fresh basis. The commercial pelleted compound feed

prepared by feed manufacturing company (Perniagaan Hasbin Jaya, Pulau Pinang, Malaysia) was purchased from a local supplier,. The feeds were offered to rabbits twice in the morning (8:00 am) and again in the afternoon (4:00 pm).

Approximate ME from daily offered pellet and grasses for T1, T2, and T3 diets were 2490.4, 2650.7, and 2266.3 kcal/kg DM, respectively. Similarly, approximate CP from daily offered pellet and grasses for T1, T2, and T3 diets were 7.5, 11.8, and 7.8%, respectively. The ME and CP values in rabbit's diet should be 2025 kcal/kg DM and 16.0%, respectively, to meet the requirements of a growing rabbit, according to National Research Council (NRC) (1977). It seems that the quantities of energy in the diets were more than required, but the CP contents in the diets were low. It was hypothesized that rabbits could take the rest of the CP from daily offered *ad libitum* pellet or grasses.

The ingredients used in the diets and their chemical compositions are shown in Table 1. Daily feed offered and refusals were recorded to estimate feed intake, and their samples were taken once a week for determining dry weight. Before feeding, rabbits were weighed at the beginning of the experiment, at 1-week interval and at the end of the experiment. Average daily gain and feed conversion ratio (FCR) were calculated to see the difference between the treatments on rabbit's performance as described by Biobaku et al. (2003). The FCR was calculated using dry matter (DM) intake by dividing the body weight (BW) gain. During the collection period, samples of offered feeds, refusals and faeces were collected and stored in a freezer. At the end of collection period, the collected faeces samples from each rabbit were thawed and mixed together to get a representative sample.

Chemical Analysis

Samples of feeds and faeces were analysed to determine the DM, crude protein (CP), ether extract (EE), and ash contents according to Association of Official Analytical Chemists (AOAC) (2000). Neutral detergent fibre (NDF) of feeds was determined following the method of Van Soest et al. (1991).

Statistical Analysis

All data were analysed through one-way ANOVA using SPSS software. They were compared between treatments using Duncan's multiple range test (DMRT) at $p<0.05$.

RESULTS AND DISCUSSION

Proximate Components

Asyastasia gangetica and *Brachiaria decumbens* contained 15.5 and 20.9% dry matter (DM), 85.7 and 92.2% organic matter (OM), 15.9 and 8.1% crude protein (CP), 1.4 and 2.8% ether extract (EE), 45.9 and 63.1% neutral detergent fibre (NDF), as well as 14.3 and 7.8% ash, respectively. The commercial pelleted compound feed contained 85.1% DM, 98.6% OM, 7.5% CP, 3.8% EE, 23.6% NDF, and 1.4% ash (Table 1). The DM content of the commercial pellet

was higher than those of the forages. The CP content of the commercial pellet was similar with the value of *B. decumbens* but not with *A. gengetica* which had a higher value (15.9%). *Asyastasia gangetica* also showed higher ash contents than the value of *B. decumbens*. In contrast, the *B. decumbens* contained comparatively higher DM, OM, EE, and NDF contents than the respective values of *A. gangetica* (Table 1). The CP content in *A. gangetica* in this study was lower than the findings of Adigun et al. (2014), who reported that the leaf meal from *A. gangetica* contained 19.3% CP. This variation may have resulted due to the use of different aged plants, because the nutrient contents in plants can be varied by plant maturity (Suhaimi et al., 2017). Besides, the differences in climate, rainfall and temperature of studied area are considered as factors that contribute in variation of CP content in plants.

Asyastasia gangetica contained 15.9% CP in this study, making it suitable to replace conventional protein sources in rabbit's diet. Owoleke et al. (2016) reported that CP needed by rabbit was about 16-18%, but 15-16% was also recommended for growing rabbit. In another study, Cheeke (1987) suggested that adult rabbits required about 12% of CP, 14% of crude fibre, and 2% of EE daily. This means that *A. gangetica* contains lower EE (1.4%) than the daily EE requirment of rabbits.

Suhaimi et al. (2017) reported that the CP content of *B. decumbens* was about 9-20%; and this value declined as the plant aged. Thus, the low CP content of *B. decumbens* found in this study (Table 1) may have resulted due to the usage of aged plants. Similarly, the NDF content in *B. decumbens* in this study was lower (63.0 vs. 69.2%) than the findings of Silva et al. (2016) respectively, which can

Table 1

Chemical composition (%) of the feed ingredients

Nutrients	Commercial pellet	*Asystasia gangetica*	*Brachiaria decumbens*
Dry matter	85.1	15.5	20.9
Organic matter	98.6	85.7	92.2
Crude protein	7.5	15.9	8.1
Ether extract	3.8	1.4	2.8
Neutral detergent fibre	23.6	45.9	63.1
Ash	1.4	14.3	7.8
Metabolisable energy (kcal/kg dry matter)[β]	2490.4	2800.6	2076.9

Note. [β] Data obtained from the secondary data (Biobaku et al., 2003; Sobayo et al., 2012; Suhaimi et al., 2017)

also be explained due to the differences in plant maturity and location of studied areas. Besides other nutrients, NDF is also important in rabbit's diet; the NDF value of commercial pellet was much lower than those of the forages, which is a positive trait as excess NDF content (>60%) can affect negatively on total DM intake in animals (Mertens, 1997). Nevertheless, rabbit has the ability to utilise the NDF efficiently due to the presence of microorganisms in their caecum.

The ash content of commercial pellet was lower than those of the forages. The ash content in *A. gangetica* was much higher than the *B. decumbens*, which can provide more minerals to the animal compared to other feed ingredients used in this study. When formulating rabbit's diet, the percentage of all nutrients must be taken into account to fulfill the nutrient requirments of rabbit.

Dry Matter and Nutrients Intakes

The feeding effect of *A. gangetica* and *B. decumbens* on daily intakes of DM and other nutritional components by rabbits is given in Table 2. Rabbits fed with T3 diet showed higher daily forage DM intake than those fed with T2 diet, whereas there was no difference on daily pellet DM intake between rabbits fed with T2 and T3 diets as their diet of commercial pellet was the same. However, replacement of half of commercial pellet by offering of test forages on *ad libitum* basis did not affect (p>0.05) the daily total DM and OM intakes by rabbits, which suggests that test forages have potential to be used as feed in rabbit's diet. This result is in agreement with Amata and Okorodudu (2016), who stated that 50:50 mixtures of concentrate (maize) and forage (*Centrocema pubescens*) led to maximum performance in rabbit. The optimum total DM and OM intakes in this study were achieved when rabbits fed with test forages *ad libitum* in combination with 50% concentrate. A certain ratio of these forages to concentrate may give optimum rabbit performance that could lead to reduced feed cost.

Rabbits fed with T2 diet showed significantly (p<0.05) higher (14.1 g/d) CP intake followed by rabbits fed with T1 (10.1 g/d) and T3 (9.6 g/d) diets, whereas no influence of CP intake was observed between rabbits fed T1 and T3 diets. This may be explained due to the lower CP contents in both commercial pellet and *B. decumbens* compared to *A. gangetica* (Table 1). Thus, *B. decumbens* can be considered as an unsuitable protein source in rabbit's diet as supply of plenty of T3 diet had no influence on rabbit's CP intake.

Khalil (2016) reported that *A. gangetica* was a suitable complementary protein and mineral source for goat. Therefore, it is also a good complementary feed for rabbit because of high CP value. Nowadays, chicken and beef meat are being replaced with rabbit meat in human diet because of its high quality protein; nevertheless rabbits require adequate amount of protein in their diet for maintenance and growth.

Rabbits fed with T1 diet showed higher (5.1 g/d) EE intake followed by T3 (3.9 g/d) and T2 (2.9 g/d) diets, which

Table 2

Feed intake and digestibility of nutritional components by rabbits fed commercial pellet with or without Asystasia gangetica *and* Brachiaria decumbens

Parameter	Treatment			*p*-value
	T1	T2	T3	
Intake (g/d)				
Grass DM	0.0^{c} ±0.0	62.0^{b} ±4.0	68.9^{a} ±4.0	0.000
Pellet DM	134.3^{a} ±27.0	53.8^{b} ±7.1	52.3^{b} ±3.1	0.000
Total DM	134.3 ±27.0	115.8 ±9.4	121.2 ±4.9	0.360
Total OM	132.5 ±26.6	107.5 ±9.1	115.1 ±4.7	0.144
Total CP	10.1^{b} ±2.0	14.1^{a} ±0.9	9.6^{b} ±0.4	0.020
Total EE	5.1^{a} ±1.0	2.9^{b} ±0.3	3.9^{b} ±0.2	0.003
Total NDF	31.7^{c} ±6.4	41.7^{b} ±2.7	55.8^{a} ±2.6	0.000
Digestibility (%)				
DM	52.5 ±6.2	58.0 ±1.9	49.1 ±6.0	0.092
OM	63.0 ±4.4	63.4 ±1.7	56.6 ±5.2	0.081
CP	71.6 ±3.4	70.9 ±1.7	68.5 ±3.3	0.328

Note. ^{abc} means with different superscripts in a row differ significantly ($p<0.05$). T1 = control diet containing 100% commercial pellet; T2 = half of the control diet plus *Asystasia gangetica* on *ad libitum* basis; T3 = half of the control diet plus *Brachiaria decumbens* on *ad libitum* basis. DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fibre

could be reflected due to the higher EE content in commercial pellet than other treatments containing test forages. There was no significant ($p>0.05$) difference on EE intake between the rabbits fed with T2 and T3 diets. Although total DM intake was reduced with increasing levels of EE in rabbit's diet as reported by Choi and Palmquist (1996), there was no influence on total DM intake among the treatments in the present study. This could be due to the presence of optimum level of EE in the experimental diets, which might be lower than the maximum level acceptable for use in rabbit's diet preventing adverse effect on their DM intake.

Total NDF intake increased ($p<0.05$) as commercial pellet was replaced by test forages. Rabbits fed T3 diet showed higher (55.8 g/d) NDF intake followed by T2 (41.7 g/d) and T1 (31.7 g/d) diets. This was attributed due to the use of different feed ingredients in the diets that contained different NDF contents (Table 1). Assis et al. (2019) reported that the NDF intake by animals could be increased when the energy density of the diet was low.

The DM and nutrient digestibility by the rabbits fed on different treatments are shown in Table 2. Similar to total DM and OM intakes, no influence ($p>0.05$) of experimental diets was observed on the

digestibility of nutritional fractions. The DM digestibility for T3 diet containing *B. decumbens* is in line with the findings of Roeder (n.d.), who reported that the DM digestibility of timothy hay was 49.1%. The DM digestibility of alfalfa hay was higher (50.7%) compared to the current DM digestibility of T3 diet, but it was lower compared to the T2 diet (58.0%). This suggests that T2 or T3 diet containing test forages had better DM digestibility when compared to other common forages that were fed to rabbits. Thus, when combining concentrates with forages, not only can it reduce the feed cost but also lead to use of locally available forages. Moreover, it can reduce dependency on the imported feeds such as timothy, alfalfa and other common forages.

Rabbits fed with T1 diet showed the highest CP digestbility (71.6%), while those fed with T3 diet showed the lowest (68.5%). Purwin et al. (2019) found that the CP digestibility of dehydrated alfalfa meal was 71.8%, which is in line with the current study. With increasing demand on animal protein, rabbit is suitable to be reared because of its high ability to convert forage into meat, low cost of production, and high quality protein meat (Amata & Okorodudu, 2016). Non-significant digestibility in DM, OM, and CP among treatments was found in this study which indicated that the rabbits were able to utilise nutrients from the concentrate, or the combination of concentrate and test forages.

Growth Performance

Effect of the experimental diets on initial BW, final BW weight, average daily gain, and FCR are presented in Table 3. The average final BW of rabbits among treatments ranged from 1200 g to 2130 g. The final BW was not influenced (p>0.05) by the diets. Rabbits fed with T1 diet showed the highest final BW, while rabbits fed with T3 diet showed the lowest. This result might be attributed to the fact that there was no effect of diets on the DM intake by the rabbits. Individual BW of rabbit for marketing is about 1.9-2.0 kg for White New Zealand or Chinchillia breed; less than this BW is considered to be in low quality. Average final BW of rabbit from T1 diet showed closer weight to the required market weight of rabbit. In the current study, low BW was achieved, and this result may have been caused by the use of mixed breed.

Unlike the final BW, the average total weight gain and the daily weight gain were affected (p>0.05) by the diets. The significantly highest BW gain and the lowest FCR were obtained with the T1 and T2 diets, whereas the significantly lowest BW gain and the highest FCR were obtained with the T3 diet. This result may have possibly been because of the significant effect of diets on the intake of CP, EE, and NDF. Rabbits fed with T3 diet showed lower BW gain, which might be due to the high fibre content, coarseness, and high DM content of the *B. decumbens*. Iyeghe-Erakpotobor et al. (2006) observed that 25% levels of soybean cheese waste/maize offal diet

Table 3

Growth performance of rabbits fed commercial pellet with or without Asystasia gangetica *and* Brachiaria decumbens

Parameter	Treatment			*p*-value
	T1	T2	T3	
Initial weight (g)	1178 ±374	1183 ±312	1165 ±258	0.997
Final weight (g)	1745 ±416	1650 ±216	1320 ±159	0.143
Total weight gain (g)	568^{a} ±61	468^{a} ±132	155^{b} ±106	0.001
Daily weight gain (g)	11.0^{a} ±1.4	9.0^{a} ±2.7	3.3^{b} ±2.2	0.002
FCR	12.3^{b} ±1.8	13.9^{b} ±4.4	30.3^{a} ±4.0	0.000

Note. ^{ab} means with different superscripts in a row differ significantly ($p<0.05$). T1 = control diet containing 100% commercial pellet; T2 = half of the control diet plus *Asyśtasia gangetica* on *ad libitum* basis; T3 = half of the control diet plus *Brachiaria decumbens* on *ad libitum* basis; FCR = feed conversion ratio

group lost body weight (-2.08 g/d) when fed with bracharia hay. In another study, Asuquo (1997) also reported that differences in BW and rate of weight gain of rabbits could be attributed due to the differences in the nutrient composition of the supplied forages. Although *B. decumbens* is palatable to rabbits, it contains saponins, an anti-nutrient. However, Faccin et al. (2016) reported that rabbits were not vulnerable to *Brachiaria* poisoning with concentrations of saponins that are toxic to sheep.

Rabbits fed with T1 and T2 diets had similar values of FCR, but their values were lower significantly ($p<0.05$) than the values of those fed T3 diet. The lower values of FCR implies that the rabbits were able to convert the concentrate and *A. gangetica* forage more efficiently to meat. The rates of daily weight gain in rabbits fed with T1 and T2 diets in this study were in line with daily growth of 5-10 g in growing rabbits as reported by Iyeghe-Erakpotobor et al. (2001) under tropical conditions.

CONCLUSION

Overall, the commercial pellet and the diet containing *Asyastasia gangetica* showed almost similar results. It is recommended for use a diet consisting of half of the concentrate and *A. gangetica ad libitum*. Not to mention that diet cintaining *Brachiaria decumbens* instead of *A. gangetica* can also be an alternative, but it is less suggested because lower rate of BW gain may occur. In rural areas where *A. gangetica* and *B. decumbens* forages are available, these could be efficiently utilised for feeding growing rabbits.

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Anti-caking Agent Effects on the Properties of Spray-dried 'Cempedak' Fruit Powder

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ABSTRACT

'Cempedak' fruit, an aromatic fruit that has a short shelf life can be converted into powder through spray-drying process. However, the spray-dried powder that was obtained had a high tendency to cake. Hence, three different anti-caking agents (calcium silicate, silicon dioxide, and calcium phosphate) were added separately at a concentration of 1.5% (w/w). It was found that calcium phosphate (1.5% w/w) yielded 'cempedak' fruit powder with lowest moisture content, water activity, hygroscopicity, and caking (change in cake height ratio), with minimal color changes in its reconstituted form and low viscosity. Different calcium phosphate concentration (0-2.00% w/w) was then applied in the production of 'spray-dried' powder. With increase of calcium phosphate addition from 0 to 0.66%, the moisture content, water activity, hygroscopicity, cake height ratio of 'cempedak' powder decreased, with no significant decrease with further addition. Calcium phosphate (0.66 % w/w) yielded powder with the best properties: lowest moisture content (4.65%), water activity

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(0.18), hygroscopicity (22.0), and change in cake height ratio (0.17). In addition, there was a minimal change in color of its reconstituted powder, with a slight change in viscosity.

Keywords: Anti-caking agent, caking properties, 'cempedak' fruit, spray-drying

INTRODUCTION

Caking of food powders results from combined factors, which include composition, particle size, temperature, pressure, and relative humidity (Lipasek et al., 2011). Anti-caking agents are substances that can prevent caking, lumping, and aggregation of hygroscopic powders by improving their flowability (Aguilera et al., 1995; Jaya et al., 2006; Phanindrakumar et al., 2005). The anti-caking agent function acts as moisture-protective barrier, by competing with powders for moisture (Barbosa-Cánovas et al., 2005). The flowability is improved and caking is inhibited by acting as surface physical barriers between particles and on its surface (Aguilera et al., 1995; Phanindrakumar et al., 2005).

Low molecular weight sugars are known to cause caking during storage (Chung et al., 2000). These sugars are very hygroscopic and tend to be sticky. They also create high agglomerates upon exposure to moisture (Cano-Chauca et al., 2005). Due to differences in the powder hygroscopicity between water-soluble amorphous and crystalline solids, re-crystallization of the metastable amorphous form free moisture (Hartmann & Palzer, 2011). The released water later influences the crystallization velocity of the residual amorphous fraction that leads to caking.

Anti-caking agents which are known to absorb excess water, was commonly added into food powder, as with addition, it will help the powder maintain their free-flowness (Lipasek et al., 2011). In addition, anti-caking agents have the capability to absorb oils and nonpolar organic compounds by encapsulating powder particles (Jaya & Das, 2004). The use of anti-caking agents also promotes the reduction in moisture migration dynamics through gradients of water activity (Castro et al., 2006).

Common anti-caking agents include calcium stearate, silicon dioxide, calcium phosphate, calcium silicate, and corn starch (Lipasek et al., 2011). These anti-caking agents are effective at low concentrations and are generally used in concentrations up to 3%, as their legal allowable concentration is restricted to a limited level, which in practice is generally within 1% or less (Hollenbach et al., 1982; Jaya & Das, 2005). The addition of calcium phosphate, calcium silicate or calcium oxide has been found to give a favorable free-flowing characteristic to powder when it is stored (Jaya & Das, 2005; Jaya et al., 2006). Chang et al. (2019) incorporated calcium silicate and tricalcium phosphate in the production of soursop powder. The addition of 0.25% calcium phosphate in the production of *Garcinia indica* powder and pineapple powder produced powders (Nayak & Rastogi, 2010; Phanindrakumar et al., 2005). In addition, the inclusion of calcium phosphate in

kiwi fruit powder was found to reduce its moisture content when added together with Arabic gum (Benlloch-Tinoco et al., 2013).

'Cempedak' fruit (*Artocarpus integer* L.) is an aromatic fruit that is popular in Southeast Asia. It can be consumed ripe or unripe, or processed into chips or creamed to make jams and cakes (Chong et al., 2008; Lim, 2012). As 'cempedak' is seasonal and has a short shelf-life, it can be converted into powder for better storage and product variability (Pui et al., 2018). The 'cempedak' powder has yield of 57.1%, moisture content of 6.11%, water activity of 0.22, hygroscopicity of 30.6g/100g, and carotenoid content of 1.43 mg/g. With water solubility index of 88.45, the 'cempedak' fruit powder can be incorporated into different food product such as pastries, cakes, or reconstituted into juice.

The present study was conducted to improve the properties of 'cempedak' fruit powder that was obtained through spray-drying. The powder obtained was found to have a high moisture content (more than 5%), which on visual observation led to caking and lumping during storage. Thus, it became necessary to add anti-caking agents and investigate whether the properties of the resulting 'cempedak' fruit powder could be improved. Three common anti-caking agents, namely calcium phosphate (CP), silicon dioxide (SIO), and calcium silicate (CS) all at 1.5% (w/w) concentration, were added separately to enzyme-liquefied 'cempedak' pulp before spray-drying and their effects on powder properties including moisture content, water activity, hygroscopicity, color of powder, and change in cake height ratio (caking) during storage were investigated. In addition, the spray-dried powder with anti-caking agents was also reconstituted with water (50 mL), whereby the change in color and viscosity were determined.

MATERIALS AND METHODS

Materials

'Cempedak' variety CH28 was procured in 3 different batches (n = 3), with ten fruits per batch) from the Department of Agriculture, Serdang, Selangor, Malaysia. The 'cempedak' fruit were wrapped with newspaper and ripened fruit were used (with strong aroma when it is ripe). The 'cempedak' fruit has total sugar and fiber content of 27-28 g in 100 mL, and 5%, respectively, with total soluble solids of 34-35°Brix (Pui et al., 2018). Celluclast® 1.5 L (for enzyme treatment of 'cempedak' pulp) was purchased from Novozymes, Denmark, while Maltodextrin 10 DE (drying aid) was purchased from Bronson and Jacobs, Kuala Lumpur, Malaysia. Food grade anti-caking agents i.e. calcium phosphate (CP), silicon dioxide (SIO), and calcium silicate (CS), were purchased from V.I.S. FoodTech, Malaysia.

Preparation of Spray-Dried 'Cempedak' Fruit Powder Containing Anti-Caking Agents

Ripe 'cempedak' fruit (weighing 1.5-2 kg each) were slit into half, and the arils removed. After the removal of seeds, the fruit pulp homogenized at low speed for

one minute using a commercial blender to obtain a homogenous puree. To prepare 'cempedak' juice, homogenized 'cempedak' puree was mixed with distilled water at 1: 2 puree: water ratio and incubated with 1.2% (v/w) Celluclast® 1.5 L for 1 hour at 45°C and 100 rpm in a shaking water bath (WNB 14, Memmert GmbH + Co. KG., Schwabach, Germany). The end product (liquefied 'cempedak' puree) was then subjected to pasteurization at 90°C for 5 min in a water bath to inactivate the added enzyme and endogenous enzymes, and/or microorganisms that might be present (Pui et al. 2018).

A Büchi B-290 mini spray-dryer (Büchi Labortechnik AG, Flawil, Switzerland) was used to convert the enzyme-treated 'cempedak' pulp obtained above into powder (Pui et al. 2020). Before spray-drying, 15% (w/w) matlodextrin and 1.5% (w/w) of an anti-caking agent (calcium silicate, calcium phosphate, or silicon dioxide) were added, and the mixture mixed thoroughly with a homogenizer (T25, IKA®-Werke Gmbh & Co., Staufen, Germany). The spray-dryer feed was stirred continuously at room temperature, and when inlet air temperature in the spray-dryer reached 160°C, the mixture was pumped into an atomizer to be spray-dried. In all experiments, the spray-dryer aspirator rate and pump rate were kept constant at 100% and 10%, respectively. The outlet spray-dryer temperature ranged from 85-95°C. The spray-dried 'cempedak' fruit powder was collected from product vessel and stored in amber glass bottles at 4°C prior to powder analysis.

After selecting the most suitable anti-caking agent, the spray-drying experiment was repeated using different concentrations (0, 0.25, 0.66, 1.50, and 2.50% w/w) of the anti-caking agent. The 'cempedak' fruit powders spray-dried with anti-caking agents were analyzed in terms of moisture content, water activity, hygroscopicity, color, caking and surface morphology. In addition, the powders were reconstituted 50 mL water and the reconstituted powder tested in the aspect of its change in color and viscosity.

Analysis of Spray-Dried 'Cempedak' Fruit Powder

Moisture Content and Water Activity. 'Cempedak' fruit powders were determined following method by Association of Official Analytical Chemists (AOAC) with drying in oven (Memmert, Germany) at 105°C for 5 hours and repeated until the weight is constant, whereas the water activity was measured using water activity meter at 25±1°C (PRE 00207, AquaLab Pre, Decagon Devices, Inc., Pullman, USA) (AOAC, 2000; Chang et al., 2020). Calibration was carried out using potassium sulfate (K_2SO_4) and potassium chloride (KCl) solution, prior to sample measurement.

Hygroscopicity. About 2 g of 'cempedak' fruit powder was placed into a pre-weighed Petri dish (100 mm × 15 mm). The dish was then placed in an airtight desiccator (that contains 500 mL of saturated solution of Na_2SO_4) for one week at room temperature. The difference in the weight after storage was used to calculate the hygroscopicity (Cai & Corke, 2000).

Color. A HunterLab ColorFlez Ultra-Scan® spectrophotometer (Hunter Associates Laboratory Inc., Reston, USA) was employed in the color determination of 'cempedak' fruit powder (Chang et al., in press; Wong et al., 2015). The instrument was first calibrated against a white tile and black tile, respectively. Color analysis was conducted at room temperature and its readings were expressed in *L** (lightness-darkness), *a** (greenness-redness), and *b** value (blueness-yellowness).

Caking Test (Change in Cake Height Ratio). Cake height ratio was determined using the caking test according to Janjatović et al. (2012), using a powder rheometer, TA.HDplus Powder Flow Analyzer (Stable Micro Systems, Godalming, England). The apparatus' powder column was filled with 'cempedak' fruit powder until it reached the 70 mm mark. In the first two conditioning cycles, the blade of the apparatus leveled the top of the powder column and the blade measured the height of the column, followed by moving down of bladed through the height of the column and then moving down again through the column at a tip speed of 20 mms^{-1}. The powder was then compacted to 200 g force before being sliced by blade at 10 mms^{-1}. This movement of compaction was repeated four more times. Cake strength is defined as the work (g.mm) required to cut the cake, while the mean cake strength on the other hand, is the average force to cut the cake expressed in grams. The measurement of settlement and powder column compaction is defined as the change in cake height ratio (current cycle cake height divided by initial column height).

Surface Morphology. The surface morphology of 'cempedak' fruit powder was evaluated using a scanning electron microscope (SEM) (LEO 1455 Variable Pressure SEM, Carl Zeiss, Germany). The 'cempedak' fruit powders were attached using a two-sided carbon-conducting tape, and covered with a thin layer of gold using sputter coater (BAL-TEC SCD 005, Japan) (Tonon et al., 2008). Scanning electron micrographs were obtained at an accelerating voltage of 5 kV and digital images were captured at magnifications of 2000×.

Reconstitution of Spray-Dried 'Cempedak' Fruit Powder. 'Cempedak' fruit powder was reconstituted with water to the same solids content as the spray-drier feed (10%). The powder (2.5 g) was mixed with water (25 mL) and mixed with vortex for 2 min (Grabowski et al., 2008). The color of reconstituted 'cempedak' fruit powder was evaluated using a HunterLab UltraScan ColorFlez colorimeter (Hunter Associates Laboratory Inc., Reston, USA) and its change in color (ΔE) was calculated according to Eq. 1.

$$\Delta E = \sqrt{((L - Lo)2 + (a - ao)2 + (b - bo)2)} \quad (1)$$

HunterLab values *L**, *a**, and *b** represents lightness-darkness, greenness-redness, and blueness-yellowness, respectively.

The viscosity of the reconstituted 'cempedak' fruit powder was measured using a Brookfield viscometer (DV-II+Pro, Brookfield Viscometer Ltd., Harlow, England), RheocalcT 3 software programme and small sample adaptor. The viscometer was auto-zeroed prior to analysis. The reconstituted 'cempedak' powder (15 mL) was poured into the sample cup, and analysis was carried out at 20 rpm rotational speed. Viscosity value is expressed in centipoise (cP).

Statistical Analysis

Data obtained from this study was analyzed using one-way ANOVA (Minitab software 17, Minitab Inc., Pennsylvinia, USA) where significant differences in the various treatments were determined using Tukey's test ($p \leq 0.05$). Results were expressed as means ± respective standard deviations of three replicates.

RESULTS AND DISCUSSION

Effects of Different Anticaking Agents on Properties of 'Cempedak' Fruit Powder

In this study, the effect of three different anti-caking agents (calcium phosphate, silicon dioxide, and calcium silicate) were on the properties of the spray-dried 'cempedak' fruit powder was examined. The moisture content, water activity, hygroscopicity, color, change in cake height ratio of 'cempedak' fruit powder after spray-drying are shown in Table 1. Moisture is recognized as a factor that affects caking the most (Chen & Chou, 1993). When powders are stored in a contained packaging, besides the transfer of moisture from environment; if the temperature changes, there may be condensation and evaporation of moisture, which eventually leads to crystallization of powder (Hartmann & Palzer, 2011). Moisture collected on the surfaces of powders causes moisture re-distribution or absorption and contributes to the stickiness of the surfaces. This encourages inter-particle binding, formation of clusters, and inter-particles fusion which eventually leads to caking (Jaya & Das, 2003).

From Table 1, the addition of calcium phosphate caused the biggest reduction of moisture content (17.3 %), followed by silicon dioxide (9.8%), and calcium silicate (3.6%). On the other hand, the 'cempedak' fruit powders had water activities ranging from 0.18-0.21, indicating that all the powders may be considered as stable (Fitzpatrick et al., 2007).

The higher reduction of moisture content in 'cempedak' fruit powder incorporated with calcium phosphate may be due to its ability that can take water up 10% of its weight. Although both silicon dioxide and calcium silicate were reported to have the capability to absorb moisture, the function was not obvious as compared to calcium phosphate in this study (Chung et al., 2001).

The uptake of water by calcium phosphate in kiwi fruit powder, where there was a reduction of moisture content, with slight decrease in water activity (Benlloch-Tinoco et al., 2013). Calcium phosphate was previously added into mango juice to obtain a non-sticky and free-flowing

Table 1
Effects of different anti-caking agents on the properties of 'cempedak' fruit powder

Anti-caking agents	No anti-caking agent (control)	Calcium silicate (1.5% w/w)	Silicon dioxide (1.5% w/w)	Calcium phosphate (1.5% w/w)
Moisture content (%)	5.91±0.11[a]	5.70±0.26[ab]	5.33±0.13[b]	4.89±0.07[c]
Water activity	0.22±0.00[a]	0.21±0.01[a]	0.19±0.00[b]	0.18±0.01[b]
Hygroscopicity (g/100 g)	25.00±1.00[a]	26.03±0.55[a]	25.70±0.73[a]	22.67±0.58[a]
L^*_p	72.63±0.58[a]	70.55±0.61[a]	74.0±1.20[a]	72.83±0.99[a]
a^*_p	9.17±0.30[a]	7.65±0.95[a]	8.63±1.16[a]	8.03±1.01[a]
b^*_p	32.11±0.51[a]	28.86±0.50[b]	28.30±0.99[b]	30.38±1.00[b]
Change in cake height ratio	0.31±0.05[a]	0.34±0.05[a]	0.29±0.01[b]	0.20±0.01[b]

Each value represents the mean of triplicate samples ± standard deviation. L^*_p, a^*_p, and b^*_p = Color of powder. Values within the same row with different superscripts (a-c) are significantly different at $p \leq 0.05$, as measured by Tukey's HSD test

freeze-dried mango powder (Jaya & Das, 2004). Silicon dioxide (2% w/w) and calcium silicate (2% w/w) were able to improve the physical stability in terms of particle size of powdered sodium ascorbate (Vitamin C) (Lipasek et al., 2011). In the work of Chang et al. (2019), it was reported that calcium phosphate was a more stable anticaking agent as compared to calcium silicate. Calcium phosphate has a higher glass transition temperature that causes better prevention of moisture adsorption as it forms a protection barrier on the dried powder.

In addition, a combination of calcium oxide, calcium silicate and calcium phosphate at the concentration of 0.25% were applied into pineapple powder (Phanindrakumar et al., 2005). It was found that the addition of anti-caking agents caused a decrease of the water uptake (hygroscopicity) when subjected to 43% relative humidity for 12 hours, as compared to control (without anti-caking agents). However, the mechanism and the difference among the effects of each anti-caking agent were not discussed. From Table 1, it is noted that the addition of anti-caking agents did not show significant increase ($p>0.05$) in powder hygroscopicity.

The color of 'cempedak' fruit powder incorporated with different anti-caking agents was also recorded in Table 1. The addition of silicon dioxide produced 'cempedak' fruit powder with an increase of 2.57 in L^*value, indicating that the powder was slightly lighter than the 'cempedak' fruit powder with no added anti-caking agent and powder incorporated with calcium silicate and calcium phosphate. The addition of all three anti-caking agents did not show any significant effect ($p>0.05$) on powder redness, while decreasing its yellowness (Table 1). However, there were also no significant difference observed in the b^* of all powder incorporated with different anti-caking agents. In general, with the addition of anti-caking agents, the total change in color with the incorporation of calcium phosphate (2.22), calcium silicate (4.15),

and silicon dioxide (4.63), were of lower range and these small differences were not visible to eye.

Changes in cake height ratio portray the caking characteristics of a powder, where an increase in cake height ratio indicates that a powder has a high tendency to cake (Tze et al., 2012). From Table 1, it can be observed that the addition of calcium phosphate managed to reduce the change in cake height ratio drastically (reduction of 35.4%), while calcium silicate and silicon dioxide did not decrease the change in cake height ratio. This indicates that addition of calcium phosphate reduced the powder's tendency to cake, which is an important criterion for powder storage. The result is in agreement with the addition of calcium phosphate (0.01-0.02 kg/kg) in mango powder, giving the desired free-flowing property to the powder during storage (Tainter & Grenis, 2001).

The SEM morphological images of 'cempedak' fruit powder produced with different anti-caking agents (2000x magnifications) are shown in Figure 1. From the figure, it can be seen that the addition of anti-caking agents improved the surface appearances of 'cempedak' fruit powder. It is also noted that silicon dioxide produced a powder with the smoothest surface, followed by calcium silicate, and lastly, calcium phosphate. From Figure 1, it is found that 'cempedak' fruit powder added with anti-caking agents had lesser holes and dents as compared to the 'cempedak' fruit powder without anti-caking agents. However, the 'cempedak' fruit powder added with silicon dioxide had a smooth surface yet was agglomerated together. Slight wrinkle was found on the surface of 'cempedak' fruit powder incorporated with calcium phosphate.

Dents that are present on the surface of powder, which is attributed to the shrinkage of particles during drying and cooling, have adverse effect on the flow properties of powder particles (Benkovic & Bauman, 2009). The faster degradation rate is often linked with the wrinkled particles, which occurs due to the fast water evaporation, which leads to collapsed hollow spherical structures and thus the formation of microfissures (Ferrari et al., 2013).

The 'cempedak' fruit powder was reconstituted in order to determine its change in color compared to control (without anti-caking agents) as shown in Table 2. From the table, it is found that there was no significant difference (p>0.05) among the color values of 'cempedak' fruit powders (L*, a*, b*) incorporated with different anti-caking agents.

The addition of anti-caking agents increased the viscosity of reconstituted 'cempedak' fruit powder, as shown in Table 2. Although the addition of different anti-caking agents caused a slight increase of viscosity, these viscosities were considered low, indicating that the increase might not be observed. The increase in total solid (with the addition of anti-caking agents), increased the viscosity of reconstituted powder. This accounts for the suspending stabilization of particles in dispersion system (Ferrari et al., 2013).

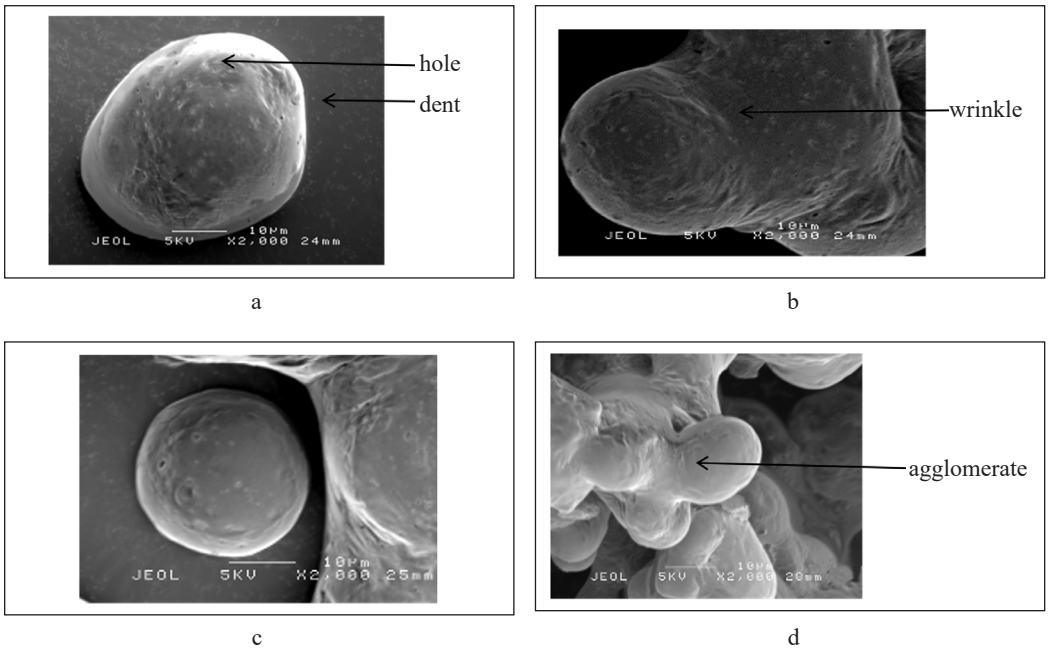

Figure 1. Morphological images of 'cempedak' fruit powder produced with different anti-caking agents (2000× magnifications)

Table 2
Effects of different anti-caking agents on the properties of reconstituted 'cempedak' fruit powder

Anti-caking agents	No anti-caking agent (control)	Calcium silicate (1.5% w/w)	Silicon dioxide (1.5% w/w)	Calcium phosphate (1.5% w/w)
L^*_{RP}	53.20±1.17^a	54.11±1.10^a	55.16±1.03^b	55.16±0.99^{ab}
a^*_{RP}	11.48±0.58^a	11.78±0.55^a	10.82±0.05^a	10.78±0.34^a
b^*_{RP}	37.74±1.09^a	34.20±0.61^b	31.93±0.06^c	34.60±0.63^b
ΔE_{RP}	0.00±0.00^a	3.71±0.60^b	6.97±0.63^c	3.85±0.30^b
Viscosity of reconstituted powder (cP)	3.1±0.0^a	4.4±0.1^d	3.8±0.1^c	3.6±0.1^b

Each value represents the mean of triplicate samples ± standard deviation. L^*_{RP}, a^*_{RP}, and b^*_{RP} = Color of reconstituted powder, ΔE_{RP} = Change in the color of reconstituted powder. Values within the same row with different superscripts (a-c) are significantly different at $p \leq 0.05$, as measured by Tukey's HSD test

In general, from the results in Table 2, it is indicated that calcium phosphate was most suitable anti-caking agents as compared to silicon dioxide and calcium silicate in improving the moisture content, water activity, and reduction of caking tendency (low change in cake height ratio).

Effects of Different Concentration of Calcium Phosphate on Properties of 'Cempedak' Fruit Powder and Reconstituted Powder

It was found that the mulberry leaf extract (50 mg/mL) had free radical scavenging activity of 49.88±1.23%. A different range

of calcium phosphate was applied to analyze its effect in the spray-dried 'cempedak' fruit powder. Results obtained are shown in Table 3. From Table 3, it can be seen that the moisture content of spray-dried 'cempedak' fruit powder was reduced with the addition of 0.66-2.00% calcium phosphate, with a reduction of 17.3-21.3%. Higher solid concentrations produced powders with lower moisture contents (Genovese & Lozano, 2000). The moisture content of less than 5% indicates the acceptable range of fruit powder moisture content (Gallo et al., 2011). Calcium phosphate has been applied in different fruit powders such as kiwi, mango, and pineapple (Benlloch-Tinoco et al., 2013; Jaya et al., 2006; Phanindrakumar et al., 2005).

The water activity with the addition of calcium phosphate (0.18-0.22) indicates the powder is safe microbiologically with water activity values that are in close approximation to 0.2 (Fitzpatrick et al., 2007). The increase in calcium phosphate concentrations led to a reduction in the hygroscopicity of spray-dried 'cempedak' fruit powder (Table 3).

The hygroscopicity of 'cempedak' fruit powder did not reduce further (p>0.05) with a further increase in calcium phosphate concentration after 0.66% (w/w), with the total reduction of 9.3-12% in the hygrocsopicity values. This is due to the increase anti-caking agent concentration that acts to create moisture protection barrier for the powder (Phanindrakumar et al., 2005). Thus, the best concentration of calcium phosphate to be incorporated for spray-drying of 'cempedak' fruit powder is 0.66% (w/w).

Table 3
Effects of different calcium phosphate concentrations on properties of 'cempedak' fruit powder

Calcium phosphate concentration (% w/w)	0.00	0.25	0.66	1.50	2.00
Moisture content (%)	5.91 ±0.11^{a}	5.44 ±0.14^{b}	4.65 ±0.13^{c}	4.89 ±0.07^{c}	4.85 ±0.30^{c}
Water activity	0.22 ±0.00^{a}	0.20 ±0.05^{b}	0.18 ±0.00^{bc}	0.18 ±0.01^{b}	0.18 ±0.00^{c}
Hygroscopicity (g/100 g)	25.00 ±1.00^{a}	23.67 ±0.6^{ab}	22.0 ±0.03^{b}	22.67 ±0.58^{b}	22.00± 0.00^{b}
L^*_p	72.63 ±0.58^{a}	71.11 ±0.58^{a}	71.54 ±0.60^{a}	72.83 ±0.99^{a}	73.75 ±1.00^{a}
a^*_p	9.17 ±0.30^{a}	8.77 ±0.85^{a}	8.39 ±1.02^{a}	8.03 ±1.01^{a}	7.64 ±0.95^{a}
b^*_p	32.11 ±0.51^{a}	31.72 ±1.03^{ab}	31.64 ±1.00^{ab}	30.38 ±1.00^{ab}	30.05 ±0.4^{b}
Cake height ratio change	0.31 ±0.05^{a}	0.26 ±0.02^{b}	0.17 ±0.02^{c}	0.20 ±0.01^{c}	0.18 ±0.01^{c}

Each value represents the mean of triplicate samples ± standard deviation. L^*p, a^*p, and b^*p = Color of powder. Values within the same row with different superscripts (a-c) are significantly different at $p\leq0.05$, as measured by Tukey's HSD test. P = powder

Microencapsulation of anthocyanin pigment present in *Garcinia indica* Choisy containing 0.25% calcium phosphate was found to have the lowest hygroscopic moisture content of 4.38% (Nayak & Rastogi, 2010). Calcium phosphate (0.01 to 0.02 kg per kg solid) were added on mango in vacuum drying of mango powder, and that optimum amount of calcium phosphate in their study was found to be 0.015kg per kg solid that had low hygroscopicity of 8.33-10.27, however, these values were measured using hygroscopicity measurement apparatus, which is different from this study (Tainter & Grenis, 2001).

There is no significant increase (p>0.05) in the aspect of 'cempedak' fruit powder lightness and redness (Table 3). The addition of 2% calcium phosphate decreased powder yellowness by 4%. In general, the total color difference in powder that ranges from 1.47 to 2.8, indicating that the color difference cannot be detected visually (Obón et al., 2009). It is also found that increase in calcium phosphate concentration leads to the increase in overall color difference (Tainter & Grenis, 2001). The reduction of change in cake height ratio with the addition of 0.66-2.00% (w/w) calcium phosphate is noted in Table 3. The powder flow and degree of caking of mango powder are found to exhibit decreasing trend with the increase of calcium phosphate concentration (0.1 to 0.2 kg/kg) (Jaya & Das, 2004).

Figure 2 shows the SEM morphological images of 'cempedak' fruit powder produced with 0.66% (w/w) calcium phosphate (2000× magnifications). From the figure, it is shown that the surface appearances of 'cempedak' fruit powder with 0.66% (w/w) calcium phosphate were with lesser dent. Table 4 shows the effects of calcium phosphate concentration on total color change and viscosity of reconstituted 'cempedak' fruit powder. It is observed that the total color change of reconstituted 'cempedak' fruit

Table 4
Effects of different calcium phosphate concentrations on properties of reconstituted 'cempedak' fruit powder

Calcium phosphate concentration (% w/w)	0.00	0.25	0.66	1.50	2.00
L^*_{RP}	53.20 ±1.17^{a}	54.97 ±0.04^{ab}	55.16 ±0.61^{ab}	555.16 ±0.99^{ab}	56.88 ±1.03^{b}
a^*_{RP}	11.48 ±0.58^{a}	11.09 ±0.63^{a}	11.00 ±0.71^{a}	10.68 ±0.43^{a}	10.63 ±0.87^{a}
b^*_{RP}	37.74 ±1.09^{a}	36.65 ±0.69^{ab}	36.49 ±0.62^{ab}	34.60 ±0.63^{b}	32.93 ±1.06^{b}
ΔE_{RP}	0.00 ±0.00^{a}	2.01 ±0.10^{b}	2.52 ±0.64^{b}	3.85 ±0.30^{c}	4.1 3±0.32^{c}
Viscosity (cP)	3.1 ±0.0^{a}	3.3 ±0.1^{b}	3.5 ±0.1^{bc}	3.6 ±0.1^{bc}	3.8 ±0.1^{c}

Each value represents the mean of triplicate samples ± standard deviation. L^*_{RP}, $a^*_{RP,}$ and b^*_{RP} = Color of reconstituted powder, ΔE_{RP} = Change in the color of reconstituted powder. Values within the same row with different superscripts (a-c) are significantly different at $p \leq 0.05$, as measured by Tukey's HSD test. RP = reconstituted powder

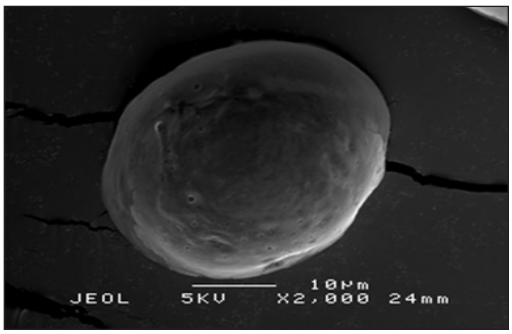

Figure 2. Morphological images of 'cempedak' fruit powder produced with 0.66% (w/w) calcium phosphate (2000× magnifications)

powder increased with the increase of calcium phosphate concentration. The total color changes values that is lesser than 5, obtained from all reconstituted powder, indicates that the color changes were not obvious (Obón et al., 2009). On the other hand, the addition of different concentration of maltodextrin causes a slight increase of viscosity. However, the increase may not be observed as the viscosity values are low.

CONCLUSIONS

This study demonstrated the effects of three anti-caking agents: calcium silicate, silicon dioxide, and calcium phosphate on the characteristic of spray-dried 'cempedak' fruit powder, with the aim of improving powder properties to reduce clumping and caking. Addition of calcium phosphate significantly decreased the moisture content, water activity, and degree of caking of the 'cempedak' fruit powder. The optimal amount of calcium phosphate was calculated as 0.66% (w/w), with a significant reduction of hygroscopicity of 'cempedak' powder. Therefore, 0.66% (w/w) calcium phosphate can successfully be used to improve the 'cempedak' fruit powder properties. However, the packaging material to better protect the powder from caking and absorption of water has to be further examined.

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Reproductive Biology and Fruit Setting of *Passiflora quadrangularis* L. (Giant Granadilla) in East Malaysia

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ABSTRACT

Passiflora quadrangularis L., also known as giant granadilla, belongs to the family Passifloraceae together with the well-known species *Passiflora edulis* Sims. This species has received attention from growers in recent years due to its aromatic flowers, unique and excellent fruit flavor and phytotherapeutic properties. Despite the numerous health benefits of this *Passiflora* species, information on their agronomical features and production is scarce. Therefore, the objective of this study was to examine the reproductive biology and fruit setting of *P. quadrangularis* cultivated in Sarawak, Malaysia. The findings revealed that the giant granadilla was a steady-state species that produced flowers lasting for only one day. *Passiflora quadrangularis* started to produce flowers 3 months after transplantation, followed by fruiting two months after anthesis. In East Malaysia, the flowers started blooming at dawn (0625±0.17 hours) followed by anthesis at 0806±0.23 hours and remained open until sunset. In contrast to *P. edulis* which flowered all year round, this species only

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exhibited two peaks, with a minor peak recorded from January-March and a major peak from September-December, coinciding with warm temperatures and moderate rainy season. Good fruit yields were recorded which was attributed to the ability for self-pollination due to autogamy and geitonogamy, in addition to the presence of effective pollinators. The yearly production of *P. quadrangularis,* produced relatively larger fruit with weights ranging from 884.4-2892.7 g, was 20,151.36 kg ha^{-1} (8993 fruits). Detailed information on the reproductive behavior of *P. quadrangularis* growing in local climates can be used for commercial cultivation and future breeding studies.

Keywords: Floral, giant granadilla, *Passiflora quadrangularis,* passion fruit**,** phenology, self-pollination

INTRODUCTION

Passion flowers are known to be one of the most alluring and appealing plants in the tropics. They produce exotic flowers that are unique and extremely mesmerizing (Ulmer & MacDougal, 2004). *Passiflora* L. consists of more than 500 species and is the largest genus in the Passifloracea family (Perez et al., 2007). *Passiflora* species are mainly distributed in the Neotropics and only 23 species are native in Southeast Asia, Australia and Oceania (Perez et al., 2007; Ulmer & MacDougal, 2004). The genus has approximately 50 species that bear edible fruits, however only two species; *P. edulis* Sims (purple passion fruit) and *P. edulis* f. *flavicarpa* (yellow passion fruit), are widely cultivated for fresh fruit and juice production (Patel et al., 2011). In addition, the lesser known *Passiflora quadrangularis* L., known as the giant granadilla, is also cultivated on a small scale for local consumption in certain countries (Kishore et al*.,* 2010) including Malaysia.

The world production of passion fruit increased from 1.05 million MTs in 2005 to 1.47 million MTs in 2017 (Altendorf, 2018), with the market price in Malaysia ranging from RM 15-16/kg. The current passion fruit market has a tendency towards healthy and functional products at affordable prices. Passion fruit juice is highly concentrated, and it makes a highly palatable beverage when diluted and sweetened (Das et al., 2013). The juice is preferably consumed alone or mixed with other fruit juices to improve the aroma. The demand for passion fruit has increased not only because of its organoleptic properties but also due to its essential nutrient composition, multivitamin content and antioxidant properties (Li et al., 2016; Phamiwon et al., 2016; Ramaiya et al., 2019). In many countries (i.e., India, Brazil and the United States) the juice is also used for flavour in many food products, such as desserts, jam, jelly, ice-cream and yogurt (Joy, 2010; Salazar et al., 2016; Shivanna, 2012;). Apart from its edible uses, passion fruit also possesses considerable medicinal properties. *Passiflora* species are rich in phytochemicals, e.g., flavonoids, tannins, phenols, glycosides, fatty acids and alkaloids (Ingale & Hivrale, 2010). These essential

elements are used in anti-inflammatory (Li et al., 2016), anticonvulsant, antimicrobial (Ripa et al., 2009), anticancer (Martina et al., 2007) and anti-diabetic (Phamiwon et al., 2016) treatments to treat osteoarthritis (Min et al., 2011) and insomnia (Fructose et al., 2017) because of their high antioxidant effects (Martinez et al., 2012).

Passion fruit was first planted in Malaysia by the Department of Agriculture in Negeri Sembilan in 1914. Then in 1960, it was planted for commercial purposes in Johor and Pahang (Chai, 1979). However, disease outbreaks affected the production and restricted further expansion of the use of fruit for commercial production. Passion fruit has the potential to be produced on a large scale in Malaysia as the local climate of this country is ideal for growing this fruit (Ramaiya et al., 2013). To date, only 8 species have been recorded in Malaysia with two widely cultivated species; the purple and yellow passion fruit. Recently, the non-native *P. quadrangularis* has gained attention from local growers due to its relatively large fruit size (~1-3 kg) (Ramaiya et al., 2013), aromatic flavor and health benefits (Das et al., 2013). This species has been cultivated on a small scale locally and internationally and it is the only passion fruit that bears edible mesocarp (Catalina et al., 2018).

It is important to study the adaptability and biology of *P. quadrangularis*. This plant is not native to Malaysia and it has been claimed that plants from different geographical locations can produce plants phenotypically different from those in their native environment (Gratani, 2014). Success in plant adaptability is reflected in the quantity and quality of the offspring produced. However, this process is not simple and producing well-adapted flowering plants involves complex ecological interactions between species and the environment, which affect plant phenology and physiology. In addition, the features of plants are not only caused by their genetic properties but also highly influenced by external factors such as geographic regions, climate conditions, and the availability of nutrients and pollinators (Catalina et al., 2018; Morellato and Haddad, 2000). To date, no published data is available on the cultivation and use of *P. quadrangularis* in Malaysia. Therefore, the objective of this study was to examine the reproductive biology and fruit setting of *P. quadrangularis* cultivated in Sarawak, East-Malaysia. Knowledge of the plant biology of this non-native species is crucial for local management and commercial production.

MATERIALS AND METHODS

Study Location and Plant Cultivation

The experiment was conducted at the passion fruit farm at Universiti Putra Malaysia Bintulu Campus (N 03° 12.45', E 113° 4.68'), Sarawak. The soil was categorized as Bekenu series, (*Typic Paleudults*) with a sandy loam texture and the pH was 5.36 for depths of 0-15 cm. The monthly weather data on rainfall and temperature obtained from the Malaysian Meteorological Department, Sarawak Branch (Kuching, Sarawak) is presented in Figure 1. The means temperature of the region ranged

from 26ºC to 28.0ºC. The monthly rainfall varied from 94.8 mm to 638.8 mm.

The planting materials used in this study were cuttings collected from Gold Rabbit Farm, Bintangor (N 02° 32.71', E 111.6° 56.9'), Sarawak, where passion fruit is planted at a commercial scale. Vertical trellis system with twenty rows, each 25 m long, was constructed. The trellis system consisted of 2.0 m tall posts set at 5 m intervals along the rows. Three-gauge galvanized wires were strung along the rows and attached to the posts. Five months-old seedlings were chosen for transplanting and a planting distance of 2.0 X 2.5 m was used. Plant maintenance, consisting of weeding, fertilization, and pruning, was performed accordingly.

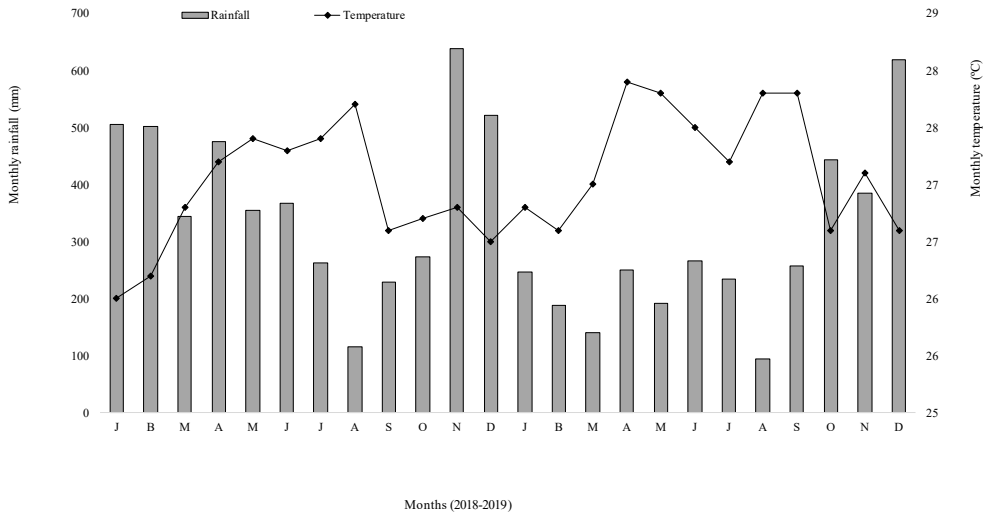

Figure 1. Meteorological data for monthly rainfall and temperature year 2018 and 2019

Observation of Phenology and Fruits Productivity

The vegetative shoots were tagged and their developmental changes were observed up to the fruiting stage. The phenological records were correlated with metrological data (i.e., monthly rainfall and temperature). The plant development stages recorded in this experiment were based on the extended BBCH scale-Growth stages of mono-and dicotyledonous plants (Meier et al., 2009). The observations that were recorded were the occurrence, duration and frequency of vegetative growth, flower blooming and fruit setting. The major bloom and minor bloom were classified based on the number of flowers open per day. Major blooms were characterized as more than half of the vines producing five or more flowers per day for 10 days in a month (Kishore et al*.,* 2010), whereas the minor bloom referred to less than half of the vines producing less than

5 flowers per day for at least 5-10 days per month. Additionally, detailed observations of the flowering phenology, blooming time, changes in the position of pistil and stamen and time of anthesis until the flowers closed were recorded. The initial fruit set was observed after 3 days of pollination. If pollution is successful, the flower ovary will begin to enlarge within 3 days (Das et al., 2013). The fruit development and ripening stages were also recorded. The total production was calculated on a hectare basis.

Pollinator and Nectar Composition

The insects visiting the *P. quadrangularis* flowers were collected, photographed, and identified with the taxonomic keys from Gonzalez et al. (2009). Observations were carried out from the pre-anthesis stage until anthesis was complete. Additionally, the nectar composition of *P. quadrangularis* flowers was examined using High Performance Liquid Chromatography (HPLC) following method by Shaffiq et al. (2013). The flowers of *P. quadrangularis* were randomly collected during blooming and immediately brought to the laboratory. The flowers were then dissected, the nectar was extracted using a sterile micropipette, and the volume was recorded for individual flowers. The nectar was kept in microcentrifuge tubes and stored at 4ºC prior to sugar analysis. A total of 20 µL nectar was diluted to a ratio of 1:100 with a mobile phase consisting of ultrapure water with 0.0001 M Ca-EDTA.

Three stock standard sugar solutions (HPLC grade ≥ 99.5%), namely sucrose, glucose and fructose, were prepared in the mobile phase. Prior to injection, the standard solutions and nectars were passed through a membrane filter with a 0.45 µm pore size and 10 µL was used per injection. The sugar compositions were determined by HPLC system (Waters Corp., Milford, MA, USA) equipped with a Waters Delta 600 pump controller and an in-line degasser AF connected with a Waters 2414 refractive index detector. A Waters Sugar-Pak I column (300 x 6.5 mm), packed with a microparticulate cation-exchange gel in calcium form was used in this study. The flow rate was 0.4 mL min^{-1} in the optimized working phase. The optimized temperature of the detector was 40ºC and the column was set at 70ºC. The sugar compounds in nectar were identified by comparing their retention times. The standard retention times for sucrose, glucose and fructose were 10.3, 12.7 and 15.1 min, respectively.

RESULTS AND DISCUSSION

Passiflora quadrangularis Flower Blooming

The flowering of *P. quadrangularis* is classified as "steady-state"; meaning that this species exhibits constant production of few flowers each day with each flower lasting only one day. The flowers of this species are solitary, produce relatively stronger fragrances and faced downward. Five main stages were recognized in *P. quadrangularis* flower development; stage

1-bracts formation, stage 2-bud initiation, stage 3-bud development, stage 4-complete bud formation, and stage 5-flower blooming. The first flower bloom in *P. quadrangularis* was observed on 5 September 2019, 4 months after transplanting. The first bloom occurred 122 days after transplanting. This species required a slightly longer period (16.8±0.84 days) than that of other species, *e.g*., *P. edulis* (13.4±0.55 days), before flowers opened after their appearance. Montero et al. (2013) reported that the development time of the flower buds was 13 days and differed in areas outside the native range of Brazil, the plant at 16-18 days in Malaysia and 21 days in Venezuela (Haddad & Figueroa, 1972). The maximum size of the flower bud recorded in this study was 6.45±0.07 cm. Flowers of this species started to open early in the morning (0615±0.12 hours) and the flowers remained opened until sunset at approximately 1600-1700 hours. This is contradictory to *P. edulis* in which the flowers are in full bloom from 1215 to 1340 hours in a local tropical climate (Ramaiya et al., 2020). Full flower blooming was recorded at approximately 0715±0.20 hours, and it took approximately 30 min for the styles to be completely curved and be in contact with the anthers, with anthesis starting at 0815±0.23 hours.

Flower Blooming Stages and Stigma and Anther Positions

In *Passiflora* flowers, the position and movement of the stigma and anther are very important for the role of self-pollination (Banu et al., 2009). Three phenological stages have been recognized in flowers of *P. quadrangularis*. In this study, no significant changes in color or odor was observed in any stage of flower development. Phase 1 is known as pre-anthesis. During this time, which corresponds to the stage immediately preceding anthesis, the flowers are in the bud stage. At this stage, the reproductive structure was not visible; the gynoecium and androecium were positioned towards the center of the flower and were fully covered by the sepals.

Phase 2 is known as flower homogamy and herkogamy. Generally, *Passiflora* flowers are homogamous, with the anthers and stigmas maturing simultaneously, and present herkogamy, leading to a spatial separation of the anthers and stigma. During this stage, the flowers started to open, the reproductive structures were fully exposed to the external environment, and the sepals, petals and corona spread out rapidly. During flower opening, the upright facing anthers drooped, causing the dehisced side of the anther to face downward. The styles which are in an upright position began to tilt until the receptive stigmas came into contact with the anthers, thereby transferring pollen grains onto the stigma surface. In *Passiflora* flowers, this important phenomenon reduces the distance between the stigmas and anthers, which aid in successful pollination. As the pollen grains were large and white, the presence of pollen grains on the stigma surface could be clearly seen.

Additionally, during stage 2 of flower blooming, *P. quadrangularis* flowers exhibited 3 types of style curvature: a) styles without curvature (WC), in which the styles stand erect; b) partially curved (PC) styles, in which the styles are partially curved and stigmas do not touch the anthers; and c) completely curved (CC) styles in which the styles are fully curved and the stigma was in contact with the anther. The percentage (%) of the different types of styles in *P. quadrangularis* flowers are presented in Figure 2. The majority of the flowers exhibited the CC style (80.00%), which provided assurance for successful reproduction in *P. quadrangularis*. The WC flowers were relatively less common, as such flowers do not get pollinated and bear fruits (6.67%).

Phase 3 is known as flower senescence; and during this time, the closure of the flower occurs. The petals and corona wilted, and the surfaces lost turgidity and started closing. The sepals returned to an upright position while the stigmas shifted backward, and the bracts remained attached. The pollinated flowers showed rapid enlargement of the ovary and developing fruit could be observed in 3 days. Non-pollinated flowers were abscised within 2-3 days without showing enlargement of the ovary.

In its native habitat and most of the other places in the world, *Passiflora* species are thought to be self-incompatible, and carpenter bees help to pollinate the flowers (Bruckner et al., 1995; Catalina et al., 2018; Madureira et al., 2014). In addition to the presence of effective pollinators, the

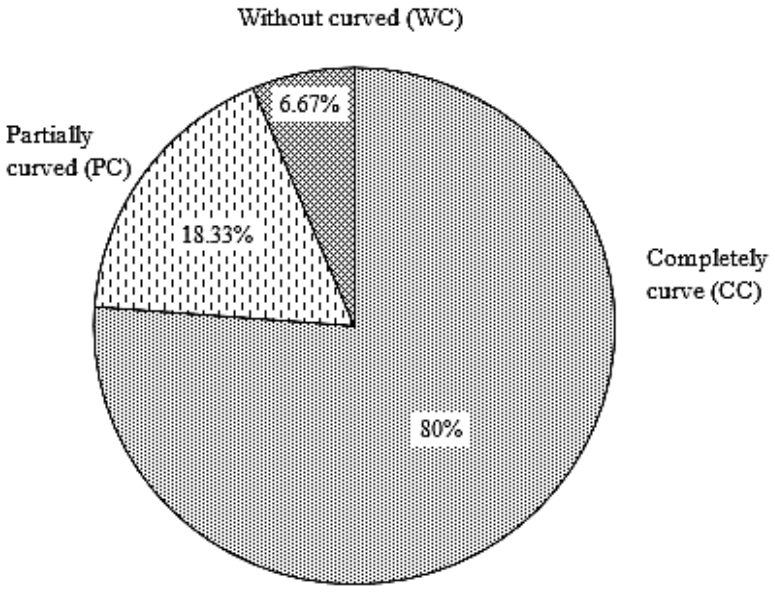

Figure 2. Percentage (%) of different types of style observed among *P. quadrangularis* flowers

unique movement of the styles and position of stigmas and anthers are important for self-pollination, as discussed above. At the present study site, there were good fruit sets, despite pollinators being relatively infrequent and sometimes absent. There were several visitors that helped with pollination; the most common visitors were ant species, which are nectar feeders and not pollinators. Therefore, the studied *P. quadrangularis* species mostly exhibited unique autonomous self-pollination; involving the movement of the styles, causing the stigmas to face the anthers during flower blooming and providing reproductive assurance to the species. The plants were self-compatible and able to produce constant fruit sets during the study period when biotic pollination was limited. According to Knight and Sauls (1994) and Sicard and Lenhard (2011), changes from self-incompatibility to self-compatibility frequently occur in cultivated flowering species. A number of flowering plants (*e.g.,* species of Zingiberaceae such as *Roscoea schneideriana* (Zhang & Li, 2008) and orchids (Chen et al., 2012) grow in areas with an insufficient presence of pollinators. The evolution of self-compatibility has generally been interpreted as a strategy for reproductive assurance in the absence or scarcity of pollinators.

Flowers Visitors and Nectar Composition

The genus of *Passiflora* includes several cultivated species of economic importance, with Colombia and Brazil as the center of diversification (Cerqueira-Silva et al., 2016). The pollen of *P. quadrangularis* has a sticky texture and is heavy, making wind pollination ineffective (Aguiar-Menezes et al., 2002; Souza et al., 2004). Insects are essential pollinators in certain species of *Passiflora* as autonomous self-pollination only has a small contribution to the fruit set. For example, autonomous self-pollination only contributed to 4% of the *P. ligularis* fruit set, as reported by Cataline et al. (2018). The solitary flowers of *P. quadrangularis* bear a single annular ridge-like nectary at the base of the corona, which stores nectar and is covered by an operculum. Sugary nectar is produced for pollinators that visit the flowers. The pollinators observed in this study were classified into three groups based on their sizes. The large pollinators that were observed were butterflies (*Cethosia hypsea hypsina*) and carpenter bees (*Xylocopa sonorina*). The medium pollinators were honeybees (*Apis millifera*) and moths (*Amata huebneri*). Stingless bees (*Trigona* sp.) were the only small pollinators recorded among the main visitors. As the pollinators landed on the flat corona and fed on the nectar, their upper bodies had contact with the exposed pollen-bearing surface of the anther. The pollen grains deposited on their wings and thorax then touched the stigma and transferred the pollen grains. It was also observed that ants frequently fed on the nectar produced by *P. quadrangularis* vines. Five ant species were identified as nectar feeders; *Technomyrmex* sp., *Pristomyrmex* sp., *Componotus* sp., *Polyrhachis* sp., and *Pachycondyla* sp.

Each flower produced approximately 22.7-58.0 µL of nectar during phase 2. Sucrose, glucose, and fructose were the predominant sugar components in *P. quadrangularis* flower nectar. The sugar composition of *P. quadrangularis* was compared with other *Passiflora* species (Figure 3). The nectar of *P. quadrangularis* was dominated by sucrose (40.78-58.34 g 100 g^{-1}), followed by glucose (4.56-6.43 g 100 g^{-1}) and fructose (4.32-6.21 g 100 g^{-1}). Similar trend was recorded in the nectar composition of *P. maliformis*. In contrast, *P. incarnata* and *P. edulis* (purple) possessed higher reducing sugars; fructose and glucose compared to sucrose. On average, the nectar of *P. quadrangularis* and *P. maliformis*; sucrose (83-86%) was dominant followed by glucose (7-8%) and fructose (7-8%), while the average composition in *P. edulis* and *P. incarnata* was dominated by glucose (34-37%) and fructose (35- 37%) with least sucrose (26-31%). According to Garcia and Gottsberger (2009), there are variations in the nectar sugar composition of *Passiflora* species with fructose and glucose being dominant in *P. caerulea* and *P. foetida,* while sucrose was the dominant sugar in *P. suberosa*. According to the classification by Perez and d'Eeckenbrugge (2017), *P. edulis*

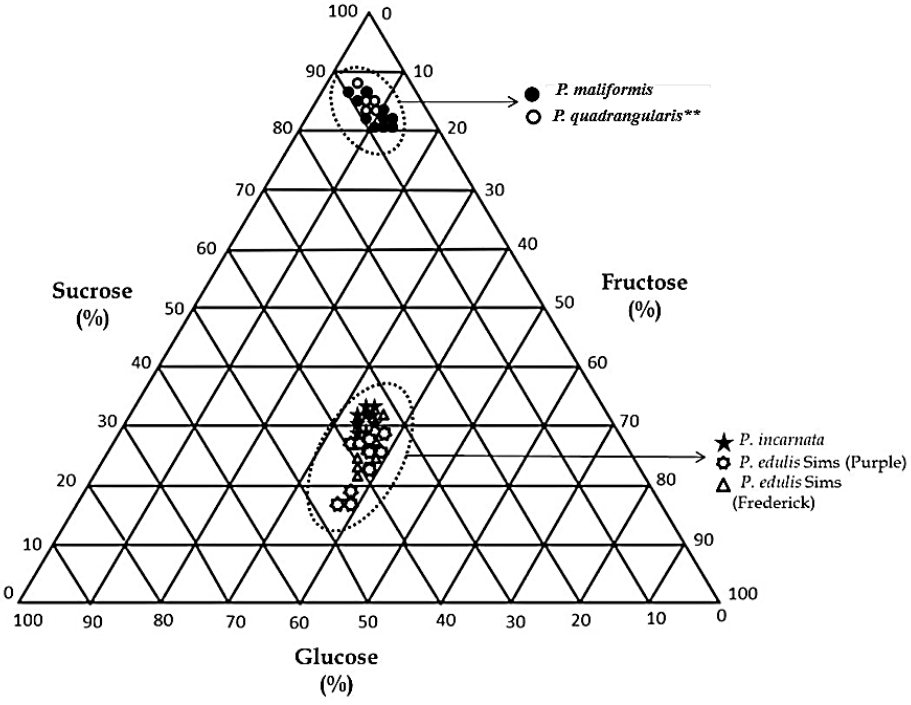

Figure 3. Nectaries sugars (sucrose, glucose and fructose) composition of *P. quadrangularis*** in comparison with other passion fruit species, illustrated by the ternary diagram

and *P. incarnata* belong to the Passiflora supersection and *P. quadrangularis* and *P. maliformis* belong to the Laurifolia super-section. The similarity between *P. edulis* and *P. incarnata* and that between *P. quadrangularis* and *P. maliformis* was reflected by their nectar chemistries. According to the authors, the nectar of *P. edulis* and *P. incarnata* flowers were dominated by glucose and fructose, while those of *P. quadrangularis* and *P. maliformis* flowers were dominated by sucrose. Nevertheless, the composition of sugar may be related to the pollinator type. Koschnitzke and Sazima (1997) mentioned the nectar of *P. suberosa* (Decoloba subgenus), which is pollinated by wasps in its native distribution, tended to be rich in sucrose, while flowers pollinated by short-tongued bee, *e.g., P. foetida* had a hexose (i.e., glucose and fructose) rich nectar.

Passiflora quadrangularis Fruit Development

The fruit developed from the ovary was oblong in shape when it matured and showed marked variation in their size, weight, and number of seeds per fruit. After the anthesis, the ovary enlarged, and the fruits developed. Simultaneously, seeds and pulp were also developed. The days required for *P. quadrangularis* fruit ripening was approximately 2 months after anthesis. Upon maturity, the fruit exocarp turned greenish yellow. The pericarp of fruit, which contains the aerial, is formed by three distinct tissues; i) an external epidermis which is smooth and a thin layer of rind; ii) a mesocarp, edible thick, juicy and sweet flesh and iii) the endocarp, which is separated from the mesocarp by an aerenchymatous tissue and has parenchyma tissue. *Passiflora quadrangularis* produces larger fruit; fruit length (20.75±2.26 cm) X fruit width (12.24±1.22 cm) compared to the common passion fruit, i.e., *P. edulis* with fruit length (7.48±0.96 cm) X fruit width (6.61±0.49 cm). The mesocarp of this fruit is edible and taste similar to honeydew. The unripen mesocarp can be cooked as a vegetable (Fischer et al., 2018). While the ripen fruit's pulp is orange in color, sweet in taste and less acidic than the common passion fruit and suitable to make juice and other processed products.

Fruit development phase for *P. quadrangularis* is presented in Figure 4. There are three stages of fruit development. The first stage is the initiation of fruit to begin with cell division and cell expansion. Fruit weights increases rapidly after day 10 of anthesis and attains half of the weight of a ripe fruit on day 20. Stage 2 is called the fruit expansion and maturation. During this stage, arils expand to form a bag which is termed juice-sac, containing yellow to orange juicy pulp and development of seed. *Passiflora quadrangularis* has four longitudinal rows at the position of the endocarp wall. Stage 3 is the ripening, softening and coloring of fruit and accumulation of soluble solid and acid. At this stage, all chloroplast are masked by color pigments. The sac is its maximum size and weight at this stage. Juice sac increases its size and volume and juice concentration. Our results revealed that *P. quadrangularis*

achieved complete maturity after 62.6±1.94 days (~2 months) of anthesis. Similarly, *P. edulis* also attained maturity after 2 months of anthesis (Nave et al., 2010). Fruit maturity of a crop is influenced by genetic make-up, physiological condition of the specific crop as well as environmental factors such as rainfall, humidity, temperature and day length (Das et al., 2013).

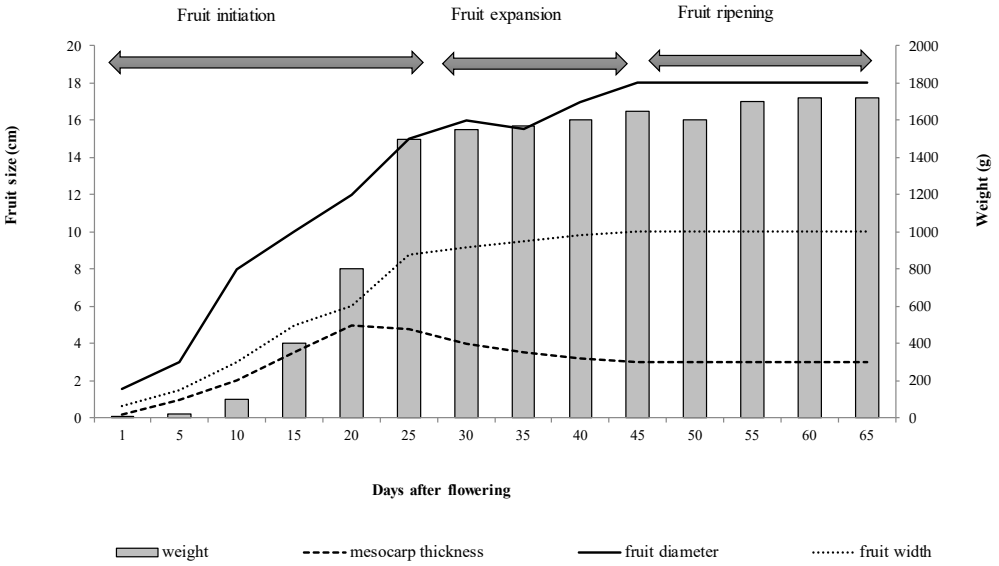

Figure 4. Curve for development of *P. quadrangularis* fruit with time

Passiflora quadrangularis was observed to only flower and fruit at certain months (Figure 5). The major peak was observed in September-November, coinciding with warm temperatures and moderate raining season, and a minor peak in February-March. This contrasts with *P. edulis* which produces fruits all year around with three major peaks recorded in April-May, July-September and November-January. Kishore et al. (2010) from India stated that *P. quadrangualris* had three major periods of bloom in March-April, July-August and September-October and this is in contrast to the present finding where there was only a single major peak served in a year.

In its center of origin (South America), the plant produced fruits throughout the year. There were long vegetative periods observed from April to August in *P. quadrangularis* (Ulmer & MacDougal, 2004). The long vegetative phase in plants may allow the accumulation of carbohydrate which is later used during flowering and fruiting to

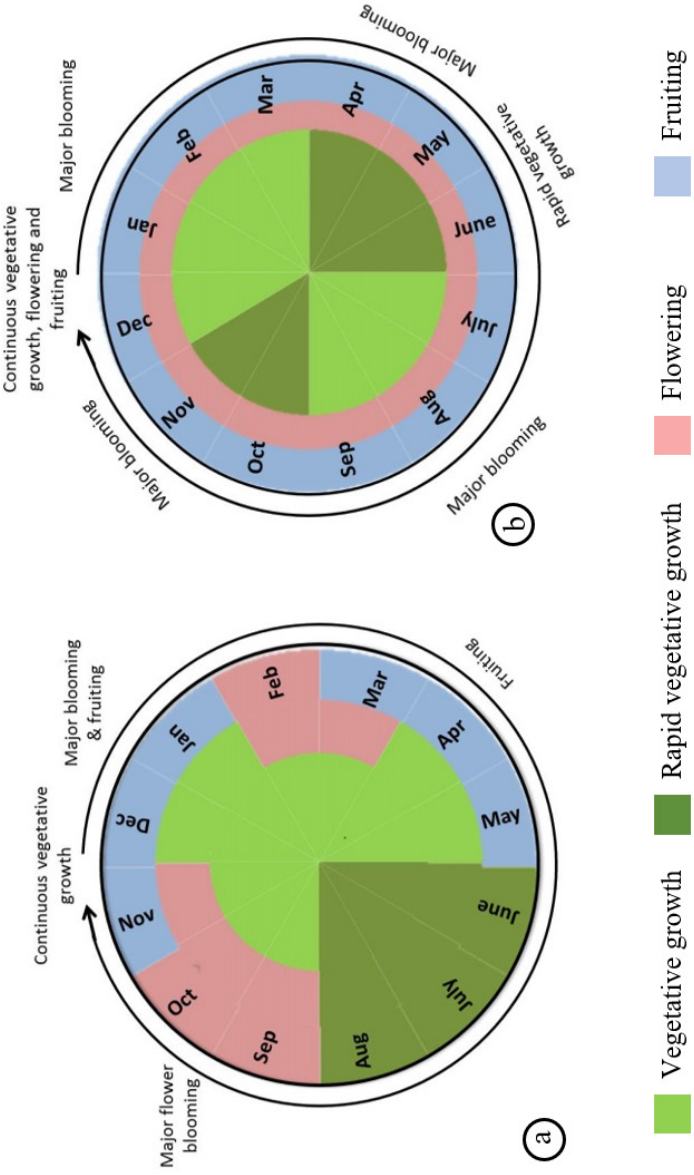

Figure 5. Phenological pattern per year cycle in (a) *P. quadranguaris* compared to the (b) common passion fruit, *P. edulis* (Purple)

produce heavier fruits. Knight and Sauls (1994) stated this species began to flower in spring and its fruit matured in summer and the fruits weigh 225-450 g under Florida conditions. The reproductive phenological pattern of *Passiflora* plant was affected by climatic conditions as well as geographic region of cultivation. Moreover, different cultivated *Passiflora* species respond differently to environmental factors and soil type. The irradiance of the environment in which the plants grow is of fundamental importance, because the adaptation of the plants to this environment depends on the adjustment of their photosynthetic path, so the light is used in a more efficient way (Das et al., 2013; Koehler-Santos et al., 2006). In this study, the production of *P. quadrangularis* produced bigger fruits, which was 20,151.36 kg ha^{-1} (8993 fruits) with weights ranging from 884.4 to 2892.7 g. Comparatively, the annual production of *P. edulis* at local climate in East Malaysia is 119,174 fruits of 11,103.90 kg ha^{-1} with the weight range of 56.4-156.5 g (Ramaiya et al., 2020).

CONCLUSION

According to the findings of the present study, *P. quadrangularis* was well adapted to the local climate, and its success was reflected in the quantity and quality of the fruit produced. There was no environmental selective pressure constraining the timing of the flowering and fruiting of *P. quadrangularis.* The flowers of this species are known as "steady-state"; meaning that they constantly produce few flowers each day, with each flower lasting only a day. This species undergoes unique autonomous self-pollination involving the movement of the styles and anthers, which assures fruit production regardless of the presence of pollinators. The detailed reproductive biology of *P. quadrangularis* reported here can provide visual clues that farmers can utilize to time farming operations in local cultivation practices and planting management. In addition, this information will also help further research on breeding and increasing production of *P. quadrangularis*.

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Photosynthetic Performance in Improved 'KDML105' Rice (*Oryza sativa* L.) Lines Containing Drought and Salt Tolerance Genes under Drought and Salt Stress

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ABSTRACT

Rice (*Oryza sativa* L.) 'KDML105' is the most popular aromatic rice originating in Thailand. This cultivar is highly susceptible to abiotic stresses, especially drought and salt stress during the seedling stage. The objective of this study was to investigate the photosynthetic performance in response to drought and salt stress of four improved breeding lines, specifically CSSL94 and CSSL103 (containing drought-tolerant quantitative trait loci: DT-QTLs) and RGD1 and RGD4 (containing a salt-tolerance gene, *SKC1*), with 'KDML105' (susceptible) and DH103 (tolerant to drought and salt stress) as the controls. Rice seedlings were grown for 21 days in hydroponic solutions and then exposed to salt stress (150 mM NaCl) or drought stress (20% PEG6000) for 10 days. The results indicated that when subjected to drought and salt stress, all rice lines/cultivar exhibited significant

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reductions in net photosynthesis rate (P_N), stomatal conductance (g_s), transpiration rate (E), the maximal quantum yield of PSII photochemistry (F_v/F_m), the effective quantum yield of PSII photochemistry (F_v'/F_m'), photosynthetic pigments, and SPAD readings, whereas water use efficiency (WUE) and non-photochemical quenching (NPQ) increased. Compared with 'KDML105', CSSL94, and CSSL103 were more tolerant to both drought and salinity, showing less reduction in all photosynthetic parameters. For RGD1 and RGD4, it was confirmed that these lines had a higher level of salt tolerance than 'KDML105' based on better photosynthetic performance under salt stress, demonstrating that these lines were also more tolerant to drought stress.

Keywords: Chlorophyll fluorescence, drought stress, photosynthetic performance, rice, salt stress

INTRODUCTION

Rice is the most important staple food crop for almost all countries in Asia including Thailand, and the demand for rice is ever-increasing with the growing global population (Lin et al., 2018). Rice production is associated with a whole range of influencing factors such as variety, terrain, climate, fertilization, and soil properties (Ran et al., 2018). Moreover, various types of stress, biotic, and abiotic, adversely affected the survival, growth, and performance of rice (Anami et al., 2020). Among abiotic stress factors, drought and salinity dramatically reduced the growth and yield of rice, particularly in rainfed ecosystems (Sekar & Pal, 2012). In Thailand, approximately 76% of the total of 9.2 million ha of rice-growing areas are under rainfed conditions. The majority of the rainfed lowland areas are found in the northeast (4.8 million ha) and the north (1.4 million ha) regions. These areas are drought-prone, and rice yield is low and fluctuates between 1.5 and 2.2 t ha^{-1} (Jongdee et al., 2006). Recently, it was estimated that 55-68% yield loss occurred in two study areas in Northeast Thailand in the drought year of 2012 (Polthanee et al., 2014). Northeast Thailand is also affected by saline soils, where 18% of the agricultural land (1.84 million ha) is affected by salts to varying degrees (Arunin & Pongwichian, 2015). A study involving a total of 51 farmers' fields in this region from 2002-2005 revealed an average of 20% reduction in rice yield even under slightly saline soils having the electrical conductivity of saturated soil extract (EC_e) between 3 and 5 dS m^{-1} (Clermont-Dauphin et al., 2010).

Photosynthesis is the fundamental metabolic process determining crop growth and yield, but it is strongly inhibited by drought and salinity (Pandey & Shukla, 2015). In rice, a strong positive relationship between photosynthesis capacity and vegetative growth (Murata, 1981) as well as grain yield (Ambavaram et al., 2014) has been reported. Therefore, more tolerant rice genotypes are better able to maintain a more efficient photosynthetic capacity

under drought (Ambavaram et al., 2014) and salt stress (Li et al., 2017). Drought and salinity negatively influence photosynthetic performance through decreased stomatal conductance, transpiration rate, photosystem II efficacy, and photosynthetic pigments (Hungsaprug et al., 2019; Netondo et al., 2004). The photosynthetic rate is also adversely affected by limited CO_2 diffusion into the chloroplast, via limitations on stomatal conductance and the mesophyll transport of CO_2, as well as alterations in leaf photochemistry and carbon metabolism (Chaves et al., 2009). These effects vary according to the intensity, duration, and frequency of stress as well as the plant species (Rötzer et al., 2012). Photosystem II is the most susceptible component of the photosynthetic machinery that carries the brunt of abiotic stress (Gururani et al., 2015). Thus, abiotic stress usually leads to photoinhibition through damage to PSII reaction centers (Nishiyama et al., 2006).

'Khao Dawk Mali 105' or 'KDML105', known in the world market as 'Thai Hom Mali' or 'Thai jasmine rice', has a unique fragrance and good eating/cooking quality. 'KDML105' is mostly cultivated in northeast Thailand; hence, its growth and yield are under threats from both drought and salinity stress. 'KDML105' is susceptible to abiotic stresses, especially drought (Kanjoo et al., 2012) and salinity (Kanawapee et al., 2012). Efforts have been made to improve rice cultivars to obtain better resistance to salinity and water deficit stresses through molecular marker-assisted backcross breeding (MABC). Several rice lines were developed as donors for drought-tolerance quantitative trait loci (DT-QTL), including DH103 and DH212 (Lanceras et al., 2004). Subsequently, through MABC, 90 chromosome segment substitution lines (CSSLs) with a 'KDML105' genetic background (CSSL no. 1–90) were developed, which carried DT-QTL from chromosomes 1, 3, 4 and 9 of DH212and chromosome 8 of DH103 (Kanjoo, 2011). These CSSLs lines were evaluated for agronomic traits in a field condition under drought stress compared with 'KDML105', and it was found that these improved lines showed higher grain yield than 'KDML105' (Kanjoo et al., 2012). Two selected CSSL lines (CSSL1 and CSSL4), which carried DT-QTL from chromosome 1 of DH212, were evaluated for physiological responses under drought stress. Only CSSL4 revealed superior tolerance over 'KDML105' through its ability to maintain stable net photosynthesis rates (Hungsaprug et al., 2019). However, detailed physiological drought responses of other CSSL lines introgressed with DT-QTL from other chromosomes have not been evaluated.

To improve the salt tolerance of rice, the *SKC1* gene controlling salt tolerance in rice has been identified (Gregorio et al., 2002). The *SKC1* gene functions in controlling ion homeostasis by increasing K^+ content and lowering Na^+/K^+ in rice shoots, thereby increasing yields under salinity stress (Ren et al., 2005; Thomson et al., 2010). Introgression lines with the 'KDML105' rice

genetic background carrying QTL and *SKC1* for salt tolerance were derived by crossing 'KDML105' with salt-tolerant FL496 (IR66946-3R-196-1-1) or FL530 (IR66496-3R-230-1-1). These backcross introgression lines of 'KDML105' were proven to have higher salt tolerance than 'KDML105', as indicated by lower shoot Na^+/K^+ and higher yield under salt stress (Punyawaew et al., 2016; Vanavichit et al., 2018). However, detailed studies on the effects of drought and salt stress on physiological responses of these improved lines have not been reported. These studies are expected to provide an insight into how physiological and biochemical mechanisms are differentially enhanced by the introgressed genes, which enable higher tolerance of the improved lines compared with the sensitive parent 'KDML105'.

Photosynthetic performance under stress is one of the most important processes determining stress tolerance ability, growth, and yield. In this study, we evaluated the photosynthetic performance under drought and salt stress of four improved lines with a 'KDML105' genetic background introgressed with DT-QTL (from chromosome 8 of DH103) or the *SKC1* gene. Various photosynthetic parameters, including net photosynthesis rate, chlorophyll fluorescence, and leaf greenness index, were analyzed to clarify the drought- and salt-tolerance mechanisms. The information provided in this study may be applied for selecting appropriate introgression lines for breeding rice cultivars with tolerance to both drought and salt stresses.

MATERIALS AND METHODS

Plant Materials and Growth Condition

Six (6) rice lines/cultivars were used in this study, including CSSL94 and CSSL103, which are CSSLs with a 'KDML105' genetic background carrying DT-QTL on chromosome 8; RGD14376 (RGD1) and RGD12150-B-21-MS3 (RGD4), which are backcross introgression lines of 'KDML105' carrying a salt tolerance gene (*SKC1*) on chromosome 1; 'KDML105', the original parental cultivar, which is susceptible to drought and salt stress; and the double-haploid line, DH103, which is known to be tolerant to both drought and salt stress (Lanceras et al., 2004; Nounjan et al., 2016). Seeds were kindly provided by the Rice Gene Discovery Unit, BIOTEC, Thailand. The experiment was conducted in the greenhouse at the Crop Station, Faculty of Agriculture, Khon Kaen University, Thailand. Seeds were soaked in 3% calcium hypochlorite for 30 min and then washed several times with distilled water. Seeds were then germinated in distilled water on filter paper in the dark at room temperature. After 3 days, germinated seeds were transferred to a plastic net placed over 15 L of Yoshida nutrient solution (Yoshida et al., 1976) in a plastic container (50 × 60 × 11 cm). In each container, 20

germinated seeds per line/cultivar were randomly arranged and allowed to grow for 21 days, during which the nutrient solution was replaced every 4 days. On day 21, the plants were separated into 3 treatment groups i.e. control, drought stress, and salt stress. For drought stress, plants were treated with Yoshida solution containing 20% polyethylene glycol 6000 (PEG6000). For salt stress, plants were fed with Yoshida solution containing 150 mM NaCl. For the control, plants continued to be fed with the Yoshida solution. The physiological traits were determined at 10 days after drought and salt treatments. The chosen concentration of PEG6000 had an osmotic potential of -0.7 MPa, corresponding to moderate water stress (Osmolovskaya et al., 2018). The 150 mM NaCl solution had the same osmotic potential as the 20% PEG6000 solution; therefore, it imposes a similar level of osmotic stress. The NaCl solution had an approximate EC value of 14.8 dS m^{-1}.

Determination of Leaf Gas Exchange Parameters

Net photosynthesis rate (P_N), stomatal conductance (g_s), transpiration rate (E) and water use efficiency (WUE) were evaluated on the youngest fully expanded leaf of a randomly selected rice plant in each replicate by using a portable infrared gas exchange analyzer (LI-6400, LI-COR, NE, USA) from 9.00 to 11.00 a.m. The net photosynthesis rate was determined under the following conditions: 30ºC, CO_2 concentration at 400 ppm, and 30–70% relative humidity. The photosynthesis photon flux density was maintained at 1500 µmol (photon) m^{-2} s^{-1}.

Measurement of Chlorophyll Fluorescence Parameters

As soon as the above gas exchange measurement was completed, the photosynthetic efficiency of PSII was measured on the same leaf using a chlorophyll fluorometer (Hansatech, Kings Lynn, UK). The maximal quantum yield of PSII efficiency (F_v/F_m) was calculated from minimal fluorescence in the dark-adapted state (F_0) and maximal fluorescence in the dark-adapted state (F_m), as described by Schreiber (2004). Steady-state fluorescence in the light-adapted state (F_s) and maximal fluorescence in the light-adapted state (F_m') were determined in the light condition. The effective quantum yield of PSII efficiency (F_v'/F_m') was calculated as described by Schreiber (2004). Non-photochemical quenching (NPQ) was computed as described by Bilger and Bjorkman (1990).

Measurement of Leaf Greenness

Leaf greenness of the same youngest fully expanded leaf used for gas exchange and chlorophyll fluorescence measurements was measured at 3 positions along the leaf blade from 9.00 to 11.00 a.m. using a SPAD-502 chlorophyll meter (Minolta Corp., Ramsey, New Jersey, U.S.A.).

Determination of Photosynthetic Pigments

Chlorophyll and carotenoid contents were determined by a modified method, as outlined by Arnon (1949) and Lichtenthaler (1987), using 0.1 g of leaf tissue. The absorbance of the pigment extract was estimated at 470, 645, and 663 nm by using a spectrophotometer (Hanon, Model i3, China) with 80% acetone as a blank. The content of pigments was expressed as mg g^{-1} tissue fresh weight. The pigment contents were calculated using the following equations:

Total chlorophyll (TC) = $[20.2\ A_{645} + 8.02\ A_{663}] \times [V/(1000 \times W)]$

Chlorophyll *a* (Chl *a*) = $[12.7\ A_{663} - 2.69\ A_{645}] \times [V/(1000 \times W)]$

Chlorophyll *b* (Chl *b*) = $[22.9\ A_{645} - 4.68\ A_{663}] \times [V/(1000 \times W)]$

Carotenoid (CA) = $[1000\ A_{470} - (1.82 \times \text{Chl } a) - (85.02 \times \text{Chl } b)]$

where: V = total volume of extract (ml); W = leaf weight (g)

Experimental Design and Statistical Analysis

The experiment was laid out as 3 × 6 factorials with a completely randomized design with four replications. The data were subjected to analysis of variance. Duncan's multiple range test (DMRT) was used to compare means at a significant difference of $p \leq 0.05$ and paired-samples t-test was performed at a significant difference of $p \leq 0.05$ and 0.01. Pearson's correlation was calculated for the relationships among photosynthesis traits under drought and salt stress conditions. All calculations and data analyses were performed using SPSS for Windows version 19. In addition, cluster analysis based on fourteen physiological parameters was analyzed using PC-ORD version 5.10.

RESULTS

Changes in Leaf Gas Exchange Parameters

Drought generally imposed stronger inhibitory effects on leaf gas exchange parameters than salt stress. Under non-stressed control conditions, RGD4 showed the highest P_N (25.87 µmol CO_2 m^{-2} s^{-1}), whereas DH103 showed the lowest P_N (19.26 µmol CO_2 m^{-2} s^{-1}). All improved lines (CSSL94, CSSL103, RGD1, and RGD4) and 'KDML105' showed significantly higher P_N than DH103 (Figure 1A). In contrast, under drought stress, DH103 showed the highest P_N (5.43 µmol CO_2 m^{-2} s^{-1}; 71.8% reduction when compared with control plants), whereas 'KDML105' showed the lowest P_N (3.97 CO_2 m^{-2} s^{-1}; 83.05% reduction when compared with control plants) (Figure 1A). For the improved lines, P_N under drought stress decreased by 79.7, 79.8, 78.8, and 79.2% for CSSL94, CSSL103, RGD1, and RGD4, respectively, when compared with control plants. Under salt stress, RGD4 showed the highest P_N (12.82 µmol CO_2 m^{-2} s^{-1}), while 'KDML105' showed the lowest P_N (7.89 µmol CO_2 m^{-2} s^{-1}) (Figure 1A). This suggested that RGD4 had higher salt tolerance compared to CSSL94, CSSL103, RGD1, and DH103. A similar trend was

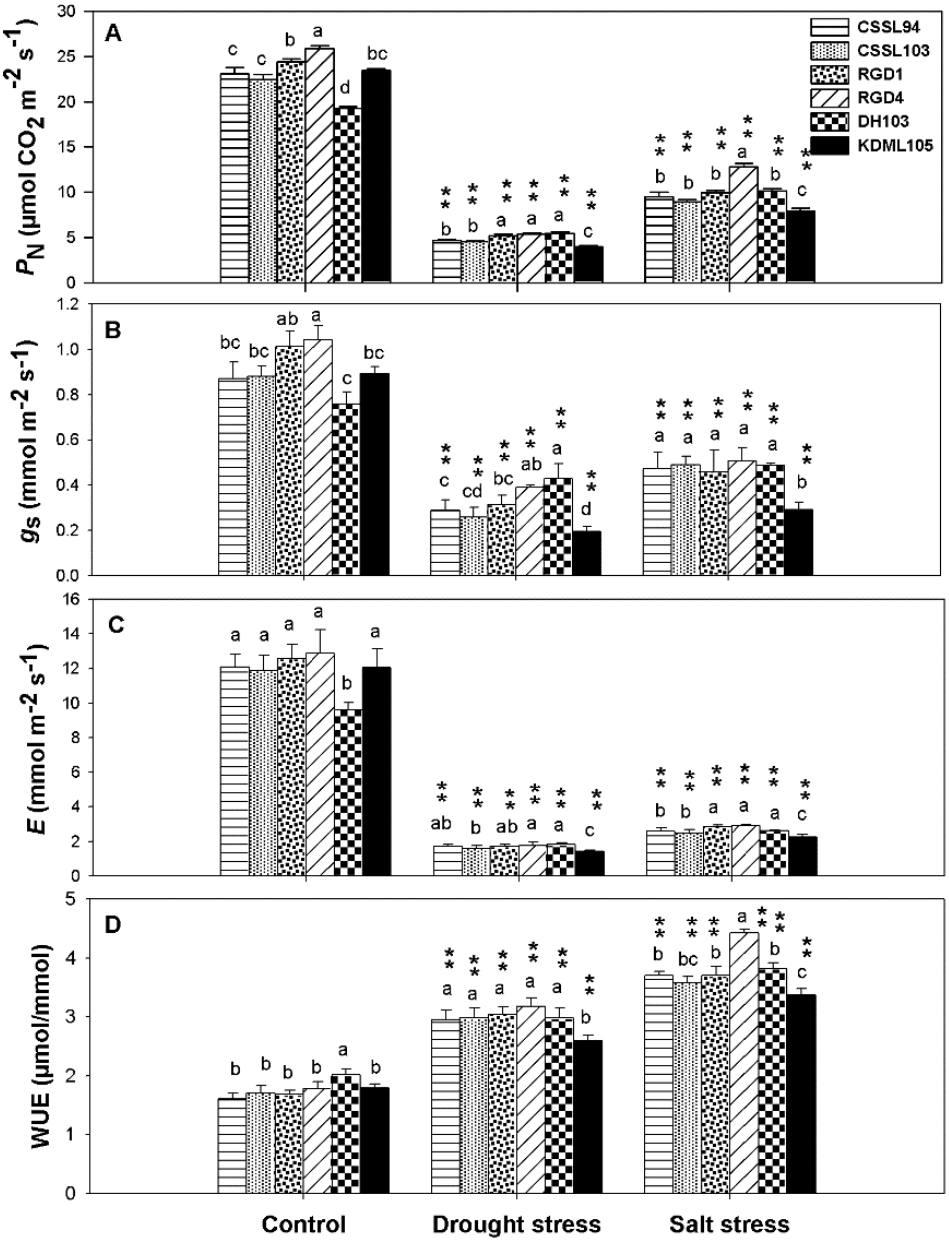

Figure 1. (A) Net photosynthesis rate (P_N), (B) stomatal conductance (g_s), (C) transpiration rate (E), and (D) water use efficiency (WUE) under control, salt, and drought stress for 10 days. Bars with different letters within each treatment group are significantly different according to DMRT ($p \leq 0.05$). The asterisk (*, **) indicates a significant difference ($p \leq 0.05$ and $p \leq 0.01$) in the mean values between control and stress (drought or salt stress) conditions of each line/cultivar

found in g_s (Figure 1B) and E (Figure 1C). Conversely, drought, and salt stress led to a large and significant increase in WUE in all rice lines/cultivars, particularly in RGD4 under salt stress (Figure 1D). Analysis of variance showed that P_N, g_s, E, and WUE were significantly affected by treatments (T), lines/cultivar (C), and treatments and lines/cultivar interaction (T × C), as shown in Table 1.

Table 1

Analysis of variance of studied parameters in six lines/cultivars of rice seedlings treated with drought treatment (20% PEG6000) and salt treatment (150 mM NaCl)

Parameter	Lines/Cultivars (C) (df = 5)	Treatment (T) (df = 2)	Lines/Cultivars × Treatment (C×T) (df = 10)
P_N	16.99**	2133.05**	7.97**
g_s	0.05**	2.53**	0.03**
E	2.56*	782.65**	2.51**
WUE	0.3*	24.70**	0.148*
F_0	128.39**	3376.042**	119.45**
F_m	221836.55**	77857.62**	13945.70**
Fv/Fm	0.0001**	0.03**	0.00002ns
F_v'/F_m'	0.12**	0.006**	0.01ns
NPQ	0.052**	0.055**	0.005**
SPAD	51.61**	24.23**	7.06**
TC	0.339**	1.666**	0.129**
Chl *a*	0.167**	0.637**	0.032**
Chl *b*	0.167**	0.637**	0.07**
CA	0.032**	0.268**	0.15**

Note. * and ** are means with significant differences at $p \leq 0.05$ and $p \leq 0.01$, respectively ns are means with no significant differences

Changes in Chlorophyll Fluorescence Parameters

Under drought stress, F_0 significantly increased in all lines/cultivar (except DH103) when compared to control plants. Of the lines/cultivar, 'KDML105' showed the highest F_0 whereas DH103 had the lowest, and all four improved lines had intermediate values. Under salt stress, 'KDML105' also showed the highest F_0, which was significantly higher than all other lines (Figure 2A). For F_m, only RGD1, RGD4, and 'KDML105' showed a significant reduction under drought stress;

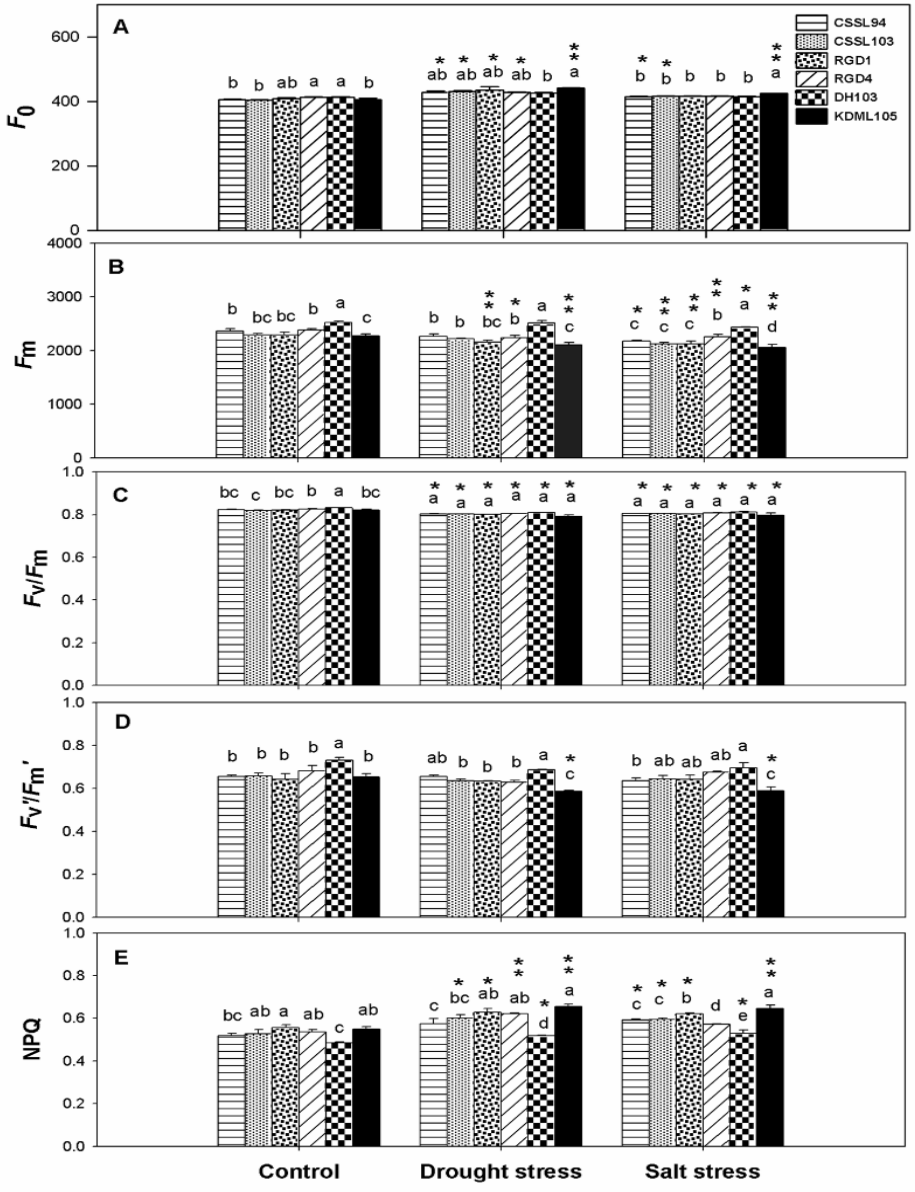

Figure 2. (A) Minimal fluorescence in the dark-adapted conditions (F_0), (B) maximal fluorescence in the dark-adapted conditions (F_m), (C) maximum quantum yield of PSII efficiency (F_v/F_m), (D) effective quantum yield of PSII efficiency (F_v'/F_m'), and (E) non-photochemical quenching (NPQ) under control, salt and drought stress for 10 days. Bars with different letters within each treatment group are significantly different according to DMRT ($p \leq 0.05$). The asterisk (*, **) indicates a significant difference ($p \leq 0.05$ and $p \leq 0.01$) in the mean values between control and stress (drought or salt stress) conditions of each line/cultivar

however, under salt stress, all six lines/cultivar showed a significant reduction in F_m (Figure 2B). Both drought and salt stress resulted in significant reductions ($p \leq 0.05$) in F_v/F_m of all rice lines/cultivar, with 'KDML105' showing the highest percent reduction (Figure 2C). However, for F_v'/F_m', only 'KDML105' showed a significant reduction ($p \leq 0.05$) when exposed to both stress conditions (Figure 2D). Under both types of stress, compared with the controls, NPQ increased in all genotypes, with 'KDML105' showing the highest percent increase (Figure 2E). Furthermore, all improved lines (CSSL94, CSSL103, RGD1, and RGD4) showed higher F_v/F_m, F_v'/F_m', and F_m, and lower F_0 and NPQ under drought and salt stress than 'KDML105' (Figures 2A-2D). Analysis of variance showed that differences among lines/cultivar (C), treatments (T) and treatments and lines/cultivar interaction (T × C) were significant for F_0, F_m, F_v/F_m, and F_v'/F_m', except for F_v/F_m and F_v'/F_m', which did not show a significant difference in (T x C) (Table 1).

Changes in the Content of Photosynthetic Pigments

In general, drought had stronger effects on pigment concentrations than salt stress. Under drought stress, a significant reduction in TC was observed in CSSL94 (14.0%), CSSL103 (9.6%), RGD1 (19.5%), RGD4 (20.6%), and 'KDML105' (33.6%) when compared with control plants (Figure 3C). The tolerant line DH103 exhibited stable TC, Chl *a*, and Chl *b* contents under both types of stress (Figures 3A, 3B, and 3C). Under salt stress, only 'KDML105' showed significant reductions in TC (19.6%), Chl *a* (12.8%), and Chl *b* (34.3%), where the only exception was CSSL103, which showed a significant reduction in Chl *b* content (18.3%). Under drought stress, all four improved introgression lines had significantly higher contents of TC, Chl *a*, and Chl *b* than 'KDML105'. Under salt stress, only RGD1 and RGD4 had significantly higher TC, Chl *a*, and Chl *b* contents than 'KDML105'. RGD4 had the highest pigment contents under salt stress (Figures 3A, 3B, and 3C). When subjected to drought stress, compared with the controls, carotenoid contents were significantly reduced in all lines/cultivar, with 'KDML105' being the most reduced (27.6%). However, under salt stress, carotenoid contents were significantly reduced in CSSL94, CSSL103, RGD1, and 'KDML105', but remained unchanged for RGD4 and DH103 (Figure 3D). RGD4 again had the highest carotenoid content under salt stress. For SPAD readings, under non-stressed conditions, DH103 showed the highest SPAD reading (37.08), whereas CSSL94, CSSL103, RGD1, RGD4 and 'KDML105' showed SPAD readings of 34.38, 33.68, 33.55, 35.20, and 34.40, respectively (Figure 3E). When subjected to drought and salt stress, leaf greenness of 'KDML105' was the most adversely affected among the six lines/cultivar. The percentage of reduction in SPAD readings under drought stress was 5.5, 1.6, 5.6, 7.0, and 15.3% in CSSL94, CSSL103, RGD1, RGD4, and 'KDML105', respectively, compared to the controls. Under salt stress,

only KDML105 showed a significant reduction ($p \leq 0.01$) in SPAD readings (decreased 9.85% compared to control), as shown in Figure 3E. Analysis of variance showed that differences among lines/cultivar (C), treatments (T), and treatments and lines/cultivar interaction (T × C) were significant for all pigment parameters (Table 1).

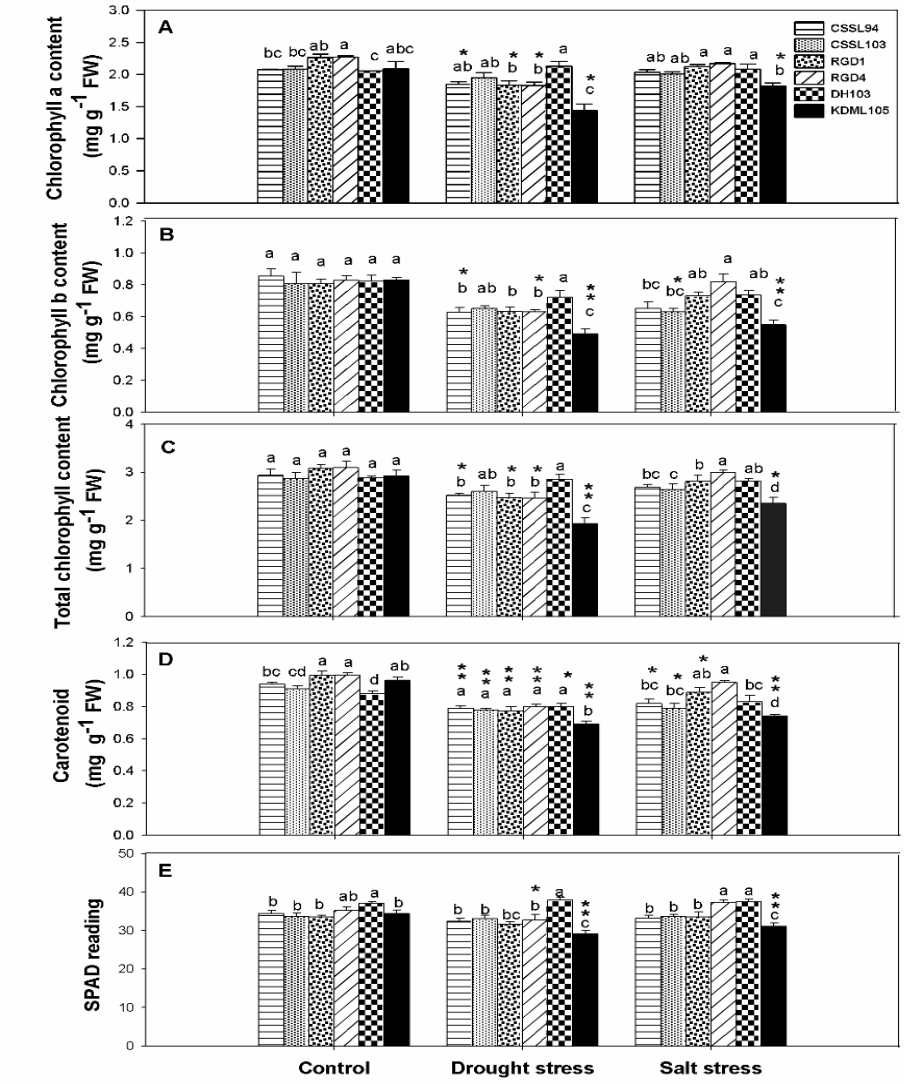

Figure 3. (A) Chlorophyll *a*, (B) chlorophyll *b*, (C) total chlorophyll, (D) carotenoid, and (E) SPAD readings under control, salt, and drought stress for 10 days. Bars with different letters within each treatment group are significantly different according to DMRT ($p \leq 0.05$). The asterisk (*, **) indicates a significant difference ($p \leq 0.05$ and $p \leq 0.01$) in the mean values between control and stress (drought or salt stress) conditions of each line/cultivar

The Correlation among Photosynthetic Traits

Relationships among fourteen photosynthetic traits (P_N, g_s, E, WUE, F_0, F_m, F_v/F_m, F_v'/F_m', NPQ, SPAD, TC, Chl *a*, Chl *b*, and CA) under stress conditions were determined using Pearson's correlation analysis. Under drought stress (Table 2), highly significant ($p \leq 0.01$) positive correlations were found between P_N and other leaf gas exchange parameters (g_s, E, and WUE). Furthermore, highly significant ($p \leq 0.01$) positive correlations were exhibited between P_N and fluorescence parameters (F_m, F_v/F_m, and F_v'/F_m') and between P_N and pigment parameters (SPAD, TC, Chl *a*, and Chl *b*). In contrast, significant negative correlations were observed between P_N and F_0 ($p \leq 0.05$) as well as NPQ ($p \leq 0.01$). Similarly, under salt stress (Table 3), significant positive correlations were found between P_N and g_s, E, WUE, F_m, F_v/F_m, SPAD, TC, Chl *a*, Chl *b*, and CA (all traits; $p \leq 0.01$, except for F_v/F_m, and CA; $p \leq 0.05$). In contrast, negative correlations were observed between P_N and F_0 ($p \leq 0.01$) as well as NPQ.

Table 2

Pearson's correlation coefficients (r values) among physiological parameters in rice seedling exposed to drought stress (20% PEG6000)

Parameter	P_N	g_s	E	WUE	F_0	F_m	F_v/F_m
P_N	1						
g_s	0.787**	1					
E	0.730**	0.687**	1				
WUE	0.715**	0.488*	0.534**	1			
F_0	-0.444*	-0.482*	-0.381	-0.645**	1		
F_m	0.541**	0.610**	0.536**	0.284	-0.422*	1	
*F*v/*F*m	0.521**	0.366	0.680**	0.534**	-0.423*	0.553**	1
F_v'/F_m'	0.542**	0.585**	0.764**	0.408*	-0.564**	0.602**	0.594**
NPQ	-0.458**	-0.565**	-0.569**	-0.245	0.509**	-0.840**	-0.600**
SPAD	0.571**	0.671**	0.567**	0.413*	-0.464*	0.743**	0.395
TC	0.620**	0.556**	0.505**	0.414*	-0.416*	0.617**	0.506*
Chl *a*	0.549**	0.527**	0.427*	0.368	-0.441*	0.557**	0.443*
Chl *b*	0.641**	0.461*	0.589**	0.424*	-0.196	0.602**	0.539**
CA	-0.172	0.018	-0.122	0.539**	0.056	0.111	-0.245

Table 2 *(Continued)*

Parameter	F_v'/F_m'	NPQ	SPAD	TC	Chl *a*	Chl *b*	CA
P_N							
g_s							
E							
WUE							
F_0							
F_m							
*F*v/*F*m							
F_v'/F_m'	1						
NPQ	-0.745**	1					
SPAD	0.686**	-0.758**	1				
TC	0.647**	-0.501*	0.555**	1			
Chl *a*	0.600**	-0.451*	0.518**	0.978**	1		
Chl *b*	0.583**	-0.495*	0.491*	0.733**	0.575**	1	
CA	-0.060	-0.134	-0.138	0.095	0.140	- 0.085	1

Note. Each value indicates the Pearson's correlation coefficient of a pair of parameters. * and ** denote correlations that are significantly different at $p \leq 0.05$ and $p \leq 0.01$, respectively

Table 3

Pearson's correlation coefficients (r values) among physiological parameters in rice seedling exposed to salt stress (150 mM NaCl)

Parameter	P_N	g_s	E	WUE	F_0	F_m	F_v/F_m
P_N	1						
g_s	0.576**	1					
E	0.755**	0.639**	1				
WUE	0.828**	0.436*	0.539**	1			
F_0	-0.555**	-0.710**	-0.623**	-0.441*	1		
F_m	0.585**	0.473*	0.692**	0.652**	-0.642**	1	
*F*v/*F*m	0.448*	0.677**	0.532**	0.386	-0.746**	0.688**	1
F_v'/F_m'	0.306	0.493*	0.576**	0.250	-0.614**	0.651**	0.693**
NPQ	-0.383	-0.639**	-0.537**	-0.371	0.714**	-0.638**	-0.769**
SPAD	0.646**	0.601**	0.726**	0.519**	-0.687**	0.852**	0.659**
TC	0.677**	0.603**	0.646**	0.611**	-0.568**	0.627**	0.689**
Chl *a*	0.533**	0.568**	0.494*	0.463*	-0.492*	0.452*	0.743**
Chl *b*	0.627**	0.416*	0.619**	0.594**	-0.457*	0.645**	0.331
CA	0.443*	0.372	0.525**	0.495**	-0.418*	0.752**	0.576**

Table 3 *(Continued)*

Parameter	F_v'/F_m'	NPQ	SPAD	TC	Chl *a*	Chl *b*	CA
P_N							
g_s							
E							
WUE							
F_0							
F_m							
*F*v/*F*m							
F_v'/F_m'	1						
NPQ	-0.663**	1					
SPAD	0.466*	-0.651**	1				
TC	0.467*	-0.635**	0.596**	1			
Chl *a*	0.478*	-0.619**	0.401	0.902**	1		
Chl *b*	0.264	-0.406*	0.656**	0.750**	0.391	1	
CA	0.593**	-0.542**	0.558**	0.521**	0.450*	0.421*	1

Note. Each value indicates the Pearson's correlation coefficient of a pair of parameters. * and ** denote correlations that are significantly different at $p \leq 0.05$ and $p \leq 0.01$, respectively

Cluster Analysis among Lines/Cultivars under Drought and Salt Stress

All six rice lines/cultivar were classified into three groups as indicated by the PC-ORD program at 75% similarity based on fourteen photosynthetic traits. Under drought stress, the dendrogram divided the rice into three groups. 'KDML105' and DH103 each formed a group individually, whereas all four improved lines were clustered into one group (Figure 4A). Under salt stress, the six rice lines/cultivar were also divided into three groups: the first group included DH103 and RGD4, the second group included 'KDML105', and the third group included CSSL94, CSSL103, and RGD1 (Figure 4B).

DISCUSSION

The ability of plants to tolerate, survive, and grow in adverse environments depends directly on the efficiency of photosynthesis. The major components limiting photosynthesis under drought and salt stress include a reduction in chlorophyll content, decrease in photochemical efficiency of PSII, limitation of CO_2 diffusion due to stomatal closure, and reduction in the activity of photosynthetic enzymes (Ashraf & Harris, 2013; Pandey & Shukla, 2015; Sudhir & Murthy, 2004). In this study, photosynthesis-related traits, including leaf greenness, photosynthetic pigments, PSII photochemical efficiency, and

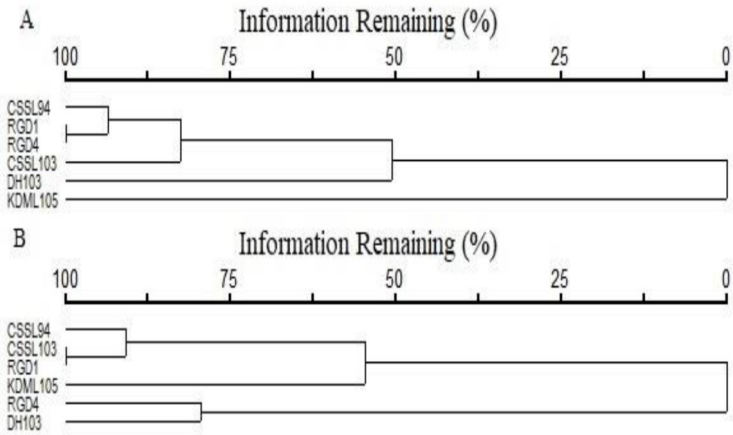

Figure 4. Dendrogram showing similarity among improved lines of rice 'KDML105' (CSSL94, CSSL103, RGD1, and RGD4), 'KDML105' and DH103 based on leaf photosynthetic parameters under (A) drought stress (20% PEG6000) and (B) salt stress (150 mM NaCl) at the seedling stage

stomatal conductance, had strong positive correlations with the net CO_2 fixation rate under stress conditions (Tables 2 and 3). This indicates the strong influences of pigment contents, energy transfer efficiency in the photosystem, and stomatal limitation on photosynthetic performance (Moradi & Ismail, 2007).

The leaf greenness values (SPAD reading) and photosynthetic pigments are indicators that can be related to photosynthetic performance. As indicated in this study, P_N was highly significantly correlated with chlorophyll contents and SPAD readings (Tables 2 and 3). A decrease in chlorophyll content under salt stress is a commonly reported phenomenon in various studies because of the adverse effects of sodium ions on the stability of the chloroplast membrane (Ashraf & Bhatti, 2000; Pongprayoon et al., 2019). A drought stress-induced reduction in photosynthetic pigments occurred in conjunction with damage to chloroplast structure and the photosynthetic apparatus, consequently inhibiting net CO_2 assimilation (Kalefetoğlu Macar & Ekmekçi, 2009; Li et al., 2006; Wang et al., 2018). Photosynthetic pigments and SPAD readings were used as indicators of abiotic stress tolerance, where less reduction in pigment contents or SPAD readings under stress indicated a higher level of plant stress tolerance (Jinwen et al., 2009; Munns et al., 2006). For rice, under both drought (Kumari et al., 2019) and salt stress (Lee et al., 2013), more sensitive varieties showed a greater reduction in chlorophyll content than the more tolerant varieties. In the present study, drought and salt stress decreased photosynthetic pigments and SPAD readings, and these parameters were closely related (Tables 2 and 3). Moreover,

all improved lines of 'KDML105', which carried DT-QTLs (CSSL94 and CSSL103) or *SKC1* gene (RGD1 and RGD4), had higher chlorophyll, carotenoids and SPAD readings than the sensitive parent 'KDML105' under both stress conditions. Co-expression network analysis of genes located in the DT-QTL that was introgressed into CSSL94 (Nounjan et al., 2018) revealed that the gene *Os08g41990* involved in chlorophyll biosynthetic processes might play a role in maintaining chlorophyll content under salt stress.

The effects of drought and salt stress on the leaf PSII photochemical efficiency were expressed in terms of F_0, F_m, F_v/F_m, F_v'/F_m', and NPQ (Moradi & Ismail, 2007; Li et al., 2006). It was previously reported that drought and salt stress reduced F_m, F_v/F_m, and F_v'/F_m' and increased F_0 and NPQ (Dongsansuk et al., 2013; Mishra & Panda, 2017). A reduction in F_m, F_v/F_m, and F_v'/F_m' indicates that the thylakoid membrane and chloroplast are damaged due to photoinhibition leading to a decrease in P_N (Murata et al., 2007; Ranjbarfordoei et al., 2006). Similar results were found in this study, where drought and salt stress lowered the leaf PSII photochemical efficiency in rice seedlings of all lines/cultivar. Chlorophyll fluorescence analysis of 232 diverse rice genotypes clearly showed that less reduction in F_m, F_v/F_m, and F_v'/F_m' and less increase in F_0 and NPQ were associated with higher salt tolerance ability (Tsai et al., 2019). Under both drought and salt stress, all improved lines had significantly higher F_v'/F_m' than 'KDML105'. A reduction in PSII photochemical efficiency was reported to be highly correlated with a reduction in net photosynthesis rate under drought (Iqbal et al., 2019), which is like the results in this study (Table 2). In contrast, F_0 and NPQ increased when plants were subjected to drought and salt treatments. An increase in F_0 was associated with photoinactivation and damage to PSII reaction centers, while NPQ indicated the photoprotective processes that removed excess excitation energy to prevent the formation of harmful free radicals (Murchie & Lawson, 2013). All improved lines (CSSL94, CSSL103, RGD1, and RGD4) showed higher F_v'/F_m' and lower NPQ than the parental line 'KDML105', indicating more efficient use of light energy in photochemical reaction and therefore less non-photochemical energy dissipation. Similar findings by Dongsansuk et al. (2013) reported that salt stress also caused a dramatic reduction in F_v'/F_m', while NPQ was increased in 'KDML105'. In contrast, leaf PSII photochemical efficiency was unaffected by salt stress in Pokkali (salt-tolerant variety). Mishra and Panda (2017) also reported that the tolerant variety N22 showed higher F_m and F_v/F_m as well as lower F_0 and NPQ under drought stress than the susceptible variety IR64. In comparison with 'KDML105', all improved lines in this study also showed higher F_m and F_v/F_m as well as lower F_0 and NPQ under drought and salt stress.

A decline in photochemical efficiency under drought and salt stress which leads to reduced light-dependent NADPH and ATP synthesis, combined with a reduction in CO_2

uptake and diffusion due to stomatal closure, results in lower CO_2 fixation and Calvin cycle activities (Chaves et al., 2009). Hence, the P_N of rice in this study was dramatically reduced from an average (across genotypes) of 23.08 µmol (CO_2) m^{-2} s^{-1} in the controls to 4.86 µmol (CO_2) m^{-2} s^{-1} under drought and 9.87 µmol (CO_2) m^{-2} s^{-1} under salt stress (Figure 1A; Tables 2 and 3). Based on the observation of Ramegowda et al. (2014), a similar level of reduction in P_N of rice at the vegetative growth stage was demonstrated, dropping from 21 µmol (CO_2) m^{-2} s^{-1} under control conditions to approximately 6 µmol (CO_2) m^{-2} s^{-1} under drought stress. For salt stress, Nounjan et al. (2018) found that the P_N of 'KDML105' declined from 24 µmol (CO_2) m^{-2} s^{-1} to 4 µmol (CO_2) m^{-2} s^{-1} when subjected to salt stress at 16.50 dS m^{-1} for 9 days. Under slightly lower stress levels in this study (14.8 dS m^{-1} for 10 days), P_N of 'KDML105' decreased from 23.45 to 7.90 µmol (CO_2) m^{-2} s^{-1} (Figure 1A).

Simultaneous investigations on the effects of drought and salt stress on rice have rarely been reported, and this study demonstrated that drought imposed a stronger inhibitory effect than salt stress on photosynthesis in rice. Although the PEG and NaCl solutions had an equal osmotic potential of -0.7 MPa and were expected to induce similar levels of osmotic stress, a greater reduction in pigment contents and most photosynthetic parameters was recorded in the drought-induced PEG solutions (Figures 1A and 1B). This could be due to some intrinsic limitations of using PEG to simulate drought conditions, such as the viscosity of the solution and absorption of PEG by roots, thus resulting in less water uptake, root damage, and leaf dysfunction (Osmolovskaya et al., 2018). Moreover, PEG solutions caused more rapid leaf rolling, compared to NaCl solution, leading to lower leaf surface area exposed to sunlight, hence lower photosynthesis rate. On the other hand, the negative effects of salt stress on photosynthesis were due to osmotic stress, which mainly induced stomatal closure as well as ion toxicity stress, which occurs as a result of excess Na^+ accumulation in the chloroplasts (Munns & Tester, 2008). The direct influences of excess Na^+ on photosynthesis were associated with the disruption of the proton motive force necessary for ATP production as well as its interference with CO_2 fixing enzymes (van Zelm et al., 2020).

In this study, all improved lines of 'KDML105', namely CSSL94, CSSL103, RGD1, and RGD4, showed greater values in P_N and all related gas exchange parameters (g_s, E, and WUE) than the parental cultivar 'KDML105' under both drought and salt stress. In the case of CSSL94, similar results were demonstrated by Nounjan et al. (2016), where CSSL94 had higher P_N under salinity stress compared to 'KDML105'. Co-expression network analysis of genes located in the DT-QTL introgressed into CSSL94 (Nounjan et al., 2018) revealed that the gene *Os08g41990* involved in chlorophyll biosynthetic process might play a role in maintaining chlorophyll content under salt stress. Consequently, this may lead to the higher photosynthetic

performance of CSSL94. However, photosynthesis of this line under drought stress has not been observed. In a study using a related near-isogenic line from the same CSSL population, Chutimanukul et al. (2018) reported that rice line CSSL16 (improved 'KDML105' introgressed with DT-QTL segments from chromosome 1 of DH212) showed a much lower reduction in photosynthesis under salt stress than 'KDML105'. From the co-expression analysis study, ten hub genes on the DT-QTL were found, of which six functioned in chloroplasts. One of these genes, *PsbS1*, which encodes the CP22 protein located in PSII, was proven to play a protective role through its activity in non-photochemical quenching under salt stress (Chutimanukul et al., 2018). The *SKC1* gene, also known as *OsHKT1;5* in rice, is responsible for the regulation of Na^+ in the shoot and is more highly expressed in rice genotypes with higher salt tolerance associated with good maintenance of various physiological status, including higher photosynthetic capacity (Ueda et al., 2013). Transfer of the *HKT1;5* genes from maize into tobacco resulted in transgenic tobacco with enhanced salt tolerance (Jiang et al., 2018). The crucial role of this gene in enhancing salt tolerance was confirmed in the present study. The introgression lines (RGD1 and RGD4) carrying this gene, which was transferred from a salt-tolerant donor into the genetic background of 'KDML105', showed higher salt tolerance, as indicated by a significantly higher P_N and other related gas exchange parameters of these lines compared to 'KDML105' under salt treatments (Figure 1). Moreover, the photosynthetic performance of these lines under drought stress was also superior to 'KDML105'. This is the first report on physiological studies, particularly in relation to photosynthesis, of these lines under salt and drought stress.

The results in this study clearly showed that introgression of DT-QTLs into the drought-sensitive 'KDML105' not only conferred drought tolerance ability to the improved lines (CSSL94 and CSSL103) but also enhanced salt resistance. Similar work by Basu et al. (2017) revealed two drought-tolerant breeding lines with superior drought and salinity tolerance based on several physiological parameters, including photosynthesis. Moreover, introgression lines (RGD1 and RGD4) of 'KDML105' carrying *SKC1* gene were more tolerant not only to salt stress but also to drought stress, as indicated by enhanced photosynthetic performance. As suggested by Nounjan et al. (2016), photosynthesis is an important target trait for the selection of breeding lines tolerant to drought and salt stress, and improving crop yield relies largely on photosynthetic performance (Zhu et al., 2010). Thus, these improved lines with high photosynthetic capacity are potentially useful as genetic resources for developing improved Thai jasmine rice lines for multiple stress tolerance via gene pyramiding.

CONCLUSION

Thai jasmine rice 'KDML105' is sensitive to drought and salinity. This study highlighted a search for improved rice breeding lines with a 'KDML105' genetic background that better tolerate drought and salinity stress based on photosynthesis-related characters, including better maintenance of pigments, PSII photochemical efficiency, and net photosynthesis rate. The improved photosynthetic performance under drought and salt stress of CSSL94, CSSL103, RGD1, and RGD4 indicated that these lines have the potential to be used in the rice breeding program and targeted for improvement of multiple stress tolerance.

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Characterization of Peat Microbial Functional Diversity in Aerobic Rice Rhizosphere

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ABSTRACT

Microorganisms in the rhizosphere possess numerous metabolic activities. The addition of inorganic substance such as fertilizer could affect the microbial functional diversity. This study was conducted to evaluate the effect of different rate of NPK fertilizer on microbial functional diversity in the rhizosphere of local aerobic rice variety. Aerobic rice variety MRIA 1 was used in this study. Peat was taken from a non-agricultural area in Klang, Malaysia. The effect of fertilizer rate was determined with 4 different rates (C = non-fertilized; T1 = 100 kg/ha NPK; T2 = 200 kg/ha NPK; T3 = 400 kg/ha NPK). Microbial functional diversity was performed using Biolog™ Ecoplate System and measured by microbial activities, such as average well color development (AWCD), species richness (*R*), Shannon-Weaver index (*H* index) and species evenness (*E*). As a result, microbial activity increased to 5.7% when fertilizer applied at T2, while fertilizer rate at T3 increased species richness by 3.2%. However, addition of fertilizer did not affect the *H* index while species evenness slightly decreased by 1.1% when applied at T3. Bacteria population was reduced when fertilizer added at T1. Fertilizer addition to the peat soil decreased the culturable population of nitrogen-fixing microbes while no effect was found on culturable fungal, actinomycetes and phosphate-solubilizing microbe population. Microbes in T2 utilized many carbon

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sources. Variation in carbon sources used by microbes was found when fertilizer was applied at different rates. D-cellobiose, pyruvic acid methyl ester, and L-serine were the carbon sources that influenced the microbial function in soil. It is concluded that fertilizer has an effect on microbial functional diversity in the peat rhizosphere of local aerobic variety. The recommended fertilizer rate (T2) increased the microbial activity while high fertilizer rate (T3) increased species richness and decreased species evenness.

Keywords: Carbon source, fertilizer, microbial functional diversity, rhizosphere

INTRODUCTION

Fertilizer is one of essential component for agricultural crop production. The acidification of soil and nutrients disparity was among the bad effects of high amount of fertilizer in soil. Nitrogen and phosphorus in fertilizer could affect the functional diversity and community structure of rhizosphere microorganisms, which play a vital role in plant growth and soil fertility (Dotaniya & Meena, 2015). A decrease in microbial community due to inorganic disturbance such as fertilizer could potentially limit the services that they provide for the soil and plant. Therefore, it is important to study how microbial community in the rhizosphere responds towards environmental changes. Rhizosphere has been regarded as a remarkable ecosystem because microorganisms inhabited this area showed a variety of metabolic activities (Saleem, 2015). Microorganisms in rhizosphere are capable to do vast functions, for instance, constructing below and above multitrophic interactions and nutrient cycling covering different trophic levels.

To date, there is inadequate microorganism research in aerobic rice. Prior studies on aerobic rice only emphasized on useful microorganism for enhancing plant growth and as soil enrichment (Nahi et al., 2016; Nasarudin et al., 2018). On the other hand, constant flooding and raised water inputs are needed in the usual paddy rice field. These will be challenging since it is predicted that economic water insufficiency will affect 22 million irrigated dry-season rice in Southeast Asia by 2025 (Tuong & Bouman, 2003). Henceforth, aerobic rice was initiated to preserve and improve water use efficiency. The trait from lowland and upland rice were integrated to produce aerobic rice (Tuong & Bouman, 2003), which can be grown in non-flooded fields with additional irrigation and high input. Researches on microbial community using restriction fragment length polymorphism were already performed (Sutradhar, 2015; Vishwakarma & Dubey, 2019). However, to our knowledge, there is no study on the response of microbial functional diversity towards fertilizer specifically in local aerobic rice. The response of microbial functional diversity towards fertilizer is important because as mentioned earlier microbes play a vital role in plant growth and soil fertility. Microbial functional diversity reflects genetic diversity (Carolina,

2018) and it is important to determine the analytical level of diversity to sustain ecosystem function from disturbances. Aerobic rice is a potential crop for future food production. The ability of aerobic rice to grow in non-saturated condition is suitable to address water scarcity which is beneficial for food security. Thus, this study was conducted with the objective to evaluate the effect of NPK fertilizer on microbial functional diversity in the rhizosphere of local aerobic rice variety using the Biolog™ Ecoplate System.

MATERIALS AND METHOD

Experimental Design

This study was carried out in the glasshouse at the Malaysian Agricultural Research and Development Institute (MARDI). The temperature of the glasshouse when the study was conducted was 32 ^{o}C. MRIA 1 was selected for aerobic rice variety and was planted in a planter box (620 mm x 230 mm x 188 mm). Peat soil was selected because aerobic rice was previously being planted in peat areas in order to test the planting suitability of aerobic rice MRIA 1 (Othman et al., 2014). Peat was taken from a non-agricultural area in Klang, Malaysia. The peat is classified as hemic and collected from an oligotrophic area. A total of 4 planter boxes used for each fertilizer rate representing 4 replicates. Each planter box was planted with 5 aerobic rice and composite as 1 replicate. The experiment was arranged in a randomized complete block design (RCBD).

A standard manual by Othman (2013) was adapted for the growth of aerobic rice. Prior to planting, soil was prepared using rack, shovel, and trowel. Aerobic rice seed germination was performed by soaking the seeds in tap water for 24 hours. The seed were sown with the rate of 130 kg/ha using the dry direct seeding method. Each seed was sown with the distance between 25 cm. The NPK compound fertilizer rate used for aerobic rice is 150:60:60 kg/ha of N, P_2O_5, and K_2O (Othman, 2013). The application of compound fertilizer was done at 5 days, 25 days, and 45 days after sowing. The optimum water depth for soil watering was 40 mm which occured during the vegetative stage. The effect of fertilizer rate was determined with 4 different rates as follows:

a. non-fertilized (control)
b. 100 kg/ha NPK compound fertilizer (T1)
c. 200 kg/ha NPK compound fertilizer, recommended dosage (T2)
d. 400 kg/ha NPK compound fertilizer (T3)

Soil Sampling

Rhizosphere was collected during the mature stage of aerobic rice growth, which was after 90 days of sowing. The soil strongly adhered to the roots after gentle shaking was considered as rhizosphere soil.

Soil Properties

A standard method by Chapman and Pratt (1978) was used for measuring soil pH

in soil/water extract with the ratio of 1:10. Cation exchange capacity (CEC) was performed according to the proposed method by Harada and Inoko (1980). The determination of soluble phosphorus was performed according to Bray and Kurtz (1945). A total of 2 g of soil was added to 20 ml of extracting solution which consisted of NH_4F and HCl to remove soluble phosphorus. Flow injection analyzer (Lachat Instruments, USA) was used to measure CEC and soluble phosphorus. Total carbon and total nitrogen were measured according to the combustion method (Lagen, 1996) by using elemental analyzer (Flash 2000, Thermo Scientific, USA).

Microbial Functional Diversity Analysis

Microbial functional diversity was evaluated by measuring the microbial activity and community level physiological profile. Biolog Ecoplate™ system was used to assess these two criteria. The Biolog Ecoplate™ consists of 31 different carbon sources pertinent to ecosystem. A metabolic fingerprint was produced from the interaction between the microbial community and carbon sources (Garland, 1997). Soil suspensions (soil 100 g to 1 litre of distilled water) were agitated for 30 min followed by filtration using 0.45 µm filter paper. A total of 100 µl aliquots were inoculated in the Ecoplate™ and incubated at 27°C. The consumption of carbon source by microbes resulted in cell respiration and thus reduces tetrazolium dye to purple color. Color intensity in each well was recorded as optical density (OD) at 590 nm wavelength. To ensure that only carbon source-use response was obtained, the OD was measured at 48 hours to allow the microbial usage of any soluble organic carbon obtained from the rhizosphere (Gomez et al., 2006).

Microbial Activity

Average well color development was validated by measuring the OD values (590 nm) after 48 hours of incubation. Average well color development was calculated as follows (Gomez et al., 2006):

$$AWCD = \sum OD_i / 31$$

where, OD_i is the optical density value from each Ecoplate™ well.

Community-level Physiological Profile

Community-level physiological profile was determined by assessing the richness (*R*), Shannon diversity index (*H*), and Shannon evenness (*E*) (Garland, 1997). The values were calculated from the OD values of Ecoplate wells incubated at 48 hours. Richness was determined as the total number of oxidized carbon substrates (Biolog EcoPlate™ well with OD ≥ 0.25 as threshold for positive response of oxidized carbon substrates). Shannon diversity index was calculated as follows:

$$H = \Sigma p_i (\ln p_i)$$

where, p_i is the ratio of the activity on each substrate (OD_i) to the sum of activities on all substrates (ΣOD_i) in

Biolog EcoPlate™ well whereas ln is the natural logarithm.

Shannon evenness was examined from the value of *H* and *R* (Zak et al., 1994) and calculated as follows:

$$E = H / \ln R$$

Carbon Source Utilization

The utilization of carbon source of microbes was measured using OD in Ecoplate well after 48 hours of incubation. Carbon source in Ecoplate was categorized into 5 guilds as according to Weber and Legge (2009).

Enumeration of Culturable Microorganism

Total plate count was used to evaluate culturable aerobic microorganism. The abundance of bacteria, fungi, actinomycetes, nitrogen-fixing microbes and phosphate-solubilizing microbes were measured by using nutrient agar (NA), potato dextrose agar (PDA), starch casein agar (SCA), Burks's agar (NF), and Pikovskaya's agar (PKA), respectively. Nutrient agar and PDA were prepared as specified by the manufacturer (Merck, Germany). Burk's agar, PKA, and SCA were prepared as according to Ali Hassan et al. (2019). A total of 10 g of soil was added into 90 ml of sterile distilled water to create a soil suspension. A tenfold dilution series was performed on the soil suspension and aliquots of 1 ml were inoculated on selective media and incubated at 27°C for 48 hours for NA and PDA while NF, PKA, and SCA were incubated for 168 hours (7 days). Colony forming unit (CFU) was used to quantify total plate count and expressed as $\log_{10}$ CFU/ml.

Statistical Analysis

Analysis of variance (ANOVA) and multiple comparisons of means Fisher's least significant difference (LSD) test were used for the interpretation of AWCD, *R*, *H*, *E*, and aerobic culturable microorganism. The amount of change in AWCD, *R*, *H*, *E*, and aerobic culturable microorganism were calculated using the percentage of change. Principal component analysis was performed for substrates in Ecoplate™ well. All statistical analyses were performed with Minitab 17.

RESULT AND DISCUSSION

Soil Properties

The average of total carbon, total nitrogen, total and soluble phosphorus, CEC and pH were 45.09%, 2.36%, 853.87 ppm, 57.35 ppm, 75.57 meq/100g, and 4.91, respectively (Table 1). Fertilizer that was applied at 50% lower than recommended rate (T1) increased the total nitrogen. This indicates that fertilizer application at 100% recommended rate (T2) and high rate (T3) can decrease total nitrogen and this might be explained by high nitrogen mineralization. A previous study had shown that the addition of high NPK fertilizer could cause high nitrogen mineralization in peat (Maftu'ah et al., 2019).

However, total carbon, total phosphorus, and CEC were increased when fertilizer was

applied at 50% lower than the recommended rate (T1) and at 100% recommended rate (T2). This indicates that excessive fertilizer (T3) reduces total phosphorus in peat that might be caused by the low capacity of peat organic colloid to adsorb phosphorus (Ahmad et al., 2013). Prior study had shown that high amount of inorganic fertilizer reduced the soil CEC (Zhang et al., 2017). The decrease of CEC might be explained by the enhanced of the soil organic matter decomposition when high NPK fertilizer was applied as demonstrated by previous research (Bhatt et al., 2019).

Table 1
Soil chemical properties

Fertilizer rate	TC (%)	TN (%)	TP (ppm)	SP (ppm)	CEC (meq/100 g)	pH
C	37.08c	1.77c	598.51c	50.5c	67.88c	4.54c
T1	51.16a	2.68a	847.25b	65.9b	70.11b	4.86b
T2	39.01b	2.03b	860.48a	48.8d	81.02a	4.96a
T3	24.34d	1.42d	581.89d	74.7a	60.86d	4.96a

Note. CEC = Cation exchange capacity; SP = Soluble phosphorus; TC = Total carbon; TN = Total nitrogen; TP = Total phosphorus. Mean separation of soil chemical properties using Fisher's least significant difference. Mean in each column with the same letter is not significantly different ($p \leq 0.05$) among the fertilizer rate

Table 1 shows that the addition of fertilizer from T1 and T3 had increased the pH from 4.54 (non-fertilized) to 4.96 (heavily fertilized). The increase in pH will result in a conducive environment for microbial population as more microbes that prefer pH 5 and above may thrive in this condition as compared to lower pH condition. Earlier studies have shown that pH is the main driver of microbial diversity (Ren et al., 2018; Too et al., 2018).

Apart from that, it can be deduced from the results of total carbon and nitrogen that the C/N ratio was the lowest in T3 (17.14) and the highest in C (20.9) due to no addition of nitrogen from fertilizer was incorporated into the soil in C. According to previous studies (Chen et al., 2014; Leifeld et al., 2020), a C/N ratio of less than 25 will result in decomposition to proceed at maximum rate under suitable environmental condition. It is noted that high fertilizer (T3) reduced almost all of the selected soil chemical properties (total carbon, nitrogen, phosphorus, and CEC) compared to other treatments.

Microbial Activity

The ability of microbes to use specified substrate in Biolog Ecoplate is expressed as AWCD and can be applied as microbial activity. Microbial activity was increased to 5.7% when fertilizer was applied at the

recommended rate (Figure 1). Figure 1 shows that microbial activity was higher in T2 and T3 compared to C and T1. This suggests that the addition of NPK fertilizer increased the activity of microbes in peat soil. Such finding is supported by the results in soil chemical properties where at the highest rate of fertilizer application most of the soil chemical properties were reduced.

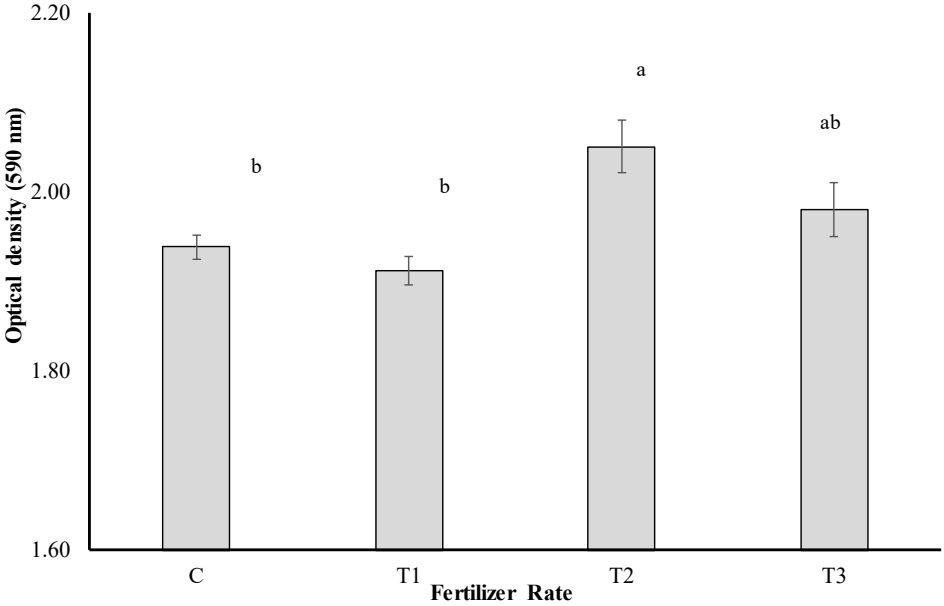


Figure 1. Microbial activity in peat

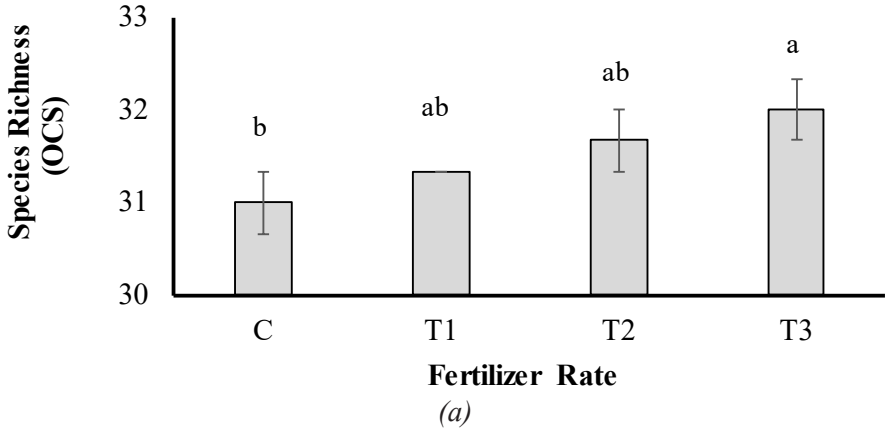
Furthermore, this result also justified by the data in soil chemical properties where at higher rate of fertilizer application, C/N ratio was reduced. Thus, this led to decomposition process resulting in higher microbial activity.

Community-level Physiological Profile

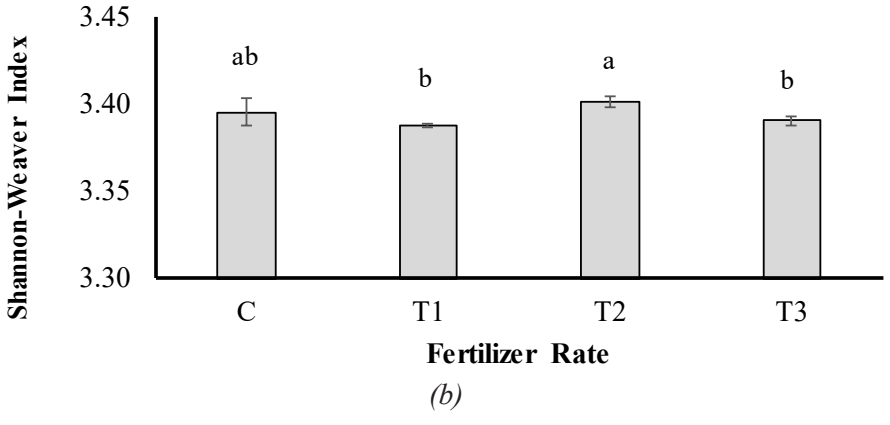
Figure 2a illustrates that high fertilizer rate (T3) increased peat species richness by 3.2%. The addition of fertilizer rate of T3 might increase microorganisms that were able to adapt to the new rhizosphere environment. For example, based on the results of soil properties, total nitrogen was reduced at T3 indicated that high nitrogen mineralization might occur. A previous report showed that archaea were significantly involved the nitrogen mineralization in peat soil (Espenberg et al., 2018). This might explain on the increase of species richness.

Shannon-Weaver index (*H* index) was used to measured catabolic diversity. It was noted that fertilizer did not affect the *H* index (Figure 2b) while Figure 2c showed that microbial evenness was slightly decreased

by 1.1% when applied to highest fertilizer rate (T3). Although species evenness was reduced, the increase in richness managed to elevate microbial activity and thus not affecting the catabolic diversity. Species that had been elevated was able to perform catabolic function in peat. The slight decrease of species evenness at T3 might be caused by the increase of specific microorganisms that favored this environment. The results from soil chemical properties showed that most of the soil chemical properties were reduced at T3. This indicated that there were microorganisms that reduced and became dormant while there are others that were enhanced from these conditions thus decreasing the species evenness.

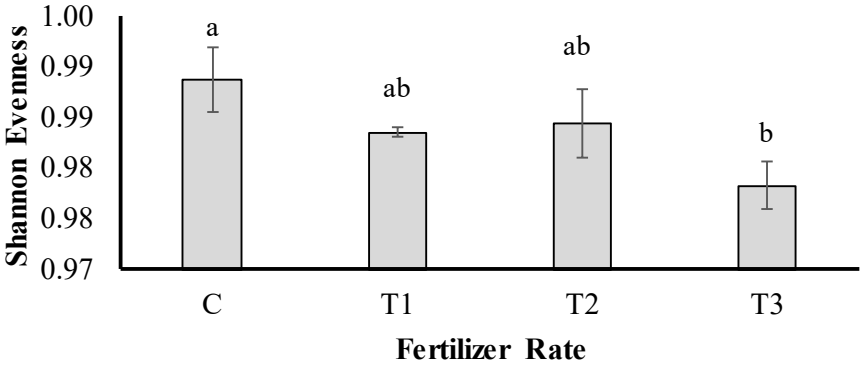


(a)



(b)

Figure 2. Community-level physiological profile in peat is evaluated by measuring (a) species richness, (b) Shannon-Weaver index, and (c) Shannon evenness. Mean separation of community-level physiological profile using Fisher's least significant difference. Mean in each column chart with the same letter is not significantly different ($p \leq 0.05$) among the fertilizer rate



(c)

Figure 2. (Continued)
Note. OCS = Total number of oxidized carbon substrate

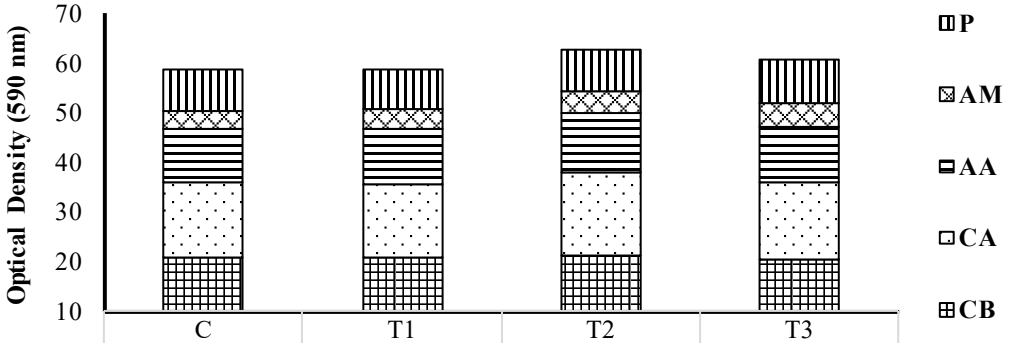
Microbial functional diversity was characterized by measuring the microbial activity and community-level physiological profile (species richness, catabolic diversity, and microbial evenness). Microbial activity in peat was increased when added with recommended fertilizer rate (T2). Species richness was elevated with excessive fertilizer application (T3). Fertilizer addition reduced species evenness but did not influence their catabolic diversity. These results indicate that microbial functional diversity in peat was enhanced with the addition of fertilizer rate tested in this study.

Carbon Source Utilization

Figure 3 shows that fertilizer application did not influence the consumption of carbohydrates by microorganisms. This indicates that the rhizosphere microorganisms in non-fertilized and fertilized peat utilized many of the given carbohydrates and might explained on the insignificant effect of fertilizer towards carbohydrate consumption. A recent study by Tang et al. (2020) had demonstrated that carbohydrates were the main carbon source that was utilized by rhizosphere microorganism. In peat soil, carbohydrates are involved in peat formation and act as an indicator for organic matter reactivity for humification (Hodgkins et al., 2018), which showed the importance of carbohydrate in peat. Thus, although there was a difference in soil chemical properties, microorganisms that were able to use carbohydrates were still present in the rhizosphere.

However, the utilization of amines and amides as well as amino acid was increased after fertilizer (T1, T2, and T3) was applied compared to non-fertilized peat (C). Numerous studies have shown that the free pool of amino acids is small in soil (Hu et al., 2017; Warren, 2014). The addition of fertilizer might increase the amino acid and amines in the rhizosphere and this

enhanced microorganisms that prefer amino acid and amines as their substrates for growth. Amino acid and amines are known to be involved in microbial metabolism as two of the main nitrogen sources for soil microorganisms which may explain the increased consumption of these two groups by microorganisms.



	C	T1	T2	T3
P	8.17	7.81	8.64	8.84
AM	3.57	4.16	4.51	4.68
AA	10.69	10.97	11.65	11.19
CA	15.41	14.92	16.79	15.57
CB	20.71	20.70	21.33	20.37

Figure 3. Carbon source utilization in peat

Note. P = Polymer; AM = Amines and amides; AA = Amino acid; CA = Carboxylic and acetic acid; CB = Carbohydrate

It was noted that for total guild consumption, microbes in recommended fertilizer rate utilized the highest carbon sources. The effect of fertilizer on microbial consumption of total carbon source had the same pattern with catabolic diversity in community-level physiological profile. It was also observed that the total guild consumption coincided with their microbial activities. This shows that the recommended fertilizer rate has enriched the microbes that can utilize different types of carbon sources as shown by using Biolog. This scenario was expected since Biolog Ecoplate was used in the quantification of carbon source utilization.

The optical densities of 31 carbon sources from the rhizosphere at different fertilizer rate were used to create a heatmap (Figure 4). This study showed that microbes in non-fertilized peat (C) were able to heavily utilize 7 carbon sources (β-methyl-

D-glucoside, pyruvic acid methyl ester, D-galacturonic acid, 4-hydroxybenzoic acid, α-cyclodextrin, N-acetyl-D-glucosamine, and D-glucosaminic acid). Microbes in low fertilizer rate (T1) heavily used 10 carbon sources (β-methyl-D-glucoside, D-galactonic acid γ-lactone, L-arginine, D-galacturonic acid, D-mannitol, 4-hydroxybenzoic acid, L-serine, N-acetyl-D-glucosamine, D-cellobiose, and putrescine). Microbes was able to consume 10 carbon sources when fertilizer was added at recommended rate (T2) (D-galactonic acid γ-lactone, L-arginine, D-galacturonic acid, L-phenylalanine, D-mannitol, 4-hydroxybenzoic acid,

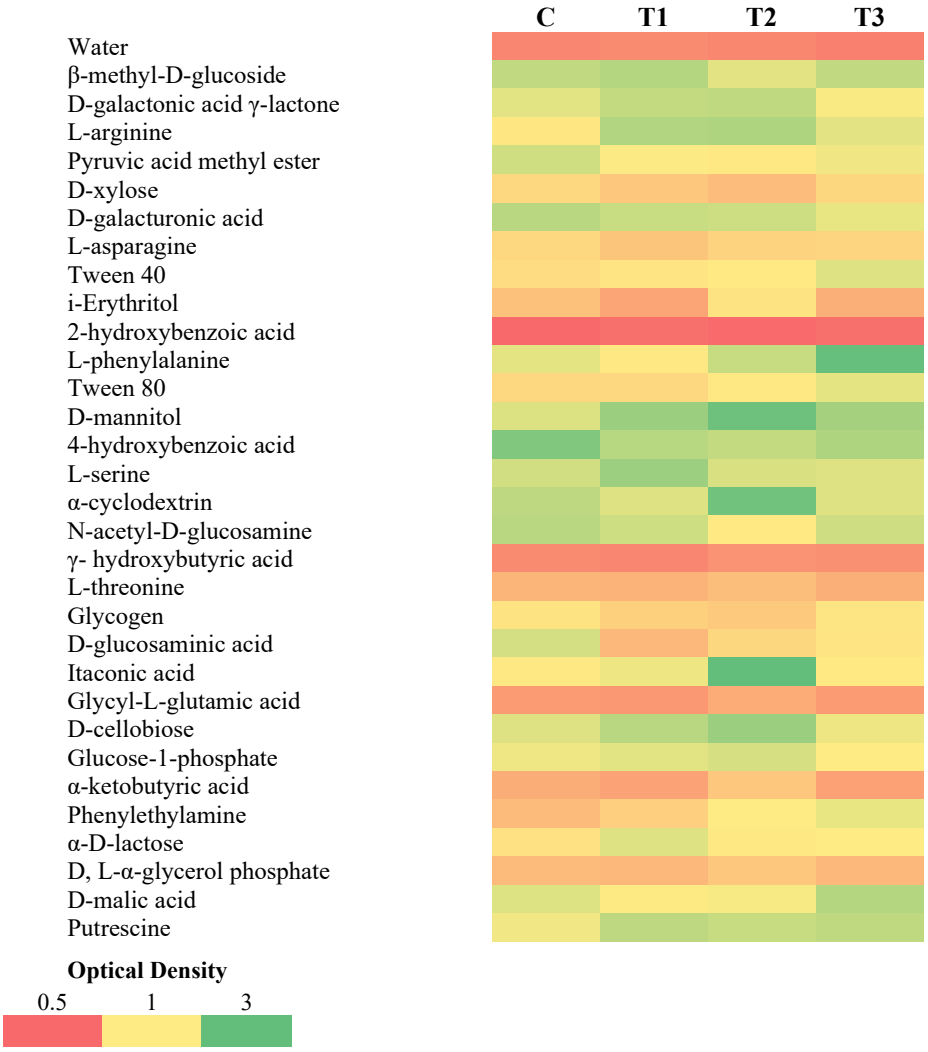


Figure 4. The heatmap of microbial communities from aerobic rice rhizosphere at different fertilizer rate depending on the carbon source utilization

α-cyclodextrin, itaconic acid, D-cellobiose, glucose-1-phosphate, and putrescine). A total of 7 carbon sources was heavily utilized by microbes in peat applied excessive fertilizer rate (T3) (β-methyl-D-glucoside, L-phenylalanine, D-mannitol, 4-hydroxybenzoic acid, N-acetyl-D-glucosamine, D-malic acid, and putrescine). This study is in agreement with previous studies (Furtak et al., 2019; Li et al., 2018). These results suggested that there was variation in the carbon source consumption by the microbes at different fertilizer rate. The changes in soil properties after the addition of fertilizer could influence the microbial community.

The principal component analysis was conducted in which data is represented to reduce dimensionality while preserving the variance of the data. For peat, principal component one (PC1), principal component 2 (PC2), and principal component 3 (PC3) explained 51.0, 30.4, and 18.6% of the variation which corresponded to eigenvalues of 16.313, 9.720, and 5.967, respectively (Table 2). A carbohydrate, D-cellobiose with coefficient 0.247 received the greatest weight in the PC1. For PC2, the greatest coefficient (0.310) occurred at pyruvic acid methyl ester, followed by 4-hydroxybenzoic acid, N-acetyl-D-glucosamine, L-arginine, and putrescine with coefficients greater than 0.25. L-serine had the greatest weight in PC3 (-0.370), followed by L-asparagine, i-Erythritol, β-methyl-D-glucoside, α-D-lactose, D-glucosaminic acid, and α-cyclodextrin. The greatest factors contributing to PC1, PC2, and PC3 in peat were mainly carbohydrates.

Principal component analysis revealed that soil microbial communities in peat utilized mostly carbohydrates. D-cellobiose which has greatest contribution in PC1 is a major carbon source for soil bacterial communities (Schellenberger et al., 2011). Cellulose is a biopolymer that is easily available due to its abundance in peat soil, since peat is formed by partially and completely decayed plant materials that by nature contain high amounts of cellulose. Hence, microbes converted cellulose to cellobiose and later use it as a carbon source.

Table 2

Principal component 1, 2, and 3. Eigenvalues (including percentage of variation), substrates loaded in each PC, substrate type, and eigenvector for substrate in each PC obtained from Ecoplates in peat

Component	Eigenvalue	Substrate	Type	Coefficient
PC1	16.313 (51.0%)	D-cellobiose	CB	- 0.247
PC2	9.720 (30.4%)	Pyruvic acid methyl ester	CA	- 0.310
		4-hydroxybenzoic acid	CA	- 0.306
		N-acetyl-D-glucosamine	CB	- 0.284
		L-arginine	AA	0.276
		Putrescine	AM	0.265

Table 2 *(Continued)*

Component	Eigenvalue	Substrate	Type	Coefficient
PC3	5.967 (18.6%)	L-serine	AA	- 0.370
		L-asparagine	AA	0.366
		i-Erythritol	CB	0.335
		β-methyl-D-glucoside	CB	- 0.324
		α-D-lactose	CB	- 0.308
		D-glucosaminic acid	CA	0.300
		α-cyclodextrin	P	0.293

Note. AA = Amino acid; AM = Amines and amides; CA = Carboxylic acid and acetic acid; CB = Carbohydrates; P = Polymer

Pyruvic acid methyl ester which has the greatest contribution in PC2 is part of citric acid cycle which is important for generating energy for microbes. L-serine contributed largely in PC3 since this amino acid is plentiful in soil and involved in nitrogen metabolism (Pascual et al., 2016; Vranova et al., 2011). Carbohydrates explained the most variation in carbon source utilization in peat suggesting that microbial functional diversity was influenced by these carbohydrates particularly D-cellobiose, N-acetyl-D-glucosamine, β-methyl-D-glucoside, i-Erythtritol, and α-D-lactose.

Population of Culturable Microorganism

The population of culturable aerobic microorganism was measured using 5 different selective media. Bacteria population was reduced when fertilizer was added at lower rate (T1), but did not affect by other fertilizer rate (T2 and T3) applied (Table 3). This can be explained by the resilience ability possessed by soil bacteria. Prior study has demonstrated that soil bacteria were resilient to inorganic fertilizer disturbances (Čuhel et al., 2019).

Fertilizer did not have an effect on culturable fungal, actinomycetes and phosphate-solubilizing microbe population. Phosphate-solubilizing microbe population was not affected by the fertilizer because of the increase of soluble phosphorus in peat after fertilizer application. It is reported that plant growth reduced the relative abundance of *Actinobacteria*, *Acidobacteria*, and *Firmicutes* because of alteration in the rhizosphere (Shi et al., 2015). However, the increase in soluble phosphorus after fertilizer application could benefit these phyla and thus increase their proportion. The ability of *Actinobacteria*, *Acidobacteria*, and *Firmicutes* to solubilize phosphorus was reported in previous studies (Cao et al., 2018; Zheng et al., 2017, 2018).

The actinomycetes population in peat was not influenced by fertilizer addition when applied at different rates. Soil acidophilic actinomycetes such as *Streptomyces* genus

were able to withstand soil chemical changes and thus might explained for the insignificant effect of fertilizer towards the population of actinomycetes. Earlier studies had shown that actinomycetes spores could be the factor for the resilience towards environmental changes (de Lima Procópio et al., 2012).

It was noted that fertilizer addition lowered the culturable population of nitrogen-fixing microbes in peat. This study measured free-living nitrogen-fixing microbes and it is known that these microbes require a low nitrogen environment to improve their activity (Wagner, 2011). Total nitrogen was increased with the addition of fertilizer and this could be the factor that contributes to the decrease of nitrogen-fixing microbes in peat.

Table 3

Population of culturable aerobic microorganism

Fertilizer Rate	Bacteria (Log_{10} CFU/ml)	Fungi (Log_{10} CFU/ml)	Actinomycetes (Log_{10} CFU/ml)	Nitrogen-fixing microbes (Log_{10} CFU/ml)	Phosphate-solubilizing microbes (Log_{10} CFU/ml)
C	8.74 ± 0.32a	7.32 ± 0.16a	5.90 ± 0.03a	7.03 ± 0.04a	5.60 ± 0.00a
T1	4.87 ± 2.44b	7.10 ± 0.10a	6.01 ± 0.15a	6.26 ± 0.19b	3.33 ± 1.67ab
T2	7.20 ± 0.20ab	2.33 ± 2.33a	5.70 ± 0.11a	6.17 ± 0.01b	3.75 ± 1.88ab
T3	8.05 ± 0.28ab	4.67 ± 2.33a	6.18 ± 0.06 a	6.39 ± 0.13 b	3.97 ± 1.99ab

Note. Mean Log_{10} CFU/ml measured based on different selective medium and data are expressed in means ± SE (standard error). Mean comparison was performed using Fisher's least significant difference. Mean in each column with the same letter is not significantly different ($p \leq 0.05$) among the fertilizer rate

CONCLUSION

It is concluded that fertilizer has an effect on microbial functional diversity in the peat rhizosphere of local aerobic variety. Certain species in microbial community such as actinomycetes were not affected while there were others such as nitrogen-fixing microbes that decreased to adapt to changes and thus maintain the microbial functional diversity. Microbial activity was enhanced when added with recommended fertilizer rate while species richness was elevated with excessive fertilizer application. Although fertilizer addition reduces species evenness, it did not influence the catabolic diversity. Microbial community treated with recommended fertilizer rate was able to utilize many carbon sources. There was variation in the carbon source consumption by the microbes at different fertilizer rate.

D-cellobiose, pyruvic acid methyl ester, and L-serine were the carbon sources that influence the microbial function in soil.

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Short Communication

A Report on Introduced Amazon Sailfin Catfish, *Pterygoplichthys pardalis* in Gombak Basin, Selangor, with Notes on Two Body Patterns of the Species

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ABSTRACT

Invasive introduced fish species are well known for their deleterious impacts on aquatic biodiversity and environment. This study provides the first report on the occurrence of introduced Amazon sailfin catfish, *Pterygoplichthys pardalis* from the Gombak basin, Selangor, Malaysia, where the suckermouth catfish, *Hypostomus plecostomus* and vermiculated sailfin catfish, *Pterygoplichthys disjunctivus* had been previously reported. Besides, selected morphometric and meristic measurements between *P. pardalis* and *P. disjunctivus* from the Pusu River, Gombak basin were compared. Moreover, we also described two body patterns of the *P. pardalis* collected from the river. The body pattern which does not fit entirely with the known characteristics of *P. pardalis* or *P. disjunctivus* is suspected to be a result of hybridization between

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both species, but deeper study should be conducted to confirm this claim.

Keywords: Biological invasion, conservation, Gombak basin, invasive fish, Klang Valley

INTRODUCTION

In many countries, fish species belonging to the family Loricariidae have become intentionally or inadvertently introduced into natural habitats, where they have become established (Wu et al., 2011). For example, such introductions have been reported in the Puerto Rico (Bunkley-Williams et al., 1994), Taiwan (Liang et al., 2005), United States (Gibbs et al., 2008), and Bangladesh (Hoosain et al., 2008). In Southeast Asia, introductions of these fishes have been reported in the Philippines (Chávez et al., 2006), Singapore (Page & Robins, 2006), Vietnam (Levin et al., 2008), Thailand (Chaichana & Jongphadungkiet, 2012), Indonesia (Qoyyimah et al., 2016), and Malaysia (Khairul-Adha et al., 2013; Samat et al., 2016).

Currently, a total of 17 valid species under genus *Pterygoplichthys* were reported (Fricke et al., 2020). Some of the species of the genus *Pterygoplichthys* have now invaded five continents and 21 countries around the world (Orfinger & Goodding, 2018), and are known for their impacts on economies and the environment. Specifically, fishes of the genus *Pterygoplichthys* have been reported to reduce native fish populations due to food competition (Hubilla et al., 2008), increase in the water turbidity as a result of the burrowing males (Gibbs et al., 2010), and predate on eggs (Chaichana & Jongphadungkiet, 2012).

The Amazon sailfin catfish, *Pterygoplichthys pardalis* is native to the Amazon River basin. However, it has since expanded its range to North America, the Caribbean, and more recently to South Asia (Hossain et al., 2018). *Pterygoplichthys pardalis* successfully invades new habitats presumably due to its modified scales and strong spines that help to protect it from predation, tolerance to low concentrations of dissolved oxygen, nest construction, parental care, and tolerance to varying levels of salinity (Armbruster & Page, 2006; Capps et al., 2011; Rueda-Jasso et al., 2013).

The importation of *P. pardalis* into Malaysia has been due to the ornamental fish industry (Khairul-Adha et al., 2013). In Malaysia, *P. pardalis* was previously reported in Langat River, Selangor (Samat et al., 2008), Perak River, Perak (Hashim et al., 2012) and Muar River, Negeri Sembilan (Hasyimah et al., 2013). In this study, we provide the first report on the occurrence of introduced *P. pardalis* in Gombak basin, Selangor, Malaysia. Moreover, we also describe two body patterns of *P. pardalis* collected from the area.

MATERIALS AND METHODS

The study was conducted at Pusu River, a small tributary of Gombak basin, Selangor, which is located near the International Islamic University, Gombak Campus, Selangor, Malaysia, with the coordinates

of 3°15'01.5"N, 101°43'45.8"E (Figure 1). The study area has a river width that ranges between 2.5 to 10.0 m and is shallow with depth ranging between 0.2 to 1.2 m. The river flows moderately over a sandy substrate with murky waters and low canopy cover, and the riverbank is covered with tall grasses. The Pusu River is located within Klang Valley, an urban area with many aquarium stores. Klang Valley has a population of over 4 million people, and this represents about 16% of Malaysia's population (Naji et al., 2014). The sampling took place on 24th of February 2020.

Two cast nets, each of which is 150 cm long, 305 cm in diameter and 2 cm of mesh size were used for the fish sampling. In each sampling point, two hours were given for the fish sampling activity. Collected fishes were fixed in 10% of formalin and then preserved in 70% of alcohol for long storage. Voucher specimens were deposited in the Museum of Zoology, University Malaya, Kuala Lumpur (UMKL).

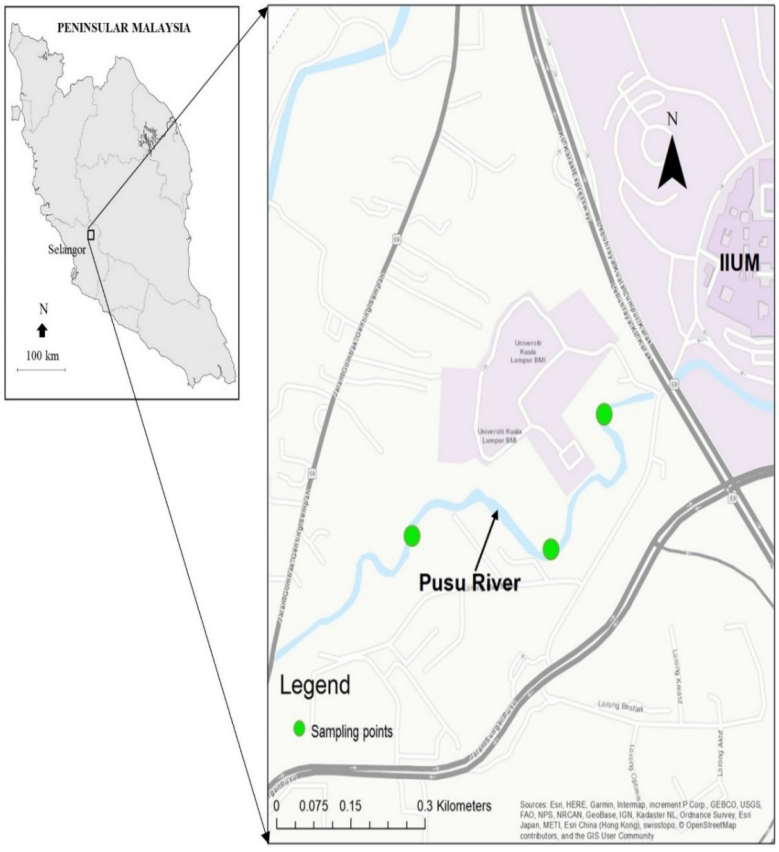

Figure 1. Locations of the occurrence of the Amazon sailfin catfish, *Pterygoplichthys pardalis* in Pusu River, Gombak basin, Selangor are indicated by green dots. Map was designed using ArcMap, version 10.2 (www.esri.com)

Selected counts and measurements were made on the left side of the specimens following Boeseman (1968). Distances were measured in millimeters (mm) using dial calipers and were converted to percentages of standard length (SL) or head length (HL). Morphometric measurement of the collected fishes such as head length, dorsal spine length, pectoral spine length, predorsal length, percentage of head length, head depth, snout length, orbital diameter and interorbital distance were compared using one-way ANOVA followed by Tukey's post hoc test. Following dissection, the sex of the fish was easily identifiable since the gonads were easily visible and distinguishable.

Specimens were identified based on keys provided by Armbruster and Page (2006), Page and Robins (2006), as well as Golani and Snovsky (2013). The photos of freshly caught and preserved specimens were also taken for further identification.

RESULTS

Fish of the genus *Pterygoplichthys* is mainly identified based on body pattern on the ventral side. Based on the observed coloration and stripe patterns, three body patterns of *Pterygoplichthys* were found in Pusu River, namely the Amazon sailfin catfish, *P. pardalis* 'type A', *P. pardalis* 'type B', and the vermiculated sailfin catfish, *P. disjunctivus*. Table 1 summarizes the selected morphometric and meristic measurements for the three types of the *Pterygoplichthys* found in the study area. Most of the measurements fell within the same range, with the exception of the snout lengths of *P. pardalis* 'type A' that appeared to be significantly ($p < 0.05$) shorter compared to *P. pardalis* 'type B' and *P. disjunctivus*. Thus, coloration and stripe patterns were more useful in differentiating this species.

Pterygoplichthys pardalis was identified by having discrete dark spots on the ventral part of the body. This species has a pattern of uncoalesced dark spots with a light background. Its pectoral fins are stout with rough surfaces and an inferior disk-shaped protrusible mouth, which we named as *P. pardalis* 'type A' (Figure 2). In contrast, *P. disjunctivus* has dark vermiculations on a light background. The vermiculations are mostly continuous with one another, unlike the mostly unconnected spots found in *P. pardalis* (Figure 3).

However, in this study, we also found several specimens of *P. pardalis* that have more discrete and larger spots than the other specimens, and linked to form short chains, which we named as *P. pardalis* 'type B' (Figure 4).

DISCUSSION

This study presents the first report of the introduced loricariid species, *P. pardalis* in the Gombak basin, Selangor, Malaysia. This finding also has added one more fish species to the list of Fatinizzati et al. (2018), which make the current total number of fish species known to occur in Gombak basin is 35. In Malaysia, *P. pardalis* was previously reported in Langat River, Selangor (Samat et al., 2008), Perak River, Perak (Hashim et al., 2012), and Muar River, Negeri

Table 1

Selected morphometrics and meristics of Amazon sailfin catfish, Pterygoplichthys pardalis *'type A' and 'type B', and vermiculated sailfin catfish,* Pterygoplichthys disjunctivus *from Pusu River, Gombak basin, Selangor*

Species	*Pterygoplichthys pardalis* 'type A' (n = 7)		*Pterygoplichthys pardalis* 'type B' (n = 9)		*Pterygoplichthys disjunctivus* (n=7)	
	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
Characters						
Total length (in mm)	334 - 440		345 - 470		353 - 450	
Standard length (in mm)	244 -346		265 - 371		271 - 346	
Percentage of standard length						
Head length	21.9 - 25.3	(23.8 ± 1.1)	23.2 - 26.1	(24.5 ± 0.9)	23.1 - 25.2	(24.0 ± 0.7)
Dorsal spine length	16.5 - 22.5	(19.5 ± 2.1)	16.4 - 21.4	(18.8 ± 1.5)	17.2 - 19.7	(18.7 ± 0.8)
Pectoral spine length	23.7 - 29.2	(26.0 ± 2.0)	20.0 - 27.1	(24.3 ± 2.1)	22.5 - 29.4	(25.7 ± 2.5)
Predorsal length	37.2 - 42.9	(39.3 ± 1.8)	35.9 - 39.4	(37.6 ± 1.1)	36.7 - 42.7	(39.1 ± 2.2)
Percentage of head length						
Head depth	62.7 - 67.9	(65.3 ± 1.7)	58.8 - 68.2	(63.8 ± 2.8)	62.3 - 68.9	(65.0 ± 2.5)
Snout length	40.4 - 44.5	(42.3 ± 1.3)[a]	43.2 - 47.2	(45.4 ± 1.4)[b]	41.5 - 45.4	(44.2 ± 1.2)[b]
Orbital diameter	11.4 - 14.9	(13.1 ± 1.3)	10.1 - 15.2	(12.2 ± 1.6)	11.4 - 14.6	(12.7 ± 1.1)
Interorbital distance	48.8 - 55.9	(51.2 ± 2.2)	45.5 - 53.4	(49.0 ± 2.5)	47.4 - 54.0	(50.7 ± 2.8)
Dorsal-fin rays	I,11 (2), I,12 (4), I,13 (1)		I, 12 (6), I,13 (3)		I,11 (2), I,12 (5)	
Anal-fin rays	I,4 (7)		I,4 (9)		I,4 (7)	
Pectoral-fin rays	I,6 (7)		I,6 (9)		I,6 (7)	
Pelvic-fin rays	I,5 (7)		I,5 (9)		I,5 (7)	
Lateral-line plates	28 (3), 29 (2), 30 (2)		29 (5), 30 (4)		28 (2), 29 (5)	
Predorsal plates	3 (7)		3 (9)		3 (7)	

Note. [a, b] Different superscripts indicate significant different ($p < 0.05$) of the same row

Figure 2. Dorsal, lateral, and ventral views of the Amazon sailfin catfish, *Pterygoplichthys pardalis* 'type A', UMKL 12834-1, 345.0 mm SL (Photos by Muhammad-Rasul A. H.)

Figure 3. Dorsal, lateral, and ventral views of the vermiculated sailfin catfish, *Pterygoplichthys disjunctivus*, UMKL 12835-1, 342.0 mm SL (Photos by Muhammad-Rasul A. H.)

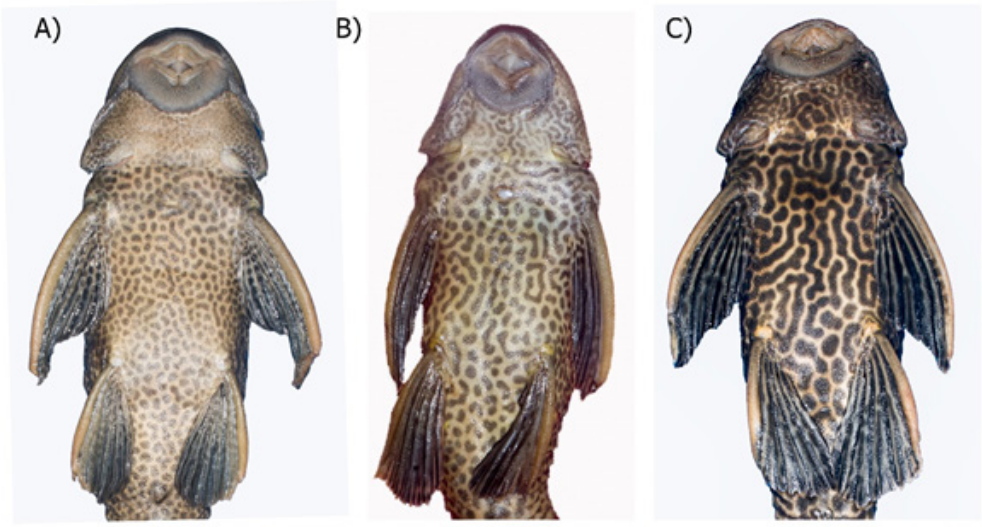

Figure 4. Comparison on the body pattern on the ventral part of *Pterygoplichthys* spp. from Pusu River, Gombak basin, Selangor. A) *Pterygoplichthys pardalis* 'type A', B) *Pterygoplichthys pardalis* 'type B', and C) *Pterygoplichthys disjunctivus* (Photos by Muhammad-Rasul A. H.)

Sembilan (Hasyimah et al., 2013), while *P. disjunctivus* was reported for Kampar River catchment, Perak (Ng et al., 2018) and Pahang River, Pahang (Mohd-Sukeri et al., 2020).

The occurrences of fish species of the genus *Pterygoplichthys* have been reported to result in negative impacts, whereas these species continue to flourish. Some of the impacts include a reduction in native fish population due to competition for food, increase in water turbidity due to the effect of the burrowing activities of the males, and predation on the eggs of co-occurring fishes (Chaichana & Jongphadungkiet, 2012; Gibbs et al., 2010; Hubilla et al., 2008). Loricariid species do not only forage along the bottoms of streams and lakes, but also bury their heads in the substrates and lash their tails occasionally making it possible to shear and uproot aquatic plants, thus reducing the abundance of submerged aquatic vegetation (Global Invasive Species Database [GISD], 2020). Their grazing activities on benthic algae and detritus may also reduce the availability of food and shelter for aquatic insects, which serve as food for other resident fish species (Ozdilek, 2007). In the long run, their activities may lead to bank structure alteration, which may cause erosion, competition with native species, alteration of the aquatic flora, and fishing gear damage (GISD, 2020; Hossain et al., 2018).

Previously, Jalal et al. (2018) reported the occurrence of the suckermouth catfish,

Hypostomus plecostomus from Pusu River, which we did not find during this study. This may be a result of the very low sampling effort deployed in this study. Meanwhile, Fatinizzati et al. (2018) only found *P. disjunctivus* in Pusu River. Species of the genus *Pterygoplichthys* are often confused with *Hypostomus*, from which they can be easily distinguished by having more dorsal fin rays 11-14 dorsal rays (*vs.* 7 in *Hypostomus*) (Fatinizzati et al., 2018; Golani & Snovsky, 2013).

In this study, we suspect that the different body patterns of *P. pardalis* 'type B' may have resulted from hybridization between *P. pardalis* and *P. disjunctivus*, which were both encountered at the same time. However, deeper investigation based on molecular analysis is needed to confirm this claim. Indeed, hybridization ability of different invasive fish species in natural waterbodies could add more to the problems of native fishes in this country. However, in this study, it is glaring that the *P. pardalis* 'type B' body pattern is distinguishable from the typical dark vermiculations on a light background which is obtainable in *P. pardalis* (Chávez et al., 2006). The morphometric and meristic measurements for the three types of the *Pterygoplichthys* in this study generally fell within the same range. Thus, coloration and stripe patterns were more useful in differentiating this species.

CONCLUSION

This study provides the first report on the occurrence of introduced Amazon sailfin catfish, *Pterygoplichthys pardalis* from the Gombak basin, Selangor. Moreover, we also described two body patterns of the *P. pardalis* collected from the river. The variant which does not fit entirely with the known characteristics of *P. pardalis* or *P. disjunctivus* is suspected to be a result of hybridization between both species, but deeper study should be conducted to confirm this claim.

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Size: Generally, each manuscript is **not to exceed 6000 words** (excluding the abstract, references, tables, and/or figures)**,** a maximum of **80 references**, and **an abstract of less than 250 words.**

2. Review article

A review article reports a critical evaluation of materials about current research that has already been published by organising, integrating, and evaluating previously published materials. It summarises the status of knowledge and outlines future directions of research within the journal scope. A review article should aim to provide systemic overviews, evaluations, and interpretations of research in a given field. Re-analyses as meta-analysis and systemic reviews are encouraged.

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(c) disseminates information and data on topical events of significant scientific and/or social interest within the scope of the journal.

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PERTANIKA JOURNALS

4. Others

Brief reports, case studies, comments, concept papers, letters to the editor, and replies on previously published articles may be considered.

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Authors' name: The names of the authors should be named **in full without academic titles.** For Asian (Chinese, Korean, Japanese, Vietnamese), please write first name and middle name before surname (family name). The last name in the sequence is considered the surname.

Authors' addresses: Multiple authors with different addresses must indicate their respective addresses separately by superscript numbers.

Tables/figures list: A **list** of the number of **black and white/colour figures and tables** should also be indicated on this page. See ***"5. Figures & Photographs"*** for details.

Example (page 2):

***In vivo* Fecundity Evaluation of *Phaleria macrocarpa* Extract Supplementation in Male Adult Rats**

Sui Sien Leong[1]* and Mohamad Aziz Dollah[2]

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[2]Department of Biomedical Sciences, Universiti Putra Malaysia, 43400 Serdang, Malaysia

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**Corresponding author*

List of Table/Figure:
Table 1.
Figure 1.

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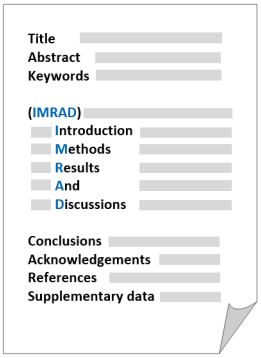

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Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words Introduction, Materials and Methods, Results, And, Discussion. It indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: Title, Authors, Keywords, Abstract, Conclusions, and References. Additionally, some papers include Acknowledgments and Appendices.

The Introduction explains the scope and objective of the study in the light of current knowledge on the subject; the Materials and Methods describes how the study was conducted; the Results section reports what was found in the study; and the Discussion section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the Journal's instructions to authors.

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PVY infected Nicotiana tabacum *plants optical density in ELISA*

- Explanatory material should be given in the table legends and footnotes.
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- Authors are advised to keep backup files of all tables.

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Book with more than 8 authors	*For all in-text references, list only the first author's family name and followed by* 'et al.' A recent study (Edge et al., 2011) concluded that… Or Edge et al. (2011) concluded that ….	For books with eight or more authors, please follow the guidelines for journal articles with eight or more authors.
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Editor	**Information prominent' (the author's name is within parentheses):** … (Zairi, 1999) … Or **'Author prominent' (the author's name is outside the parentheses):** Zairi (1999) …	Zairi, M. (Ed.). (1999). *Best practice: Process innovation management.* Oxford, United Kingdom: Butterworth-Heinemann.

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Several authors, different years referred to collectively in your work	**List sources alphabetically by the family name in the in-text reference in the order in which they appear in the Reference List.** The cyclical process (Carr & Kemmis, 1986; Dick, 2000) suggests…	Carr, W., & Kemmis, S. (1986). *Becoming critical: Education knowledge and action research.* London, United Kingdom: Falmer Press. Dick, B. (2000). *A beginner's guide to action research.* Retrieved June 1, 2019, from http://www.scu.edu.au/schools/gcm/ar/arp/guide.html

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Journals	Insertion in Text	In Reference List
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Journal article with 3 or more authors (*Pertanika*'s format)	*For all in-text references, list only the first author's family name and followed by* 'et al.' **Information prominent' (the author's name is within parentheses):** … (Erlo et al., 2008) … Or **'Author prominent' (the author's name is outside the parentheses):** Erlo et al. (2008) …	Erlo, A., Ervasti, J., Kuosma, E., & Mattila, P. (2008). Evaluation of an organizational stress management program in a municipal public works organization. *Journal of Occupational Health Psychology*, *13*(1), 10-23. doi: 10.1037/1076-8998.13.1.10

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Journal article with more than 8 or more authors	**Information prominent' (the author's name is within parentheses):** … (Steel et al., 2010) … Or **'Author prominent' (the author's name is outside the parentheses):** Steel et al. (2010) …	Steel, J., Youssef, M., Pfeifer, R., Ramirez, J. M., Probst, C., Sellei, R., ... & Pape, H. C. (2010). Health-related quality of life in patients with multiple injuries and traumatic brain injury 10+ years postinjury. *Journal of Trauma: Injury, Infection, and Critical Care, 69*(3), 523-531. doi: 10.1097/TA.0b013e3181e90c24
Journal article with DOI	**Information prominent' (the author's name is within parentheses):** … (Shaw et al., 2005) … Or **'Author prominent' (the author's name is outside the parentheses):** Shaw et al. (2005) …	Shaw, K., O'Rourke, P., Del Mar, C., & Kenardy, J. (2005). Psychological interventions for overweight or obesity. *The Cochrane Database of Systematic Reviews* (2). doi:10.1002/14651858.CD003818.pub2

Newspapers

Newspapers	Insertion in Text	In Reference List
Newspaper article – with an author	… (Waterford, 2007) ...	Waterford, J. (2007, May 30). Bill of rights gets it wrong. *The Canberra Times*, p. 11.
Newspaper article – without an author	… ("Internet pioneer", 2007) …	Internet pioneer to oversee network redesign. (2007, May 28). *The Canberra Times*, p. 15.
Article in a newsletter	… ("Australians and the Western Front", 2009) ...	Australians and the Western Front. (2009, November). *Ozculture newsletter*. Retrieved June 1, 2019, from http://www.cultureandrecreation.gov.au/newsletter/

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Conference / Seminar Papers	Insertion in Text	In Reference List
Print – *If the paper is from a book, use the book chapter citation format. If it is from regularly published proceedings (e.g. annual), use the Journal article citation format*	… (Edge, 1996) … Or Edge (1996) …	Edge, M. (1996). Lifetime prediction: Fact or fancy? In M. S. Koch, T. Padfield, J. S. Johnsen, & U. B. Kejser (Eds.), *Proceedings of the Conference on Research Techniques in Photographic Conservation* (pp. 97-100). Copenhagen, Denmark: Royal Danish Academy of Fine Arts.
Online	… (Tester, 2008) … Or Tester (2008) …	Tester, J. W. (2008). The future of geothermal energy as a major global energy supplier. In H. Gurgenci & A. R. Budd (Eds.), *Proceedings of the Sir Mark Oliphant International Frontiers of Science and Technology Australian Geothermal Energy Conference*. Canberra, Australia: Geoscience Australia. Retrieved June 1, 2019, from http://www.ga.gov. au/image_cache/ GA11825.pdf

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Government Publications	Insertion in Text	In Reference List
Government as author	**First in-text reference: Spell out the full name with the abbreviation of the body.** … (Department of Finance and Administration [DOFA], 2006) … **Subsequent in-text reference/s: Use the abbreviation of the body.** … (DOFA, 2006) …	Department of Finance and Administration. (2006). Delivering Australian Government services: Managing multiple channels. Canberra, Australia: Author.
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Abbreviations: Define alphabetically, other than abbreviations that can be used without definition. Words or phrases that are abbreviated in the *Introduction* and following text should be written out in full the first time that they appear in the text, with each abbreviated form in parenthesis. Include the common name or scientific name, or both, of animal and plant materials.

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