Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

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Pertanika Journal of Tropical Agricultural Science
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About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science is an 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.

Aims and scope

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.

Abstracting and indexing of Pertanika

Pertanika is now 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.

Citing journal articles

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

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Lag time

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Authorship

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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 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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Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.

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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

The journal is indexed in SCOPUS (Elsevier), Clarivate-Emerging Sources Citation Index (ESCI), BIOSIS, National Agricultural Science (NAL), Google Scholar, MyCite, ISC. In addition, Pertanika JSSH is recipient of "CREAM" Award conferred by Ministry of Higher Education (MoHE), Malaysia.

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Contents

Foreword

Welcome to the first issue of 2022 for the Pertanika Journal of Tropical Agricultural Science (PJTAS)!

PJTAS 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 20 articles; a review article, a short communication and the rest are regular articles. The authors of these articles come from different countries namely Ghana, India, Indonesia, Malaysia, Nigeria, Thailand, and Vietnam.

A regular article entitled "Effect of Herbal Blend and L-arginine Supplementation on Growth Performance, Intestinal Morphology, and Caecal Microflora of Growing Guinea Fowls" investigated the effect of diet containing herbal blend (HB) of turmeric (*Curcuma longa*), scent leaf (*Ocimum gratissimum*), and moringa leaf (*Morinda lucida*) supplemented with or without L-arginine (L-Arg) on growth performance, intestinal morphology, and caecal microflora of guinea fowls. Based on the results, it concluded that guinea fowls fed the diet with HB supplemented or not with L-Arg had similar growth performance with those fed with an antibiotic. L-Arg supplementation of the diet with HB resulted in increased caecal *Lactobacillus* counts of growing birds. Full information of this study is presented on page 37.

A selected article entitled "Investigating the Potential of Endophytic Lactic Acid Bacteria Isolated from Papaya Seeds as Plant Growth Promoter and Antifungal Agent" evaluated plant growth-promoting potentials further and *in vitro* antifungal activity of the lactic acid bacteria against various plant pathogens. The results highlighted the possibility of the bacterial consortium to be exploited as bioinoculant to promote plant growth and inhibit phytopathogens causing plant diseases. The further details of this study are found on page 207.

Diana Rachmawati and her teammates from Diponegoro University identified the impacts of yeast (*Saccharomyces cerevisiae*) enhanced feed on feed efficiency, growth, and survival rate of Sangkuriang catfish fingerlings. Various dosages of the commercial feed were given. The findings concluded that supplementing yeast (*S. cerevisiae*) in the commercial feed could increase feed efficiency and growth in Sangkuriang catfish fingerlings. However, it did not affect the survival rate. The detailed information of this article is available on page 273.

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 was to ensure that the quality of the papers justified 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.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers, Editor-in-Chief and Editorial Board Members of PJTAS, who have made this issue possible. PJTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

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Rumen Volatile Fatty Acids and Morphology of the Rumen Mucosa of Swamp Buffalo Raised under Semi-Intensive and Extensive System in Tropical Environment

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ABSTRACT

Swamp buffaloes are mostly raised under an extensive system because they can adapt to the harsh environment. However, exploring the rumen mucosa (RM) morphology and volatile fatty acids (VFA) of swamp buffalo associated with different production systems is still lacking. This study evaluated the rumen VFA and morphology of RM between two groups of buffalo raised under semi-intensive (SI) and an extensive system (EX). VFA was analysed using gas chromatography. The morphology of rumen mucosa was evaluated macro and microscopically for papillae length and width, surface area, density, and muscle thickness, and the microscopic evaluation for stratified squamous epithelium (SSE) and keratin thickness. SI has a greater VFA concentration than the EX. The SSE layer on the dorsal region of the rumen was thicker in the EX group than in the SI group ($p \leq 0.05$). Within the group, the SSE of the dorsal region of rumen was thicker than the ventral region ($p \leq 0.05$) in the EX group. However, the ventral region of the rumen was thicker than the dorsal region in the SI group. The thickness of the keratin layer in the EX group was significantly thicker than the SI group ($p \leq 0.05$) only on

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the dorsal region**.** In conclusion, swamp buffalo from the SI production system has a greater concentration of volatile fatty acid than the EX-group contributed by feeding management under a semi-intensive system. Nevertheless, the advantage in VFA concentration alone is not sufficient to conclude semi-intensive production system exerts a favourable effect on the morphology of the rumen mucosa.

Keywords: Morphology, production system, rumen mucosa, swamp buffalo, volatile fatty acids

INTRODUCTION

Swamp buffaloes (*Bubalus bubalis*) is an important ruminant animal in many parts of the world, especially in Eastern and Southeast Asia. They are excellent fibrous converters and well-adapted to low-quality feed under harsh environmental conditions. Although the different environments may result in different livestock productivity, Wanna et al. (2012) reported that swamp buffaloes grazing on the land produced yield better. However, they are raised under small hold-based agriculture by smallholders (Escarcha et al., 2020) for a longer period without proper forage production area (Suphachavalit et al., 2013) or fed based on agricultural residue (Savsani et al., 2017) in relatively difficult and poor feeding areas (Suhaimi et al., 2019).

Buffaloes, like other ruminants, generally have similar anatomy of the digestive system, but morphophysiological might be different in terms of digestion, absorption, and metabolism. The ratio of volatile fatty acid (VFA) composition in the rumen is affected by the type of feed, forage, species, and quantity of the microbes (L. Wang et al., 2020). This fermentation process is highly associated with ruminant species (Ferreira et al., 2017), diet, and feeding regime (Sutton et al., 2003). Ruminant productivity is generally associated with digestive efficiency, which converts the fibres into volatile fatty acids as a primary energy source by the fermentation process (Bergman, 1990). VFA is absorbed through rumen epithelium via passive transport (Steele et al., 2011) and associated with osmotic pressure, depending on the permeability of the epithelium and blood flow (Storm et al., 2012) within the mucosa (Kern et al., 2016). VFA production and rumen mucosa development are highly influenced by the diet type and composition (Celi et al., 2017; Henderson et al., 2015; Wanapat et al., 2009), the feeding regimes (Bergman, 1990), and the species of the animal (Mao et al., 2012), which might also be caused by feeding behaviour on forage utilisation (Lin et al., 2011). It was also subjected to geographical factors (Henderson et al., 2015) and seasonal influence (Ding et al., 2018), as well as the production systems (Kotresh Prasad et al., 2019), which influence the adaptive modification of the morphophysiological toward survival under extreme conditions, habitat, climate, season, and human intervention.

Many previous works agreed that the type and the quantity of VFA influence the growth of rumen papillae in various ruminant species such as in goats (Y.

H. Wang et al., 2009), sheep (Álvarez-Rodríguez et al., 2012; Baldwin, 1999), dairy cattle (Steele et al., 2011; Storm et al., 2012), beef (Kern et al., 2016), and wild ruminants (Mason et al., 2019). In addition, they also influence the growth of the earlier age of lamb (Y. H. Wang et al., 2009), kids (Kotresh Prasad et al., 2019) or calves (Consalvo et al., 2016; Suárez et al., 2007). However, there is a limited exploration of the rumen mucosa morphology and volatile fatty acids of swamp buffaloes associated with the production system in a tropical environment. Therefore, it was interesting to investigate the rumen volatile fatty acid and mucosae morphology of swamp buffaloes under different farming or production management systems. Since the animals reared under a semi-intensive system have better management and feeding system compared to the extensive system, it is hypothesised that buffalo under a semi-intensive farming system (SI) will result in a greater concentration of volatile fatty acids and improve rumen mucosal morphology. Thus, the objective of this study was to evaluate the rumen volatile fatty acid and mucosae (macro and microscopically) of swamp buffaloes raised under semi-intensive and extensive production systems in a tropical environment.

MATERIALS AND METHODS

Animals

Two groups of male swamp buffaloes (*Bubalus bubalis*) weighed 290 ± 2.90 kg at 24 months old from two different production systems were used. The first group was four animals raised under a semi-intensive system (SI), while the other group was six males raised under an extensive system (EX). SI group raised with rotation grazing in 300 acres of grazing area owned by Department Veterinary Services (DVS) (Mohd Azmi et al., 2021), disease screening conducted as prescribed by DVS, vaccinated against hemorrhagic septicemia (HS) twice/year, and anthelmintics was also given. The paddock of the SI group was fertilized with 200-300 kg of nitrogen (N), 40-60 kg of phosphorus (P), and 100-150 kg of potassium (K)/ha/year. The main grass available was *Bracharia decumbens*. The swamp buffaloes of the SI group were also supplemented with palm kernel cake (PKC) three days a week based on the calculated amount at 1.5 kg/animal/day basis when return to the holding yard (usually within 7 p.m. to 7 a.m.). However, in the EX group, the animals were on free grazing 24 hours/day on 449 acres of DVS land managed by local farmers without feed supplementation and with no vaccination or anthelmintics given, as well as no fertilization applied on the paddock. The main grass was also *Bracharia decumbens* (J. Engkias, 2018, personal communication, December 18, 2018).

Sampling Process and Determination of Nutrient Content

The feed samples taken for analysis was based on 3% dry matter bodyweight and mixed with 1.5 kg/animal/day. 200–300 gm of well-mixed feed of the SI group was used for nutrient analysis. The grass

sample [3% dry matter (DM) of body weight (BW)] was sampled using 1 m^2 quadrat from five locations for both 300 acres (SI group) and 449 acres (EX group). The DM content of the feeds was determined by heating at 105 °C for 3 h (method 930.15; Balthrop et al., 2011), and the ash contents were subsequently determined after incinerated at 550 °C for 2 h (method 942.05; Balthrop et al., 2011). The neutral and acids detergent fibres [neutral detergent fibre (NDF) and acid detergent fibre (ADF), respectively] was analysed using a fibre analyser (ANKOM Technology, USA) following the procedure suggested by Van Soest et al. (1991) without correction for residual ash. The ether extract (EE) and N content were determined by the solvent extraction and the Kjeldahl method (method 954.02 and 976.05; Balthrop et al., 2011). The detail of the nutrient contents of feed consumed by both groups is summarized in Table 1.

Table 1

Nutritive value of the diet given to the swamp buffaloes under two type of production system

Nutrient	[1]SI group (n = 4)	[1]EX group (n = 6)
[3]DM (%)	99.49	99.50
Ash (% DM)	5.69	5.09
Crude fibre (%DM)	23.73	26.03
Ether extract (%DM)	2.92	2.03
Crude protein (%DM)	8.08	6.09
[4]NDF (%DM)	57.96	64.27
[5]ADF (% DM)	28.70	33.86
[6]ADL (%DM)	3.30	3.55
Carbohydrate (%DM)	61.53	59.45
Gross energy (MJ/kg)	12.10	11.07
Hemicellulose (% DM)	29.25	30.41
Cellulose (%DM)	25.38	30.32

Note. [1]SI: Semi-intensive group grazing *Brachiaria decumbens*, [2]EX: Extensive group grazing *Brachiaria decumbens* + 1.5 kg PKC/animal/ day. [3]DM: Dry matter, [4]NDF: Neutral detergent fibre, [5]ADF: Acid detergent fibre, [6]ADL: Acid detergent lignin. Data retrieved with permission from Mohd Azmi et al. (2021)

Environmental Condition

The temperature ranges between 21 °C - 32 °C at dry (throughout the year) and southwest monsoon season (September to March) (Masud et al., 2014), while the rainfall ranges from 1500 mm – 4600 mm (Malaysian Meteorological Department [MetMalaysia], 2018).

Collection of Rumen Fluid and Volatile Fatty Acid Determination

All the sampling procedures were conducted after animals were slaughtered. Before rumen fluid was collected, an incision at 10 – 20 cm was made to insert hand to mix the rumen fluid before the collection. A 10 ml of rumen fluid was collected and immediately filtered with cheesecloth, and 2 ml of 25% of metaphosphoric Acid was added to prevent fermentation. The fluid was then centrifuged for 10 min, 4 ℃ at 15,000 x *g*. The supernatant was taken and directly injected into gas chromatography (GC) for volatile fatty acid determination using GC with HP-INNOWAX (19091N-133) polyethylene glycol (PEG) stationary phase column (30 m, 0.25 mm ID, 0.25 µm film thickness) in an Agilent 7890B gas-liquid chromatography system (Agilent Technologies, USA) equipped with a flame ionization detector (FID). The injector/detector temperature was programmed at 260 °C (Ebrahimi et al., 2017). The column temperature was set at the range of 80 - 205 °C, at the rate of 10 ℃/min increments, to achieve the best separation. The peaks were identified by comparing each VFA (Sigma-Aldrich, USA). Pivalic acid was used as an internal standard for quantification. The values obtained from the peaks were calculated and shown according to their density and molecular weight. Each sample was calculated proportionate to the volatile fatty acid of the ruminal fluid.

Collection of Rumen Mucosae for Morphometrical Evaluation

Immediately after the ruminal fluid was collected (after slaughtered), the rumen was taken out from the abdominal cavity. The dorsal and ventral region of the rumen was identified. Rumen tissue samples were taken as suggested by (Álvarez-Rodríguez et al., 2012), where five incisions of 5 cm x 5cm square from the dorsal and ventral region were taken. Tap water was used to wash this ruminal wall to remove digesta before rinsing it with phosphate buffer saline (PBS) with pH 7.4 at 37 ºC. The macro-morphometry of rumen mucosa was conducted, where ten papillae were gently extracted from 5 cm x 5 cm square of each rumen mucosa of the swamp buffaloes using forceps and scalpel blade No. 11, 4% buffered formalin was a drop on the papillae to fix the samples to ease measurements. The readings of rumen papillae were taken from well-oriented papillae of each rumen site. In contrast, the length measurement was taken from the tip to the base of the papillae along the long axis. The width of the papillae was measured at halfway perpendicular to the papillae length. In contrast, the papillae surface area was measured by circling each papilla using a stereo microscope with an image analyser (Leica M80, Leica Application suite ver. 4.3.0, Switzerland) (Figure 1). The thickness of the tunica muscularis was taken perpendicular to the muscles layer (Figure 2). The papillae density was manually counted on each incision with the help of magnifying glass,

and the readings were taken as average and expressed in papillae/cm^2. The colour score was assessed by three evaluators simultaneously as suggested by Álvarez-Rodríguez et al. (2012) (1: yellow, 2: light brown, 3: dark brown-grey).

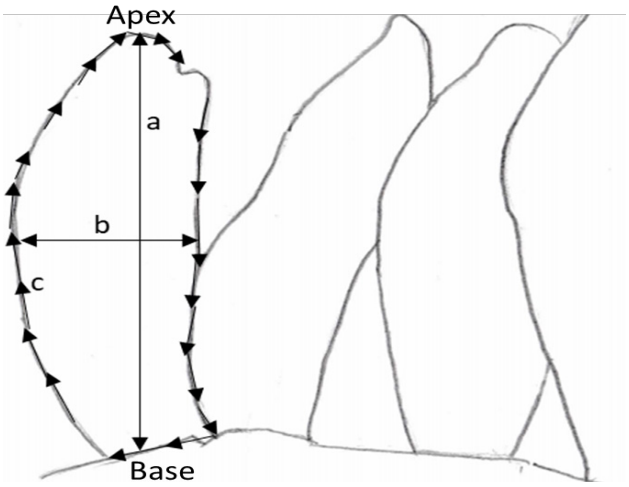

Figure 1. The illustration shows how the morphometrical measurement on the rumen papillae was conducted. The reading of the length was taken longitudinally between base and apex (a), while the width of the papillae was taken perpendicular across the papillae (b), and the surface area was measured by encircling the papillae boundaries (c). These processes were repeated on the five well-organised and prominent papillae. Less developed papillae were avoided for measurements

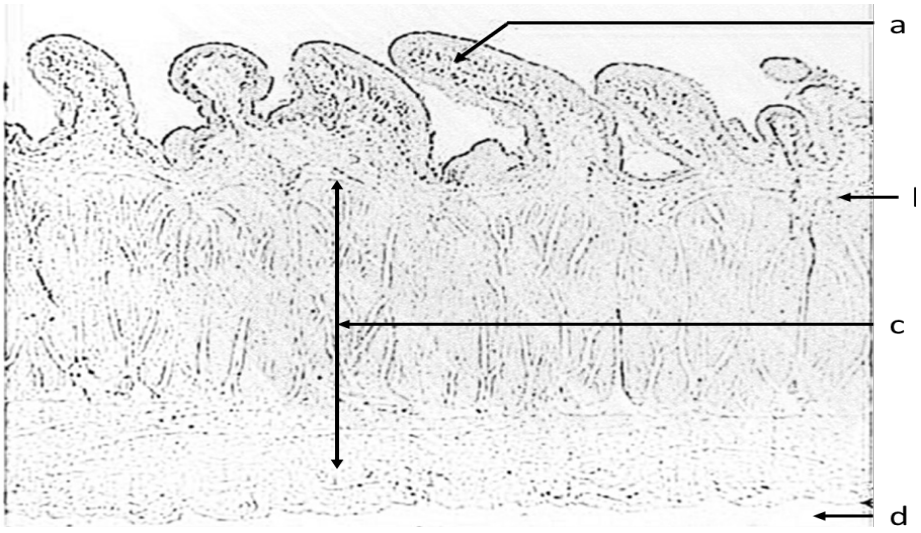

Figure 2. The illustration of the rumen wall of the ruminants; a: papillae, b: submucosa, c: tunica muscularis/ muscle layer, d: serosa layer. The morphometrical measurements of the tunica muscularis of the rumen wall should cover the inner circular and the outer longitudinal muscle (c)

Microscopic Evaluation of the Rumen Mucosae

Five pieces of the rumen mucosae at 1 cm^2 from every swamp buffalo's ventral and dorsal regions (Almeida et al., 2018) were fixed in Bouin's solution for 24 – 36 hours, dehydrated, cleared, and embedded in paraffin. Five paraffin blocks from each animal were randomly selected and cut (Feathers, Japan) using a rotary microtome (HM315R, Artisan, USA) at 5 µm, then stained with hematoxylin and eosin (H&E) for general histological and morphometrical analysis using a microscope (Leica DM2500, Leica Biosystem, Germany). Three readings of SSE and keratin layer thickness from five slides were calculated into mean.

Statistical Analysis

Data were analysed using the Statistical Product and Service Solutions (SPSS) statistical software (IBM SPSS Statistics 23, USA). A normality test was performed, and a non-parametric *t*-test (Mann-Whitney U test) was conducted on volatile fatty acid concentration, macro-morphometry (papillae length, width, surface area, density, and muscle thickness), and micro-morphometry (SSE and keratin layer thickness) to exhibit differences between the two groups. The results were reported as least square means and their associated standard errors (SE). The *p*-values lower than 0.05 ($p<0.05$) were considered significant.

RESULTS

Volatile Fatty Acids

There was no significant different ($p\geq0.05$) in pH value in rumen fluid between SI and EX groups. The acetic acid, propionic acid, butyric acid, isobutyric acid, propionic acid %, butyric acid %, and total VFA were significantly higher ($p<0.05$) in the SI group than EX group. The acetic acid was present in the highest concentration in VFA, with 77.17% in SI and 84.43% in the EX group. The acetic:propionic and acetic:butyric acid ratio was higher ($p<0.05$) in the EX group than in the SI group (Table 2). The valeric and isovaleric acid was not significantly different ($p\geq0.05$) between SI and EX groups. Isobutyric, valeric, and isovaleric acid were present in low concentrations in the rumen fluid. However, 2.11% VFA from the SI group and 0.15% from the EX group was undetected in this study.

Table 2

The concentration of the volatile fatty acids (VFA) of the rumen of the swamp buffaloes raised under semi-intensive (SI) and extensive system (EX) (mmol/ml)

Parameters	Buffalo group		
	SI group (n = 4)	EX group (n = 6)	*P*-value
pH value	6.28 ± 0.07	6.29 ± 0.09	0.557
Acetic acid (mmol/ml)	66.31 ± 3.77^{x}	65.23 ± 1.29^{y}	0.034

Table 2 *(Continue)*

Parameters	Buffalo group		
	SI group (n = 4)	EX group (n = 6)	*P*-value
Propionic acid (mmol/ml)	6.17 ± 2.14^{x}	2.910 ± 0.51^{y}	0.001
Butyric acid (mmol/ml)	14.08 ± 3.54^{x}	9.01 ± 0.88^{y}	0.008
Isobutyric acid (mmol/ml)	0.26 ± 0.15^{x}	0.11 ± 0.01^{y}	0.007
Valeric acid (mmol/ml)	0.13 ± 0.044	0.11 ± 0.01	0.609
Isovaleric acid (mmol/ml)	0.28 ± 0.173	0.23 ± 0.11	0.569
Acetic: propionic ratio	4.71 ± 1.06^{x}	7.24 ± 1.47^{y}	0.045
Acetic: Butyric ratio	10.58 ± 0.60^{x}	22.42 ± 2.50^{y}	0.007
Acetic acid (%)[1]	77.17 ± 3.44^{x}	84.43 ± 0.72^{y}	0.045
Propionic acid (%)[1]	5.95 ± 0.43^{x}	3.77 ± 0.68^{y}	0.037
Butyric acid (%)[1]	14.77 ± 0.65^{x}	11.65 ± 1.00^{y}	0.016
[2]Total VFA (mmol/ml)	85.92 ± 4.34^{x}	77.26 ± 1.47^{y}	0.033

Note. $^{x, y}$ the value in the row differed significantly at $p<0.05$
[1]The concentration of acetic, propionic, and butyric acid to the total VFA presented in percentage
[2]2.11% of VFA from the total VFA in the SI group and 0.15% in the EX group was undetected

Morphology and Morphometry of Rumen Mucosae

Figure 3 shows the morphology of the rumen mucosa, and Table 3 shows the morphometry of the rumen mucosa of both swamp buffaloes of the current study. Generally, the rumen walls are composed of papillae in the innermost layer, followed by submucosa, tunica muscularis, and serosa (Figure 3). The tunica muscularis comprises inner circular and outer longitudinal, which are not very obvious compared to the inner circular muscle layer (Figure 3). The papillae in the dorsal region are flat, smaller, and shorter than in the ventral region. As the size of the papillae of the ventral region was prominent and longer, the comparison was not statistically conducted.

Table 3 shows the measurements of the dorsal and ventral region of the rumen papillae in the swamp buffaloes raised under semi-intensive (SI) and extensive (EX) production systems. There are no significant differences ($p>0.05$) in the absorptive capacity (papillae length, width, and surface area) and motility (tunica muscularis thickness) between SI and EX groups. However, SI is showing superiority in papillae while EX is in muscle thickness in both regions.

The morphology of the rumen papillae comprises keratinized stratified squamous epithelium (SSE) as epithelium at the outermost layer and interstitium (Figure 4). The cellular proliferation at the stratum basale, where the cell mitotic occurred, is very active compared to other regions of the epithelium.

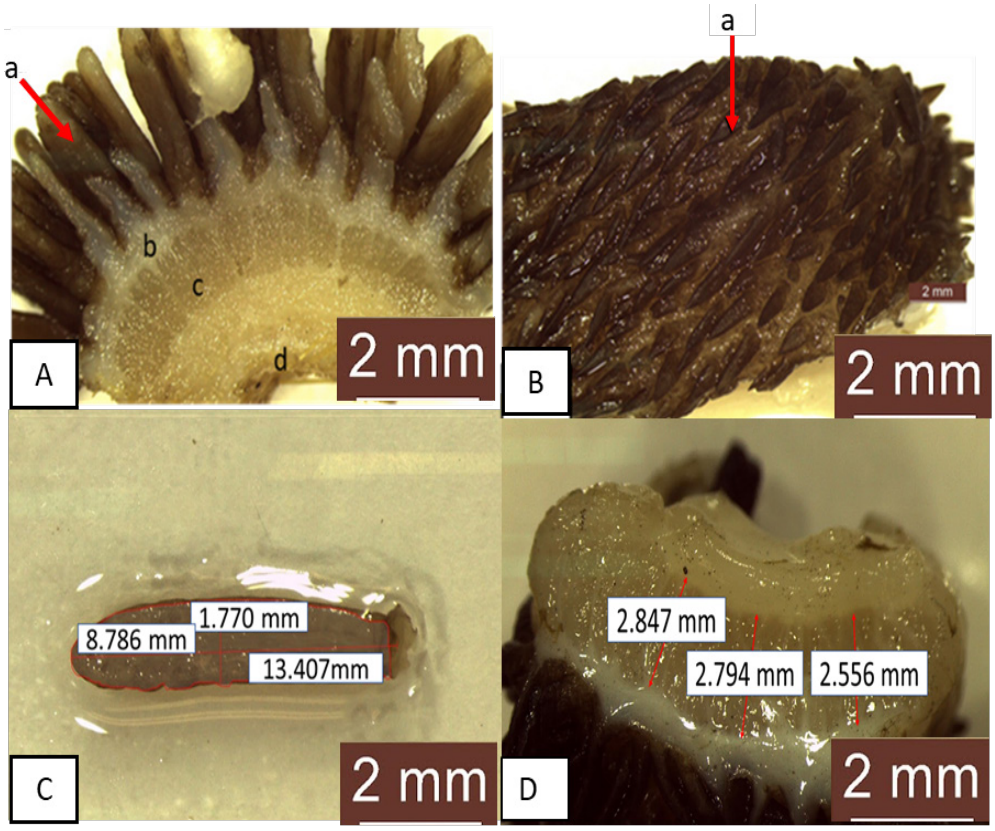

Figure 3. The morphology and morphometrical evaluation of the rumen papillae and rumen mucosae. (A) The gross morphology of the ventral region's rumen papillae and mucosal layer comprises of the papillae, muscle, and connective tissue, which comprises of a: papillae, b: submucosa layer, c: tunica muscularis, and d: serosa; (B) The surface of the rumen at the dorsal region, the papillae (e) of this region are less pronounced compared to the ventral region; (C) The measurement of the papillae (length, width, and surface area); (D) The measurement of the rumen muscle. Leica M80 equipped with Leica Application suite ver. 4.3.0, Switzerland. Scale: 2 mm

Table 3

Macro-morphometrical measurements of the dorsal and ventral region of the rumen papillae in the swamp buffaloes under semi-intensive (SI) and extensive (EX) system

Variables	Buffalo group		
	SI group (n = 4)	EX group (n = 6)	*P*-value
[2]Dorsal region			
Papillae length (mm)	1.932 ± 0.09	1.71 ± 0.12	0.171
Papillae width (mm)	11.69 ± 0.78	9.95 ± 1.39	0.300

Table 3 *(Continue)*

Variables	Buffalo group		
	SI group (n = 4)	EX group (n = 6)	*P*-value
[2]Dorsal region			
Papillae surface area (mm^2)	18.76 ± 0.84	14.66 ± 2.08	0.079
Papillae density (per cm^2)	73.22 ± 1.98	72.56 ± 2.60	0.952
Muscle layer thickness (mm)	3.81 ± 0.36	4.15 ± 0.53	0.7302
[1]Papillae colour Score	3.00	3.00	-
[2]Ventral region			
Papillae length (mm)	13.19 ± 1.05	12.06 ± 1.48	0.548
Papillae width (mm)	1.91 ± 0.059	1.74 ± 0.098	0.164
Papillae surface area (mm^2)	20.24 ± 1.818	17.61 ± 2.00	0.354
Papillae density (per cm^2)	275.44 ± 4.92	256.22 ± 5.60	0.746
Muscle layer thickness (mm)	4.41 ± 0.15	4.42 ± 0.68	0.932
[1]Papillae colour Score	3.00	3.00	-

Note. [1]The evaluation of papillae colour was based on the visual characteristics based on three individuals evaluated at the same time
There are no significant differences ($p \geq 0.05$) in papillae of the dorsal and ventral region of the rumen in the SI and EX group of swamp buffaloes

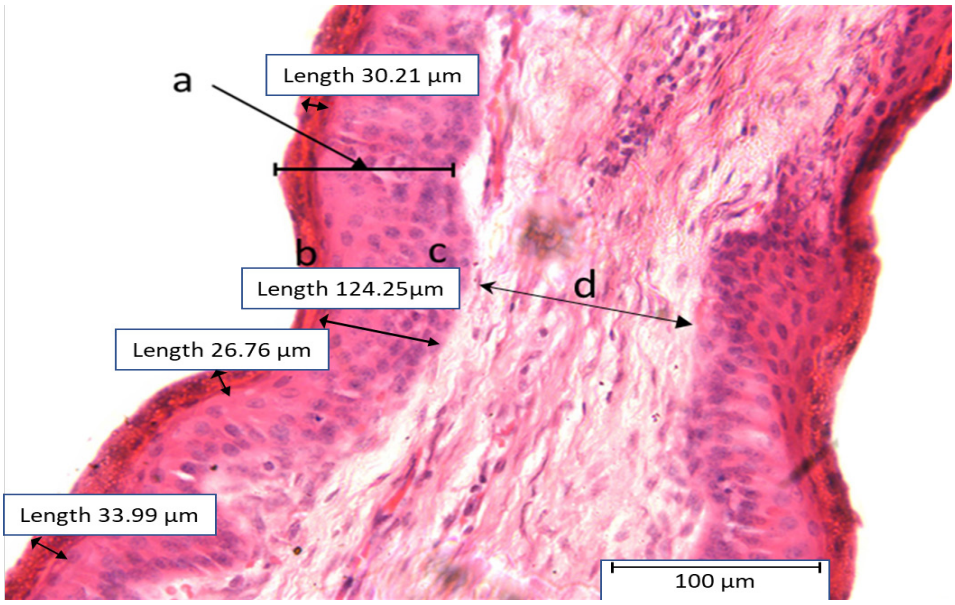

Figure 4. The morphology and measurement of the rumen papillae of the swamp buffaloes: a: SSE layer, b: keratin layer, c: stratum basale, d: interstitium of the papillae. H&E stain. Scale: 100 µm

The thickness of the stratified squamous epithelium (SSE) and keratin layer of the swamp buffaloes raised under semi-intensive (SI) and extensive (EX) production systems are shown in Tables 4 and 5, respectively. In dorsal papillae, the SSE thickness of the EX group is thicker ($p \leq 0.05$) than the SI group, however in ventral papillae, the SSE thickness between SI and EX does not differ significantly ($p \geq 0.05$). In the SI group, the thickness of SSE in the ventral is thicker ($p \leq 0.05$) than dorsal region, while in the EX group, the SSE thickness of the papillae in the dorsal region is thicker than ($p \leq 0.05$) in the ventral region (Table 4).

Table 4

The values (mean ± SE) of stratified squamous epithelium thicknesses of the rumen papillae in ventral and dorsal regions of the swamp buffaloes in semi-intensive (SI) and in extensive (EX) production system

Ruminal region	Buffalo group		
	SI group (n = 4)	EX group (n = 6)	*P*-value
Dorsal (µm)	$67.45 \pm 2.93^{a, x}$	$128.50 \pm 13.43^{b, x}$	0.002
Ventral (µm)	82.62 ± 5.38^{y}	93.03 ± 9.63^{y}	0.200
p-value	0.024	0.019	

Note. $^{a, b}$ the mean with a superscript letter in the row differs significantly at $p \leq 0.05$
$^{x, y}$ the mean with superscript a letter in the column differs significantly at $p \leq 0.05$
The analysis of data using Mann-Whitney U Test to compare two independent datasets

The thicknesses of the keratin layers in the SI and EX group are shown in Table 5. The keratin thickness of the EX group is significantly greater ($p<0.05$) than the SI group at the dorsal region of the rumen. However, in the ventral region, the thickness of keratin is greater ($p<0.05$) in the SI than in the EX (Table 5).

Table 5

The values (mean ± SE) of the keratin layer thicknesses of the rumen papillae of the swamp buffaloes under a semi-intensive (SI) and extensive (EX) production system

Ruminal Region	Buffalo group		
	SI group (n = 4)	EX group (n = 6)	*P*-value
Dorsal (µm)	$18.11 \pm 1.186^{a, x}$	$25.53 \pm 2.425^{b, x}$	0.011
Ventral (µm)	19.97 ± 0.571^{y}	$16.32 \pm 0.623^{b, y}$	0.003
p-value	0.103	0.003	

Note: $^{a, b}$ the mean with a superscript letter in the row differs significantly at $p \leq 0.05$
$^{x, y}$ the with superscript letter in the column differs significantly at $p \leq 0.05$
The analysis of data using Mann-Whitney U Test to compare two independent datasets

DISCUSSION

The present study evaluated the VFA and rumen mucosa morphology of the swamp buffaloes raised under two different production systems. Livestock production systems ensure the continuity of production to achieve adequate protein supplies by considering the rearing method, genetics, feeding management, and environmental factors. Generally, in the ruminant sector, there are three primary systems practised: extensive, semi-intensive, and intensive, which are synchronized with animal behaviour, economics, welfare, feed resources, and environment.

A ruminant diet predominantly composed (90%) of roughage (Mottet et al., 2017) is freely accessible through grazing on rangeland or pastures. It has a positive association between plant growth and rainfall and climatic condition (Marshal et al., 2005; McGrath et al., 2018), and finally may affect the production of VFA and rumen morphology. Globally, there are three different tropical climates identified according to the annual precipitation levels throughout the year. Many studies stated that tropical rangeland generally has a shorter wet but longer dry season. However, a country like Malaysia is influenced by monsoon and contains high humidity and the sunlight almost constant throughout the year. This high rainfall and sunlight in tropical monsoon (unlike other tropical regions) promote rapid growth and maturity of the grass to be low in digestibility and crude protein but higher in lignin as compared to other tropical areas (Moore & Jung, 2001). It may also cause undernutrition in livestock production (Duarte et al., 2018) to a certain extend. In tropical monsoon, an animal usually grazed at greater amount and composition during higher rainfall or wet season. (Andrew & John, 1998).

This study can be considered as the first data showing the effect of different production systems and feeding practices and their effect on the rumen VFA composition and mucosal morphology of the swamp buffaloes in the tropical region. Production systems (feeding regimes, housing, and rearing practices) are generally used to synchronize between livestock requirements, farming input, and environmental constraints. Therefore, differences in the production system may change the feeding behaviour, nutritive values, and animal-environment interactions. However, the current study only focused on the rumen VFA composition and morphology of rumen mucosae. Nevertheless, our findings revealed the difference in VFA concentration and microscopic level of the rumen papillae in different production systems, and thus this had proven our earlier hypothesis.

In digesting fibre in the rumen, the main products are VFA, ammonia, and gases. At the same time, the pH also changed accordingly. Thus, feed consumed in the rearing practices significantly influences rumen physiology (Diao et al., 2019; Ferreira et al., 2017; Kay et al., 1980; Steele et al., 2011) and the digestive morphology of the ruminants (Mason et al., 2019).

The pH range of ruminants fed on roughage should range from 6.2 to 7.0

(Wanapat & Pimpa, 1999). A pH higher than 6.5 promotes protease activity and growth rate of rumen microbes (Bach et al., 2005), while a value lesser than 6.2 will depress the growth of rumen microbes (Franzolin & Alves, 2010). However, this acidic environment was not conducive to rumen fermentation (Van Kessel & Russell, 1996) and limited the optimal VFA production. Therefore, the rumen pH has to be controlled through feeding management to prevent adverse effects on the rumen wall.

Our findings agreed with Wanapat et al. (2009), who suggested that the pH value of swamp buffalo was not influenced by diet. However, Franzolin et al. (2010) demonstrated differently where the ruminal pH might differ among diets, energy, and nitrogen source between cattle and buffaloes. This higher buffering capacity in buffaloes than in cattle, resulting in a higher pH value, as reported by Franzolin and Alves (2010), it was 6.28 in cattle and 6.29 in buffaloes, which was lower than what was suggested by Chanthakhoun et al. (2012), but in similar trend (6.51 in cattle and 6.78 in buffaloes). However, Rostini et al. (2018) reported differently where the pH was 5.62 (swamp buffalo), 5.58 (river buffalo), and 6.46 in Bali cattle.

A gap of sampling time after feeding might also affect the pH of the rumen. L. Wang et al. (2020) suggested that the lowest pH value was at four hours post-feeding, different from Goularte et al. (2011), who stated that it might take six to nine hours post-feeding. However, the feeding gap to the sample collection time was not monitored in the current study as the sample was collected immediately after slaughter. Therefore, the relationship between diet, VFA concentration, and pH value was unable to be determined accurately. Therefore, further investigation on the feeding, sampling time, and management system and their effects on the VFA in swamp buffaloes are required.

In the SI group, supplementation of PKC was given three days/week at a calculated ratio of 1.5 kg/animal/day but was not practised in the EX group. Therefore, it may affect the concentration of VFA in both groups. Dieho et al. (2016) suggested that different energy levels and nutritive value of diet cause different VFA production, which was shown in the SI group compared to the EX group. Furthermore, the tendency of different dry matter intake (DMI) may differ individually as each animal possess different feeding behaviour.

In this study, the total VFA, acetic, propionic, and butyric acid were more significant in SI than EX group indicated that the diet offered under different production systems affects the VFA concentration. Our finding agreed with all the previous studies where acetic acid was the most prominent VFA (Aluwong et al., 2013), while propionic and butyric acid were also prominent in both swamp buffalo groups. Still, it may fluctuate according to the type of diet given. High energy diet (Ma & Zhao, 2010) promotes higher production of total VFA, acetic, propionic, and butyric acid in the rumen than forage (Bergman, 1990; Kristensen, 2005; L. Wang et al., 2020; Mao

et al., 2012). Nevertheless, using different forages also caused different proportions of VFA. The total VFA produced from alfalfa was greater than bromegrass (Khorasani et al., 2001), although their digestible energy seems similar (Moyer & Hironaka, 1993). The present study was based on *Brachiaria decumbens* and PKC supplementation, which was considered very marginal to support the findings of Palmieri et al. (2012). Additional PKC as an energy source in the basal diet of less than 45% did not result in total VFA, acetic, butyric, and propionic acid changes. A higher proportion of concentrate as energy diet increased up to 70% (Suárez et al., 2007) under controlled feeding management did not observe increment of VFA production in some studies (Khorasani et al., 2001; Penner et al., 2009), which differed from the present findings. The butyric and propionic acid production seemed to be ambiguous under almost similar pH values in this study. However, according to L. Wang et al. (2006), in the micro-aerobic circumstances, the accumulation of VFA in acidogenic conditions can occur at any pH value; more investigation is required to elucidate the rumen fermentation of the swamp buffaloes.

The VFA concentration may differ between cattle and buffaloes, with the buffaloes producing less acetic acid but more propionic acid than cattle but not in butyric acid between them (Franzolin et al., 2010). Bergman (1990) suggested that acetic: propionic: butyric acid ratio was 75:15:10 to 40:20:20, which was not observed in the present study. The acetic: propionic acid and acetic: butyric acid ratio was higher in the EX group than in the SI group. It was contributed by the diet of EX, which only depended on grass and no substitution of concentrates given.

Species has a significant role in influencing VFA production. (Candyrine et al., 2019). VFA concentration was reported to be higher in buffaloes (Parmar et al., 2014) but found to be lower by Franzolin et al. (2010). Increment or reduction of VFA between cattle and buffaloes were suggested due to the supplementation introduced (Boniface et al., 1992). However, Rostini et al. (2018) showed that swamp buffaloes have the highest acetic and propionic acid compared to river buffaloes and Bali cattle. However, it has the lowest butyric acid among the three breeds studied.

Stress may also affect fermentation and VFA absorption. The authors agree with the suggestion by Lam et al. (2018) that the difference in VFA between these two groups is due to stress. Stress may disrupt VFA absorption. Generally, in an extensive farming system, human-animal contact is at the minimum where the stress factor is generated only during animal handling. Heat stress was reported to reduce the fermentation and VFA absorption process (Bernabucci et al., 2010; Silanikove, 2000). However, it may depend on the location of the tropical region (Wanapat et al., 2013). Animals raised extensively were more prone to be exposed to climatic, human-animal interaction, and feed stress (Temple & Manteca, 2020) than other production systems. Handling stress is expected to be

lesser in the SI group than the EX group as they are being exposed to the workers during feed supplementation in the holding yard. In the current study, the effect of heat stress was not obvious in both groups as the ambient temperature was almost constant. In the natural environment, wallowing behaviour is used to control body heat.

Human handling involved in the herd health program, such as disease screening, vaccination, deworming, may not impact the extensive group. However, the stress due to handling transport and slaughter cannot be avoided in both groups and maybe worsen an extensive group. Searching for food might also cause stress in the EX group because there is no food, supplementation and they have to walk a long distance. The effect of handling and rearing stress to distinguish effect on VFA and rumen mucosal morphology was not conducted in this study. Therefore, it was not discussed in detail. The study of stress factors caused by rearing practice was extensively conducted in beef cattle. However, further investigation on swamp buffaloes is still required as the buffaloes have behaviour, morphology, and genes different from the beef cattle.

Previous findings suggested that the presence of VFA as the result of carbohydrate digestion in the rumen (Dieho et al., 2016; Shen et al., 2005) and the physical structure of the feed (Suarez-Mena et al., 2016; Xu et al., 2009) were the cause to induce morphological changes of rumen mucosae. Among all the VFAs, this role was mainly played by butyric acid (Liu et al., 2019). All the above statements agree that starch altered the papillae development and rumen wall thickness at the different thresholds (Álvarez-Rodríguez et al., 2012; Y. H. Wang et al., 2009).

A better diet offered will create a better rumen mucosa morphology, as suggested in several reports (Cui et al., 2019; Diao et al., 2019; Penner et al., 2009). However, it was not observed in this study. Cui et al. (2019) demonstrated that the growth of rumen papillae positively correlated to protein and energy diet, which can be found in a well-managed farming system. As a general agreement, the papillary size is associated with the absorption capacity, which occurs in a large amount of rich in carbohydrate diet, resulting in excessive VFA concentration and low pH (de Resende-Junior et al., 2006).

Mason et al. (2019) reported that farmed deer have lesser papillae characteristics than wild deer, slightly different from the current study. The evaluation of the absorptive and motility capacities is required for macro and micro levels to support our findings as suggested by Consalvo et al. (2016) that microscopic evaluation gave a better description of rumen mucosae morphology. Our finding disagreed with the earlier suggestion that a greater concentration of VFA in the SI group can still induce the papillae's induced growth significantly. The papillae colour was also evaluated as it was initial indicator for ruminal acidosis (Consalvo et al., 2016) and keratinization (Álvarez-Rodríguez et al., 2012). The colour of the papillae started to darken as mild acidosis. Still, Nurliani et al.

(2015) suggested that the darken colour of the rumen mucosa of swamp buffaloes was unique to themselves due to blood circulation. Our study agreed with Álvarez-Rodríguez et al. (2012), where the colour was only subjected to a type of diet.

Many reports stated that the energy diet was the leading cause of papillae growth (Diao et al., 2019; Penner et al., 2009); this was supported by Cui et al. (2019) as the protein and energy decrease the growth of rumen papillae also reduced. The researchers assumed, in the beginning, this was probably due to the higher digestibility, protein, and energy of the diet offered to the SI group, which had enhanced the better growth of rumen papillae. However, this was not observed, but they hypothesized the macroscopic differences might have occurred at a younger age, as suggested by Diao et al. (2019) and Gupta et al. (2016). Still, this effect was slowly reduced as animals grew older based on Penner et al. (2009) that the response of papillae growth was subjected to time.

The macro-morphometrical comparison between the ventral and dorsal region was not performed as there is a prominent difference between the size of the rumen papillae (Clauss et al., 2009). Therefore, the effect of type and nature of the diet on muscular and mucosal development was not observed in this study at a macroscopic level. This finding agreed with Beharka et al. (1998) and Suarez-Mena et al. (2016), who proposed that the rumen muscle thicknesses were hardly influenced by the type, physical form (fine or coarse) and level of roughage.

In the current study, the stratified squamous epithelium (SSE) was thinner in SI than EX. It was probably due to the cellular response to higher VFA production to promote absorption, where at the thinner epithelium layer, VFA can be absorbed rapidly. At the same time, the thickness of keratin may be associated with the digesta mass in the rumen, as suggested by Beharka et al. (1998). Thicker in keratin layer, slowing the absorption rate of VFA. Therefore, we can hypothesize that the stratified squamous epithelium and keratin was associated with the level of VFA in the rumen. It was agreed that the thickness of the SSE layer indicates papillae growth, but it was also reflected in the thickness of the keratin layer of EX in the present study. The rumen papillae keratinization was less common in animals raised under pasture than concentrates (Barros et al., 2015), supported by Melo et al. (2013) that the grazing cattle may reduce papillae absorptive surface area and basal cell mitotic index but increase in epithelium layer and keratin thickness.

Digesta volumes in the animal raised extensively could not be expected to be full as in other systems applied. This condition seems to have a digesta attachment to the rumen wall where the mechanism of 'wear and tear' or abrasion occurred actively (Greenwood et al., 1997). This effect was also probably associated with PKC supplementation in the SI group caused by the abrasion mechanism. In Malaysia, supplementing ruminants with PKC was very common, but it can be in various forms

of particle size that farmers did not consider (Saw et al., 2012).

CONCLUSION

Swamp buffalo from the semi-intensive group (SI) has a greater concentration of volatile fatty acid (VFA), such as acetic, propionic, and butyric, and also a greater percentage of propionic, butyric, and total VFA than the extensive group (EX) due to a proper feeding and management system. Nevertheless, the advantage of greater VFA concentration alone is not sufficient to conclude that the semi-intensive production system exerts a favourable effect on the morphology of the rumen mucosa. Despite the discrepancy in the feeding and management system between the semi-intensive and extensive systems, the development of rumen mucosae of the swamp buffaloes was equivalent except for the morphological alteration found only at the microscopic level on the thickness of keratin and stratified squamous epithelium. Further investigation on the rumen mucosa of the swamp buffaloes related to the production and management system in tropical environments is required to parse the mechanism of rumen mucosa alteration in swamp buffaloes.

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REFERENCES

Almeida, M. T. C., Ezequiel, J. M. B., Paschoaloto, J. R., Perez, H. L., Carvalho, V. B., Castro Filho, E. S., & van Cleef, E. H. C. B. (2018). Rumen and liver measurements of lambs fed with high inclusions of crude glycerin in adaptation and finishing period of feedlot. *Small Ruminant Research*, *167*, 1–5. https://doi.org/10.1016/j.smallrumres.2018.08.001

Aluwong, T., Kobo, P. I., & Abdullahi, A. (2013). Volatile fatty acids production in ruminants and the role of monocarboxylate transporters: A review. *African Journal of Biotechnology*, *9*(38), 6229–6232. https://doi.org/10.4314/ajb.v9i38

Álvarez-Rodríguez, J., Monleón, E., Sanz, A., Badiola, J. J., & Joy, M. (2012). Rumen fermentation and histology in light lambs as affected by forage supply and lactation length. *Research in Veterinary Science*, *92*(2), 247–253. https://doi.org/10.1016/j.rvsc.2011.03.010

Andrew, J., & John, G. (1998). How season of grazing and herbivore selectivity influence monsoon tall-grass communities of northern Australia. *Journal of Vegetation Science*, *9*, 123–132.

Bach, A., Calsamiglia, S., & Stern, M. D. (2005). Nitrogen metabolism in the rumen. *Journal of Dairy Science*, *88*(S), E9–E21. https://doi.org/10.3168/jds.S0022-0302(05)73133-7

Baldwin, R. L. (1999). Sheep gastrointestinal development in response to different dietary treatments. *Small Ruminant Research*, *35*(1), 39–47. https://doi.org/10.1016/S0921-4488(99)00062-0

Balthrop, J., Brand, B., Cowie, R., Danier, J., Boever, J. D., Jonge, L. D., Jackson, F. S., Makkar, H., & Piotrowski, C. (2011). *Quality assurance for animal feed analysis laboratories*. Food and Agriculture Organization.

Barros, S. S., da Cruz, R. S., de Melo Junior, L. M., de Souza, D. P. M., Moron, S. E., Alexandrino, E., Missio, R. L., Neiva, J. N. M., Restle, J., Maruo, V. M., Sousa, L. F., & Ramos, A. T. (2015). Queratinização das papilas ruminais, glicogênio celular e composição química da carne de tourinhos alimentados com níveis de concentrado e farelo do mesocarpo do babaçu [Rumen papillae keratinization, cell glycogen and chemical composition of the meat from young bulls fed different levels of concentrate and babassu mesocarp bran]. *Semina: Ciências Agrárias*, *36*(3), 1671–1683. https://doi.org/10.5433/1679-0359.2015v36n3p1671

Beharka, A A, Nagaraja, T. G., Morrill, J. L., Kennedy, G. A, & Klemm, R. D. (1998). Effects of form of the diet on anatomical, microbial, and fermentative development of the rumen of neonatal calves. *Journal of Dairy Science*, *81*(7), 1946–1955. https://doi.org/10.3168/jds.S0022-0302(98)75768-6

Bergman, E. N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological Reviews*, *70*(2), 567–590. https://doi.org/10.1046/j.1461-0248.2001.00230.x

Bernabucci, U., Lacetera, N., Baumgard, L. H., Rhoads, R. P., Ronchi, B., & Nardone, A. (2010). Metabolic and hormonal acclimation to heat stress in domesticated ruminants. *Animal*, *4*(7), 1167–1183. https://doi.org/10.1017/S175173111000090X

Boniface, A. N., Murray, R. M., & Muller, D. (1992). Intake and digestion in swamp buffaloes and cattle. 2. The comparative response to urea supplements in animals fed tropical grasses. *The Journal of Agricultural Science*, *119*(2), 243–254. https://doi.org/10.1017/S0021859600014179

Candyrine, S. C. L., Jahromi, M. F., Ebrahimi, M., Chen, W. L., Rezaei, S., Goh, Y. M., Abdullah, N., & Liang, J. B. (2019). Oil supplementation improved growth and diet digestibility in goats and sheep fed fattening diet. *Asian-Australasian Journal of Animal Sciences*, *32*(4), 533–540. https://doi.org/10.5713/ajas.18.0059

Celi, P., Cowieson, A. J., Fru-nji, F., Steinert, R. E., Kluenter, A., & Verlhac, V. (2017). Gastrointestinal functionality in animal nutrition and health: New opportunities for sustainable animal production. *Animal Feed Science and Technology*, *234*, 88–100. https://doi.org/10.1016/j.anifeedsci.2017.09.012

Chanthakhoun, V., Wanapat, M., Kongmun, P., & Cherdthong, A. (2012). Comparison of ruminal fermentation characteristics and microbial population in swamp buffalo and cattle. *Livestock Science*, *143*(2-3), 172–176. https://doi.org/10.1016/j.livsci.2011.09.009

Clauss, M., Hofmann, R. R., Fickel, J., Streich, W. J., & Hummel, J. (2009). The intraruminal papillation gradient in wild ruminants of different feeding types: Implications for rumen physiology. *Journal of Morphology*, *270*(8), 929–942. https://doi.org/10.1002/jmor.10729

Consalvo, S., Mirabella, N., Pero, M. E., Grazioli, R., & Calabrò, S. (2016). Weaning techniques for buffalo calves: Pre-stomachs development and

functionality. *Journal of Nutritional Ecology and Food Research*, *3*(2), 116–124. https://doi.org/doi:10.1166/jnef.2016.1134

Cui, K., Qi, M., Wang, S., Diao, Q., & Zhang, N. (2019). Dietary energy and protein levels influenced the growth performance, ruminal morphology and fermentation and microbial diversity of lambs. *Scientific Reports*, *9*, 16612. https://doi.org/10.1038/s41598-019-53279-y

de Resende-Junior, J. C., Alonso, L. D. S., Pereira, M. N., Roca, M. M. G., Duboc, M. V., de Oliveira, E. C., & de Melo, L. Q. (2006). Effect of the feeding pattern on rumen wall morphology of cows and sheep. *Brazilian Journal of Veterinary Research and Animal Science*, *43*(4), 526–536. https://doi.org/10.11606/issn.1678-4456.bjvras.2006.26469

Diao, Q., Zhang, R., & Fu, T. (2019). Review of strategies to promote rumen development in calves. *Animals*, *9*(8), 490. https://doi.org/10.3390/ani9080490

Dieho, K., Bannink, A., Geurts, I. A. L., Schonewille, J. T., Gort, G., & Dijkstra, J. (2016). Morphological adaptation of rumen papillae during the dry period and early lactation as affected by rate of increase of concentrate allowance. *Journal of Dairy Science*, *99*(3), 2339–2352. https://doi.org/10.3168/jds.2015-9837

Ding, B. A., Ma, S. Q., Li, Z. R., Li, X. L., & Madigosky, S. R. (2018). Seasonal changes of rumen and intestine morphology of the Qinghai yak (*Bos grunniens*). *Veterinary world*, *11*(8), 1135–1138. https://doi.org/10.14202/vetworld.2018.1135-1138

Duarte, E. R., Abrão, F. O., Ribeiro, I. C. O., Vieira, E. A., Nigri, A. C., Silva, K. L., Júnior, G. F. V., Barreto, S. M. P., & Geraseev, L. C. (2018). Rumen protozoa of different ages of beef cattle raised in tropical pastures during the dry season. *Journal of Applied Animal Research*, *46*(1), 1457–1461. https://doi.org/10.1080/09712119.2018.1530676

Ebrahimi, M., Rajion, M. A., Adeyemi, K. D., Jafari, S., Jahromi, F., Oskoueian, E., Goh, Y. M., & Ghaffari, M. H. (2017). Dietary n-6: n-3 fatty acid ratios alter rumen fermentation parameters and microbial populations in goats. *Journal of Agricultural and Food Chemistry*, *65*(4), 737–744. https://doi.org/10.1021/acs.jafc.6b04704

Escarcha, J. F., Lassa, J. A., Palacpac, E. P., & Zander, K. K. (2020). Livelihoods transformation and climate change adaptation: The case of smallholder water buffalo farmers in the Philippines. *Environmental Development*, *33*, 100468. https://doi.org/10.1016/j.envdev.2019.100468

Ferreira, L. M. M., Hervás, G., Belenguer, A., Celaya, R., Rodrigues, M. A. M., García, U., Frutos, P., & Osoro, K. (2017). Comparison of feed intake, digestion and rumen function among domestic ruminant species grazing in upland vegetation communities. *Journal of Animal Physiology and Animal Nutrition*, *101*(5), 846–856. https://doi.org/10.1111/jpn.12474

Franzolin, R., & Alves, T. C. (2010). The ruminal physiology in buffalo compared with cattle. *Revista Veterinaria*, *21*(1), 104–111. https://doi.org/10.13140/2.1.1501.1522

Franzolin, R., Rosales, F. P., & Soares, W. V. B. (2010). Effects of dietary energy and nitrogen supplements on rumen fermentation and protozoa population in buffalo and zebu cattle. *Revista Brasileira de Zootecnia*, *39*(3), 549–555. https://doi.org/10.1590/s1516-35982010000300014

Goularte, S. R., Ítavo, L. C. V., Santos, G. T., Ítavo, C. C. B. F., Oliveira, L. C. S., Favaro, S. P., Dias, A. M., Torres Junior, R. A. A., & Bittar, C. M. M. (2011). Ácidos graxos voláteis no rúmen de vacas alimentadas com diferentes teores de concentrado na dieta [Volatile fatty acids in rumen cows fed with different levels of concentrate in diet]. *Arquivo Brasileiro de Medicina Veterinaria e Zootecnia*, *63*(6), 1479–1486. https://doi.org/10.1590/S0102-09352011000600027

Greenwood, R. H., Morrill, J. L., Titgemeyer, E. C., & Kennedy, G. A. (1997). A new method of measuring diet abrasion and its effect on the development of the forestomach. *Journal of Dairy Science*, *80*(10), 2534–2541. https://doi.org/10.3168/jds.S0022-0302(97)76207-6

Gupta, M., Khan, N., Rastogi, A., Haq, Z. U., & Varun, T. K. (2016). Nutritional drivers of rumen development: A review. *Agricultural Reviews*, *37*(2), 148-153. https://doi.org/10.18805/ar.v37i2.10740

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Janssen, P. H., Abecia, L., Angarita, E., Aravena, P., Arenas, G. N., Ariza, C., Attwood, G. T., Avila, J. M., Avila-Stagno, J., Bannink, A., Barahona, R., Batistotti, M., Bertelsen, M. F., Brown-Kav, A., & Zunino, P. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific Reports*, *5*, 14567. https://doi.org/10.1038/srep14567

Kay, R. N. B., Engelhardt, W. V., & White, R. G. (1980). The digestive physiology of wild ruminants. In Y. Ruckebusch & P. Thivend (Eds.), *Digestive physiology and metabolism in ruminants* (pp. 743-761). Springer. https://doi.org/10.1007/978-94-011-8067-2_36

Kern, R. J., Lindholm-Perry, A. K., Freetly, H. C., Kuehn, L. A., Rule, D. C., & Ludden, P. A. (2016). Rumen papillae morphology of beef steers relative to gain and feed intake and the association of volatile fatty acids with *kallikrein* gene expression. *Livestock Science*, *187*, 24–30. https://doi.org/10.1016/j.livsci.2016.02.007

Khorasani, G. R., Okine, E. K., & Kennelly, J. J. (2001). Effects of forage source and amount of concentrate on rumen and intestinal digestion of nutrients in late-lactation cows. *Journal of Dairy Science*, *84*(5), 1156–1165. https://doi.org/10.3168/jds.S0022-0302(01)74576-6

Kotresh Prasad, C., Abraham, J., Panchbhai, G., Barman, D., Nag, P., & Ajithakumar, H. M. (2019). Growth performance and rumen development in Malabari kids reared under different production systems. *Tropical Animal Health and Production*, *51*(1), 119–129. https://doi.org/10.1007/s11250-018-1666-8

Kristensen, N. B. (2005). Splanchnic metabolism of volatile fatty acids in the dairy cow. *Animal Science*, *80*(1), 3–10. https://doi.org/10.1079/asc41250003

Lam, S., Munro, J. C., Zhou, M., Guan, L. L., Schenkel, F. S., Steele, M. A., Miller, S. P., & Montanholi, Y. R. (2018). Associations of rumen parameters with feed efficiency and sampling routine in beef cattle. *Animal*, *12*(7), 1442–1450. https://doi.org/10.1017/S1751731117002750

Lin, M. F., Ang, S. L., Yangb, C. W., Hsua, J. T., & Wang, H. T. (2011). Study on the characteristics of gastrointestinal tract and rumen ecology of Formosan Reeves'. *Journal of Applied Animal Research*, *39*(2), 142–146. https://doi.org/10.1080/09712119.2011.565560

Liu, L., Sun, D., Mao, S., Zhu, W., & Liu, J. (2019). Infusion of sodium butyrate promotes rumen papillae growth and enhances expression of genes related to rumen epithelial VFA uptake and metabolism in neonatal twin lambs. *Journal of Animal Science*, *97*(2), 909–921. https://doi.org/https://doi.org/10.1093/jas/sky459

Ma, S. C., & Zhao, G. Y. (2010). Effects of acetic, propionic and butyric acids given intraruminally at different molar proportions or individually on rumen papillae growth and IGF-I and IGFBP-3 in plasma, liver and rumen tissue in growing sheep nourished by total intragastric infusions. *African Journal of Biotechnology*, *9*(16), 2468–2473. https://doi.org/10.4314/ajb.v9i16

Malaysian Meteorological Department. (2018) *Laporan tahunan* [Annual report]. https://www.met.gov.my/content/pdf/penerbitan/laporantahunan/laporantahunan2018.pdf

Mao, S., Zhang, R., Wang, D., & Zhu, W. (2012). The diversity of the fecal bacterial community and its relationship with the concentration of volatile fatty acids in the feces during subacute rumen acidosis in dairy cows. *BMC Veterinary Research*, *8*, 237. https://doi.org/10.1186/1746-6148-8-237

Marshal, J. P., Krausman, P. R., & Bleich, V. C. (2005). Rainfall, temperature, and forage dynamics affect nutritional quality of desert mule deer forage. *Rangeland Ecology and Management*, *58*(4), 360–365. https://doi.org/https://doi.org/10.2111/1551-5028(2005)058[0360:RTAFDA]2.0.CO;2

Mason, F., Fotschki, B., Di Rosso, A., & Korzekwa, A. (2019). Influence of farming conditions on the rumen of red deer (*Cervus elaphus*). *Animals*, *9*(9), 601. https://doi.org/10.3390/ani9090601

Masud, M. M., Rahman, M. S., Al-Amin, A. Q., Kari, F., & Filho, W. L. (2014). Impact of climate change: An empirical investigation of Malaysian rice production. *Mitigation and Adaptation Strategies for Global Change*, *19*(4), 431–444. https://doi.org/10.1007/s11027-012-9441-z

McGrath, J., Duval, S. M., Tamassia, L. F. M., Kindermann, M., Stemmler, R. T., de Gouvea, V. N., Acedo, T. S., Immig, I., Williams, S. N., & Celi, P. (2018). Nutritional strategies in ruminants: A lifetime approach. *Research in Veterinary Science*, *116*, 28–39. https://doi.org/10.1016/j.rvsc.2017.09.011

Melo, L. Q., Costa, S. F., Lopes, F., Guerreiro, M. C., Armentano, L. E., & Pereira, M. N. (2013). Rumen morphometrics and the effect of digesta pH and volume on volatile fatty acid absorption. *Journal of Animal Science*, *91*(4), 1775–1783. https://doi.org/10.2527/jas.2011-4999

Mohd Azmi, A. F., Abu Hassim, H., Mohd Nor, N., Ahmad, H., Goh, Y. M., Abdullah, P., Abu Bakar, M. Z., Vera, J., Mohd Deli, N. S., Salleh, A., & Zamri-Saad, M. (2021). Comparative growth and economic performances between indigenous swamp and murrah crossbred buffaloes in Malaysia. *Animals*, *11*(4), 957. https://doi.org/10.3390/ani11040957

Moore, K. J., & Jung, H. J. G. (2001). Lignin and fiber digestion. *Journal of Range Management*, *54*(4), 420–430. https://doi.org/10.2307/4003113

Mottet, A., de Haan, C., Falcucci, A., Tempio, G., Opio, C., & Gerber, P. (2017). Livestock: On our plates or eating at our table? A new analysis of the feed/food debate. *Global Food Security*, *14*, 1–8. https://doi.org/10.1016/j.gfs.2017.01.001

Moyer, J. R., & Hironaka, R. (1993). Digestible energy and protein content of some annual weeds, alfalfa, bromegrass, and tame oats. *Canadian Journal of Plant Science*, *73*(4), 1305–1308. https://doi.org/10.4141/cjps93-169

Nurliani, A., Budipitojo, T., & Kusindarta, D. L. (2015). Morphological characteristics of the stomach of the swamp buffalo (*Bubalus bubalis*). *Aceh International Journal of Science and Technology*, *4*(3), 78–82. https://doi.org/10.13170/aijst.4.3.3011

Palmieri, A. D., Oliveira, R. L., Ribeiro, C. V. D. M., Ribeiro, M. D., Ribeiro, R. D. X., Leão, A. G., Agy, M. S. F. A., & Ribeiro, O. L. (2012). Effects of substituting soybean meal for sunflower cake in the diet on the growth and carcass traits of crossbred Boer goat kids. *Asian-Australasian Journal of Animal Sciences*, *25*(1), 59-65. https://doi.org/10.5713/ajas.2011.11140

Parmar, N. R., Solanki, J. V., Patel, A. B., Shah, T. M., Patel, A. K., Parnerkar, S., Kumar, J. I., N., & Joshi, C. G. (2014). Metagenome of Mehsani buffalo rumen microbiota: An assessment of variation in feed-dependent phylogenetic and functional classification. *Journal of Molecular Microbiology and Biotechnology*, *24*(4), 249–261. https://doi.org/10.1159/000365054

Penner, G. B., Taniguchi, M., Guan, L. L., Beauchemin, K. A., & Oba, M. (2009). Effect of dietary

forage to concentrate ratio on volatile fatty acid absorption and the expression of genes related to volatile fatty acid absorption and metabolism in ruminal tissue. *Journal of Dairy Science*, *92*(6), 2767–2781. https://doi.org/10.3168/jds.2008-1716

Rostini, T., Zakir, I., & Biyatmoko, D. (2018). Different in quantity of microbial rumen fluid of river buffalo and swamp buffalo. In *Proceedings of the International Conference on Applied Science and Engineering* (Vol. 175, pp. 118–119). Atlantis Press. https://doi.org/https://doi.org/10.2991/icase-18.2018.32

Savsani, H. H., Murthy, K. S., Gajbhiye, P. U., Vataliya, P. H., Dutta, K. S., Gadariya, M. R., & Bhadaniya, A. R. (2017). Economics of rumen bypass fat feeding on cost of milk production, feeding and realizable receipts in lactating Jaffrabadi buffaloes. *Buffalo Bulletin*, *36*(1), 193–198.

Saw, H. Y., Janaun, J. S., Kumaresan, S., & Chu, C. M. (2012). Characterization of the physical properties of palm kernel cake. *International Journal of Food Properties*, *15*(3), 536–548. https://doi.org/10.1080/10942912.2010.492543

Shen, Z., Kuhla, S., Zitnan, R., Seyfert, H. M., Schneider, F., Hagemeister, H., Chudy, A., Löhrke, B., Blum, J. W., Hammon, H. M., & Voigt, J. (2005). Intraruminal infusion of n-butyric acid induces an increase of ruminal papillae size independent of IGF-1 system in castrated bulls. *Archives of Animal Nutrition*, *59*(4), 213–225. https://doi.org/10.1080/17450390500216894

Silanikove, N. (2000). Effects of heat stress on the welfare of extensively managed domestic ruminants. *Livestock Production Science*, *67*(1–2), 1–18. https://doi.org/10.1016/S0301-6226(00)00162-7

Steele, M. A., Croom, J., Kahler, M., AlZahal, O., Hook, S. E., Plaizier, K., & McBride, B. W. (2011). Bovine rumen epithelium undergoes rapid structural adaptations during grain-induced subacute ruminal acidosis. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *300*(6), R1515–R1523. https://doi.org/10.1152/ajpregu.00120.2010

Storm, A. C., Kristensen, N. B., & Hanigan, M. D. (2012). A model of ruminal volatile fatty acid absorption kinetics and rumen epithelial blood flow in lactating Holstein cows. *Journal of Dairy Science*, *95*(6), 2919–2934. https://doi.org/10.3168/jds.2011-4239

Suárez, B. J., Van Reenen, C. G., Stockhofe, N., Dijkstra, J., & Gerrits, W. J. J. (2007). Effect of roughage source and roughage to concentrate ratio on animal performance and rumen development in veal calves. *Journal of Dairy Science*, *90*(5), 2390–2403. https://doi.org/10.3168/jds.2006-524

Suarez-Mena, F. X., Heinrichs, A. J., Jones, C. M., Hill, T. M., & Quigley, J. D. (2016). Straw particle size in calf starters: Effects on digestive system development and rumen fermentation. *Journal of Dairy Science*, *99*(1), 341–353. https://doi.org/10.3168/jds.2015-9884

Suhaimi, A., Bustami, Y., & Saihani, A. (2019). Assessment of comparative advantage and development strategy for swamp buffalo livestock in Hulu Sungai Utara regency, South Kalimantan. *Asian Journal of Scientific Research*, *12*(2), 271–278. https://doi.org/10.3923/ajsr.2019.271.278

Suphachavalit, S., Sricharoen, P., Luesopha, T., Srisakdi, T., Na-Chiangmai, A., & Boonprong, S. (2013). Swamp buffalo production system and needs for extension on local scale farmers in the lower northeast of Thailand. *Buffalo Bulletin*, *32*, 1204–1207.

Sutton, J. D., Dhanoa, M. S., Morant, S. V, France, J., Napper, D. J., & Schuller, E. (2003). Rates of production of acetate, propionate, and butyrate in the rumen of lactating dairy cows given normal and low-roughage diets. *Journal of*

Dairy Science, *86*(11), 3620–3633. https://doi.org/10.3168/jds.S0022-0302(03)73968-X

Temple, D., & Manteca, X. (2020). Animal welfare in extensive production systems is still an area of concern. *Frontiers in Sustainable Food Systems*, *4*, 154–172. https://doi.org/10.3389/fsufs.2020.545902

Van Kessel, J. A. S., & Russell, J. B. (1996). The effect of pH on ruminal methanogenesis. *FEMS Microbiology Ecology*, *20*(4), 205–210. https://doi.org/10.1016/0168-6496(96)00030-X

Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, *74*(10), 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Wanapat, M., & Pimpa, O. (1999). Effect of ruminal NH3-N levels on ruminal fermentation, purine derivatives, digestibility and rice straw intake in swamp buffaloes. *Asian-Australasian Journal of Animal Sciences*, *12*(6), 904–907. https://doi.org/10.5713/ajas.1999.904

Wanapat, M., Kang, S., & Polyorach, S. (2013). Development of feeding systems and strategies of supplementation to enhance rumen fermentation and ruminant production in the tropics. *Journal of Animal Science and Biotechnology*, *4*, 32. https://doi.org/10.1186/2049-1891-4-32

Wanapat, M., Pilajun, R., & Kongmun, P. (2009). Ruminal ecology of swamp buffalo as influenced by dietary sources. *Animal Feed Science and Technology*, *151*(3–4), 205–214. https://doi.org/10.1016/j.anifeedsci.2009.01.017

Wang, L., Zhang, G., Li, Y., & Zhang, Y. (2020). Effects of high forage / concentrate diet on volatile fatty acid production and the microorganisms involved in VFA production in cow rumen. *Animals*, *10*(2), 223–235. https://doi.org/https://doi.org/10.3390/ani10020223

Wang, L., Zhou, Q., & Zheng, G. H. (2006). Comprehensive analysis of the factors for propionic acid accumulation in acidogenic phase of anaerobic process. *Environmental Technology*, *27*(3), 269–276. https://doi.org/10.1080/09593332708618640

Wang, Y. H., Xu, M., Wang, F. N., Yu, Z. P., Yao, J. H., Zan, L. S., & Yang, F. X. (2009). Effect of dietary starch on rumen and small intestine morphology and digesta pH in goats. *Livestock Science*, *122*(1), 48–52. https://doi.org/10.1016/j.livsci.2008.07.024

Wanna, M., Pisai, W., & Sorachai, K. (2012). Production efficiency of swamp buffaloes and Mehsana river buffalo. *Buffalo Bulletin*, *31*(1), 40–45.

Xu, M., Dong, Y., Du, S., Hao, Y. S., Wang, Y. H., Wang, F. N., & Yao, J. H. (2009). Effect of corn particle size on mucosal morphology and digesta pH of the gastrointestinal tract in growing goats. *Livestock Science*, *123*(1), 34–37. https://doi.org/10.1016/j.livsci.2008.10.00

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Phyllanthus debilis Methanolic Extract Reduces the Viability of Human Colorectal Adenocarcinoma (HT-29) Cells and Increases *LINE-1* and *Alu* DNA Methylation

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ABSTRACT

Phyllanthus debilis was shown to have a strong anti-proliferative effect on cancer cells with less effect in normal cells. However, its mechanism on the epigenetic mechanism at repeat sequences is unknown. This study was carried out to determine the effect of *P. debilis* extract on long interspersed nuclear element-1 (*LINE-1*) and *Alu* DNA methylation. The anti-proliferative effect of *P. debilis* methanolic extract on human colorectal adenocarcinoma (HT-29) at 24 hours was done using trypan blue assay. *LINE-1* and *Alu* methylation measurement on the HT-29 cell line was done after 72 hours of treatment using Pyrosequencing. The effect of *P. debilis* methanolic extract at 24 hours on the viability of HT-29 cells was dose-dependent with the half-maximal inhibitory concentration (IC_{50}) concentration of 0.1 mg/mL. Treatment with *P. debilis* methanolic extract showed significantly higher *Alu* DNA methylation when compared with the untreated HT-29 cells (37.0 ± 2.5% vs 32.3 ± 4.3%, $p<0.05$). Similarly, treatment with 5-aza-2-deoxycytidine also significantly increased the *Alu* DNA methylation compared with the untreated HT-29 cells (46.0 ± 2.3% vs 37.0 ± 2.5%, $p<0.05$). For *LINE-1*, there was a significant increase of *LINE-1* methylation when treated with *P. debilis* extract (80.3 ± 1.3% vs 76.3 ± 2.1%, $p<0.05$) and with 5-aza-2-deoxycytidine (81.8 ± 4.3% vs 76.3 ± 2.1%, $p<0.05$) when compared with untreated cells. In conclusion, treatment of *P. debilis* methanolic extract on HT-29 cell line reduces the viability of HT-29 cells and increases the methylation of *Alu* and *LINE 1*. Similar changes in methylation were also seen in the 5-aza treatment. These epigenetic changes by *P. debilis* methanolic extract may contribute to its anti-cancer properties.

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INTRODUCTION

Aberrant DNA hypomethylation, which was frequently seen in the cancer cells, can induce activation of oncogenes and loss of imprinting (Sharma et al., 2010). Overall genomic methylation in carcinogen-induced cancer cells was reduced by 20–60% compared to normal cells, and demethylation of repetitive sequences' methylation accounts for 20–30% of the human genome (Ehrlich, 2002). Hypomethylation of the genome occurs early in the development of cancer. It accumulates throughout all tumorigenic steps, from benign proliferation to invasive cancer (Fraga et al., 2004). Although gene-specific demethylation occurs in the context of global DNA hypomethylation, many of the effects are thought to be caused by the activation of transposable elements and endogenous retroviruses found in the human genome as by loss of imprinting (Whitelaw & Martin, 2001). Potentially, the reactivation of the strong promoters associated with transposable elements can globally modify the expression levels of transcription factors and/or the gene expression levels of the growth regulatory genes in which these factors reside (Whitelaw & Martin, 2001). Human DNA comprises a significant number of transposable elements. The most studied sequences were *LINE-1*, and *Alu* repeats. *LINE-1* and *Alu* repeat sequences are highly methylated in somatic tissues (Ehrlich, 2002). These repeat genetic elements are sequences that can change places on a chromosome and be exchanged between chromosomes that need to be repressed by methylation to prevent disruption to the genetic sequences and transcriptome (Rodić & Burns, 2013). Studies have shown that changes in methylation at these repeat sequences affected the changes of genome-wide methylation, and these changes can be measured as a surrogate marker for epigenetic changes in genome-wide methylation status (Lisanti et al., 2013).

Phyllanthus debilis, a less common *Phyllanthus* species, is usually used to substitute other popular *Phyllanthus* species, such as *Phyllanthus amarus* (Kumaran & Karunakaran, 2007). This herb shows anti-inflammatory, anti-microbial, anti-diabetic, anti-cancer, and antihepatotoxic properties (Ahmed et al., 2009; Chandrashekar et al., 2005). The aqueous extract of the plant shows an antihyperglycemic property (Wanniarachchi et al., 2009). *Phyllanthus debilis* has been shown to possess higher antioxidant activity than *Phyllanthus amarus*, *Phyllanthus maderaspatensis*, *Phyllanthus urinaria*, and *Phyllanthus virgatus* (Kumaran & Karunakaran, 2007). Many bioactive compounds had been found in *P. debilis*, such as β-sitosterol, debelalactone, and phyllanthin. These compounds were shown to have anti-inflammatory, antihepatotoxic, and anti-cancer (Sarin et al., 2014). Based on traditional usage but limited scientific evidence, *Phyllanthus* sp. could be explored for their potential anti-cancer activity through an epigenetic mechanism in colorectal cancer cells line (HT-29).

METHODS

Sample Preparation

Herbal Specimen Collection and Identification. *Phyllanthus debilis* was collected from a local collection at Tasek Gelugor, Penang, Malaysia. The species was identified by the herbarium unit, School of Biology, Universiti Sains Malaysia. The voucher specimen was deposited at the Universiti Sains Malaysia herbarium unit (*Phyllanthus debilis* specimen number: 11623)

Sample Extraction

The whole sample of *P. debilis* was cleaned and washed before being dried in the oven at 50 °C for three days. The dried sample was then ground and prepared in powder form. First, a five-gram sample was extracted with 100 mL of methanol (Fisher Scientific, USA) in an ultrasonic bath (Power-Sonic 405 Model, Hwashin, Korea) for 20 min and then filtered. The procedure was carried out twice more with the remaining residual extract. Finally, a rotary evaporator was used to dry the extract (Rotary Evaporator RII, Büchi, Switzerland). Prior to use, the dried extracts were kept at a temperature of -20 °C.

Cell Culture for *Phyllanthus debilis* Methanolic Extract on HT-29 Cells Viability

Passage 19 of human colorectal adenocarcinoma (HT-29) was used in this study and was grown in the Roswell Park Memorial Institute (RPMI)-1640 medium. The medium was supplemented with 10% fetal bovine serum (FBS) (Gibco™, USA) and 1% penicillium-streptomycin (10,000 U/mL, Gibco™, USA) to ensure cell growth and viability. The cells were incubated in a humidified atmosphere with 5% carbon dioxide at 37 °C for growth and maintenance.

Viability of HT-29 Cell Lines

Cells were seeded at their optimal cell density (0.05 x 10^6 cells/well) into a 24-well plate and were incubated in 5% carbon dioxide at 37 °C for 36 to 48 hours to allow cell attachment and make them reach the growth of 80% confluency. The cells were then treated *P. debilis* methanol extracts at 6 different concentrations (0.03125, 0.0625, 0.125, 0.25, 0.5, and 1 mg/mL). The experimental control was prepared with only cells and cultured medium without treatment with *Phyllanthus* extract. The experimental plates were incubated at 37 °C, 5% carbon dioxide for another 24 hours.

The cells were then washed using phosphate-buffered saline (PBS) to remove any floating cells and then incubated with trypsin for 5 min to detach the cells from the well. Next, the medium was added to the well to deactivate the trypsin and thoroughly mixed. Once mixed, the cell suspension was then mixed with 0.4% trypan blue solution in the ratio of 1:1. The cells in 10 µL of this suspension were then directly counted on a haemocytometer. The average cell count of four fields represented the number of cells per mL of cell solution and determined the total number of cells from each well.

The number of cells was counted in each of the four quadrants by using the following formula:

$$\text{Number of cell} = \left(\frac{A + B + C + D}{4}\right) \text{x } 10^4 \text{ x 2 x sample dilution}$$

The viability of the cell is determined by comparing viable cells for the treatment with the untreated cell:

$$\text{Percentage of cell viability (\%)} = \left(\frac{\text{Number of viable cells from treatment}}{\text{Number of viable cell from experimental control}}\right) \text{x } 100$$

The percentage of cell inhibition is determined by using the following formula:

$$Percentage\ of\ cell\ inhibition\ (\%) = 100 - Viability\ of\ cell\ (\%)$$

The percentage of cell inhibition (%) versus treatment concentration (mg/mL) was plotted based on the data obtained. The concentration that inhibits 50% of the cells (IC_{50}) was obtained by extrapolating the graphs constructed on the viability test.

Treatment of Cells with *Phyllanthus debilis* for Determination of DNA Methylation at *LINE-1,* and *Alu* Repeats Sequence

HT29 cells were seeded at their optimal cell density (0.4 x 10^6 cells/well) in a 6-well plate in RPMI-1640 medium (Gibco™, USA) supplemented with 10% fetal bovine serum (Gibco™, USA) and 1% penicillin-streptomycin (Gibco™, USA). Cells were maintained at 37 °C in a 5% carbon dioxide (CO_2) atmosphere. IC_{50} of the *P. debilis* (0.1 mg/mL) was used to treat the HT-29 cell line. Stock solution (0.1 mg/mL) was prepared by diluting the crude extracts in RPMI for treatment. 5-aza-2-deoxycytidine (Acros, USA) was used as a positive control in methylation study as the drug act as an epigenetic modulator. 5-aza-2-deoxycytidine stock solution (4.382 mM) was prepared by dissolving 1 mg of 5-aza-2-deoxycytidine (molecular weight = 228.208 g/mol) in 1 mL dimethyl sulphoxide (DMSO) (Sigma Aldrich, USA). The stock solution was then diluted into 100 µM in PBS (Gibco™, USA). Then, the stock was diluted with cultured medium to the concentration of 0.5 µM. The concentration of 5-aza-2-deoxycytidine at 0.5 µM used in this study was shown previously to demethylate genes at genome-wide scale (Ishiguro et al., 2007; Khamas et al., 2012). The working solutions of the drug were freshly prepared before usage. When the cells reached 80% confluency, the cells were then treated with *P. debilis* extract and 5-aza-2-deoxycytidine at the concentration

of 0.1 mg/mL and 0.5 µM, respectively. Treatments were continued for three days (72 hours) while replacing the RPMI-1640 medium and the treatment daily. Cells were harvested after completing 72 hours of treatments.

Bisulfite Modification of DNA

Genomic DNA of the HT-29 cells was isolated using Wizard SV Genomic DNA purification kit (Promega, USA) following manufacturer protocol with slight modifications. The cells were then bisulfite modified by treating them to sodium bisulfite, which selectively converts unmethylated cytosine to uracil. Still, the 5-methylcytosine is protected from deamination and is preserved in the downstream reactions. The bisulfite modification was conducted using EZ DNA Methylation-Gold™ Kit (Zymo Research, USA) by following the manufacturer's protocol with slight modification. Briefly, 500 ng of DNA was incubated with CT conversion reagent at the following temperatures: 98 °C for 10 min, 64 °C for 2.5 h, held at 4 °C. Once completed, the DNA was transferred to a spin column, washed, and desulphonated. It was further purified using wash buffer before being eluted in 20 µL deionised water. The bisulfite modified DNA was kept at 4 °C for further use.

Polymerase Chain Reaction (PCR) and Pyrosequencing

HT-29 cells were assayed at the repeat sequences of *Alu* and *LINE-1*. Each sample was biologically and technically replicated four times. *LINE-1* and *Alu* sequences and primers followed Lisanti et al. (2013). Two microliters (2 µL) of bisulfite modified DNA were used in a polymerase chain reaction (PCR) containing a total volume of 25 µL reaction. The reaction includes 12.5 µL GoTaq Green master mix (Promega, USA), 400 nM forward primer, and 400 nM biotin-labelled reverse primer. The biotinylated labelled reverse primer was used to capture a single-stranded DNA template for the Pyrosequencing assay later. PCR condition was carried out using the following a three-step protocol: an initial denaturation step at 95 °C for 15 min, then 50 cycles of 95 °C for 15 s and annealing temperature 50 °C for 30 s, followed with 72 °C for 30 s, and final elongation step at 72 °C for 5 min. About 5 µL of PCR product was subjected to gel electrophoresis to confirm the amplicon size of *LINE-1* and *Alu*.

The biotin-labelled PCR product was then captured with Streptavidin Sepharose beads (Qiagen, Germany) using a Pyrosequencing Vacuum Prep Tool (Qiagen, Germany). It was made into single-stranded DNA. The single-stranded DNA was then annealed to the sequencing primer and was heated to 80 °C for 5 min and allowed to cool to room temperature. Pyrosequencing was then carried out on a PyroMark ID (Qiagen, Germany). The percentage of methylation at the target cytosine-guanine dinucleotide (CpG) sites was quantified using PyroMark Q96 2.5.8 software (Qiagen, Germany). The primers used in PCR and Pyrosequencing and sequence to analyse *LINE-1* and *Alu* were shown in Tables 1, 2, and 3.

Table 1

The primers sequences of LINE-1 *and* Alu *repeat sequences used in the PCR reaction and Pyrosequencing*

Assay	Forward primer	Biotinylated labelled reverse primer
LINE-1	5'-TTTTGAGTTAGGTGTGGGATATA-3'	5'-AAAATCAAAAAATTCCCTTTC-3'
ALU	5'-TTTTTTTTTAAAGGTTATG-3'	5'-TCTATCCCTAAAATTAAAA-3'

Table 2

The sequencing primer of LINE-1 *and* Alu *repeat sequences used in the Pyrosequencing assay*

Assay	Sequencing primer
LINE-1	5'-AGTTAGGTGTGGGATATAGT-3'
Alu	5'-TTTTTTTTTAAAGGTTATG-3'

Table 3

Sequence to analyse for PyroMark CpG assays

Assay	Sequence to analyse
LINE-1	5'-TTC/TGTGGTGC/TGTC/TG-3'
Alu	5'-TC/TG-3'

Statistical Analysis

Comparison between 2 groups of treatments was made using student *t*-test (SPSS Version 24). All the data presented as mean ± standard deviation (SD).

RESULTS

The Effect of *Phyllanthus debilis* Methanolic Extract Towards Viability of HT-29 Cell Line

The effect of *P. debilis* methanolic extract on the viability of HT-29 was evaluated. Figure 1 shows the results of the effect of *P. debilis* methanolic extract on the viability of HT29 cells. The effect of *P. debilis* methanolic extract on the viability of HT-29 cells was dose-dependent manner. The IC_{50} concentration of *P. debilis* methanolic extract was 0.1 mg/mL.

Measurement of *Alu* and *LINE-1* DNA Methylation in HT29 Cells

The methylation changes of *Alu* and *LINE-1* repeat elements as a surrogate marker for global methylation were measured. DNA methylation was measured at one CpG site in the *Alu* sequence (Figure 2) and 3 CpG sites in the *LINE-1* repeat sequence. For *LINE-1*, data were presented as mean methylation of all 3 CpG sites (Figure 3). The methylation changes at targeted CpG sites of the untreated cells were compared with the methylation changes of the treated cells with *P. debilis* and 5-aza-2-deoxycytidine for 72 hours.

For *Alu* methylation, treatment with 5-aza-2-deoxycytidine showed higher DNA methylation when compared with the untreated HT-29 cells (46.0 ± 2.3% vs 32.3 ± 4.0, $p<0.05$). Similarly, treatment with *P.*

debilis also significantly increased the DNA methylation compared with the untreated HT-29 cells (37.0 ± 2.8% vs 32.3 ± 4.3%, $p<0.05$).

For *LINE-1*, compared with untreated cells, the average DNA methylation showed a significant increase of *LINE-1* methylation when treated with 5-aza-2-deoxycytidine (81.8 ± 4.3% vs 76.3 ± 2.1%, $p<0.05$) and with *P. debilis* (80.3 ± 1.3% vs 76.3 ± 2.1%, $p<0.05$).

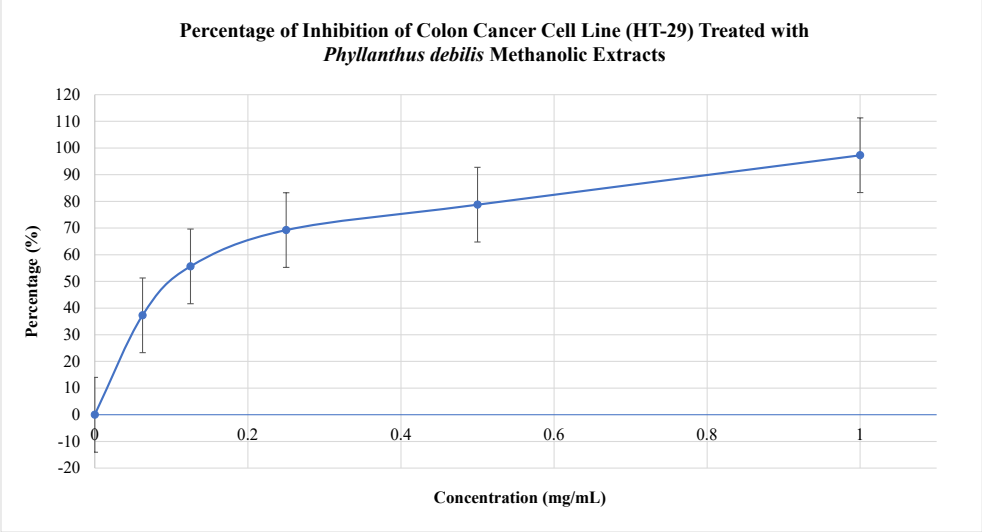

Figure 1. The inhibition of *Phyllanthus debilis* methanolic extract on HT-29 cell line. HT-29 were treated with different concentrations of *P. debilis* methanolic extract for 24 hours

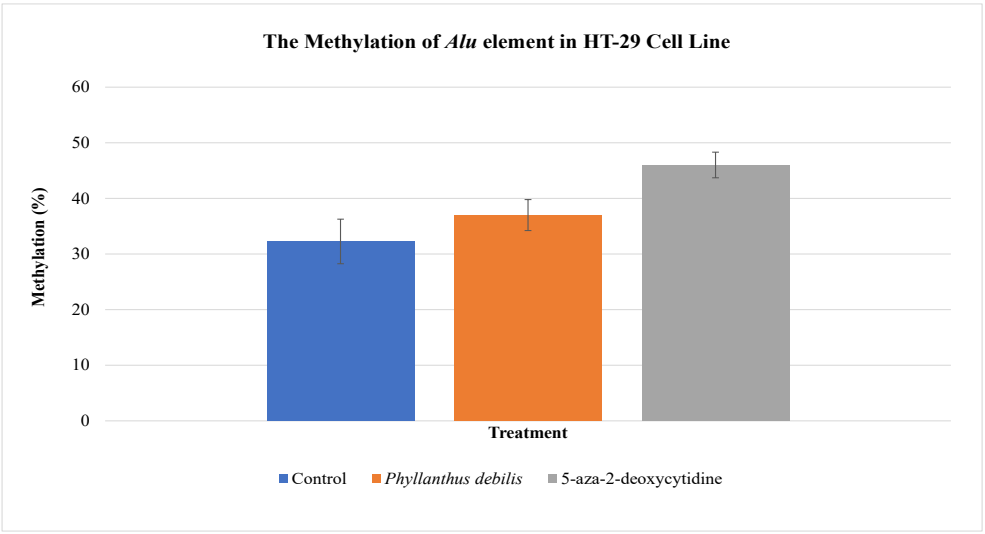

Figure 2. The methylation of *Alu* element in the untreated cells and cells treated with *Phyllanthus debilis* and 5-aza-2-deoxycytidine

Note. * Denotes significant difference in DNA methylation of *Alu* when compared with untreated cells ($p<0.05$)

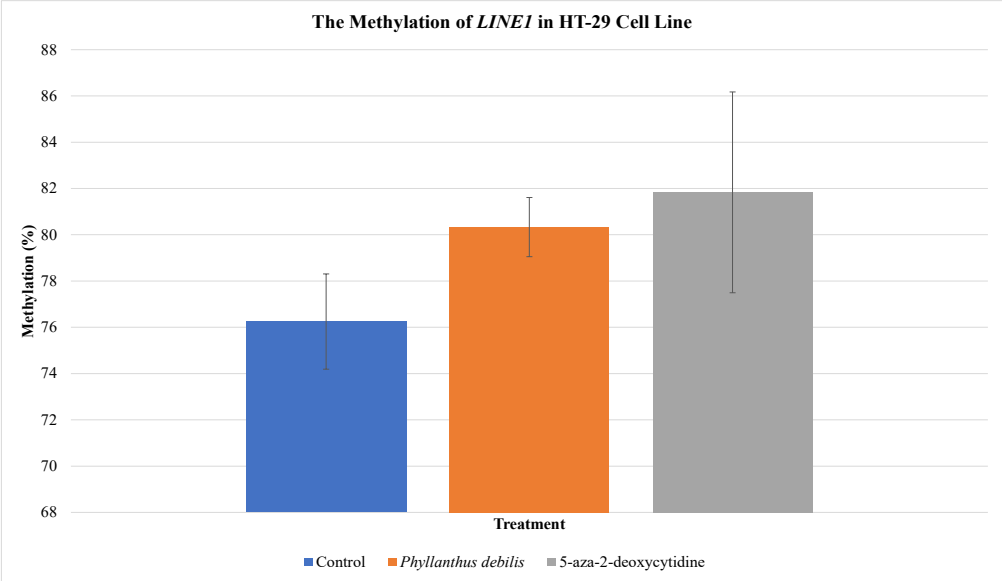

Figure 3. The methylation of *LINE-1* in the untreated cells and cells treated with *Phyllanthus debilis* and 5-aza-2-deoxycytidine

Note. * Denotes significant difference in DNA methylation of *LINE-1* when compared with untreated cells (p<0.05)

DISCUSSION

Genome-wide DNA hypomethylation plays an important role in epigenomic and genomic instability and colorectal carcinogenesis (Natsume et al., 2008). Human DNA contains large numbers of non-coding repeat sequences, the most studied sequences being *LINE-1* and *Alu* repeats. *LINE-1* repeat sequences constitute about 17% of the human genome, while *Alu* repeats constitute about 11% of the human genome (Lander, 2001; Sellis et al., 2007). Changes in methylation at these repeat sequences may affect genome-wide methylation changes, and these changes could be monitored and measured as a surrogate marker for genome-wide methylation status (So et al., 1996). A simple method was used to measure global DNA methylation using bisulfite PCR of DNA repetitive elements, and pyrosequenced was carried out by Lisanti et al. (2013). Lisanti et al. (2013) showed a strong correlation between *Alu* and *LINE-1* methylation with global methylation, analysed by high-performance liquid chromatography (HPLC), in which HPLC was considered a gold standard in measuring genome global DNA methylation.

Genomic hypomethylation demonstrated by downregulation of methylated CpG dinucleotides, which disperse throughout the genomes in non-coding repetitive sequences, has been recognised as a common epigenetic change during cancer development (Feinberg & Tycko, 2004; Sugimura & Ushijima,

2000). It has been proposed that genome hypomethylation contributes to oncogenesis by activating oncogenes, such as *c-Myc* and *H-RAS75*, activating latent retrotransposons, or causing chromosome instability (Das & Singal, 2004). Global hypomethylation in colon, liver, bladder, oesophagus, head and neck, prostate, stomach, breast, and lung carcinomas tissues was common observations compared to their normal tissue counterparts (Chalitchagorn et al., 2004).

Global DNA methylation, measured by HPLC, *LINE-1* and *Alu* assays were lower in colorectal tumour tissue than in paired normal tissue (Natsume et al., 2008). Hypomethylation of *Alu* and *LINE-1* has been reported as early events in the multistep carcinogenesis of colorectal cancer (Chalitchagorn et al., 2004; Kwon et al., 2010; Lee et al., 2009). Hypomethylation of transposable elements, such as *Alu* and *LINE-1*, causes transcriptional activation, resulting in transposable element retrotransposition, chromosome alteration, and thus genomic instability (Bae et al., 2012; Saito et al., 2010). On the other hand, CpG sites within *Alu* and *LINE-1* are usually methylated in normal cells, thus maintaining transcriptional inactivation and inhibiting retrotransposition (Yoder et al., 1997).

This study showed that the treatment with *P. debilis* induces anti-proliferation to HT-29 cells at 0.1 mg/mL with an observable small significant increase in DNA methylation of *LINE-1* and *Alu* element in HT-29 cells compared with the untreated cells. Furthermore, the changes observed due to the treatment of *P. debilis* were also seen when the HT-29 cells were treated with 5-aza-2-deoxycytidine at a low dose (0.5 µM), which showed a similar increase in DNA methylation of *LINE-1* and *Alu* but with higher methylation when compared with *P. debilis*.

Although 5-aza-2-deoxycytidine is a drug that can demethylate global and gene-specific regions (Momparler, 2013), this study showed a reverse pattern of methylation where it was found that 5-aza-2-deoxycytidine at the dose of 0.5 µM increase the *LINE-1* and *Alu* methylation. Whether the observations made on the effect of 5-aza-2-deoxycytidine at a low dose and *P. debilis* methanolic extract happened only in HT-29 cells or whether the findings were specifically at the *LINE-1* and *Alu* target sequences in this study need further evaluation.

A previous study on breast cancer cell lines and normal cell lines showed that *P. debilis* methanolic extract targets the cancer cells while sparing the normal cells (Omar & Zain, 2018). Furthermore, at the gene-specific level, *P. debilis* methanolic extract was able to reduce the *TAC1* DNA methylation (Zain & Omar, 2020). Thus, the specific target of *P. debilis* methanolic extract on cancer cells, and the increasing level of global methylation as observed in this study with increasing *LINE-1* and *Alu* methylation may reduce the proliferative ability of the colorectal cancer cells. Whether these changes may sensitise the cell to undergo apoptosis should further be tested and studied.

CONCLUSION

Treatment of *Phyllanthus debilis* methanolic extract on HT-29 cell line induced anti-proliferation of HT-29 cells with an increase of the methylation of *Alu* and *LINE-1*. Furthermore, increased methylation of *Alu* and *LINE-1* was also seen in the 5-aza-2-deoxycytidine treatment. Thus, the methylation changes in *Alu* and *LINE-1* by *P. debilis* methanolic extract may contribute to its anti-cancer properties, and its regulation on the DNA methylation should be further studied.

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REFERENCES

Ahmed, B., Khan, S., Verma, A., & Habibullah. (2009). Antihepatotoxic activity of debelalactone, a new oxirano-furanocoumarin from *Phyllanthus debilis*. *Journal of Asian Natural Products Research*, *11*(8), 687–692. https://doi.org/10.1080/10286020802621864

Bae, J. M., Shin, S., Kwon, H., Park, S., Kook, M. C., Kim, Y., Cho, N., Kim, N., Kim, T., Kim, D., & Kang, G. H. (2012). *ALU* and *LINE-1* hypomethylations in multistep gastric carcinogenesis and their prognostic implications. *International Journal of Cancer*, *131*(6), 1323-1331. https://doi.org/10.1002/ijc.27369

Chalitchagorn, K., Shuangshoti, S., Hourpai, N., Kongruttanachok, N., Tangkijvanich, P., Thong-ngam, D., Voravud, N., Sriuranpong, V., & Mutirangura, A. (2004). Distinctive pattern of *LINE-1* methylation level in normal tissues and the association with carcinogenesis. *Oncogene*, *23*(54), 8841-8846. https://doi.org/10.1038/sj.onc.1208137

Chandrashekar, K. S., Joshi, A. B., Satyanarayana, D., & Pai, P. (2005). Analgesic and anti-inflammatory activities of *Phyllanthus debilis*. Whole plant. *Pharmaceutical Biology*, *43*(7), 586-588. https://doi.org/10.1080/13880200500301670

Das, P. M., & Singal, R. (2004). DNA methylation and cancer. *Journal of Clinical Oncology*, *22*(22), 4632-4642. https://doi.org/10.1200/jco.2004.07.151

Ehrlich, M. (2002). DNA methylation in cancer: Too much, but also too little. *Oncogene*, *21*(35), 5400-5413. https://doi.org/10.1038/sj.onc.1205651

Feinberg, A. P., & Tycko, B. (2004). The history of cancer epigenetics. *Nature Reviews Cancer*, *4*(2), 143-153. https://doi.org/10.1038/nrc1279

Fraga, M. F., Herranz, M., Espada, J., Ballestar, E., Paz, M. F., Ropero, S., Erkek, E., Bozdogan, O., Peinado, H., Niveleau, A., Mao, J., Balmain, A., Cano, A., & Esteller, M. (2004). A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. *Cancer Research*, *64*(16), 5527-5534. https://doi.org/10.1158/0008-5472.can-03-4061

Ishiguro, M., Iida, S., Uetake, H., Morita, S., Makino, H., Kato, K., Takagi, Y., Enomoto, M., & Sugihara, K. (2007). Effect of combined therapy with low-dose 5-aza-2′-deoxycytidine and irinotecan on colon cancer cell line HCT-15. *Annals of Surgical Oncology*, *14*(5), 1752-1762. https://doi.org/10.1245/s10434-006-9285-4

Khamas, A., Ishikawa, T., Mogushi, K., Iida, S., Ishiguro, M., Tanaka, H., Uetake, H., & Sugihara, K. (2012). Genome-wide screening for methylation-silenced genes in colorectal

cancer. *International Journal of Oncology*, *41*(2), 490-496. https://doi.org/10.3892/ijo.2012.1500

Kumaran, A., & Joel Karunakaran, R. (2007). *In vitro* antioxidant activities of methanol extracts of five *Phyllanthus* species from India. *LWT - Food Science and Technology*, *40*(2), 344-352. https://doi.org/10.1016/j.lwt.2005.09.011

Kwon, H., Kim, J. H., Bae, J. M., Cho, N., Kim, T., & Kang, G. H. (2010). DNA methylation changes in ex-adenoma carcinoma of the large intestine. *Virchows Archiv*, *457*(4), 433-441. https://doi.org/10.1007/s00428-010-0958-9

Lander, E. S. (2011). Initial impact of the sequencing of the human genome. *Nature*, *470*(7333), 187-197. https://doi.org/10.1038/nature09792

Lee, H. S., Kim, B., Cho, N., Yoo, E. J., Choi, M., Shin, S., Jang, J., Suh, K., Kim, Y. S., & Kang, G. H. (2009). Prognostic implications of and relationship between CPG island hypermethylation and repetitive DNA hypomethylation in hepatocellular carcinoma. *Clinical Cancer Research*, *15*(3), 812-820. https://doi.org/10.1158/1078-0432.ccr-08-0266

Lisanti, S., Omar, W. A., Tomaszewski, B., De Prins, S., Jacobs, G., Koppen, G., Mathers, J. C., & Langie, S. A. (2013). Comparison of methods for quantification of global DNA methylation in human cells and tissues. *PLOS One*, *8*(11), e79044. https://doi.org/10.1371/journal.pone.0079044

Momparler, R. L. (2013). Epigenetic therapy of non-small cell lung cancer using decitabine (5-aza-2′-deoxycytidine). *Frontiers in Oncology*, *3*, 188. https://doi.org/10.3389/fonc.2013.00188

Natsume, A., Wakabayashi, T., Tsujimura, K., Shimato, S., Ito, M., Kuzushima, K., Kondo, Y., Sekido, Y., Kawatsura, H., Narita, Y., & Yoshida, J. (2008). The DNA demethylating agent 5-aza-2′-deoxycytidine activates NY-ESO-1 antigenicity in orthotopic human glioma. *International Journal of Cancer*, *122*(11), 2542-2553. https://doi.org/10.1002/ijc.23407

Omar, W. A. W., & Zain, S. N. D. M. (2018). Therapeutic index of methanolic extracts of three Malaysian *Phyllanthus* species on MCF-7 and MCF-10A cell lines. *Pharmacognosy Journal*, *10*(6s), s30-s32. https://doi.org/10.5530/pj.2018.6s.5

Rodić, N., & Burns, K. H. (2013). Long interspersed element–1 (*LINE-1*): Passenger or driver in human neoplasms?. *PLOS Genetics*, *9*(3), e1003402. https://doi.org/10.1371/journal.pgen.1003402

Saito, K., Kawakami, K., Matsumoto, I., Oda, M., Watanabe, G., & Minamoto, T. (2010). Long interspersed nuclear element 1 hypomethylation is a marker of poor prognosis in stage IA non–small cell lung cancer. *Clinical Cancer Research*, *16*(8), 2418-2426. https://doi.org/10.1158/1078-0432.ccr-09-2819

Sarin, B., Verma, N., Martín, J. P., & Mohanty, A. (2014). An overview of important ethnomedicinal herbs of *Phyllanthus* species: Present status and future prospects. *The Scientific World Journal*, *2014*, 839172. https://doi.org/10.1155/2014/839172

Sellis, D., Provata, A., & Almirantis, Y. (2007). *Alu* and *LINE-1* distributions in the human chromosomes: Evidence of global genomic organization expressed in the form of power laws. *Molecular Biology and Evolution*, *24*(11), 2385-2399. https://doi.org/10.1093/molbev/msm181

Sharma, G., Mirza, S., Parshad, R., Srivastava, A., Gupta, S. D., Pandya, P., & Ralhan, R. (2010). Clinical significance of promoter hypermethylation of DNA repair genes in tumor and serum DNA in invasive ductal breast carcinoma patients. *Life Sciences*, *87*(3-4), 83-91. https://doi.org/10.1016/j.lfs.2010.05.001

So, F. V., Guthrie, N., Chambers, A. F., Moussa, M., & Carroll, K. K. (1996). Inhibition

of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. *Nutrition and Cancer*, *26*(2), 167-181. https://doi.org/10.1080/01635589609514473

Sugimura, T., & Ushijima, T. (2000). Genetic and epigenetic alterations in carcinogenesis. *Mutation Research/Reviews in Mutation Research*, *462*(2-3), 235-246. https://doi.org/10.1016/s1383-5742(00)00005-3

Wanniarachchi, K. K., Peiris, L. D., & Ratnasooriya, W. (2009). Antihyperglycemic and hypoglycemic activities of *Phyllanthus debilis* aqueous plant extract in mice. *Pharmaceutical Biology*, *47*(3), 260-265. https://doi.org/10.1080/13880200802435754

Whitelaw, E., & Martin, D. I. (2001). Retrotransposons as epigenetic mediators of phenotypic variation in mammals. *Nature Genetics*, *27*(4), 361-365. https://doi.org/10.1038/86850

Yoder, J. A., Walsh, C. P., & Bestor, T. H. (1997). Cytosine methylation and the ecology of intragenomic parasites. *Trends in Genetics*, *13*(8), 335-340. https://doi.org/10.1016/s0168-9525(97)01181-5

Zain, S. N. D. M., & Omar, W. A. W. (2020). The effect of *Phyllanthus debilis* methanolic extract on DNA methylation of *TAC1* gene in colorectal cancer cell line. *Pharmacognosy Magazine*, *16*(67), 57. https://doi.org/10.4103/pm.pm_226_19

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Effect of Herbal Blend and L-arginine Supplementation on Growth Performance, Intestinal Morphology, and Caecal Microflora of Growing Guinea Fowls

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ABSTRACT

This experiment was carried out to investigate the effect of diet containing herbal blend (HB) of turmeric (*Curcuma longa*), scent leaf (*Ocimum gratissimum*), and moringa leaf (*Morinda lucida*) supplemented with or without L-arginine (L-Arg) on growth performance, intestinal morphology, and caecal microflora of guinea fowls. Three hundred and sixty 28-day-old male guinea fowls were randomly allotted in a completely randomized design to six treatment groups of sixty birds; each treatment group consisted of six replicates of ten birds each. Dietary treatments were laid out in a 3 × 2 factorial arrangement of the basal diet (control), diet containing enrofloxacin (1 g/kg), HB (1 g/kg diet), and each supplemented with or without L-Arg at 1 g/kg. Notwithstanding dietary supplementation with L-Arg, guinea fowls fed the diets with HB, and their counterparts fed the diets with antibiotics had similar weight gain during the growing period. Dietary L-Arg supplementation with HB increased (p<0.05) feed intake. Feed conversion ratio (p<0.05) was improved in guinea fowls fed the diets with HB and their counterparts fed with antibiotic supplemented or not with L-Arg. L-Arg supplementation of the diet with HB resulted in the longest (p<0.05) duodenal villi height

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and the shortest (p<0.05) duodenal apical width in young turkeys. The caeca content of growing guinea fowls fed the diet with HB supplemented with L-Arg had the least (p<0.05) *Clostridium* count and the highest (p<0.05) *Lactobaccillus* count. In conclusion, guinea fowls fed the diet with HB supplemented or not with L-Arg had similar growth performance with those fed with an antibiotic. L-Arg supplementation of the diet with HB resulted in increased caecal *Lactobacillus* counts of growing birds.

Keywords: Antimicrobial, growth promoter, *Lactobacillus*, phytogenics

INTRODUCTION

With the advent of drug resistance and adverse effects of chemosynthetic drugs, the interest in medicinal herbs and plant extracts/metabolites has augmented, both among the public and researchers worldwide (Abo Ghanima et al., 2020; Dhama et al., 2021; Saeed et al., 2021). In poultry production, the use of phytogenic plants as alternative growth promoters has attracted several interests due to their ability to exhibit growth-promoting, digestive stimulating, immune-enhancing, and anti-oxidative properties (Alagawany et al., 2021; Ebrahim et al., 2020; Lu et al., 2014).

Turmeric (*Curcuma longa*) is a culinary spice containing curcumin, demethoxycurcumin, bisdemethoxycurcumin, and tetrahydrocurcuminoids (Al-Sultan, 2003). Curcumin is regarded as the most active ingredient in turmeric, exhibiting strong antioxidant, anti-inflammatory, and antimicrobial properties (Alagawany et al., 2020; Quiles et al., 2002). Scent leaf (*Ocimum gratissimum*) is rich in eugenol, cinamate, camphor, and thymol (Matasyoh et al., 2007). It exhibits strong antimicrobial properties against most pathogenic bacteria, such as *Staphylococcus aureaus*, *Escherichia coli*, *Streptococcus fecalis*, *Psudomonas aeruginosa*, and *Lactobacilli* (Prabhu et al., 2009). *Morinda lucida* Benth. (Rubiaceae) leaf extract contains saponin, tannins, alkaloids, anthraquinones, and anthraquinols. It has been shown to exhibit anticoccidial, antioxidant, and antimicrobial properties (Ogundare & Onifade, 2009; Ogunlana et al., 2008).

Various studies have reported combining two or more herbal plants as a blend, resulting in a wider range of biological activity, promoting synergism effects of various bioactive ingredients (Bassolé & Juliani, 2012; Cetin et al., 2016). In addition, previous studies reported that a combination of two or more phytogenic plants as blends yield improved and better response than single inclusion (Ertas et al., 2005; Thiruvengadarajan et al., 2011).

The metabolism of L-Arg to yield nitric oxide demonstrated its ability to enhance the immune system. Nitric oxide (NO) has been reported to be involved in the development of intestinal mucosa (Kochar et al., 2011) and the elimination of pathogenic microbes (P. Li et al., 2007). L-Arg has improved immune system, growth performance, and carcass yield (Al-Daraji & Salih, 2012). The combination of the herbal blend was such that it contains phytogenics, which exhibits antioxidant, antimicrobial, and digestive stimulant properties. The herbal

blend used in this study contained *Curcuma longa*, which was used as an antioxidant (Dono, 2013), *Morinda lucida* for its antimicrobial properties (Ola-Fadunsin & Ademola, 2013), and *Ocimum gratissimum* as a digestive stimulant (Ndelekwute et al., 2015). This study evaluated the effect of the herbal blend (containing *Curcuma longa*, *Ocimum gratissimum*, *Morinda lucida*) and L-arginine supplemented diets on growth performance, gastrointestinal tract morphology, and caecal microflora of guinea fowls.

MATERIALS AND METHODS

The feeding trial was carried out according to the guidelines approved by the Animal Welfare Committee of the Federal University of Agriculture, Abeokuta, Nigeria.

Preparation of Herbal Blend and Chemical Composition

Fresh turmeric rhizomes (*Curcuma longa*) were harvested, rinsed in clean water to remove adhering dirt, chipped, and dried at 45 °C in an oven to a constant weight. The dried chips were ground (2 mm sieve) to yield turmeric powder (TP), stored at 4 °C before use in the experimental diets.

Fresh scent leaves (*Ocimum gratissimum*) and moringa leaves were plucked without the petioles and stalks. The leaves were rinsed in water to remove dirt, spread evenly, and air-dried at room temperature until they became crispy while retaining their greenish color. The dried scent and moringa leaves were ground (2 mm sieve) separately to yield scent leaf powder (SLP) and moringa leaf meal (MLM), respectively. The SLP and MLM prepared were stored at 4 °C in an air-tight container before use. To formulate the herbal blend (HB), TP, SLP, and MLM were mixed thoroughly at equal proportions. The resultant blend (HB) was stored at 4 °C in an air-tight container before use. Qualitative screening of the phytochemical compounds (tannins, alkaloids, flavonoids, terpenes, saponins, and cyanogenic glycosides) present in representative samples was conducted using the method described by Harbone (1973).

Management of Experimental Birds

A total of 500 one-day-old guinea fowl keets were brooded together for 28 days in a deep litter house using wood shavings as bedding material. During the 28-day brooding period, no antimicrobial and anticoccidial drugs were administered to the birds. Guinea fowls were fed a starter diet containing 242 g/kg crude protein and 12.13 MJ/kg metabolizable energy according to National Research Council (NRC) (1994) requirements. Feed and clean water were provided *ad libitum* during the pre-experimental and experimental periods. The feeding trial commenced at the end of the brooding period.

Dietary Treatments

On day 29, 360 male guinea fowls were selected, weighed individually, and allotted six treatments on a weight equalization basis, with each treatment group having six pens as replicates. A total of 36 similar floor pens were used in this study. Maize-soybean meal-based diets were formulated according

to NRC (1994) nutritional requirements for younger (29 to 56 d) and growing (57 to 84 d) guinea fowls (Table 1). The six treatment groups were laid out in a 3 × 2 factorial arrangement of the basal diet (control), diet containing enrofloxacin (at 1 g/kg), and HB (at 1 g/kg diet), each supplemented with or without L-Arg (Shanghai TECH Chemical Industry, China) at 1 g/kg. Diets were fed in mash form. Each treatment group was replicated six times in a completely randomized design. No medication and vaccination was administered during the experimental period (29 to 84 d).

Table 1

Basal composition of experimental diets (g/kg as fed)

Ingredients	Starter (29 – 56 d)	Finisher (57 – 84 d)
Maize	492	555
Soybean	350	280
Wheat offal	85.0	100.0
Fish meal	30.0	20.0
Bone meal	18.0	20.0
Oyster shell	15.0	16.0
*Premix	2.50	2.50
Salt	2.50	2.50
L-lysine HCL	2.0	2.0
DL-Methionine	3.0	2.0
Total	1000	1000
Determined nutrient composition (% DM)		
Dry matter	89.14	89.62
Crude protein	22.62	21.12
Ether extract	3.55	3.46
Ash	8.66	8.35
Crude fibre	3.11	3.24
Calculated nutrient composition		
Metabolizable energy (MJ/kg)	11.94	12.75
Ca (%)	1.22	1.31
P (%)	0.65	0.62
Digestible arginine (%)	1.47	1.24
Lysine (%)	1.44	1.35
Methionine (%)	0.69	0.61

Note. *Supplied per kilogram of diet: retinol acetate, 4.12 mg; cholecalciferol, 87.5 mg; DL-alpha-tocopherol acetate, 44.7 mg; menadione, 2 mg; thiamine mononitrate, 2 mg; riboflavin, 6 mg; pyridoxol, 5 mg; cyanocobalamin, 0.2 mg; D-biotin, 0.1 mg; niacin, 50 mg; pantothenic acid, 12 mg; folic acid, 2 mg; zinc [Zn (zinc sulphate monohydrate: $ZnSO_4$. H_2O)], 90 mg; manganese [Mn (manganese (II) sulfate monohydrate: $MnSO_4$. H_2O)], 80 mg; iron [Fe (iron (II) sulfate monohydrate: $FeSO_4$. H_2O)], 60 mg; copper [Cu (copper sulfate pentahydrate: $CuSO_4$. $5H_2O$)], 8 mg; iodine [I (potassium iodide: KI)], 1 mg; cobalt [Co (cobalt (II) sulfate monohydrate: $CoSO_4$. H_2O)], 0.3 mg; and molybdenum [Mo (sodium molybdate: Na_2Mo_4. $2H_2O$)], 1 mg

Data and Sample Collection

Growth Performance. Guinea fowls were weighed per replicate weekly, while the weight gain was calculated. Daily feed consumption was calculated as the difference between the feed offered and leftovers, while feed conversion ratio was calculated as feed intake to weight gain. No mortality occurred during the experimental period.

Intestinal Morphology. At 56 d and 84 d of the birds, six birds per treatment group were randomly selected, slaughtered, and eviscerated. Tissue samples from the mid-region of the three segments of the small intestine (duodenum, jejunum, and ileum) were collected according to the method as described by Gava et al. (2015). The samples were placed in 10% formalin and dehydrated in a graded ethanol series (xylene). Each segment was embedded in paraffin wax and stained with hematoxylin and eosin. A total of eight slides were recorded per observation. The villi length and crypt depths of the samples were examined under a microscope and measured from the tip to the villi base and from the villi base to the crypt base, respectively.

Caecal Microflora. Fresh content from the caeca of selected guinea fowls following intestinal morphology at 56 and 84 d were poured in labeled sterile bottles and put on ice to determine the microbial population. Caecal samples collected were serially diluted and plated on De Man, Rogosa, and Sharpe (MRS) agar plates, incubated at 37 ^{o}C for 24 hours. *Lactobacillus*, *Coliform*, *Clostridium*, and *Salmonella* counts were estimated as described by Xia et al. (2004) and were expressed as colony-forming units (Cfu) per gram of fresh sample.

Statistical Analyses

Microbial counts (Cfu/g) were transformed to logarithm$_{10}$. Data obtained were subjected to the general linear model procedure of the Statistical Analysis System (SAS) (2000) to determine the main effect of HB inclusion (control, diet with enrofloxacin, diet with HB), the main effect of L-Arg supplementation (0, 1 g/kg), and their respective interactions (HB × L-Arg). Statistically significant means were separated using Tukey's test at a probability of 5 %.

RESULTS

The effect of dietary inclusion of HB supplemented with or without L-Arg on the growth response of guinea fowls is shown in Table 2. Guinea fowls fed a control diet without L-Arg supplementation had the least (p=0.003) body weight on day 84. Dietary inclusion of HB supplemented with or without L-Arg had no effect (p>0.05) on the average weight gain of younger guinea fowls (28–56 days). Notwithstanding dietary supplementation with L-Arg, guinea fowls fed the diet with HB. Their counterparts fed the diet with antibiotics had similar weight gain during the growing and overall (28–84 days) rearing periods. In growing and overall periods, guinea fowls fed a control diet without L-Arg supplement had

Table 2

Growth performance of growing guinea fowls fed diets supplemented with herbal blend and L-arginine

Parameters	Without L-Arg supplementation			With L-Arg supplementation			SEM	*P*-values		
	Control	Antibiotic	HB	Control	Antibiotic	HB		Treatment	L-Arg	Treatment × L-Arg
Body weight (g/bird)										
d 28	230	229	230	229	230	230	4.22	0.679	0.708	0.815
d 56	746	752	730	765	746	774	20.45	0.811	0.395	0.067
d 84	1305^{c}	1476^{ab}	1446^{b}	1588^{a}	1485^{ab}	1556^{a}	142.71	0.346	0.005	0.003
Average feed intake (g/bird)										
d 28 to 56	1264^{a}	1002^{b}	984^{c}	1269^{a}	979^{c}	1030^{b}	122.22	0.001	0.265	0.001
d 56 to 84	2007^{bc}	2005^{bc}	1961^{c}	2405^{a}	2024^{bc}	2111^{b}	149.44	0.004	0.005	0.003
d 28 to 84	3272^{b}	3008^{d}	2945^{e}	3674^{a}	3004^{d}	3141^{c}	202.52	0.001	0.005	0.001
Average weight gain (g/bird)										
d 28 to 56	516	522	500	535	516	544	6.49	0.823	0.100	0.062
d 56 to 84	559^{c}	724^{ab}	715^{b}	823^{a}	739^{ab}	781^{ab}	55.40	0.280	0.138	0.006
d 28 to 84	1075^{c}	1246^{ab}	1216^{b}	1358^{a}	1257^{ab}	1326^{ab}	102.55	0.347	0.005	0.003
Feed conversion ratio										
d 28 to 56	2.45^{b}	1.92^{a}	1.97^{a}	2.37^{ab}	1.90^{a}	1.89^{a}	0.91	0.001	0.209	0.001
d 56 to 84	3.59^{b}	2.77^{a}	2.74^{a}	2.92^{ab}	2.70^{a}	2.70^{a}	0.99	0.072	0.083	0.001
d 28 to 84	3.04^{b}	2.41^{a}	2.42^{a}	2.70^{ab}	2.39^{a}	2.37^{a}	0.95	0.001	0.254	0.001

Note. All values within rows having the same or no superscripts are not significantly different (p>0.05); HB = Herbal blend, SEM = Standard error of the means

the least (p<0.05) weight gain. Guinea fowls fed the diet with HB containing no L-Arg supplementation, and birds fed the diet with antibiotic supplemented with L-Arg had the least (p=0.001) feed intake during the younger period. During the growing and overall rearing period, guinea fowls fed a control diet supplemented with L-Arg had the highest (p=0.003) feed intake. Meanwhile, L-Arg supplementation of the diet with HB resulted in increased (p=0.001) feed intake in all the rearing periods compared to their counterparts fed the diet with HB containing no supplemental L-Arg. This trend was noticed at the growing and overall phase in birds fed control diet compared with the group fed Arg-supplemented control diet. In all the rearing periods, guinea fowls were fed the diet with HB. Those fed the diet with antibiotic supplemented or not with L-Arg had a better (p=0.001) feed conversion ratio than their counterparts fed control diet containing no supplemental L-Arg. Guinea fowls fed diets containing supplemental L-Arg irrespective of dietary treatment had a similar feed conversion ratio.

The effect of dietary inclusion of HB and L-Arg supplementation on the gut morphology of younger guinea fowls is presented in Table 3. L-Arg supplementation of the diet with HB resulted in the longest (p=0.001) duodenal villi height and the shortest (p=0.001) duodenal apical width. In the jejunum, guinea fowl fed the diet with HB supplemented with L-Arg had the longest (p=0.001) jejunal villi height and the least (p=0.001) basal width. Jejunal villi height increased (p=0.006), while basal width reduced (p=0.051) following L-Arg supplementation. Guinea fowls fed a control diet containing no supplemental L-Arg had the longest (p=0.006) ileal laminal propria depth. The effect of dietary inclusion of HB and L-Arg supplementation on gut morphology of growing guinea fowls is as presented in Table 4. In the duodenum and jejunum, L-Arg supplementation of the diet with HB resulted in the longest (p<0.05) villi heights. Birds fed the diets with HB and antibiotics supplemented with L-Arg had the longest (p=0.002) villi height in the ileum.

The effect of dietary inclusion of HB and L-Arg supplementation on caecal microflora of young and growing guinea fowls is presented in Table 5. Dietary inclusion of HB supplemented with or without L-Arg had no effect (p>0.05) on the caecal microflora count of younger guinea fowls. In growing guinea fowls, the least (p=0.001) *Clostridium* count and the highest (p=0.001) *Lactobacillus* count were observed in the caeca content of birds fed the diet with HB supplemented with L-Arg. Notwithstanding the dietary treatment imposed, L-Arg supplementation in growing guinea fowls reduced (p=0.035) caecal *Clostridium* count.

Table 3

Intestinal morphology of growing guinea fowls fed diets supplemented with herbal blend and L-arginine at 56 days of age

Parameters	Without L-Arg supplementation			With L-Arg supplementation			SEM	*P*-values		
	Control	Antibiotic	HB	Control	Antibiotic	HB		Treatment	L-Arg	Treatment × L-Arg
Duodenum										
Villus height (µm)	975^{c}	979^{c}	997^{c}	998^{c}	1100^{b}	1222^{a}	102.40	0.002	0.023	0.001
Apical width (µm)	49.13^{a}	40.35^{b}	35.95^{c}	49.72^{a}	39.75^{b}	30.95^{d}	9.11	0.418	0.401	0.001
Basal width (µm)	105	106	105	102	109	107	3.33	0.098	0.643	0.175
Laminal propia depth (µm)	205	207	202	206	207	202	6.24	0.683	0.439	0.136
Jejunum										
Villus height (µm)	1024^{f}	1650^{d}	1739^{e}	1220^{e}	1820^{b}	2020^{a}	182.55	0.001	0.006	0.001
Apical width (µm)	41.60	37.40	35.72	40.20	37.70	36.50	2.75	0.163	0.725	0.403
Basal width (µm)	237^{b}	247^{a}	180^{e}	202^{d}	222^{c}	173^{f}	19.12	0.117	0.051	0.001
Laminal propia depth (µm)	367	370	385	365	372	388	7.52	0.309	0.118	0.215
Ileum										
Villus height (µm)	370	375	365	377	395	360	6.22	0.338	0.268	0.276
Apical width (µm)	52.70	52.20	51.90	50.90	50.60	52.20	2.42	0.278	0.362	0.218
Basal width (µm)	105	114	117	103	107	109	3.02	0.133	0.112	0.119
Laminal propia depth (µm)	525^{a}	495^{c}	505^{bc}	505^{bc}	472^{d}	475^{d}	28.22	0.451	0.154	0.006

Note. All values within rows having the same or no superscripts are not significantly different (p>0.05); HB = Herbal blend, SEM = Standard error of the means

Table 4

Intestinal morphology of growing guinea fowls fed diets supplemented with herbal blend and L-arginine at 84 days of age

Parameters	Without L-Arg supplementation			With L-Arg supplementation			SEM	*P*-values		
	Control	Antibiotic	HB	Control	Antibiotic	HB		Treatment	L-Arg	Treatment × L-Arg
Duodenum										
Villus height (µm)	1530^{d}	1564^{c}	1596^{b}	1570^{c}	1599^{b}	1720^{a}	119.51	0.004	0.011	0.007
Apical width (µm)	122	125	120	120	122	121	6.22	0.451	0.162	0.410
Basal width (µm)	255	252	250	250	249	252	3.77	0.406	0.486	0.187
Laminal propia depth (µm)	505	502	499	500	503	502	9.56	0.528	0.079	0.724
Jejunum										
Villus height (µm)	1680^{d}	1690^{d}	1970^{b}	1780^{c}	1775^{c}	2030^{a}	199.62	0.001	0.964	0.001
Apical width (µm)	99	102.60	104	99	100	102	2.22	0.401	0.183	0.471
Basal width (µm)	202	199.55	201	200	191	188	1.79	0.167	0.113	0.127
Laminal propia depth (µm)	305	306.70	302	300	303	303	3.45	0.193	0.061	0.482
Ileum										
Villus height (µm)	399^{b}	399^{b}	395^{b}	402^{b}	484^{a}	490^{a}	26.21	0.119	0.074	0.002
Apical width (µm)	101	104	107	100	102	100	1.44	0.791	0.065	0.101
Basal width (µm)	155	156	153	152	155	152	2.01	0.412	0.436	0.602
Laminal propia depth (µm)	655	662	659	652	665	665^{d}	6.02	0.694	0.074	0.125

Note. All values within rows having the same or no superscripts are not significantly different (p>0.05); HB = Herbal blend, SEM = Standard error of the means

Table 5

Caecal microfloral of growing guinea fowls fed diets supplemented with herbal blend and L-arginine

Parameters	Without L-Arg supplementation			With L-Arg supplementation			SEM	*P*-values		
	Control	Antibiotic	HB	Control	Antibiotic	HB		Treatment	L-Arg	Treatment × L-Arg
Younger phase										
Clostridium (Cfu/g)	7.00	6.80	6.50	6.90	6.50	6.40	0.25	0.220	0.204	0.084
Coliform (Cfu/g)	6.40	6.20	6.10	6.20	6.00	6.00	0.28	0.382	0.251	0.343
Lactobacillus (Cfu/g)	6.70	6.90	7.00	6.90	7.00	7.30	0.32	0.076	0.082	0.605
Salmonella (Cfu/g)	4.80	4.40	4.30	4.60	4.30	4.10	0.08	0.347	0.693	0.507
Grower phase										
Clostridium (Cfu/g)	6.90^{a}	5.50^{b}	5.10^{bc}	4.40^{c}	4.50^{c}	4.40^{c}	0.21	0.456	0.035	0.001
Coliform (Cfu/g)	6.20	6.00	6.00	6.00	6.00	6.30	0.05	0.032	0.045	0.629
Lactobacillus (Cfu/g)	6.00^{d}	6.90^{c}	7.90^{b}	7.00^{c}	7.20^{c}	8.90^{a}	0.23	0.021	0.002	0.001
Salmonella (Cfu/g)	4.20	4.00	4.00	4.10	4.20	4.00	0.03	0.222	0.631	0.343

Note. All values within rows having the same or no superscripts are not significantly different ($p>0.05$); HB = Herbal blend, SEM = Standard error of the means

DISCUSSION

The non-significant effects of HB inclusion and L-Arg supplementation on weight gain of younger guinea fowls, which later revealed a significant effect at the growing phase of the birds in the present study, corroborated earlier studies revealing age-dependent response of poultry birds to phytogenic feed additives (Hernández et al., 2004; Jamroz et al., 2005). The similar weight gain obtained for guinea fowls fed HB diet and those fed the diet with antibiotic during the growing and overall rearing period notwithstanding dietary supplementation with L-Arg confirmed the ability of HB to replace in-feed antibiotics without compromise on weight gain. Significant improvements in body weight gain in the HB group have been documented (Murugesan et al., 2015).

Increased feed intake of young and growing guinea fowls following L-Arg supplementation of the diet with HB, when compared to their counterparts fed a similar diet but not supplemented with L-Arg in the present study, could be linked with the secretagogue activity of Arg inducing the release of insulin and glucagon, which influences feed intake and efficiency of utilization (Woods et al., 2006). Previous studies confirmed that NO generated via the L-Arg pathway is actively involved in feeding behavior through the hypothalamus (Malfatti et al., 2015), acts as a central mediator and physiological modulator of food intake (Mancuso et al., 2010). L-Arg infusion in rats also promoted satiety quantified by increased food intake after 24 hours from L-Arg administration (Malfatti et al., 2015).

The improved feed conversion ratios obtained for guinea fowls fed the diet with HB and birds fed the diet with antibiotic notwithstanding L-Arg supplementation than birds fed control diet having no supplemental L-Arg supported previous findings that inclusion of phytogenic feed additive (Brenes & Roura, 2010; Jamroz et al., 2005) and L-Arg supplementation improved feed conversion ratio of broilers when compared with the control group (Filho et al., 2021; Pramujo et al., 2019). In addition, Wang et al. (2021) reported an improved feed conversion ratio of broilers fed a diet supplemented with phytogenic feed additives (containing oregano, cinnamon, citrus peel, and fructooligosaccharides). Better feed conversion ratio obtained for guinea fowls fed the diet with HB could be attributed to the additive effect of antimicrobial properties exhibited by constituent scent leaf (Prabhu et al., 2009), antioxidant and antimicrobial properties of turmeric (Quiles et al., 2002), and moringa leaf (Ogundare & Onifade, 2009) contained in the blend. Previous studies also reported improved growth of broilers following dietary inclusion of *Morinda lucida* leaf meal (Lala et al., 2017) and turmeric powder (Ahmadi, 2010).

Intestinal morphological changes following the inclusion of HB and L-Arg supplementation and the longest duodenal and jejunal villi height recorded for young and growing guinea fowl fed the diet with HB supplemented with L-Arg suggests

increased surface area. Increased villus height has been linked with increased surface area for nutrient absorption (Kamboh et al., 2015), resulting in good gut health (Viveros et al., 2011). Reduced apical width in the duodenum and basal width in the jejunum recorded for younger guinea fowl fed the diet with HB supplemented with L-Arg suggests reduced cellular turnover and improved intestinal health. Reduced apical width has been suggested to increase mature enterocytes, thereby increasing enzyme activity in the villus brush border (Chen et al., 2011). Shallower apical width has been linked with improved gut health and growth since cellular turnover is an energy-consuming process that uses resources that might otherwise be utilized toward growth (Markovic et al., 2009). In separate studies, dietary inclusion with phytogenic blend (Geyra et al., 2001; Reisinger et al., 2011) and L-Arg supplementation (Zhan et al., 2008) have improved intestinal morphology. Geyra et al. (2001) reported increased villus height and surface area following dietary supplementation with phytogenic blend (Tecnaroma Herbal Mix PL® Tecnessenze, Saudi Arabia). Dietary inclusion with a blend of oregano, anise, and citrus peel improved intestinal morphology (Reisinger et al., 2011). Dietary supplementation with L-Arg in turkeys (Oso et al., 2017) and weaned pigs (Zhan et al., 2008) has improved intestinal morphology.

The mechanism through which phytogenic additives and L-Arg supplementation improve intestinal morphology has been elucidated. Bioactive compounds present in phytogenic plants may stimulate secretions and activity of endogenous digestive enzymes leading to increased absorption surface area in the intestine and improved intestinal morphology (Lee et al., 2004). The major bioactive component in scent leaf (cinnamate) and turmeric powder (curcumin) used in the formulation of HB in the current study has been reported to stimulate pancreatic digestive enzyme activities (Hernández et al., 2004).

Increased *Lactobacillus* growth within the gut will lead to enhanced production of lactic acid (Harley & Prescott, 2002), reduced gut pH, and thereby inhibit normal growth of enteropathogens leading to improved animal health (Z. Li et al., 2018).

The mechanism through which PFA and L-Arg supplementation improve gut microflora has been described. Phytogenic feed additives reduce gut pathogenic bacteria by disrupting the cellular membrane of pathogens, affecting their hydrophobicity and virulence capacity, stimulating the immune system by activating lymphocytes and macrophages, protecting intestinal mucosa from pathogen colonization, and promoting the growth of beneficial bacteria, such as *Lactobacilli* and *Bifidobacteria* (Windisch & Kroismayr, 2007). In addition, plant bioactive compounds may improve the antioxidant status in the intestinal mucosa due to the antioxidant activities of the herbal feed additives and the reduction of pathogenic microbes, which can lead to better intestinal microstructures (Patra et al., 2019). For example, higher lactobacillus

and lower *Clostridium* count in the caeca of broiler chickens fed phytogenic feed additives supplemented with arginine had been attributed to nitric oxide produced by arginine (Eriksson et al., 2003; Ren et al., 2014) and phytogenic antimicrobial properties (Wati et al., 2015), respectively.

L-Arg supplementation produces NO, a component of the immune system, thereby limiting pathogenic activity (Wink et al., 2011), thus, preventing the multiplication of diseases (Ren et al., 2014). L-Arg supplementation has been reported in previous studies to alleviate the negative effect of *Salmonella typhimurium* (Eriksson et al., 2003), *Eimeria tenella* (Allen, 1999), and infectious bursal disease virus (Emadi et al., 2010) in broiler chickens.

CONCLUSION

Guinea fowls fed the diet with HB supplemented or not with L-Arg had similar growth performance with those fed with antibiotics. However, L-Arg supplementation of the diet with HB improved intestinal morphology of both younger and growing birds and increased caecal *Lactobacillus* counts of growing birds.

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REFERENCES

Abo Ghanima, M. M., Alagawany, M., Abd El-Hack, M. E., Taha, A., Elnesr, S. S., Ajarem, J., Allam, A. A., & Mahmoud, A. M. (2020). Consequences of various housing systems and dietary supplementation of thymol, carvacrol, and euganol on performance, egg quality, blood chemistry, and antioxidant parameters. *Poultry Science*, *99*(9), 4384-4397. https://doi.org/10.1016/j.psj.2020.05.028

Ahmadi, F. (2010). Effect of turmeric (*Curcumin longa*) powder on performance, oxidative stress state and some of blood parameters in broiler fed on diets containing aflatoxin B1. *Global Veterinaria*, *5*(6), 312–317.

Alagawany, M., El-Saadony, M. T., Elnesr, S. S., Farahat, M., Attia, G., Madkour, M., & Reda, F. M. (2021). Use of lemongrass essential oil as a feed additive in quail's nutrition: Its effect on growth, carcass, blood biochemistry, antioxidant and immunological indices, digestive enzymes and intestinal microbiota. *Poultry Science*, *100*(6), 101172. https://doi.org/10.1016/j.psj.2021.101172

Alagawany, M., Farag, M. R., Abdelnour, S. A., Dawood, M. A., Elnesr, S. S., & Dhama, K. (2020). Curcumin and its different forms: A review on fish nutrition. *Aquaculture*, *532*, 736030. https://doi.org/10.1016/j.aquaculture.2020.736030

Al-Daraji, H. J., & Salih, A. M. (2012). The influence of dietary arginine supplementation on blood traits of broiler chickens. *Pakistan Journal of Nutrition*, *11*(3), 258-264. https://doi.org/10.3923/pin.2012.258.264

Allen, P. C. (1999). Effects of daily oral doses of L-Arg on coccidiosis infections in chickens. *Poultry Science*, *78*(11), 1506-1509. https://doi.org/10.1093/ps/78.11.1506

Al-Sultan, S. I. (2003). The effect of *Curcuma longa* (turmeric) on overall performance of broiler chickens. *International Journal of Poultry*

Science, *2*(5), 351-353. http://doi.org/10.3923/ijps.2003.351.353

Bassolé, I. H. N., & Juliani, H. R. (2012). Essential oils in combination and their antimicrobial properties. *Molecules*, *17*(4), 3989-4006. https://doi.org/10.3390/molecules17043989

Brenes, A., & Roura, E. (2010). Essential oils in poultry nutrition: Main effects and modes of action. *Animal Feed Science and Technology*, *158*(1-2), 1–14. https://doi.org/10.1016/j.anifeedsci.2010.03.007

Cetin, E., Yibar, A., Yesilbag, D., Cetin, I., & Cengiz, S. S. (2016). The effect of volatile oil mixtures on the performance and ileo-caecal microflora of broiler chickens. *British Poultry Science*, *57*(6), 780–787. https://doi.org/10.1080/00071668.2016.1214682

Chen, J., Wang, M., Kong, Y., Ma, H., & Zou, S. (2011). Comparison of the novel compounds creatine and pyruvate on lipid and protein metabolism in broiler chickens. *Animal*, *5*(7), 1082-1089. https://doi.org/10.1017/S1751731111000085

Dhama, K., Sharun, K., Gugjoo, M. B., Tiwari, R., Alagawany, M., Iqbal Yatoo, M., Thakur, P., Igbal, M. N., Chaicumpa, W., Michalak, I., & Elnesr, S. S. (2021). A comprehensive review on chemical profile and pharmacological activities of *Ocimum basilicum*. *Food Reviews International*. https://doi.org/10.1080/87559129.2021.1900230

Dono, N. D. (2013). Turmeric (*Curcuma longa* Linn.) supplementation as an alternative to antibiotics in poultry diets. *Indonesian Bulletin Animal and Veterinary Sciences*, *23*(1), 41-49.

Ebrahim, A. A., Elnesr, S. S., Abdel-Mageed, M. A. A., & Aly, M. M. M. (2020). Nutritional significance of aloe vera (*Aloe barbadensis* Miller) and its beneficial impact on poultry. *World's Poultry Science Journal*, *76*(4), 803-814. https://doi.org/10.1080/00439339.2020.1830010

Emadi, M., Jahanshiri, F., Jalalian, F. A., Kaveh, K., Bejo, M. H., Ideris, A., Assumaidaee, A. A., & Alimon, R. A. (2010). Immunostimulatory effects of arginine in broiler chickens challenged with vaccine strain of infectious bursal disease virus. *Journal of Animal and Veterinary Advances*, *9*(3), 594-600. http://doi.org/10.3923/javaa.2010.594.600

Eriksson, S., Chambers, B. J., & Rhen, M. (2003). Nitric oxide produced by murine dendritic cells is cytotoxic for intracellular *Salmonella enterica* sv. Typhimurium. *Scandinavian Journal of Immunology*, *58*(5), 493–502. https://doi.org/10.1046/j.1365-3083.2003.01330.x

Ertas, O. N., Güler, T., Çiftçi, M., Dalkilic, B., & Simsek, U. G. (2005). The effect of an essential oil mix derived from oregano, clove and anise on broiler performance. *International Poultry Science*, *4*(11), 879-884. https://doi.org/10.3923/ijps.2005.879.884

Filho, T. S., Lima, E. C., Oliveira, D. H., Abreu, M. L. T., Rosa, P. V., Laurentiz, A. C., Naves, L. P., & Rodrigue, P. B. (2021). Supplemental L-arginine improves feed conversion and modulates lipid metabolism in male and female broilers from 29 to 42 days of age. *Animal*, *15*(2), 100120. https://doi.org/10.1016/j.animal.2020.100120

Gava, M. S., Moraes, L. B., Carvalho, G. Z., Fallavena, L. C. B., Moraes, H. L. S., Herpich, J., & Salle, C. T. P. (2015). Determining the best sectioning method and intestinal segment for morphormetric analysis in broilers. *Brazilian Journal of Poultry Science*, *17*(2), 145-150. http://doi.org/10.1590/1516-635x1702145-150

Geyra, A., Uni, Z., & Sklan, D. (2001). Enterocyte dynamics and mucosal development in the post hatch chick. *Poultry Science*, *80*(6), 776–782. https://doi.org/10.1093/ps/80.6.776

Harbone, J. B. (1973). *Phytochemical methods*: *A guide to modern techniques of plant analysis*. Chapman and Hall.

Harley, J. P., & Prescott, L. M. (2002). *Laboratory exercises in microbiology*. McGraw-Hill.

Hernández, F., Madrid, J., Garcia, V., Orengo, J., & Megias, M. D. (2004). Influence of two plant extracts on broiler performance, digestibility, and digestive organ size. *Poultry Science*, *83*(2), 169–174. https://doi.org/10.1093/ps/83.2.169

Jamroz, D., Wiliczkiewicz, A., Wertelecki, T., Orda, J., & Skorupińska, J. (2005). Use of active substances of plant origin in chicken diets based on maize and locally grown cereals. *British Poultry Science*, *46*(4), 485-493. https://doi.org/10.1080/00071660500191056

Kamboh, A. A., Arain, M. A., Mughal, M. J., Zaman, A., Arain, Z. M., & Soomro, A. H. (2015). Flavonoids: Health promoting phytochemicals for animal production - A review. *Journal of Animal health Production*, *3*(1), 6-13. http://doi.org/10.14737/journal.jahp/2015/3.1.6.13

Kochar, N. I., Chandewal, A. V., Bakal, R. L., & Kochar, P. N. (2011). Nitric oxide and the gastrointestinal tract. *International Journal of Pharmacology*, *7*(1), 31-39. https://doi.org/10.3923/ijp.2011.31.39

Lala, A. O., Ajayi, O. L., Okwelum, N., Oso, A. O., Fakorede, T. V., Adebayo, T. A., & Jagbojo, J. E. (2017). Haematological, biochemical and organ changes in broiler chickens fed varying levels of *Morinda lucida* (Brimstone) leaf meal supplementation in the diets. *Tropical Animal Health and Production*, *50*(5), 1005-1010. https://doi.org/10.1007/s11250-018-1524-8

Lee, K. W., Everts, H., & Beynen, A. C. (2004). Essential oils in broiler nutrition. *International Journal of Poultry Science*, *3*(12), 738–752. http://doi.org/10.3923/ijps.2004.738.752

Li, P., Yin, Y. L., & Li, D. F. (2007). Amino acids and immune function. *British Journal of Nutrition*, *98*(2), 237–252. https://doi.org/10.1017/S000711450769936X

Li, Z., Wang, W., Liu, D., & Guo, Y. (2018). Effects of *Lactobacillus acidophilus* on the growth performance and intestinal health of broilers challenged with *Clostridium perfringens*. *Journal of Animal Science and Biotechnology*, *9*, 25. https://doi.org/10.1186/s40104-018-0243-3

Lu, C., Zhang, J., Shi, X., Miao, S., Bi, L., Zhang, S., Zhou, Q. Y., Zhang, M., Xie, Y., Miao, Q., & Wang, S. (2014). Neuroprotective effects of tetramethylpyrazine against dopaminergic neuron injury in a rat model of Parkinson's disease induced by MPTP. *International Journal of Biological Sciences*, *10*(4), 350–357. https://doi.org/10.7150/ijbs.8366

Malfatti, C. R. M., da Silva, L. A., Pereira, R. A., Michel, R. G., Snak, A. L., & Santos, F. S. (2015). Acute hypothalamic administration of L-arginine increases feed intake in rats. *Revista de Nutrição*, *28*(1), 55–63. https://doi.org/10.1590/1415-52732015000100005

Mancuso, C., Navarra, P., & Preziosi, P. (2010). Roles of nitric oxide, carbon monoxide and hydrogen sulfide in the regulation of the hypothalamic-pituitary-adrenal axis. *Journal of Neurochemistry*, *113*(3), 563–575. https://doi.org/10.1111/j.1471-4159.2010.06606.x

Markovic, R., Sefer, D., Krstic, M., & Petrujkic, B. (2009). Effect of different growth promoters on broiler performance and gut morphology. *Archivos de Medicina Veterinaria*, *41*(2), 163–169. https://doi.org/10.4067/S0301-732X2009000200010

Matasyoh, L. G., Josphat, C. M., Francis, N. W., Miriam, G. K., Anne, W. T. M., & Titus, K. W. (2007). Chemical composition and antimicrobial activity of the essential oil of *Ocimum gratissimum* L. growing in eastern Kenya. *African Journal of Biotechnology*, 6(6), 760-765.

Murugesan, G. R., Syed, B., Haldar, S., & Pender, C. (2015). Phytogenic feed additives as an

alternative to antibiotic growth promoters in broiler chickens. *Frontiers in Veterinary Science*, *2*, 21. https://doi.org/10.3389/fvets.2015.00021

National Research Council. (1994). *Nutrient requirements of domestic animals: Nutrient requirements of poultry*. Academy Press.

Ndelekwute, E. K., Afolabi, K. D., Uzegbu, H. O., Unah, U. L., & Amaefule, K. U. (2015). Effect of dietary black pepper (*Piper nigrum*) on the performance of broiler. *Bangladesh Journal of Animal Science*, *44*(2), 120-127. https://doi.org/10.3329/bjas.v44i2.26012

Ogundare, A. O., & Onifade, A. K. (2009). The antimicrobial activity of *Morinda lucida* leaf extract on *Escherichia coli*. *Journal of Medicinal Plants Research*, *3*(4), 319-323. https://doi.org/10.5897/JMPR.9001185

Ogunlana, O. E., Ogunlana, O., & Farombi, O. E. (2008). *Morinda lucida*: Antioxidant and reducing activities of crude methanolic stem bark extract. *Advances in Natural and Applied Sciences*, *2*(2), 49-54.

Ola-Fadunsin, S. D., & Ademola, I. O. (2013). Anticoccidial effects of *Morinda lucida* acetone extracts on broiler chickens naturally infected with *Eimeria* species. *Pharmaceutical Biology*, *52*(3), 330-334. https://doi.org/10.3109/13880209.2013.836545

Oso, A. O., Bamgbose, A. M., Adebayo, A. O., Olowofeso, V. O., Pirgozliev, A. A., Adegbenjo, A. O., Osho, S. O., Alabi, J. O., Li, H., Liu, G., Yao, K., & Xin, W. (2017). Effect of dietary supplementation with arginine on haematological indices, serum chemistry, carcass yield, gut microflora and lymphoid organs of growing turkeys. *Livestock Science*, *198*, 58-64. https://doi.org/10.1016/j.livsci.2017.02.005

Patra, A., Amasheh, S., & Aschenbach, J. R. (2019). Modulation of gastrointestinal barrier and nutrient transport function in farm animals by natural plant bioactive compounds - A comprehensive review. *Critical Reviews in Food Science and Nutrition*, *59*(1), 3237-3266. https://doi.org/10.1080/10408398.2018.1486284

Prabhu, K. S., Lobo, R., Shirwaikar, A. A., & Shirwaikar, A. (2009). *Ocimum gratissimum*: A review of its chemical, pharmacological and ethnomedicinal properties. *The Open Complementary Medicine Journal*, *1*(1), 1-15. http://doi.org/10.2174/1876391X00901010001

Pramujo, M., Mutia, R., & Wijayanti, I. (2019). Effect of chitosan oligosaccharide (COS) and l-arginine supplementation on broiler performance. In *IOP Conference Series: Earth and Environmental Science* (Vol. 251, p. 012060). IOP Publishing Ltd. https://doi.org/10.1088/1755-1315/251/1/012060

Quiles, J. L., Mesa, M. D., Ramirez-Tortosa, C. L., Aguilera, C. M., Battino, M., Gil, A., & Ramirez-Tortosa, M. C. (2002). *Curcuma longa* extract supplementation reduces oxidative stress and attenuates aortic fatty streak development in rabbits. *Arteriosclerosis Thrombosis and Vascular Biology*, *22*(7), 1225–1231. https://doi.org/10.1161/01.ATV.0000020676.11586.F2

Ren, W., Chen, S., Yin, J., Duan, J., Li, T., Liu, G., Feng, Z., Tan, B., Yin, Y., & Wu, G. (2014). Dietary arginine supplementation of mice alters the microbial population and activates intestinal innate immunity. *The Journal of Nutrition*, *144*(6), 988–995. https://doi.org/10.3945/jn.114.192120

Reisinger, N., Steiner, T., Nitsch, S., Schatzmayr, G., & Applegate, T. J. (2011). Effects of a blend of essential oils on broiler performance and intestinal morphology during coccidial vaccine exposure. *Journal of Applied Poultry Research*, *20*(3), 272-283. https://doi.org/10.3382/japr.2010-00226

Saeed, M., Arain, M. A., Ali Fazlani, S., Marghazani, I. B., Umar, M., Soomro, J., Bhutto, Z. O.,

Soomro, F., Noreldin, A. E. Abd El-Hack, M. E. Elnesr, S. S., Faraq, M. R., Dhama, K., Chao, S., & Alagawany, M. (2021). A comprehensive review on the health benefits and nutritional significance of fucoidan polysaccharide derived from brown seaweeds in human, animals and aquatic organisms. *Aquaculture Nutrition*, *27*(3), 633-654. https://doi.org/10.1111/anu.13233

Statistical Analysis System. (2000). *SAS user's guide statistics* (8.1 ed.). SAS Institute Inc.

Thiruvengadarajan, V. S., Ellaiah, P., & Madhusudhanachetty, C. (2011). *In vitro* antioxidant and alpha-amylase inhibitory activity of isolated compounds from ethyl acetate extract of *Cynodon dactylon* and *Piper betle*. *International Journal Pharmaceutical and Indian Research*, *1*(4), 261–265.

Viveros, A., Chamorro, S., Pizarro, M., Arija, I., Centeno, C., & Brenes, A. (2011). Effects of dietary polyphenol-rich grape products on intestinal microflora and gut morphology in broiler chicks. *Poultry Science*, *90*(3), 566–578. https://doi.org/10.3382/ps.2010-00889.

Wang, J., Su, S., Pender, C., Murugesan, R., Syed, B., & Kim, W. K. (2021). Effect of a phytogenic feed additive on growth performance, nutrient digestion, and immune response in broiler-fed diets with two different levels of crude protein. *Animals*, *11*(3), 775. https://doi.org/10.3390/ani11030775

Wati, T., Ghosh, T. K., Syed, B., & Haldar, S. (2015). Comparative efficacy of a phytogenic feed additive and an antibiotic growth promoter on production performance, caecal microbial population and humoral immune response of broiler chickens inoculated with enteric pathogens. *Animal Nutrition*, *1*(3), 213-219. https://doi.org/10.1016/j.aninu.2015.08.003

Windisch, W., & Kroismayr, A. (2007). Natural phytobiotics for health of young piglets and poultry mechanisms and application. *Poultry Science*, *86*(Suppl. 1), 643.

Wink, D. A., Hines, H. B., Cheng, Y. S., Switzer, C. H., Flores-Santana, W., Vitek, M.P., Ridnour, L. A., & Colton, C. A. (2011). Nitric oxide and redox mechanisms in the immune response. *Journal of Leukocyte Biology*, *89*(6), 873-891. https://doi.org/10.1189/jib.1010550

Woods, S. C., Lutz, T. A., Geary, N., & Langhans, W. (2006). Pancreatic signals controlling food intake; insulin, glucagon and amylin. *Philosophical Transactions of the Royal Society B*, *361*(1471), 1219-1235. https://doi.org/10.1098/rstb.2006.1858

Xia, M. S., Hu, C. H., & Xu, Z. R. (2004). Effect of copper-bearing montmorillonite on growth performance, digestive enzyme activities, and intestinal microflora and morphology of male broiler. *Poultry Science*, *83*(11), 1868–1875. https://doi.org/10.1093/ps/83.11.1868

Zhan, Z., Ou, D., Piao, X., Kim, S. W., Liu, Y., & Wang, J. (2008). Dietary arginine supplementation affects microvascular development in the small intestine of early-weaned pigs. *Journal of Nutrition*, *138*(7), 1304–1309. https://doi.org/10.1093/jn/138.7.1304

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Outbreak and Insecticide Susceptibility of Pod Feeding-larvae on Cocoa in Ghana

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ABSTRACT

Cocoa is an important foreign exchange earner and a major source of income for several households in Ghana. In 2018, a larval outbreak on cocoa pods was reported in Ghana. Although the origin is unknown, it was perceived to be a secondary pest outbreak. In this study, a survey was conducted in the outbreak areas to identify and determine the occurrence of the pest and its susceptibility to a commonly used insecticide. Field and laboratory studies were conducted to identify the pest, determine the level of infestation, insecticide susceptibility, and field management. The outbreak was mainly caused by larvae of *Anomis leona* (~96% infestation of cocoa trees in some communities) with extensive feeding damage (chewing channels/tunnels) on the pericarp of pods. Field populations of *A. leona* larvae from districts in the Central region subjected to bifenthrin were susceptible at the recommended field rate (0.0245%) for mirids after 48 h of exposure under laboratory conditions. The insecticide induced a median lethal concentration (LC_{50}) of ≤0.0061% and ≤0.0018% on *A. leona* larvae from Jukwa and Twifo Praso in the Central region at 24 and 48 h of exposure, respectively. Field application of bifenthrin was able to suppress infestation. The findings show that *Anomis* larvae were responsible for the outbreak, inducing extensive damage on pods. Bifenthrin was toxic to the larvae and could be used to manage them on the field. The outbreak indicates the need to develop an integrated management and monitoring strategy for cocoa pests to minimize future outbreaks.

Keywords: *Anomis leona*, cocoa, Ghana, insecticide, pest outbreak

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INTRODUCTION

Although non-native to West Africa, cocoa (*Theobroma cacao* L.) is widely grown in the sub-region, with Cote D'Ivoire and Ghana being the two leading producers of the crop globally (Food and Agriculture Organization Corporate Statistical Database [FAOSTAT], 2019). It is the largest agricultural foreign exchange earner for Ghana raking in nearly US $2 billion annually (Ghana Cocoa Board [COCOBOD], 2019) and a major source of employment for several individuals (Ghana Living Standards Survey [GLSS], 2014). With an estimated 800,000 households involved in cocoa production in Ghana (COCOBOD, 2019), market dynamics, especially concerning the price of cocoa, substantially affect the financial health of several households (Kolavalli et al., 2012; Vigneri & Kolavalli, 2018). Sustaining the productivity of this sector is therefore of utmost importance to the Ghanaian economy.

However, cocoa production is severely affected by pests and diseases, resulting in substantial yield losses and invariably reducing the income of cocoa cultivation households and the nation's foreign exchange. The pests of cocoa include insects, such as mirids, stink bugs, mealybugs, coreid bugs, termites, and other lepidopteran larvae, which attack different parts and phenological stages of the crop (Awudzi et al., 2009). Crop losses due to these pests vary from about 18% for stink bugs (Owusu-Manu, 1976) to a reported 30-40% for mirids (Awudzi et al., 2016).

Management of cocoa insect pests is dominated by applying conventional insecticides (Adu-Acheampong et al., 2015). For instance, to mitigate the activities of pests and diseases and increase production, the Government of Ghana instituted the Cocoa High Technology (Hi-Tech) and Cocoa Disease and Pest Control (CODAPEC) programs, with the dual aim of subsidizing fertilizers and applying (spraying) pesticides for farmers respectively. However, these flagship programs have encountered some challenges, and CODAPEC is unable to completely meet the insecticide requirement of cocoa farmers leading to its augmentation by farmers with insecticides sourced from the open market (Anang et al., 2013; Aneani et al., 2012), a pathway for the usage of unapproved chemicals (Denkyirah et al., 2016).

Despite implementing various pest management methods, insect pest outbreaks occur due to several factors (Obeng-Ofori & Afreh-Nuamah, 2007). In late 2018, farmers reported a larval outbreak on cocoa pods in some cocoa growing areas in Ghana. It was purported that this was due to applying an insecticide product containing fipronil to control cocoa insect pests. The larvae were reported to be chewing the pods of cocoa. Prior to the reported outbreak, fall armyworm had invaded Ghana. As a result, this larval outbreak was initially reported by farmers and extension personnel to be fall armyworms on cocoa. Hence it was important to identify the pest to aid in its control. Therefore, this study aimed to identify the pest responsible for the outbreak, the level of infestation and insecticide susceptibility of the pest to aid in remedial measures to suppress the outbreak and assist in mitigating future outbreaks.

MATERIALS AND METHODS

Survey Area

The study was conducted in districts with reported pest outbreaks on cocoa pods in the Ashanti, Central, and Western regions of Ghana from September to December 2018 (Supplementary Data 1, Figure 1). Farms were selected within two communities per district (unless indicated otherwise) in heavily infested areas with the aid of District Officers/Community Extension Agents of the Cocoa Health and Extension Division (CHED) for assessment. Communities with the reported outbreak were in the following cocoa districts; Nyinahin, Enchi, Breman Asikuma, Twifo Praso, Assin Fosu, and Jukwa (Supplementary Data 1). These districts have bimodal rainfall peaks usually occurring from April-July (major season) and August-November (minor season). The communities surveyed were Awiseso (Ny1), Achiase (Ny2), and Asuontaa (Ny3) in Nyinahin, Homaho (Homaho Farm 1 [AF1]), and Homaho Farm 2 [AF2]) in Assin Fosu, Kwamo (Kwamo Farm 1 [BA1] and Kwamo Farm 2 [BA2]) in Breman Asikuma, Watreso (Jk1), Asuoyenu (Jk2), and Bekawopa (Jk3) in Jukwa, Koraso (TP1) and Assin Adiembra (TP2) in Twifo Praso and Mile 4 (Enchi) in Enchi. The temperature and rainfall levels in these districts are presented in Supplementary Data 1.

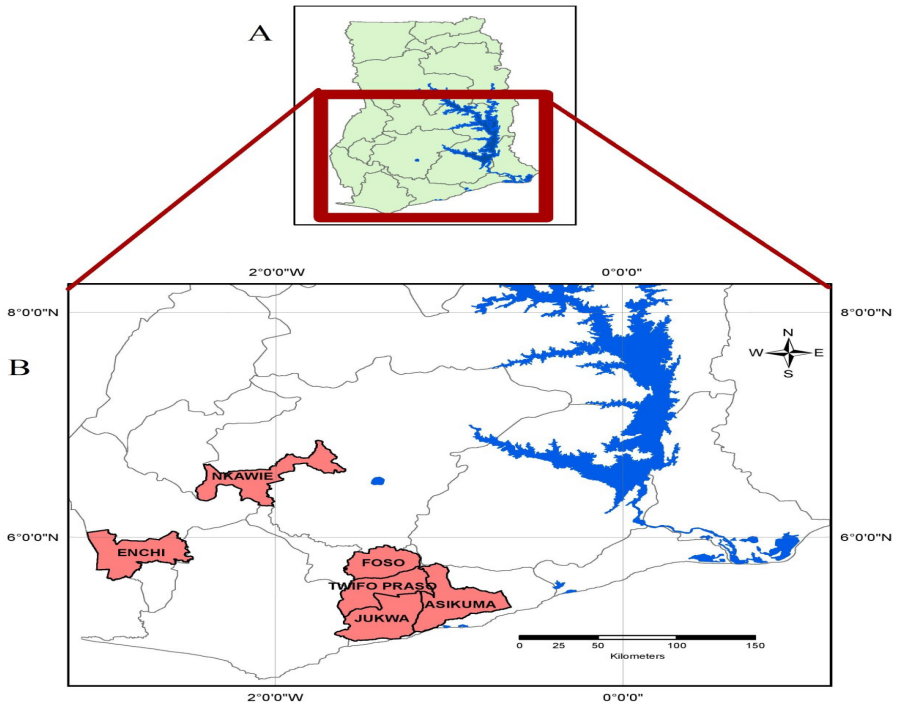

Figure 1. Map of Ghana (A) showing the cocoa districts (B) with the reported outbreak. Nyinahin district was recently carved out from Nkawie district

Note. Foso: Assin Fosu; Asikuma: Breman Asikuma

Insect Pest Assessment and Collection

Thirty trees were randomly sampled per 0.5 acres in each farm (3 replicates per farm) and visually assessed at 2 m above ground level for insects and insect damage (Collingwood, 1971). All potential insect-inhabiting sites like pods, pod peduncles, flower cushions, stem, and their interfaces within the sampling height were inspected. A tree was considered infested if the following was observed; damage by an insect pest, insect pest presence or both. The level of infestation by a particular insect pest was estimated as below:

% Tree infestation = (Number of trees infested by pest/Number of trees sampled) x100%

Larvae [via brushing into collection containers (top diameter = 19 cm, height = 21.6 cm, and bottom diameter = 16 cm)] and larvae-infested cocoa pods (30 per farm, thus 10 *Anomis*-infested, 10 *Earias*-infested, and 10 *Characoma*-infested pods) were collected from the farms and reared to adult in containers (same dimension as collection container) in the insectary at Cocoa Research Institute of Ghana (CRIG), New Tafo-Akim at 25-30 °C, 70-85% relative humidity (RH), and 12: 12 h light: dark (L: D) photoperiod. Green succulent cocoa leaves (daily) and pods (every three days) were fed to the larvae until adult emergence. Adult emergence for a specific insect pest was estimated as follows:

$$\text{Adult emergence (\%)} = \frac{\text{Number of pod-infested larvae with adult emergence}}{\text{Total number of pod-infested larvae sampled for the pest}} \times 100\%$$

Identification was made based on morphological features of the larvae and adults using the keys and images in Awudzi et al. (2009), Entwistle (1972), Ferreira et al. (2015), Mkhize (1971), and Schuh (2013).

Insecticide Susceptibility Tests of Field Populations

Larval samples from the study area except the Central region were insufficient for the susceptibility tests. Hence, *Anomis* larvae from this region were used for the tests. Larvae (4th instar) of *A. leona* from farms in each district were pooled for the district and used for the tests. An insecticide product containing bifenthrin (27 g/L) was selected for the susceptibility tests due to bifenthrin's toxicity to insects (Johnson et al., 2010), approval for use against cocoa mirids in Ghana and wide usage across the cocoa landscape (Awudzi et al., 2009). The product's recommended field concentration (0.0245%) for mirids was used (1 ml) to impregnate Whatman No. 1 filter paper in Petri dishes. Larvae (5) of *A. leona* were exposed to the papers (Ackonor & Adu-Acheampong, 2007) for 24 and 48 h to observe mortality. Samples from Jukwa and Twifo Praso were used for the dose-mortality test due to the inadequate number of larvae from the other sites. The dose-mortality response was determined

as follows: four concentrations (0.03768%, 0.0205%, 0.006%, and 0.0015%) of the insecticide were prepared and used (1 ml) to impregnate Whatman No. 1 filter paper in Petri dishes. The concentrations were selected based on their ability to induce larval mortality of 10–100% in a preliminary test. Larvae (5) of *A. leona* were exposed to the insecticide-treated papers for 24 and 48 h and mortality was noted afterwards. Larvae exposed to filter paper impregnated with distilled water (1 ml) for 24 and 48 h were used as controls. Three replicates were performed for each concentration and control. The testing condition was 25 °C – 27 °C, 70–85% RH, and 12: 12 h L: D photoperiod.

Field Application of Insecticide

The extensive nature of the outbreak required chemical control to suppress the pest population quickly. Based on the results of the susceptibility tests, the bifenthrin-based insecticide was applied at 100 ml in 11 L of water for 0.5 acres at monthly intervals for two months in the outbreak areas. Application of the insecticide was made using a motorized mist blower with every row application technique (Awudzi et al., 2012). Assessment of *Anomis* larvae infestation of cocoa was done before and at 48 h after each application. It was done by randomly selecting 30 cocoa trees per 0.5 acres and visually inspecting the pods and trees from 0 to 2 m above ground level for the presence of the larvae or signs of their damage.

Data Analysis

The proportion of infested cocoa trees for each insect pest was computed for each farm as the ratio of infested trees to the total number of sampled cocoa trees. Larval mortality at the recommended concentration was arcsine transformed and subjected to analysis of variance (ANOVA) using IBM SPSS ver. 20. Mortality data for the dose-response effect was used to compute the median lethal concentration (LC_{50}) using Insecticide Resistance Monitoring Application-QCal (IRMA-QCal) (Lozano-Fuentes et al., 2012). LC_{50} values with overlapping 95% confidence intervals (CIs) were considered not significantly different (Asekunowo et al., 2018). Finally, the number of trees infested by *Anomis* larvae before and after insecticide application on the field was computed using the tree infestation (%) formula above.

RESULTS

Insect Damage and Species Composition

Insect damage on the pod include feeding channels/tunnels on the pericarp due to chewing activity (horizontal and vertical) by *Anomis leona* and *Earias biplaga* and holes on pods with many frasses covering the entrance caused by the boring activity of the pod borer *Characoma* spp. (Supplementary Data 2). Feeding lesions (water-soaked depressions) were also observed on pods, and this was caused by the nymphs and adults of mirids (*Sahlbergella singularis* Haglund, *Distantiella theobroma* Distant,

and *Helopeltis* spp.) and coreid bugs (*Pseudotheraptus devastans* Distant) when they suck sap from the pod. The feeding lesions could coalesce to form a large patch. The lesions caused by coreid bugs were larger than those of mirids. Some of the pods also looked deformed/malformed, a symptom of coreid bug infestation. Some immature pods were observed to show signs of ripening, a condition known as premature ripening, and this is caused by the feeding (sucking) activity of stink bugs (*Bathycoelia thalassina* Herrich-Schaeffer) (Supplementary Data 2).

Holes were also observed on the trunk and branches of the cocoa tree within the sampling height, with many frasses at the entrance of the hole and base of the tree. This is a typical damage due to the boring activity of the larvae of the stem borer. Cocoa trees were also defoliated by the chewing activity of *A. leona* and *E. biplaga* (Supplementary Data 2).

Insects observed to be feeding on cocoa pods on the farms include nymphs and adults of mirids (*S. singularis*, *D. theobroma*, and *Helopeltis* spp.), larvae of lepidopterans (*A. leona*, *E. biplaga*, and *Characoma* spp.), nymphs and adults of stink bug (*B. thalassina* Herrich-Schaeffer) and nymphs and adults of coreid bug (*P. devastans* Distant).

Infestation Level

About 96% of the sampled cocoa trees in Mile 4 in Enchi district in the Western region were infested with *Anomis*, while mirids and *Earias* infestation of the trees were 60% and 68%, respectively. Pod borer, coreid, and stink bugs infestations were less than 20%. Stem borer infestation was not observed on this farm (Figure 2). Pod borer infestation was highest in Ny1 while *Earias* infestation dominated (96% and 100%) in the other two farms in Nyinahin district in the Ashanti region (Figure 2). *Anomis* infestation was the second-highest in all the three farms in the district. Infestation by mirids was observed in all the sampled farms in this district, while stink bug and stem borer infestations were observed in only Ny3 and Ny2 at 4% each, respectively.

Anomis infestation dominated in seven out of the nine farms in the Central region, with coreid bug and *Earias* infestation dominating the other two farms (Figure 3). High *Anomis* infestations (88%–96%) were observed in farms in Breman Asikuma (BA2), Twifo Praso (TP1 and TP2), and Jukwa (Jk2). In Assin Fosu, *Anomis* (76%), *Earias* (52%), and *Characoma* caused the most infestations at AF1. However, in AF2, infestations were mostly caused by coreid bugs (72%), *Anomis* (40%), and *Earias* (24%). The most common infestations in Breman Asikuma were caused by *Anomis* (68%), *Earias* (52%), and stink bugs (20%) in BA1 and *Anomis* (96%), *Earias* (40%), and pod borer (28%) in BA2. Infestation of cocoa in the Jukwa district was dominated by *Anomis*, *Earias*, and coreid bug at 40%, 48%, and 32% in Jk1, 88%, 12%, and 44% in Jk2 and 80%, 24%, and 8% at Jk3. *Anomis*, *Earias*, and coreid bug infestations occurred at 88%, 56%, and 20% in TP1 and 96%, 44%, and 36% in TP2.

Generally, *Anomis* infestation was the most dominant on cocoa pods in the outbreak communities, followed by *Earias*

(Figure 4). Lower infestation levels by the other insect pests were also observed on cocoa pods. Insects that emerged from the reared larvae/incubated pods were the lepidopterans *A. leona* (8.5%), *E. biplaga* (3.1%), and *Characoma stictigrapha* (2.7%) (Supplementary Data 2).

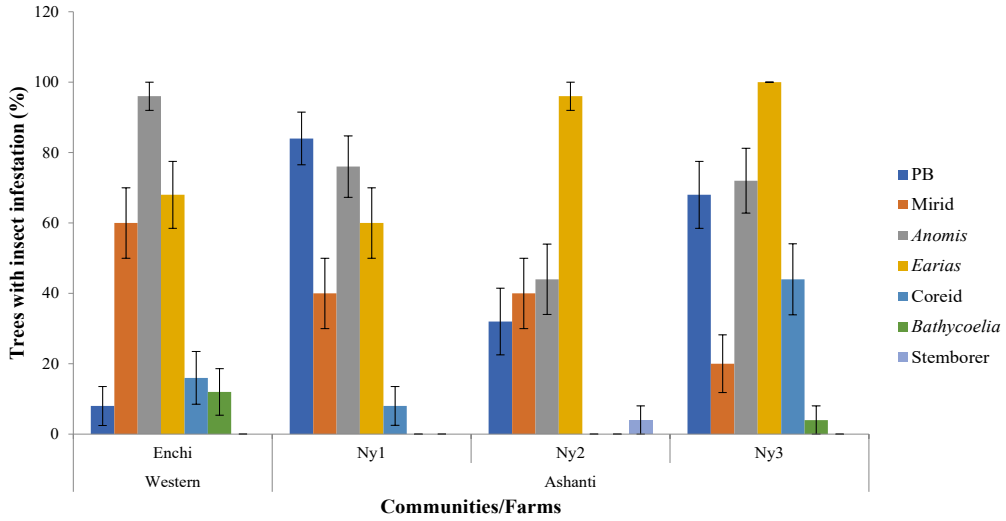

Figure 2. Mean insect pest infestation level of cocoa trees in Ashanti and Western regions of Ghana. The error bars denote standard error

Note. Ny1: Nyinahin Farm 1; Ny2: Nyinahin Farm 2; Ny3: Nyinahin Farm 3; PB: Pod borer

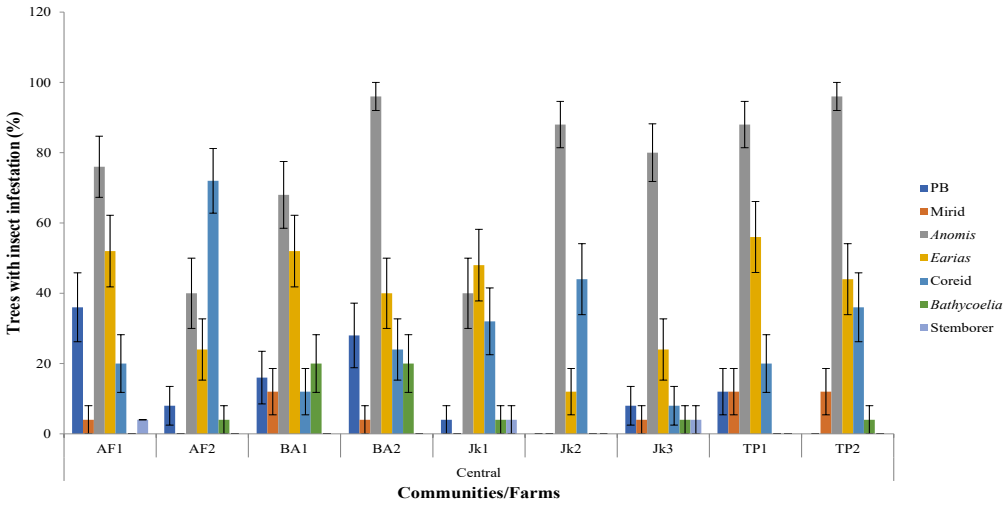

Figure 3. Mean insect pest infestation level of cocoa trees in the Central region of Ghana. The error bars denote standard error

Note. AF1: Assin Fosu Farm 1; AF2: Assin Fosu Farm 2; BA1: Breman Asikuma Farm 1; BA2: Breman Asikuma Farm 2; Jk1: Jukwa Farm 1; Jk2: Jukwa Farm 2: Jk3: Jukwa Farm 3; TP1: Twifo Praso Farm 1; TP2: Twifo Praso Farm 2; PB: Pod borer

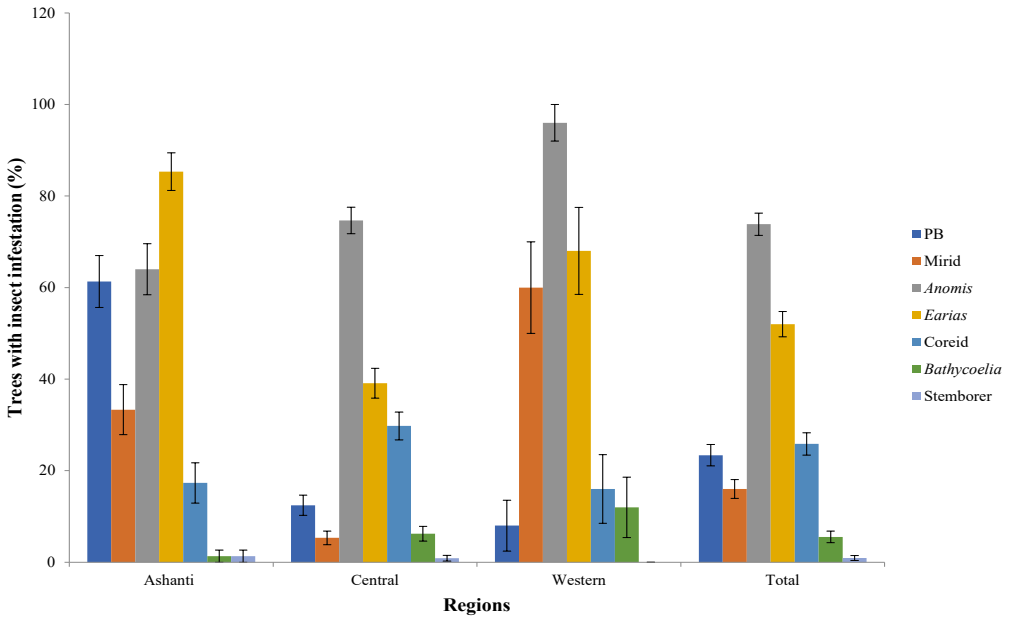

Figure 4. Mean insect pest infestation level of cocoa trees in the outbreak areas. The error bars denote standard error. Ashanti region (C = 3, N = 3), Central region (C = 7, N = 9), Western region (C = 1, N = 1)

Note. C = Number of communities; N = Number of farms sampled; PB: Pod borer

Susceptibility to Bifenthrin

Larval mortality for the controls was 0%. At the recommended label concentration of the bifenthrin product, larvae of *A. leona* from Breman Asikuma were highly susceptible at an exposure period of 24 h (Table 1). Larval mortality for the other localities ranged between 73% and 88%. When insecticide exposure was prolonged to 48 h, mortality of 93% to 100% was observed (Table 1). The mortality rates for the larval populations were not significantly different at 24 (p = 0.053) and 48 h (p = 0.441). The LC_{50} at 24 h for *A. leona* larvae from Jukwa and Twifo Praso was 0.0026% and 0.0061%, respectively, but these were not significantly different (Table 2).

Table 1

Mortality of Anomis leona *larvae after exposure (24 and 48 h) to recommended label concentration of bifenthrin*

District	Mortality ± SE (%)	
	24 h	48 h
Assin Fosu	86.67 ± 6.67	100 ± 0.00
Breman Asikuma	100 ± 0.00	100 ± 0.00
Jukwa	73.33 ± 6.67	93.33 ± 6.67
Twifo Praso	88.33 ± 3.86	93.33 ± 1.67

Note. Recommended label concentration was 0.0245%; SE: Standard Error

Table 2

Bifenthrin toxicity to Anomis leona *larvae at different exposure durations*

Exposure duration (h)	Larval population	LC_{50} (%) [95% CI]	Slope ± SE	Intercept ± SE
24	Jukwa	0.0026 [0.0008-0.0085]	0.6282 ± 0.2404	3.7330 ± 1.2292
	Twifo Praso	0.0061 [0.0037-0.0099]	1.4339 ± 0.3401	7.3223 ± 1.6978
48	Jukwa	0.0003 [0-0.0095]	0.5376 ± 0.3058	4.4131 ± 1.6512
	Twifo Praso	0.0018 [0.0008-0.0037]	1.2187 ± 0.3687	7.7355 ± 1.6512

Note. LC_{50}: Median lethal concentration; CI: Confidence interval; SE: Standard error

Field Management of Pest

The insecticide was effective against *Anomis* larvae in the field, inducing knockdown and significantly reducing larval infestation of cocoa pods after 48 hours. New larval damage on pods was also not observed. There was a further reduction in *Anomis* infestation of the cocoa trees in the treated farms after the second application (Figure 5).

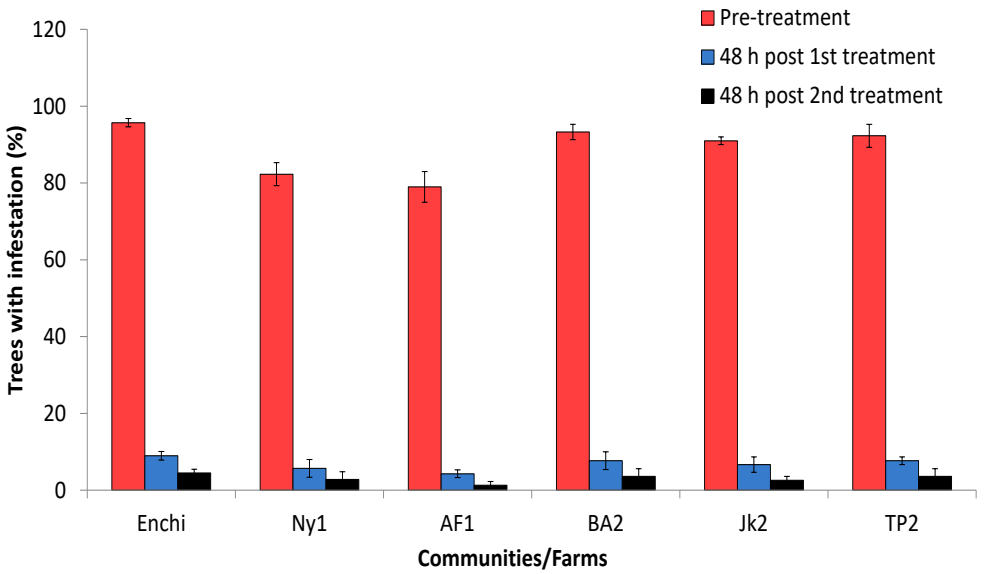

Figure 5. Mean level of infestation of cocoa by *Anomis* larvae in the study sites. The error bars denote standard error

Note. Ny1: Nyinahin Farm 1; AF1: Assin Fosu Farm 1; BA2: Breman Asikuma Farm 2; Jk2: Jukwa Farm 2; TP2: Twifo Praso Farm 2

DISCUSSION

Pest outbreak is caused by several biotic and abiotic factors, including innate biological capability, topography, host plant, plant architecture, climatic/weather conditions, and pest density (Creeden et al., 2014; Singh & Satyanarayana, 2009). An assessment of the outbreak indicated damage diagnostic of larvae of *Anomis* and *Earias*, although damage by other insect pests was also observed. The infestation levels varied among the outbreak areas for different insect pest species. Most (96%) of the cocoa trees in Mile 4 in the Enchi district were attacked by *Anomis* larvae. Damage by *Anomis* was pervasive in the Central region irrespective of the district sampled. The predominance of lepidopteran (*Characoma* spp., *E. biplaga*, and *A. leona*) damage on cocoa pods was conspicuous in the Nyinahin district in the Ashanti region. Although *Characoma* infestation dominated in one sampled site and *Earias* infestation dominated in the other two, infestation by *Anomis* in the district was higher than that of *Characoma* and only second to *Earias*. In farms in Twifo Praso and Assin Fosu, nearly all the sampled cocoa trees were attacked by *Anomis*. Herbivory activities of *Anomis* larvae were on the pods and leaves. Defoliation by *Anomis* larvae reduces the photosynthetic ability of the plant. However, of imminent concern was the pod-feeding activity of the larvae since young cocoa pods that are attacked may stop growing due to the extensive damage. Similarly, pod feeding and folivory by larvae of *Earias biplaga* can adversely affect the plant and result in yield reduction. The holes created by *Anomis* and *Earias* and the feeding lesions by mirids are likely to be exploited by fungi (Afreh-Nuamah, 1999; Sarfo & Opoku, 2006) to cause pod rot. Since the pods and specifically the beans of cocoa are the most valued parts, the activities of these insects would invariably affect yield.

Reports from extension (CHED) officers and farmers in the affected communities indicate that before the outbreak, an insecticide product containing fipronil was used to control insect pests of cocoa, particularly mirids and other Hemipterans. Although the product was effective, an upsurge in lepidopteran larvae on cocoa pods was observed afterwards and hence the perception that the said product might have eliminated natural enemies of the larvae leading to their increased population on cocoa. However, besides the toxicity of some pesticides to natural enemies (either directly and/or indirectly through consuming pesticide-tainted prey), pesticides can also increase pest populations through processes, such as hormesis (Raupp et al., 2010; Szczepaniec et al., 2011) and the enhanced nutritive value or suitability of host plants to herbivores by inducing physiological alterations in plants (Gupta & Krischik 2007; Raupp et al., 2010). Contrastingly, a farm in one of the outbreak communities (Jukwa) in which the fipronil product was also used was unaffected by the outbreak. Hence, concluding on the cause of an outbreak without concrete evidence is problematic since the factors that account for pest outbreaks are enigmatic.

Although pod feeding was more pronounced and preferred in this outbreak, similar to the observation of Sarfo and Opoku (2006), larvae of *Anomis* and *Earias* also defoliate cocoa (Awudzi et al., 2009). If uncontrolled, the outbreak of defoliators may contribute to tree decline and eventually tree mortality (Asaro & Chamberlain, 2015). Although mortality due to defoliation is difficult to establish due to the interplay of factors, a positive relationship between the frequency and severity of defoliation and tree mortality has been established (Asaro & Chamberlain, 2015). The Western region, which was the first to report the outbreak, has previously had an *A. leona* outbreak over a decade ago in Bibiani district (Sarfo & Opoku, 2006). Pest outbreaks are complex and difficult to predict, and as reviewed by Asaro and Chamberlain (2015), they could occur in the same region in subsequent or different years by the same pests. Therefore, it is important to develop a monitoring system to detect incipient population build-up and forecast outbreaks.

Although several lepidopteran larvae were found to be infesting cocoa pods, *A. leona* and *E. biplaga* were the most common and occurred simultaneously in the sampled locations. This phenomenon is not rare and is reported in other countries (Asaro & Chamberlain, 2015; Ciesla & Asaro, 2013). Despite the co-occurrence of both larvae, *A. leona* was the most dominant. The perception of secondary infestation by farmers and extension staff could result from a disruption in the cyclic pest-natural enemy interaction, which could be due to a spatial (Maron et al., 2001) or temporal escape of the pest from its natural enemies. The outbreak could have also been triggered by predisposing factors, such as reduced resistance of the trees, albeit temporary and the abundance of suitable food for the pests, such as flushes for defoliators like *A. leona* and *E. biplaga*. *Anomis* and *Earias* are of minor importance in cocoa production in Ghana compared to mirids, stink bugs and coreid bugs. The larvae of *Anomis* and *Earias* are occasional pests. They are usually observed at the onset of the rains when flushes emerge, a food substrate for the larvae. Defoliator abundance, therefore, tends to increase with the abundance of flushes; hence, the inability to initiate control measures might have led to the build-up of the pests and dispersion to other substrates, such as the pods. Simplification of agricultural ecosystems, such as cocoa through monoculture, makes them prone to pest outbreaks (Obeng-Ofori & Afreh-Nuamah, 2007). Hence, in the absence of host resistant planting varieties, insect pest outbreaks could be mitigated via mixed systems, such as agroforestry or mixed cropping (Crowder & Jabbour, 2014; Klapwijk & Bjorkman, 2018) and strict adherence to integrated crop management systems.

During outbreaks, prompt action involving insecticides is of importance since it is a quicker means of suppressing the pest if the insecticides are efficacious and help avoid escalation of the outbreak. Bifenthrin was toxic to field-collected *A. leona* larvae and, hence, suitable as an effective agent

for controlling the outbreak. The 24 h LC_{50} values of 0.0026% and 0.0061% on *A. leona* larvae from Jukwa and Twifo Praso, respectively, compared favourably with that (24 h LC_{50} of 0.00558%) of an insecticide susceptible strain of the fruit fly *Bactrocera zonata* (Ahmad et al., 2010). Bifenthrin was more toxic to the test larvae in this study compared to pests, such as the spider mite *Tetranychus urticae* (LC_{50} of 0.00753%) (Wang et al., 2014) and field strains of *B. zonata* (LC_{50}s of 0.04586 and 0.05333%), although the testing methods differed.

Bifenthrin is commonly used against mirids and other cocoa insect pests (Adu-Acheampong et al., 2015; Antwi-Agyakwa et al., 2015; Awudzi et al., 2016). It quickly knocks down insects before inducing mortality. Its efficacy on *Anomis* larvae under laboratory and field conditions indicates that it could be used as a control agent against the pest. However, documented information on *A. leona* and *E. biplaga* in Ghana is limited. The recent outbreak highlights the urgent need for investigations into this pest's population dynamics and bio-ecology and exploration for effective but eco-friendly management practices to control it on cocoa sustainably.

CONCLUSION

Larvae of *Anomis leona* predominantly caused a caterpillar outbreak on cocoa pods in Ghana in 2018. The larvae consumed the pericarp of the pods causing extensive damage that warranted control. Field populations of *A. leona* were susceptible to bifenthrin under laboratory conditions, and field application of the insecticide was able to suppress the outbreak. Developing an integrated pest management approach would help in effectively managing pests with less emphasis on synthetic insecticide usage. It is equally important to develop a monitoring and forecasting system for early pest detection and prediction of outbreaks.

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REFERENCES

Ackonor, J. B., & Adu-Acheampong, R. (2007). Standard protocol for screening conventional insecticides at the Cocoa Research Institute of Ghana for mirid control. *Journal of the Ghana Science Association*, *9*(2), 117-121. https://doi.org/10.4314/jgsa.v9i2.18020

Adu-Acheampong, R., Sarfo, J. E., Appiah, E. F., Nkansah, A., Awudzi, G., Obeng, E., Tagbor, P., & Sem, R. (2015). Strategy for insect pest control in cocoa. *American Journal of Experimental Agriculture*, *6*(6), 416-423. https://doi.org/10.9734/AJEA/2015/12956

Afreh-Nuamah, K. (1999). *Insect pests of tree crops in Ghana: Identification, damage and control measures*. Buck Press.

Ahmad, S. F., Ahmed, S., Khan, R. R., & Nadeem, M. K. (2010). Evaluation of insecticide resistance in two strains of fruit fly, *Bactrocera zonata*

(Saunders) (Tephritidae: Diptera), with fruit dip method. *Pakistan Entomologist*, *32*(2), 163-167.

Anang, B. T., Mensah, F., & Asamoah, A. (2013). Farmers' assessment of the government spraying program in Ghana. *Journal of Economics and Sustainable Development*, *1*(7), 92–99.

Aneani, F., Anchirinah, V. M., Owusu-Ansah, F., & Asamoah, M. (2012). Adoption of some cocoa production technologies by cocoa farmers in Ghana. *Sustainable Agriculture Research*, *1*(1), 103–117. https://doi.org/10.5539/sar.v1n1p103

Antwi-Agyakwa, A. K., Osekre, E. A., Adu-Acheampong, R., & Ninsin, K. D. (2015). Insecticide use practices in cocoa production in four regions in Ghana. *West African Journal of Applied Ecology*, *23*(1), 39–48.

Asaro, C., & Chamberlain, L.A. (2015). Outbreak history (1953-2014) of spring defoliators impacting oak-dominated forests in Virginia, with emphasis on gypsy moth (*Lymantria dispar* L.) and fall cankerworm (*Alsophila pometaria* Harris). *American Entomologist*, *61*(3), 174-185. https://doi.org/10.1093/ae/tmv043

Asekunowo, P. O., Haque, R. A., Razali, M. R., Avicor, S. W., & Wajidi, M. F. F. (2018). Synthesis and characterization of nitrile functionalized silver(I)-*N*-heterocyclic carbene complexes: DNA binding, cleavage studies, antibacterial properties and mosquitocidal activity against the dengue vector, *Aedes albopictus*. *European Journal of Medicinal Chemistry*, *150*, 601-615. https://doi.org/10.1016/j.ejmech.2018.03.029

Awudzi, G. K., Ackonor, J. B., Cudjoe, A. R., Dwomoh, E. A., & Sarfo, J. E. (2009). *Manual for cocoa insect pests, symptoms of their damage and methods of their control*. Cocoa Research Institute of Ghana.

Awudzi, G. K., Cudjoe, A. R., Assuah, M. K., Adu-Acheampong, R., Dwomoh, E. A., Adu-Yeboah, P., & Sarfo, J. E. (2012). *Pesticide use in cocoa cultivation in Ghana*. Cocoa Research Institute of Ghana.

Awudzi, G. K., Asamoah, M., Owusu-Ansah, F., Hadley, P., Hatcher, P. E., & Daymond, A. J. (2016). Knowledge and perception of Ghanaian cocoa farmers on mirid control and their willingness to use forecasting systems. *International Journal of Tropical Insect Science*, *36*(1), 22–31. https://doi.org/10.1017/S1742758415000247

Ciesla, W. M., & Asaro, C. (2013). *Fall cankerworm*. https://www.fs.usda.gov/Internet/FSE_DOCUMENTS/stelprdb5426975.pdf

Collingwood, C. A. (1971). A comparison of assessment methods in cocoa mirid count trials. In *Proceedings of 3rd International Cocoa Research Conference* (p. 161). Cocoa Research Institute of Ghana.

Creeden, E. P., Hicke, J. A., & Buotte, P. C. (2014). Climate, weather, and recent mountain pine beetle outbreaks in the western United States. *Forest Ecology and Management*, *312*, 239–251. https://doi.org/10.1016/j.foreco.2013.09.051

Crowder, D. W., & Jabbour, R. (2014). Relationships between biodiversity and biological control in agroecosystems: Current status and future challenges. *Biological Control*, *75,* 8–17. https://doi.org/10.1016/j.biocontrol.2013.10.010

Denkyirah, E. K., Okoffo, E. D., Adu, D. T., Aziz, A. A., Ofori, A., & Denkyirah, E. K. (2016). Modeling Ghanaian cocoa farmers' decision to use pesticide and frequency of application: The case of Brong-Ahafo Region. *SpringerPlus*, *5*(1), 1113. https://doi.org/10.1186/s40064-016-2779-z

Entwistle, P. F. (1972). *Pests of cocoa*. Longman Group Ltd.

Ferreira, P. S. F., Henry, T. J., & Coelho, L. A. (2015). Plant bugs (Miridae). In A. Panizzi & J. Grazia (Eds.), *True bugs (Heteroptera) of the Neotropics* (pp. 237-286). Springer. https://doi.org/10.1007/978-94-017-9861-7

Food and Agriculture Organization Corporate Statistical Database. (2019). *FAOSTAT*. http://www.fao.org/faostat/en/#data/QC

Ghana Cocoa Board. (2019). *Cocoa*. COCOBOD.

Ghana Districts. (2017). *Aowin Municipal Assembly*. https://www.ghanadistricts.com/Home/LinkDataDistrict/2729

Ghana Living Standards Survey. (2014). *Ghana Living Standards Survey Round 6 - Main report*. GLSS. http://www.statsghana.gov.gh/gssmain/fileUpload/Living%20conditions/GLSS6_Main%20Report.pdf

Ghana Statistical Service. (2014). *2010 Population and housing census: District analytical report - Assin North Municipal*. GSS. https://new-ndpc-static1.s3.amazonaws.com/CACHES/PUBLICATIONS/2016/06/06/Assin+North.pdf

Gupta, G., & Krischik, V. A. (2007). Professional and consumer insecticides for the management of adult Japanese beetle on hybrid tea rose. *Journal of Economic Entomology*, *100*(3), 830–837. https://doi.org/10.1093/jee/100.3.830

Johnson, M., Luukinen, B., Gervais, J., Buhl, K., & Stone, D. (2010). *Bifenthrin technical fact sheet*. http://npic.orst.edu/factsheets/archive/biftech.html

Klapwijk, M. J., & Björkman C. (2018). Mixed forests to mitigate risk of insect outbreaks. *Scandinavian Journal of Forest Research*, *33*(8), 772-780. https://doi.org/10.1080/02827581.2018.1502805

Kolavalli, S., Vigneri, M., Maamah, H., & Poku, J. (2012). *The partially liberalized cocoa sector in Ghana: producer price determination, quality control, and service provision*. International Food Policy Research Institute (IFPRI). http://www.cocoaconnect.org/sites/default/files/publication/kolavalli%20et%20al%20%282012%29%20the%20partically%20liberalized%20cocoa%20sector%20in%20Ghana.pdf

Lozano-Fuentes, S., Saavedra-Rodriguez, K., Black, W. C. IV, & Eisen, L. (2012). QCal: A software application for the calculation of dose-response curves in insecticide resistance bioassays. *Journal of the American Mosquito Control Association*, *28*(1), 59-61. https://doi.org/10.2987/11-6192.1

Maron, J. L., Harrison, S., & Greaves, M. (2001). Origin of an insect outbreak: Escape in space or time from natural enemies?. *Oecologia*, *126*(4), 595-602. https://doi.org/10.1007/s004420000558

Mkhize, S. B. V. (1971). *Studies on the lepidoptera of cocoa farms in Ghana* [Unpublished Master's thesis]. University of Ghana.

Ministry of Food and Agriculture. (n.d. a). *Atwima Mponua District*. http://mofa.gov.gh/site/directorates/district-directorates/ashanti-region/153-atwima-mponua

Ministry of Food and Agriculture. (n.d. b). *Twifo-Hemang Lower Denkyira District*. http://mofa.gov.gh/site/directorates/district-directorates/central-region/215-twifo-herman-lower-denkyira

Ministry of Food and Agriculture. (n.d. c). *Asikuma-Odoben-Brakwa District*. http://mofa.gov.gh/site/sports/district-directorates/central-region/201-asikuma-odoben-brakwa

Obeng-Ofori, D., & Afreh-Nuamah, K. (2007). Crop pests and pest management. In D. Obeng-Ofori (Ed.), *Major pests of food and selected fruit and industrial crops in West Africa* (pp. 1-44). City Publishers.

Owusu-Manu, E. (1976). Estimation of cocoa pod losses caused by *Bathycoelia thalassina* (Herrich - Schaeffer) (Hemiptera, Pentatomidae). *Ghana Journal of Agricultural Science*, *9*(2), 81-83.

Raupp, M. J., Shrewsbury, P. M., & Herms, D. H. (2010). Ecology of herbivorous arthropods in urban landscapes. *Annual Review of Entomology*, *55*, 19–38. https://doi.org/10.1146/annurev-ento-112408-085351

Sarfo, J. E., & Opoku I. Y. (2006). Report on an unusual attack on cocoa by a defoliator, *Anomis leona* Schaus (Coleoptera: Noctuidae), in Ghana. In A. Y. Akrofi & F. Baah (Eds.), *Proceedings of INCOPED 5th International Seminar on Cocoa Pests and Diseases* (pp. 114-117). Cocoa Research Institute of Ghana. https://www.incocoa.org/data/incoped_workshop_5_proceedings_2006.pdf

Schuh, R. T. (2013). *On-line systematic catalog of plant bugs (Insecta: Heteroptera: Miridae)*. http://research.amnh.org/pbi/catalog/

Singh, T. V. K., & Satyanarayana, J. (2009). Insect outbreaks and their management. In R. Peshin & A. K. Dhawan (Eds.), *Integrated pest management: Innovation-development process* (pp. 331-350). Springer. https://doi.org/10.1007/978-1-4020-8992-3_13

Szczepaniec, A., Creary, S. F., Laskowski, K. L., Nyrop, J. P., & Raupp, M. J. (2011). Neonicotinoid insecticide imidacloprid causes outbreaks of spider mites on elm trees in urban landscapes. *PLOS One*, *6*(5), e20018. https://doi.org/10.1371/journal.pone.0020018

Vigneri, M., & Kolavalli, S. (2018). *Growth through pricing policy: The case of cocoa in Ghana*. Food and Agriculture Organization of the United Nations. http://www.fao.org/3/I8329EN/i8329en.pdf

Wang, S., Tang, X., Wang, L., Zhang, Y., Wu, Q., & Xie, W. (2014). Effects of sublethal concentrations of bifenthrin on the two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae). *Systematic and Applied Acarology*, *19*(4), 481-490. https://doi.org/10.11158/saa.19.4.11

SUPPLEMENTARY DATA

Supplementary Data 1

Information on sampled communities and farms

Region	Cocoa district	Community	Farm code	Farm size (acres)	Age of farm (years)	Mean annual temperature (°C)	Mean annual rainfall (mm)
Ashanti	*Nyinahin	Awiseso	Ny1	10	20	27	1000 -1850
		Achiase	Ny2	15	>25		
		Asuontaa	Ny3	8	10		
Central	↕Assin Fosu	Homaho	AF1	5	>25	26-30	1500 -2000
		Homaho	AF2	2	15		
	ǂBreman Asikuma	Kwamo	BA1	1	7	26-34	1200 -2000
		Kwamo	BA2	3	8-9		
	♯Jukwa	Watreso	Jk1	0.6	9	26-30	1750
		Asuoyenu	Jk2	10	20		
		Bekawopa	Jk3	1.5	28		
	♯Twifo Praso	Koraso	TP1	8	7	26-30	1750
		Assin Adiembra	TP2	1.5	13		
Western	‡Enchi	Mile 4	Enchi	8	9	26	1700 -2100

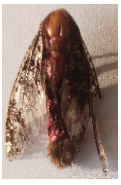
Note. Temperature and rainfall data were obtained from the following sources: *Ministry of Food and Agriculture (MoFA), (n.d. a); ↕Ghana Statistical Service (GSS) (2014); ♯MoFA (n.d. b); ǂMoFA (n.d. c); ‡Ghana Districts (2017)

Supplementary Data 2

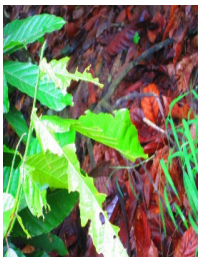
Insect damage, pest, and part of plant affected

Plant part	Damage	Insect
Pod	 Feeding lesions (black spots) on pod	 Adult (left) and nymph (right) of mirid (*Sahlbergella singularis*)
	 Feeding channel/pathway on pod	 Larva (left) and adult (right) of *Anomis leona*
	 Holes on pods (Larva boring into pod)	 Larvae of *Earias*
	 Deformed pod with feeding lesions	 Nymph (left) and adult (right) of coreid bug (*Pseudotheraptus devastans*)

Supplentary Data 2 (*Continue*)

Plant part	Damage	Insect
Pod	 Prematurely ripened pod that also has lesions	 Adult (left) and nymph (right) of stink bug (*Bathycoelia thalassina*)
	 Frass-covered hole on pod	  Larvae (left) and adult (right) of pod borer *Characoma*
	  Holes/feeding cavities/pathways on pods	Co-infestation of pods by larvae of *Anomis* and *Earias*
Stem	  Hole in trees with frass at entrance of hole and base of trees	  Larva of a stem borer (left) Adult of a stem borer (right)

Supplentary Data 2 (*Continue*)

Plant part	Damage	Insect
Leaf	  Cocoa leaves with parts chewed	Defoliators including larvae of *Anomis* and *Earias*

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Review Article

Food Waste Treatment Methods and its Effects on the Growth Quality of Plants: A Review

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ABSTRACT

Food waste and leftovers were common materials that were currently used as bio-compost or soil conditioners upon decomposition. Food waste was a source of food that has declined nutritional value and is not deemed favorable for human consumption. Leftovers were defined as uneaten edible remains of a meal. Biodegradation of these components contributes to many macronutrients, including carbon (C), hydrogen (H), nitrogen (N), phosphorus (P), and potassium (K) in the compost, which makes it a suitable growing condition for plants. In this study, the main sources of research data were one hundred thirty-one scientific articles relating to food waste treatment methods and the growth quality of plants over the last few years. This review was the consensus of the role and

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characteristics of food waste and leftovers as fertilizers. Moreover, the paper briefly discusses the different composting methods for these materials and their corresponding effects on the growth quality of plants.

Keywords: Composting, food waste, food waste treatment, leftovers, plant growth

INTRODUCTION

Food waste is a complex problem that can affect the various elements of sustainability, including climate, economic and social conditions (Food and Agriculture Organization of the United Nations [FAO], 2013), apart from directly depriving the food and nutrition availability to a huge population. Approximately 1.32 billion metric tons of food, approximately one-third of consumption food, was missing and wasted (FAO, 2011). Furthermore, municipal solid waste (MSW) management, which most developed countries face, was difficult. The world's biggest organic waste was food waste, accounting for roughly 30.7 % (Kadir et al., 2016). Table 1 shows the amount of food waste generated in different countries in 2010. Melikoglu et al. (2013) stated that Asian countries could further increase their annual urban food waste volume from 278 to 416 million tonnes between 2005 and 2025. Table 2 shows the amount of food waste generated in Malaysia. Food loss can be categorized as (1) food waste, which was generally the degraded form, and (2) food by-products that were obtained through the removal of food from the food supply chain (FAO, 2014). For developing countries, food waste in the early stage of the production chain was a problem, whereas leftovers are primarily observed as the problem for developed countries.

Table 1

Amount of food waste generated in different countries in 2010 (Melikoglu et al., 2013)

Country	Food waste generation ($x10^6$ tons)
United States	79.0
United Kingdom	11.0
Singapore	0.6
Japan	19.0
Taiwan	16.5
Korea	4.3

Table 2

Amount of food waste generated in Malaysia (Jereme et al., 2016)

Sources of food	Food waste generation ($x10^6$ tons/year)
Households	3.192
Wet and night markets	2.040
Restaurants	1.941
Hotels	0.572
Shopping malls	0.108
Hypermarkets	0.106
Food industries	0.311
Schools	0.021

Leftovers have a higher percentage of disposal than food waste; this situation can be observed in the Netherlands, in which leftovers attributed to consumers recorded at 10–15 % of the total waste (Toumi, 2017). The various research reviews aid in progressive learning, as it includes the latest approach and recommendations that can be very insightful for the research. Hence, this review paper focused on summarizing the application of food waste and leftovers as compost and the different approaches adopted by fellow researchers in studying the properties and characteristics of this organic compost. In the first part of the review, different methods used to compost the food waste were summarized based on the published articles and research papers. The second part of the review discussed the available practices to measure compost nutrient quality. The sequential order has been followed to promote a sheer focus on each stage. Furthermore, it was so that the appropriate food waste treatment method could be distinguished and selected as a convenient reference for future research studies.

Food that has been dumped and disposed of can be segregated into two categories as (a) food waste and (b) leftovers. To begin with, food waste, otherwise known as pre-consumer food, was the category of food obtained from kitchen areas of restaurants and households. These wastes largely include meal prep waste, such as vegetables and fruit seeds and peelings. Unservable food and its residues, such as coffee grounds, were also included in this category. Moreover, these wastes also include foods that perish during the production process, transportation process, and excess food that has expired in supermarkets and bakeries (Bashir et al., 2013; Blakeney, 2019; Heikal Ismail et al., 2020; Muruga et al., 2021). The category of leftovers usually includes the food residues left on plates disposed of by the consumers. These wastes were also known as post-consumer food, and they usually included domestic foods that had been discarded and were often co-mingled with yard debris upon disposal. This food waste can often be spotted in a large amount in dining restaurants, buffets, and hotels (Ebner et al., 2014).

Food waste and leftovers were biodegradable wastes discharged from various sources, including food processing industries, hospitality sectors, and households (Paritosh et al., 2017). A total amount of approximately 90 % of food waste was biodegradable. These waste products have remarkable qualities, and they have high moisture content and a physical shape that can quickly be decomposed (Paritosh et al., 2017). Food-based compost was a good alternative composting source because of the high organic content matter, high moisture content, and the ratio of carbon to nitrogen (C/N) content for waste in residues. In the cases of post-consumer food leftovers, handling these wastes has been observed as a more laborious process attributed to the separation process that needs to be conducted for efficient utilization since the wastes were often subjected to contaminants upon disposal (Risse & Faucette, 2009).

The ratio of carbon to nitrogen was one of the key dimensions that can impact the composting cycle and the product properties (Amin, 2011; Apagu, 2012). Hence, since leftovers were often subjected to contaminants, calculating the optimized ratio of C/N while including possible impurities present in the waste can be difficult. Conversion of waste to compost was also essential as it was a beneficial low-cost method and maintenance compared to incineration and land disposal.

FOOD WASTE TREATMENT METHODS

Discarding in the Landfill

Food waste treatment using the landfill approach was the most common waste management strategy (Jayaprakash et al., 2018). Based on a report by the Environmental Protection Agency (EPA) (2017) in the site of landfills, the volume of municipal solid wastes fell to 135.5 million tons in 2010, from 145.3 million tons in 1990. The fell was caused by a surge of waste recovery through recycling and composting utilized as energy recovery. However, many negative impacts have been linked to landfill disposal in which leachate contamination could contaminate rivers and seas (Abdul Jalil, 2010; Abdullah & Chin, 2010) and lead to groundwater contamination (Lih, 2015).

Hydrogen sulfide (H_2S) was one of the major odors causing compounds in landfill gas and could cause environmental and health impacts (Du et al., 2014). The concentration of H_2S ranges from 100 micrograms per cubic meter up to 1000 milligrams per cubic meter in various municipal solid waste landfill sites. Besides pollution, the high living expenses due to the growing budget for landfill management was also one of the growing concerns faced soon (Muhammad Firdaus et al., 2018). Moreover, the formation of leachate was a big issue that must be considered in the sanitary landfill. These were the wastewater extracts from the soil (Kasozi et al., 2009).

Incineration

As for incineration of waste, this method has a high capability of releasing dioxin gases, which are highly toxic and can threaten human health (Paritosh et al., 2017). Food waste treatment using incineration was also another approach that was being widely used. However, this method has been reported to consume a huge amount of energy compared to other waste treatments used to produce thermal energy that greatly reduces the wastes' volume. In addition, high moisture and salt content in food waste can reduce the lifecycle of the incinerator, and it directly accounts for the release of dioxins. Therefore, incineration was also considered a potential environmental impact and public health risk (Chen, 2016). Besides, the huge energy supply needed incineration also requires a high cost to be set up and utilized (Bong et al., 2017).

Anaerobic Digestion

Anaerobic Digestion (AD) was a process that produces biogas through microbiological digestion of organic matter in an anaerobic

condition. This treatment approach focuses on recycling, recovery, and a reduction system of landfill disposal method (Palaniveloo et al., 2020). The factors that promote this method are environmental-friendly, renewable fuel, and low cost. Anaerobic microbes' functions to convert organic wastes and biomass into biogas, which consists of 70 % methane, 30 to 40 % carbon dioxide (CO_2), and traces of hydrogen (H_2) and H_2S (Warman et al., 2009). The food-based raw material was suitable for an anaerobic digestion process due to its composition and moisture content. The by-product of this process is called digestate, or organic solids with a high content of nutrients and are suitable for use as fertilizer or compost (Yang et al., 2014). There were four main stages involved in anaerobic digestion, and the process is as shown in Figure 1.

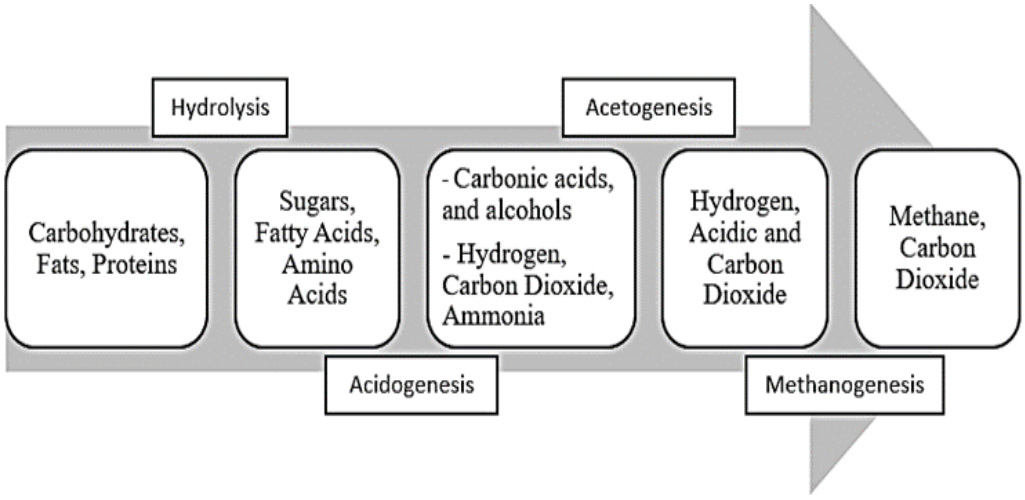

Figure 1. Process stages of anaerobic digestion

Composting

Composting was defined to create a solid humus-like substance to biologically degrade organic materials under controlled aerobic conditions (Cabanillas et al., 2013). Compared to the other waste treatment approach, composting was gaining more interest as it was economically and environmentally beneficial. Composting was one of the biological waste treatments widely treated as a natural and sustainable appropriate organic waste management (Garnett, 2012). This process occurs naturally, provided that microbial growth is possible with the right species, moisture, aerobic environments, feed, and nutrients. The composting mechanism will proceed even more easily by managing these variables. When composted, waste was broken down into a blend of soil conditioning.

It lowers the volume of deposits entered by almost 18 %. Composting was important for this solution to be developed and for reducing solid waste, including food waste.

A case study conducted in Denmark to study the life-cycle analysis and benefits of home composting of food waste suggested that this replacement of fertilizer might significantly benefit the environment. In addition, research comparing the efficacy of a few organic fertilizers and chemical fertilizers on the growth rate of watermelon (*Citrullus lanatus*) has been performed. The analysis concluded with organic fertilizers showed the highest results regarding the consistency parameters of the watermelon's redness and rind thickness (Massri & Labban, 2014). Furthermore, composting was also a technology that was simpler to incorporate and less expensive than other technical approaches (Íñiguez-Covarrubias et al., 2018).

In application, the composted food waste can be used as fertilizer, biogas, or feed mills to produce animal pellet feed. The C/N ratio of the composted substance was a critical variable that needs to be considered in composting, and the favorable ratio ranges from 25–30:1 (Hanc et al., 2014). The location consideration of composting site should be located near landfill to reduce material handling and ease operations. However, there were certain drawbacks associated with composting. The main drawback that has been observed in a Norwegian study was to produce a stable matured compost and the unpleasant odor produced from the composter (Chen, 2016). Besides that, there were arguments that state that the composting process reduces the overall greenhouse gas emissions, and others claimed that it increases greenhouse gas emissions.

Different Methods of Composting

Pit/Heap Method. The pit method was conducted by piling and turning the waste once or twice a week for adequate aeration. This method was also known as windrow or trench composting. The advantage of this method includes (Paritosh et al., 2017):

Little expense—Only requires kitchen scraps (or any other food wastes sources), the pile of leaves, and grass clippings as the materials to begin composting. Then, there will be no expenses on compost space as it will be conducted in the soil.

On-site compost—The compost will ordinarily be conducted in garden space, making it easier to transfer the compost to the plants once it has matured.

This method was primarily encouraged if there was an abundance of free land space to conduct it. In the cross-section of this composting site, the shape of the windrow diversifies from rectangular, trapezoidal, and triangle, as shown in Figure 2.

The shape and size of the pit highly depend on the composting materials and the equipment used for turning. However, based on a study conducted, the minimum area required for a composting unit was 1.25 m x 1.25 m. The pile will be typically mixed and turned with a pitchfork to lift and loosen the pile. At times, the compost pile's temperature was monitored and turned at a

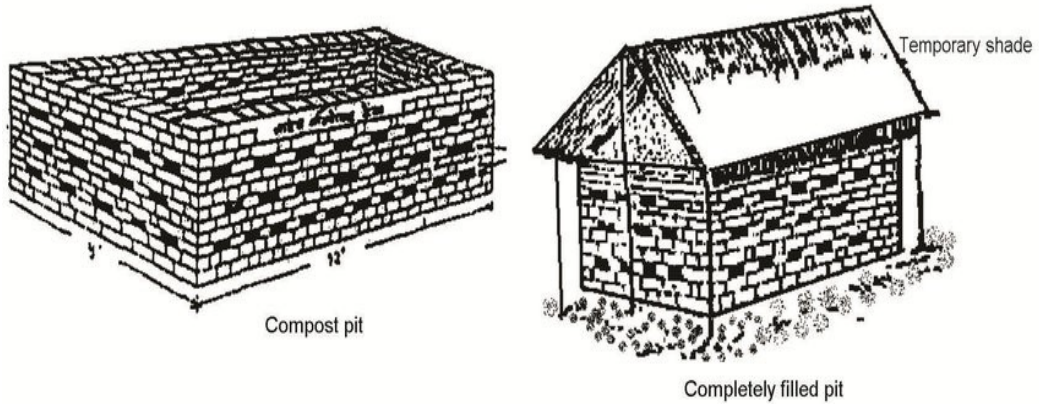

Figure 2. The visual aspect of compost pit

certain temperature range, usually around 55 °C to 60 °C (Khater, 2015). In addition, based on the research conducted by Edwards (Edwards et al., 2008), to prevent leakage of nutrient-rich solution from the pile, the bottom and sides of the base of the trench need to be watered. However, it does come along with certain drawbacks. The compost pile smell can easily attract those pests, and there was no reliable method to keep them away from it. However, the pit-based method was also labor-intensive (Praveen, 2009).

Aerated Statis Piles. Aerated static pile (ASP) was a method of compost in which air was introduced to the stack pile through perforated pipes and blowers (Figure 3). ASP was an improvised version of windrow composting in which airflow was provided underneath to supply oxygen to prevent anaerobic conditions. The need to transform the compost heap into the compost pile was removed by causing airflow. This system was well adapted for different feedstocks, such as green waste, cooking waste, biosolids, and animal dung. The aerobic condition of the compost can be maintained by controlling airflow throughout the compost pile. Composting and aeration of a manually aerated pile were primarily seen in Korean farms. Based on a study on the effects of aeration rate on compost quality, the results show that low and medium aeration rate has a higher impact in obtaining an optimal nitrogen concentration in the compost.

Proper temperature sampling in the ASP method was essential to produce a good quality compost pile. At a temperature of 44.7 °C under proper conditions of adequate oxygen supply, the pile began to decompose (Andersen et al., 2012; Apagu, 2012). The pile forming takes place on a network of drilled pipes for the front loader. The perforation of tubes was arranged in two rows of perforation placed in parallel to each other. This method commonly requires no labor. However, the ASP approach was

weather-sensitive, and this design could also cause fluctuation in pathogen concentration due to inconsistent mixing. This method also implies high cost, and it was not suitable to decompose grease-based substances and animal manure (Risse & Faucette, 2009).

Figure 3. The visual aspect of the aerated statis piles method

Compost Bin and Box. Compost bins, also known as in-vessel systems, used drums, perforated barrels, and specially designed containers to ease the composting process, as shown in Figure 4. This in-vessel system performs compost processes primarily in drums or channels designed to have optimal conditions, using a high-rate controlled aeration device. Compost bins were home composting systems that were frequently compared to the industrial composting system on the aspects of energy consumptions and environmental burdens. A home-oriented compost bin was potentially environmentally beneficial as collection and transportation of bio-waste can be avoided (Pandey et al., 2016).

An improved compost bin design needs to be produced to eliminate the indiscriminate release of greenhouse gas during the composting process for better performance. Besides bin, compost box was another approach that follows container usage to decompose food residues. The method that was prominent in this category was the Takakura portable compost boxes. This technique treated food waste in offices and universities (Jiménez-Antillón et al., 2018). The Institute for Global Environmental Strategies in Japan conceived and developed the strategy in

Figure 4. Compost bin/box

2010. This approach has been practiced in an estimated 40,000 households in the city area of Surabaya, Indonesia. The amount of solid waste in that city that has been reduced by practicing this method was about 30 % (Kurniawan et al., 2013). However, despite the benefits, there was a couple of weakness that can be observed. The weakness was as stated below (Íñiguez-Covarrubias et al., 2018; Lekammudiyanse & Gunatilake, 2008):

- Time-consuming—The process of feeding the Takakura compost box requires a long time to be conducted due to chopping materials before adding them to the box.
- Hefty maintenance—The outer part of the compost box should be cleaned frequently to prevent odors, insects, and pests.

Implementing biochar in this method has also proven to improvise the compost quality and rate of production. Biochar as an alternative additive in the compost bin helps to promote the fermentation process of compost. Dead leaves and cuttings were used to produce biochar, decorative plant waste, and cocoa leaves. Lawn waste was used as a bulking agent in the composting process. Based on the study conducted on the effectiveness of biochar on food compost, in the product, biochar had raised the concentration of ammonium (NH_4) by 37.8 to 45.6 % and nitrate (NO_3) by 50 to 62 % (Waqas et al., 2018).

Compost Tumbler. Compost tumbler was almost like compost bins. Despite the similarity, compost tumbler was a better way to aerate the compost without physically exerting energy to turn the compost itself. Figure 5 shows the aeration that occurred in the compost tumbler. This tumbler system can be formed by using trash cans or soap barrels to construct it. As for the mechanical properties, proper rotation in composting machines of this method was a successful technique that produces an actual product because of proper stirring, aeration, and compost mixtures (Rich & Bharti, 2015). The material design for this composter was made up of light metal to allow the tumbler's rotation to be easy.

For the compost tumbler method, microorganisms and enzymes were used as additives according to the recommended measurement. Compost additives help boost composting processes, soil micro- and macronutrient quality, soil nitrogen, phosphorus, and potassium concentrations (Jayaprakash et al., 2018). The tumbler compost machine was widely used because of the turning accessibility that provides equal ventilation to the compost. However, the drawback of this system was that there was no specific moisture and temperature levels measurement system essential to monitor the compost. Therefore, it was essential to observe the compost from time to time to know if it was too dry, too wet, or not to heat up. In addition, manufactured compost tumblers were costly than the compost bins and piles method (Thenabadu et al., 2015).

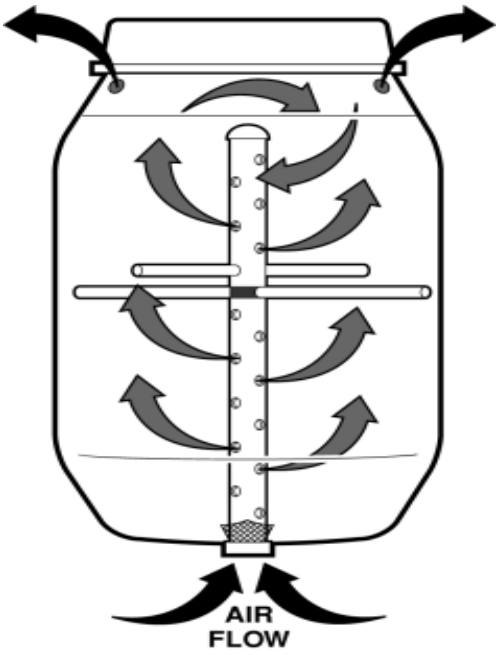

Figure 5. The visual aspect of compost tumbler

Solar Digester. The solar digester was an approach that operated well in confined spaces, such as black plastic or metal drum. The solar energy utilization stimulated the photosynthetic process in these applications. One of the prominent solar digesters that were being used was called the Green Cone technology. This compost equipment was produced and designed in Ontario, Canada since the year 1988. This equipment consists of plastic, with a top-shaped cone and attached basket in which the food was stored underground under the cone, as shown in Figure 6. In Greece, this idea of solar digester has been utilized by constructing digester below ground level and covered with flat-plate solar collectors at the roof structure. The steps for this process were to insert the food waste through the hinged lid, and it will fall into the container. The fungi, bacteria, microorganisms, worms, and insects must decompose the food scraps in this stage. The positive quality of this system was that 90 % of the input was absorbed into the surrounding soil as a compost water source to the plant. Nevertheless, the setback for this approach was that it only disperses compost within a particular area of land, and the dispersion cannot be manipulated and controlled (Mu et al., 2017).

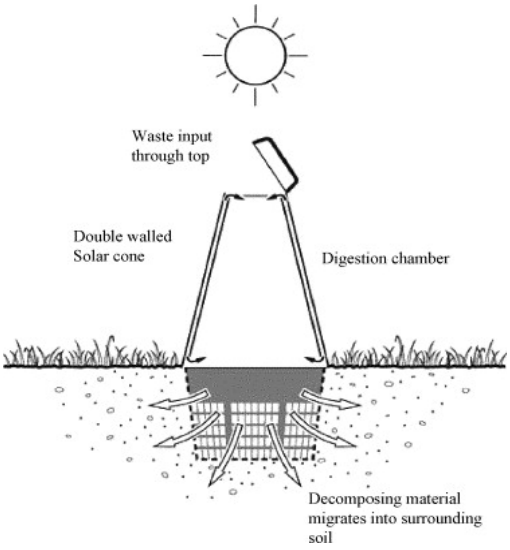

Figure 6. The visual aspect of solar digester

Vermicomposting. Vermicomposting was a composting technique that utilizes various species of worms to decompose the food scraps. This method was also widely regarded as a clean and sustainable approach to dealing with organic waste (Bong et al., 2017). Then, this approach was also an eco-friendly, eco-biotechnological, and

bio-oxidative application mechanism that stabilized the waste into functional bio-product (Saer et al., 2013). Vermicomposting amplifies the usage of worms to digest the food waste into compost. It appears to be an excellent alternative because worm casts in nutrients and microbial life were much higher and considered a higher value commodity (Adhikary, 2012). The type of worms that were commonly used were the white worms, red wigglers, and earthworms. These worms feed on the food scraps, and the material passes through the digestive tract to produce cocoons, also known as vermicompost granules. Earthworms in this method function as a crucial mediator that elevates the surface area accessibility to microorganisms, increasing the enzymatic actions and alteration of organic wastes' physical attributes (Jayaprakash et al., 2018).

Vermicompost contains various types of enzymes, such as amylase, lipase, cellulase, and chitinase. These enzymes help split the organic matter in the field and release the nutrients to the roots of plants. The secretion of chemicals from the worm's digestive tracts helps break down organic matter, so the product has higher nutrient saturation than its original form. The process of decomposition using vermicomposting was during the mesophilic phase of the process. Hence, the material that needs to be composted should be thermophilically composted before or after the worms are added to the compost pile. The product of decomposition was called vermicast (Figure 7).

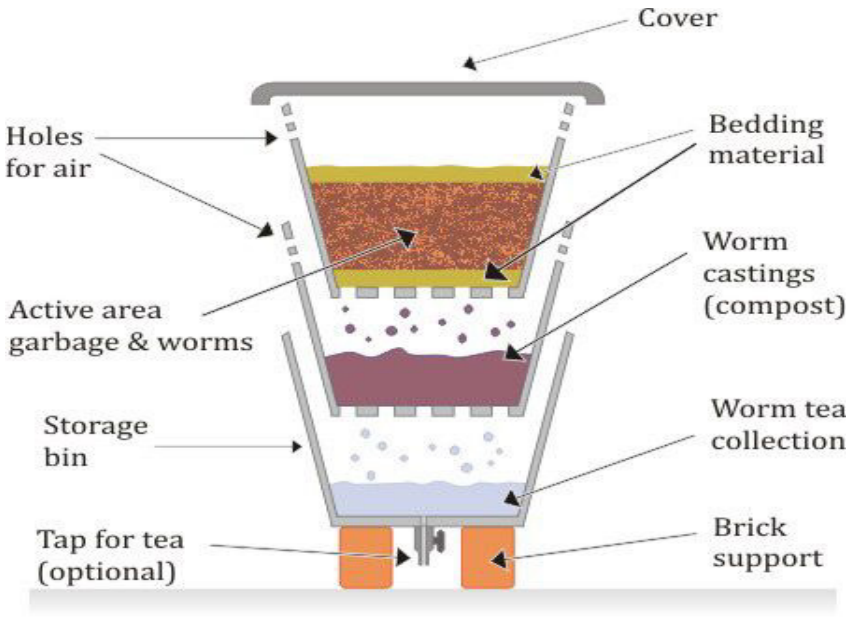

Figure 7. Vermicomposting bin using worms

Vermicompost can overcome soil-borne plant diseases, such as root rot. This approach benefits rural agricultural lands, sustains the soil system, and ensures activity and alive. In addition, vermicomposting enrich nutrients in the soil and increases soil aeration, thus facilitating the survival and dispersal of the beneficial bacteria in such systems. However, this worm casting procedure will undoubtedly cause a handful amount of investment depending on the size of the operation. The problem associated with this method was the death of worms on a large or small scale. This situation might occur if the worms do not obtain sufficient food, food is too dry or too wet, or the bin is too hot (Adhikary, 2012).

Food waste treatment based on insects has been recognized increasingly as an environmentally friendly way to recycle resources and have the advantage of low installation costs. Furthermore, by a particular extraction procedure, insects can be an excellent source of protein (Choi et al., 2017; Lee et al., 2019). However, it was vital to keep conditions suitable for insects to survive and thrive as feed components, adequate temperatures, moisture, and acidity (Yoon et al., 2020). Of the insects, considerable attention has been paid to treating food waste with black soldier fly larvae (BSFL) (Lalander et al., 2019). The larvae breached various organic wastes using the powerful mouth and digestive enzymes (Cho et al., 2020; Pastor et al., 2015). As a result, organic wastes were effectively degraded, such as debris from rotten animals and plants (A. Singh & Kumari, 2019). BSFL may pupate in two weeks under ideal conditions for food supply, temperature, and moisture. BSFL composting benefits from rapid treatment of organic waste and reducing bacterial growth and odor (Pastor et al., 2015).

Black soldier fly's (BSF) larvae and prepupae were valuable sources of animal feed since they comprised 40 % and 30 % of protein and fat, respectively (Cummins et al., 2017; St-Hilaire et al., 2007). Other studies have shown that BSFL reduces cow manure nutrient concentrations, namely 30 to 50 % nitrogen and 61 to 70 % phosphorus (Myers et al., 2008). In many Asian countries, BSF raising was subject to appropriate natural conditions. BSF raising had an optimum temperature and relative humidity of 26–27 °C and 60–70 %, respectively (Barragan-Fonseca et al., 2017). The optimal substrates moisture content was 52–70 % relative humidity (Barragan-Fonseca et al., 2017). The optimal light intensity was 135–200 μmol/m^2 (Shumo et al., 2019), heavily influenced by seasons and the weather. Given the high temperatures and humidity of many Asian countries, including those of Southeast Asia, industries linked to the growth of BSF have high potential when implemented in those areas. The most effective ratio between the number of larvae and gram of substrate was reported to be 2:1 (Pastor et al., 2015) in the cultivation of BSFL (Figure 8).

The BSFL can be monitored by analyzing larval development aspects during the treatment of organic waste. Larvae were reported as 15 to 36.7 days,

154 to 271 mg, 2.3 to 37 mg/d, and 85.6 to 97.1 %, respectively (Julita et al., 2018; Liu et al., 2018; Myers et al., 2008; Pastor et al., 2015). The larvae were identified as the larval patient. The growth of BSFL depends on the type and conditions of the substrates. For example, when fed with food waste, fruits and vegetables, and poultry feed, BSFL survival was 87 %, 90 %, and 93 %, respectively. However, the survival rate was as low as 39 % when fed with digested sludge (Lalander et al., 2019).

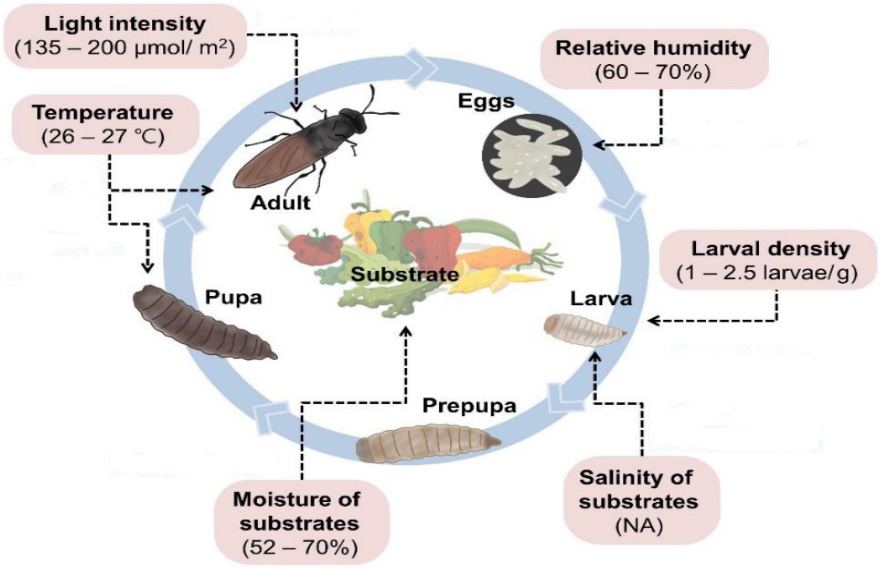

Figure 8. Vermicomposting using BSFL (Kim et al., 2021)

Berkeley Method. Berkeley method was a common and proven method to be used for green waste and food waste treatment. The method was a hot composting system that incorporates aerobic food waste treatment. This aerobic digestion process breaks up food waste and leftovers by various types of microorganisms, including bacteria and fungi, in the presence of oxygen into a hygienic, humus-rich substance. According to the Berkeley method, the procedure to conduct the composting method was by attaching a moveable mesh wire on the ground to form a compost bin. This method can produce finished compost in under a month. The rotation will continue until the compost smells sweet and, when touched, be as crumbly cold. It will then store the composted food waste in a safe place for further research. Based on the research conducted, the Berkeley method took one month or lesser to produce the bio-compost (Daud et al., 2016). Figure 9 shows the process of the Berkeley method conducted.

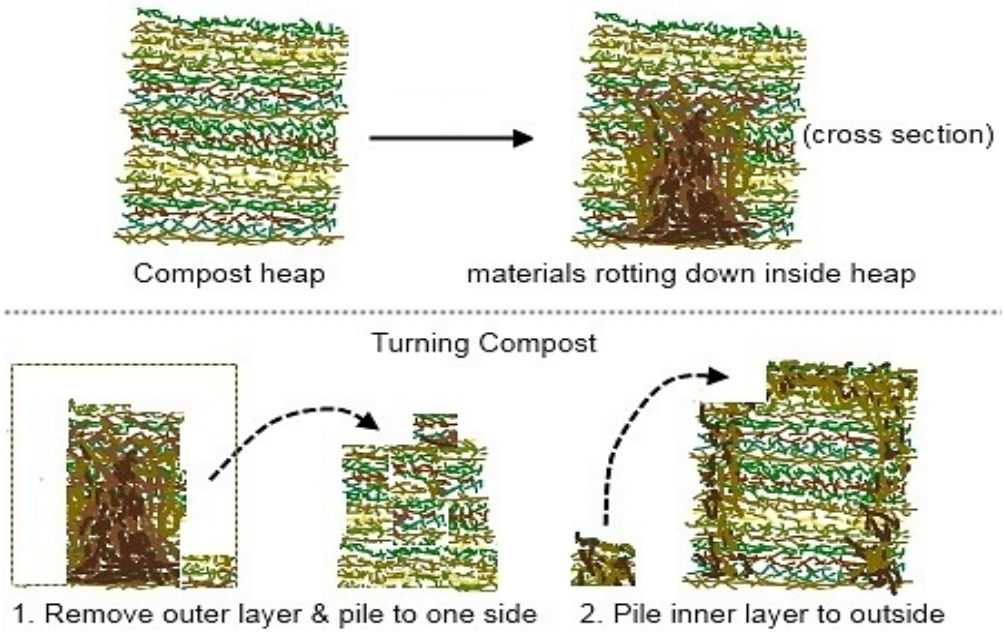

Figure 9. Berkeley working method

Bokashi Method. The Bokashi method was a fermentation approach that uses anaerobic digestion, as shown in Figure 10. This system has originated in Japan, and the fertilizer mainly emphasizes the usage of rice bran, the meal of rapeseed, husk of rice, intense microorganism, sugar molasses, and water (Dou et al., 2012). The Bokashi method applies the usage of effective microorganisms to ferment the feedstocks. It focuses on using effective microorganisms (EM) to facilitate and accelerate the decomposition process.

EM was a mixture of microorganisms that primarily constitutes lactic acid bacteria that coexist in liquid media of pH 3.5. It was understood that EM improves soil and plant microbial variety, boosts field quality, and increases crop growth and quality. The benefits of integrated municipal organic waste recycling with EM technologies have been explored. The organic fertilizer produced from this approach can be utilized as soil nourishment, helps to increase the volume of water contained in the soil (Kumar et al., 2004). Bokashi compost method was also time efficient as compared to usual decomposing methods. Generally, the decomposition process of organic matter into simple organic compounds takes two to three months to be used as compost. On the other hand, the decomposition works faster within two to four weeks for Bokashi processing and does not leave behind foul odor and heat (Vinoth Kumar & Kasturi Bai, 2008).

Other various studies supported Bokashi as soil nourishment in the agricultural

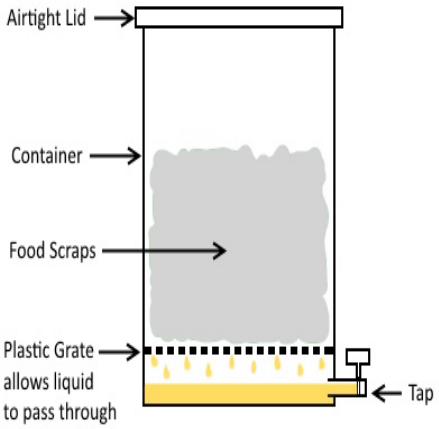

Figure 10. The visual aspect of Bokashi bin

sector. Bokashi's implementation in soil has further improved the concentration of P and K and the number of the plant stem, length, and diameter of an *Alpinia purpurata* plant. A study has also shown that Bokashi manure has elevated the growth of the okra plant. From research conducted to study Dana Mae Christel from the University of Vermont, a few advantages come with this method. Firstly, the EM for Bokashi treatments provides a longer-lasting plant-available nitrogen nutrient than thermophilic compost and vermicompost. Then, this method also increases the aboveground height and biomass of plants. It was due to the compost's capability of providing a longer-term supply of nitrogen combined with a suitable amount of phosphorus and potassium for plant growth. Finally, this research concluded that Bokashi treatment was a suitable alternative fertility amendment for the organic vegetable production system (Jayaprakash et al., 2018).

Automated Biodigesters. Automated biodigesters were machinery that digested and processed food waste into nutrient-rich fertilizers (Figure 11). The machine mixes and produces pre-compost within 24 hours process with a 90 % weight reduction of waste. Automatic kitchen waste compost bin is one of the research technologies conducted at the University of Dalian, China. The designed equipment was equipped with crushing pre-treatment and waste fermentation. However, they also added that additional research was required to upgrade and improve the functional components to cover the kitchen waste disposal system fully. The greenhouse biogas plant was another type of biodigester practiced in many countries. A good number of biogas plants have been constructed in developing countries with a hollow sunlight sheet and an aluminum alloy frame support. The hollow sunlight sheet functioned to store the energy from the sunlight to aid the digestion process (Aschonitis et al., 2014; Garnett, 2012; Ma et al., 2020).

Figure 11. The visual aspect of automated biodigester

NUTRIENT CONTENT IN BIO-COMPOST

The bio compost that has been formed using the chosen method will then be tested by comparing its capability to nurture a specific species of plant. The prime function of bio compost was to improve soil fertility. The assessment and analysis of soil was a proficient measure of soil fertility. The typical soil test provides nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), pH, and base saturation concentration. Hence, the compost produced will be assessed on the N, P, and K content as it is the prime nutrients that aids in plant growth. NPK fertilizers formed from organic waste were essential to nurture healthy soil and plant growth. In addition, the nutrient levels, such as N, P, or K, should be periodically checked and tracked in potting soil stocks used for experiments (Che Jusoh et al., 2013; Dimkpa et al., 2017).

Based on a study conducted to study the effects of NPK fertilizers on the relative growth performance of maize grown in Nigeria, the researchers concluded in a positive remark stating that agro-ecological variety in the selected farmland should be focused on organic mineral fertilizers suggested. It includes the advanced nutrient control support scheme, especially by focusing on areas of low soil fertility. A couple of methods can be implemented to get a thorough analysis of soil test quality. One of the commonly used approaches was by utilizing the atomic absorption spectrophotometer. For the sample preparation, a mixture of hydrochloric acid and sulfuric acid will be utilized to dilute the fine particles of compost collected (Lü et al., 2010).

Another method that can be implied was also by using Multiwave 3000 microwave digestion system. The microwave digestion method can be conducted by following the EPA Method 3052. This approach aimed to decompose the sample by adding a judicious choice of combinations of acids, which can be done for most matrices. Auto analyzer machine was also another main method used to study the micronutrients content in soils and compost. This method was primarily practiced in biogeochemical-ecological research to analyze the composition of water and soil samples. For this approach, the method of Murphy-Riley was commonly used to analyze the potassium content in the soil. This method focuses on the reaction between ortho-phosphates and molybdate with antimony potassium tartrate as the catalyst. This reaction was then followed by the reduction with ascorbic acid at a pH below 1. The blue component formed will be measured at 880 nm to identify the concentration of potassium. Then, to analyze the nitrogen concentration, the copper-cadmium coil was set as non-active during analysis (Massri & Labban, 2014; Moonrungsee et al., 2015).

The sample was mixed with an internal reference, lithium nitrate of fixed concentration, to identify potassium concentration in the compost. This product

was then nebulized and flushed with a mixture of propane-air. The mixture was burned in the machine, and the result obtained was emission lines for potassium and lithium. The values obtained will be compared to the intensity of the emission generated by the lithium ions. It was to compensate for fluctuations in flame temperature, flow rate, and chemical interferences. Besides that, nutrient monitoring methods based on meters were also very common in the soil testing industry. The machine opts for remotely controlled equipment operationally like its laboratory collaborators using battery-operated nutrient-specific meters containing electrode sensors or field-portable spectrometers and solution ion calibration (A. L. Singh et al., 2010; Patil & Bodhe, 2011; R. P. Singh et al., 2011).

GROWTH QUALITY OF PLANTS

Organic farming was tremendously advantageous, thus making our development more economically feasible. Organic agriculture shall track the plague or the disease without preventing environmental degradation, avoid contamination, increase the productivity of land so far crops can contain nutrients, and allow for increased marketable prices (Najar et al., 2015; Ukoje & Yusuf, 2013). Vermicompost was one of the better planting organic mediums. Vermicompost was highly organic and may not contain any chemicals, indicating that they were environmentally sustainable. It was nutritional, and it released nutrients at a time, which quickly plants could consume by reducing their need for pesticide application because herbs and fewer pesticides were also safe since their pest and disease were not present. In addition, it offers an environmentally safe approach to mineral fertilizers due to the availability of comparatively significant quantities of crop growth and production micronutrients while offering an environmentally sustainable alternative (Lazcano & Dominguez, 2014).

Organic farming, such as using vermicompost, could be a trend in sustainable agriculture to be an almost permanent practice for years to come since vermicompost releases nutrients at a slow pace, allowing fast absorption by plants and increasing the moisture keeping power of the soil resulting in improved quality of plant production. Furthermore, vermicompost usage for the significant production of vegetables can also fix the problems related to the disposal of wastes and overcome the lack of organic material on an acceptable basis. On the other hand, a reliable blend of pungent and inorganic sources of substances could have the potential to obtain an excellent financial result for follow-up plants with strong soil wellbeing (Abdelaziz et al., 2007).

Soil variation with mature and healthy compost, even with potted urban solid waste, was extensively analyzed, and the effects of breed plants on beans, black eye peas, okra, tomato, squash, eggplant, watermelon, corn, and chili pepper (Roe et al., 1993) were increased. The practices

for integrated nutrient management were an essential factor in the practical and cost-efficient management of the soil fertility in sugarcane growing. Sugar cane injectors can lead to higher soil fertility, growth, or return into merged organic and inorganics matter (including biological fertilizers).

Vermicompost can promote plant cultivation, root production, and hence nutritional uptake. With humic substances, which were the main component of soil organic matter, the shoot biomass will increase over the hormone effects on root elongation and plant development (Oworu et al., 2010). In conclusion, the addition of vermicompost will improve the availability of nutrients in the land and nutrient intake in plants. It can result in a direct or indirect influence. Direct results shall be given either by the nutrients accompanied with the composting manure. In this sense, indirect results were improved microbial action and preservation of flavor, enhanced soil structure, eating matter, and water quality. The behaviors of microbial materials may increase the mobilization of nutrients but may also contribute to the immobilization of nutrients. Thus, improving soil composition and restoring the plants' roots, with the more significant soil volumes (Zandonadi et al., 2007).

The parameters to assess the growth quality of plants that have been fertilized by different compost can be measured by measuring plant height, the number of leaves and branches, and the dry weight of the plant (Ibrahim et al., 2018). The plant height measurement was taken using a measuring strip from the floor to the tip of the highest growth point. Firstly, the height of the plants will be measured daily until the plant is fully mature. Then, the ruler was set at the base of the plant the height was recorded. The value was charged with both date and height values. Then for the number of leaves, the leaves were counted daily and recorded.

The grid counting method was an approach used to measure the size of leaves. This method will be conducted using grid paper was removed from the plant and put on a paper grid. Then, on grid paper, the outlines of the leaf were drawn by pencil. Finally, by measuring the grids occupied by the leaf, the area of the leaf was determined. This approach was precise, but it requires time and effort to place on many leaves. The leaf area and dry weight were calculated after the 30-days growth, the leaves' surface area and dry weight of the greens were measured. The leaves area was measured using an application named "Petiole." This application runs by calibrating the mobile camera with a Petiole Pad, and once the calibration is completed, the leaf is placed in front of the camera (Xiao et al., 2009). The leaf area was then measured automatically by the app, and the results obtained were recorded.

The gravimetric method was also one of the methods used for leaf area measurement (Aschonitis et al., 2014). The leaf was first removed from the plant and put on white paper for this process. Next, a paper was cut

out based on leaf shape. Now this paper's weight is contrasted with that of the known area on the same page. This approach was laborious and time-consuming when used on several leaves. In addition, image processing methods are currently being used to calculate the leaf region easily and precisely. Leaf area was calculated using the following steps: acquisition of images, image pre-processing, leaf segmentation regions, region filling, and area calculations (Feng & Chun, 2010). Other researchers use threshold-based segmentation to obtain the size of the leaf.

Kang et al. (2021) investigated the growth characteristics of Chinese cabbage using organic fertilizer (compost). As a result, the leaf length (12.6 to 12.9 cm), root length (11.8 to 15.3 cm), fresh weight (14.7 to 16.5 g), and dry weight (3.4 to 3.9 g) were increased after four weeks compared to plants without compost with lower parameters: length of leaf (11.8 cm), length of root (9.3 cm), fresh weight (13.4 g) and dry weight (2.5 g). Furthermore, in compost-treated plants, the level of stomach conductivity that measures water relations in a plant increased to 0.07 ± 0.0 mol/m^2, showing that the treated plants can stand dryness or salinity stress.

Table 3 shows the effects of Bokashi compost on bell peppers plant growth. Tong et al. (2021) declared that the plant height difference could be due to a high level of sodium ion (Na^+) in the medium of Bokashi that can cause salinity stress (López-Serrano et al., 2021). Nutrient deficiencies could also lead to slow growth. For example, Razaq et al. (2017) reported a nitrogen deficiency in soybean plants and mint plants (*Mentha piperita*). In both conventional (2.95 mm) and Bokashi-grown plants, there was no significant ($p \leq 0.05$) difference in internode length (2.65 mm). In contrast, Bokashi plants ($p \leq 0.05$) stems were significantly (10.2 mm) thicker than those of conventional plants (7.3 mm). Plant height explains that nutrient deficiencies and salinity may be associated with the Bokashi plant's thinner stem.

Shafique et al. (2021) stated that the maximum and significant increase in shoot length (14 ± 0.81 cm), number of leaves (12 ± 0.0), the diameter of leaves (4.66 ± 0.23 cm), length of leaves (9.0 ± 0.40 cm), surface areas of leaves (36.7 ± 0.87 cm^2), length of the whole plant (14.33 ± 0.47 cm), and root length (5.66 ± 0.47 cm), has been found in vermicompost germinating media. Furthermore, the results showed a significant increase of vermicompost in all vegetation growths and floral parameters, such as plant root volume (15.66 ± 0.81) and number of lateral plant growth (9.00 ± 0.81) and stemmed diameter (1.0 ± 0.0 cm), number of open flowers (2.66 ± 0.94), flower diameter (9.00 ± 0.81 cm), fresh weight (16.66 ± 0.47 g), number of flower buds (7.00 ± 0.81).

Table 3

Effect of Bokashi compost on bell pepper plant growth (Tong et al., 2021)

Parameters	Values
Plant height (mm)	611.00 ± 24.34
Stem diameter (mm)	7.32 ± 0.81
Stem internode (mm)	2.60 ± 0.10
Number of fruits per plant	2.60 ± 0.89
Fruit weight per plant (kg)	0.76 ± 46.06
Fruit circumference (mm)	233.60 ± 26.08

CONCLUSION

This review article has gathered most of the information that can be implemented in researching the effect of food waste and leftovers as compost on the growth quality of the plant. Firstly, many advantages can be obtained from composting food waste and leftover foods as a soil amendment. Furthermore, it was a promising alternative to produce fertilizer soon. From all the compost methods reviewed, the Bokashi method stands out as the most convenient approach for this research. This method tends to be more affordable and, at the same time, delivers results in a shorter period compared to the others. Although anaerobic biodigesters deliver the product within a considerably shorter period among all the approaches presented, the availability and cost seem to be the drawback for this method. However, the Bokashi method was generally affordable, and it was a technique well-practiced worldwide. The pivotal element in this composting method was Bokashi bran. Bokashi bran has a lower processing cost, and it was compatible with being used in diverse anaerobic designed environments. The function of the Bokashi bran in this process was to implement lactic acid bacteria to amplify the decomposition process in the vessel. This sequence of the procedure will be conducted by following the reference that has been reviewed in this article.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Abdelaziz, M., Pokluda, R., & Abdelwahab, M. (2007). Influence of compost, microorganisms and NPK fertilizer upon growth, chemical composition and essential oil production of

Rosmarinus officinalis L. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, *35*(1), 86–90. https://doi.org/10.15835/nbha351261

Abdul Jalil, M. (2010). Sustainable development in Malaysia : A case study on household waste management. *Journal of Sustainable Development*, *3*(3), 91–102.

Abdullah, N., & Chin, N. L. (2010). Simplex-centroid mixture formulation for optimized composting of kitchen waste. *Bioresource Technology*, *101*(21), 8205–8210. https://doi.org/10.1016/j.biortech.2010.05.068

Adhikary, S. (2012). Vermicompost, the story of organic gold: A review. *Agricultural Sciences*, *3*(7), 905–917. https://doi.org/10.4236/as.2012.37110

Amin, M. E. M. H. (2011). Effect of different nitrogen sources on growth, yield and quality of fodder maize (*Zea mays* L.). *Journal of the Saudi Society of Agricultural Sciences*, *10*(1), 17–23. https://doi.org/10.1016/j.jssas.2010.06.003

Andersen, J. K., Boldrin, A., Christensen, T. H., & Scheutz, C. (2012). Home composting as an alternative treatment option for organic household waste in Denmark: An environmental assessment using life cycle assessment-modelling. *Waste Management*, *32*(1), 31–40. https://doi.org/10.1016/j.wasman.2011.09.014

Apagu, A. B. (2012). Recycling biodegradable waste using composting technique. *Journal of Environmental Science and Resources Management*, *4*, 40–49.

Aschonitis, V. G., Papamichail, D. M., Lithourgidis, A., & Fano, E. A. (2014). Estimation of leaf area index and foliage area index of rice using an indirect gravimetric method. *Communications in Soil Science and Plant Analysis*, *45*(13), 1726–1740. https://doi.org/10.1080/00103624.2014.907917

Barragan-Fonseca, K., Dicke, M., & van Loon, J. (2017). Nutritional value of the black soldier fly (*Hermetia illucens* L.) and its suitability as animal feed – A review. *Journal of Insects as Food and Feed*, *3*(2), 105–120. https://doi.org/10.3920/jiff2016.0055

Bashir, M. T., Ali, S., Ghauri, M., Adris, A., & Harun, R. (2013). Impact of excessive nitrogen fertilizers on the environment and associated mitigation strategies. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences*, *15*(2), 213–221.

Blakeney, M. (2019). Food loss and waste and food security. In *Food loss and food waste* (pp. 1–26). Edward Elgar Publishing. https://doi.org/10.4337/9781788975391.00006

Bong, C. P. C., Goh, R. K. Y., Lim, J. S., Ho, W. S., Lee, C. T., Hashim, H., Abu Mansor, N. N., Ho, C. S., Ramli, A. R., & Takeshi, F. (2017). Towards low carbon society in Iskandar Malaysia: Implementation and feasibility of community organic waste composting. *Journal of Environmental Management*, *203*(Part 2), 679–687. https://doi.org/10.1016/j.jenvman.2016.05.033

Cabanillas, C., Stobbia, D., & Ledesma, A. (2013). Production and income of basil in and out of season with vermicomposts from rabbit manure and bovine ruminal contents alternatives to urea. *Journal of Cleaner Production*, *47*, 77–84. https://doi.org/10.1016/j.jclepro.2013.02.012

Che Jusoh, M. L., Abd Manaf, L., & Abdul Latiff, P. (2013). Composting of rice straw with effective microorganisms (EM) and its influence on compost quality. *Iranian Journal of Environmental Health Science and Engineering*, *10*, 17. https://doi.org/10.1186/1735-2746-10-17

Chen, Y. T. (2016). A cost analysis of food waste composting in Taiwan. *Sustainability*, *8*(11), 1210. https://doi.org/10.3390/su8111210

Cho, S., Kim, C. H., Kim, M. J., & Chung, H. (2020). Effects of microplastics and salinity on food waste processing by black soldier fly (*Hermetia illucens*) larvae. *Journal of Ecology and Environment*, *44*, 7. https://doi.org/10.1186/s41610-020-0148-x

Choi, B. D., Wong, N., & Auh, J. H. (2017). Defatting and sonication enhances protein extraction from edible insects. *Korean Journal for Food Science of Animal Resources*, *37*(6), 955–961. https://doi.org/10.5851/kosfa.2017.37.6.955

Cummins, V. C., Rawles, S. D., Thompson, K. R., Velasquez, A., Kobayashi, Y., Hager, J., & Webster, C. D. (2017). Evaluation of black soldier fly (*Hermetia illucens*) larvae meal as partial or total replacement of marine fish meal in practical diets for Pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture*, *473*, 337–344. https://doi.org/10.1016/j.aquaculture.2017.02.022

Daud, N. M., Khalid, S. A., Nawawi, W. N. W., & Ramli, N. (2016). Producing fertilizer from food waste recycling using berkeley and bokashi method. *Ponte Academic Journal*, *72*(4). https://doi.org/10.21506/j.ponte.2016.4.11

Dimkpa, C., Bindraban, P., McLean, J. E., Gatere, L., Singh, U., & Hellums, D. (2017). Methods for rapid testing of plant and soil nutrients. In E. Lichtfouse (Ed.), *Sustainable agriculture reviews* (pp. 1-43). Springer. https://doi.org/10.1007/978-3-319-58679-3_1

Dou, L., Komatsuzaki, M., & Nakagawa, M. (2012). Effects of Biochar, Mokusakueki and Bokashi application on soil nutrients, yields and qualities of sweet potato. *International Research Journal of Agricultural Science and Soil Science*, *2*(8), 318-327.

Du, Y., Feng, H., Zhang, K., Hu, L. F., Fang, C. R., Shen, D. S., & Long, Y. Y. (2014). Role of iron in H_2S emission behavior during the decomposition of biodegradable substrates in landfill. *Journal of Hazardous Materials*, *272*, 36–41. https://doi.org/10.1016/j.jhazmat.2014.02.040

Ebner, J., Win, S. S., Hegde, S., Vadney, S., Williamson, A., & Trabold, T. (2014). Estimating the biogas potential from colleges and universities. In *Proceedings of the American Society of Mechanical Engineers 2014 8th International Conference on Energy Sustainability* (Vol. 2, p. V002T04A005). ASME Publishing. https://doi.org/10.1115/ES2014-6433

Edwards, S., Asmelash, A., Araya, H., & Egziabher, T. (2008). *Impact of compost use on crop yields in Tigray, Ethiopia, 2000-2006 inclusive*. https://www.fao.org/3/ai434e/ai434e.pdf

Environmental Protection Agency. (2017). *United States 2030 food loss and waste reduction goal*. EPA.

Feng, T., & Chun, W. (2010). Calculating the leaf-area based on non-loss correction algorithm. In *2010 International Conference of Information Science and Management Engineering* (Vol. 1, pp. 75-78). IEEE Publishing. https://doi.org/10.1109/isme.2010.54

Food and Agriculture Organization of the United Nations. (2011). *Global food losses and food waste – Extent, causes and prevention*. FAO.

Food and Agriculture Organization of the United Nations. (2013). *Developments in forums of importance for the mandate of the Food and Agriculture Organization of the United Nations*. FAO.

Food and Agriculture Organization of the United Nations. (2014). *Save Food: Global Initiative on Food Loss and Waste Reduction*. FAO.

Garnett, A. E. (2012). *Food and yard waste compost as a nutrient source for corn* [Master's thesis, Dalhousie University]. Dalhousie Theses and Dissertations. https://dalspace.library.dal.ca/bitstream/handle/10222/14623/Garnett,%20Angela,%20MSc,%20AGRI,%20April2012.pdf?sequence=1

Hanc, A., Szakova, J., & Ochecova, P. (2014). Differences in the mobility of Cd, Cu, Pb and Zn during composting of two types of household bio-waste collected in four seasons. *Bioresource Technology*, *168*, 204–213. https://doi.org/10.1016/j.biortech.2014.01.125

Heikal Ismail, M., Ghazi, T. I. M., Hamzah, M. H., Manaf, L. A., Tahir, R. M., Mohd Nasir, A., & Ehsan Omar, A. (2020). Impact of Movement Control Order (MCO) due to coronavirus disease (COVID-19) on food waste generation: A case study in Klang Valley, Malaysia. *Sustainability*, *12*(21), 8848. https://doi.org/10.3390/su12218848

Ibrahim, M., Rahman, N., & Zain, N. (2018). Effect of nitrogen rates on growth and quality of water spinach (*Ipomea aquatica*). *Annual Research and Review in Biology*, *26*(1), 1–12. https://doi.org/10.9734/arrb/2018/40352

Íñiguez-Covarrubias, G., Gómez-Rizo, R., Ramírez-Meda, W., & de Jesús Bernal-Casillas, J. (2018). Composting of food and yard wastes under the static aerated pile method. *Advances in Chemical Engineering and Science*, *8*(4), 271–279. https://doi.org/10.4236/aces.2018.84019

Jayaprakash, S., Lohit, H. S., & Abhilash, B. S. (2018). Design and development of compost bin for Indian kitchen. *International Journal of Waste Resources*, *8*(1), 1000323. https://doi.org/10.4172/2252-5211.1000323

Jereme, I. A., Siwar, C., Begum, R. A., & Abdul Talib, B. (2016). Addressing the problems of food waste generation in Malaysia. *International Journal of Advanced and Applied Sciences*, *3*(8), 68–77. https://doi.org/10.21833/ijaas.2016.08.012

Jiménez-Antillón, J., Calleja-Amador, C., & Romero-Esquivel, L. G. (2018). Food waste recovery with Takakura portable compost boxes in offices and working places. *Resources*, *7*(4), 84. https://doi.org/10.3390/resources7040084

Julita, U., Suryani, Y., Kinasih, I., Yuliawati, A., Cahyanto, T., Maryeti, Y., Permana, A. D., & Fitri, L. L. (2018). Growth performance and nutritional composition of black soldier fly, *Hermetia illucens* (L.), (Diptera : Stratiomyidae) reared on horse and sheep manure. In *IOP Conference Series: Earth and Environmental Science* (Vol. 187, p. 012071). IOP Publishing. https://doi.org/10.1088/1755-1315/187/1/012071

Kadir, A. A., Ismail, S. N. M., & Jamaludin, S. N. (2016). Food waste composting study from Makanan Ringan Mas. In *IOP Conference Series: Materials Science and Engineering*, *Soft Soil Engineering International Conference 2015* (Vol. 136, p. 012057). IOP Publishing. https://doi.org/10.1088/1757-899x/136/1/012057

Kang, S. M., Shaffique, S., Kim, L. R., Kwon, E. H., Kim, S. H., Lee, Y. H., Kalsoom, K., Aaqil Khan, M., & Lee, I. J. (2021). Effects of organic fertilizer mixed with food waste dry powder on the growth of Chinese cabbage seedlings. *Environments*, *8*(8), 86. https://doi.org/10.3390/environments8080086

Kasozi, G. N., Nkedi-Kizza, P., & Harris, W. G. (2009). Varied carbon content of organic matter in Histosols, Spodosols, and Carbonatic soils. *Soil Science Society of America Journal*, *73*(4), 1313–1318. https://doi.org/10.2136/sssaj2008.0070

Khater, E. S. G. (2015). Some physical and chemical properties of compost. *International Journal of Waste Resources*, *5*(1), 1000172.

Kim, C. H., Ryu, J., Lee, J., Ko, K., Lee, J. Y., Park, K. Y., & Chung, H. (2021). Use of black soldier fly larvae for food waste treatment and energy production in Asian countries: A review. *Processes*, *9*(1), 161. https://doi.org/10.3390/pr9010161

Kumar, S., Mondal, A. N., Gaikwad, S. A., Devotta, S., & Singh, R. N. (2004). Qualitative assessment of methane emission inventory from municipal

solid waste disposal sites: A case study. *Atmospheric Environment*, *38*(29), 4921–4929. https://doi.org/10.1016/j.atmosenv.2004.05.052

Kurniawan, T. A., de Oliveira, J. P., Premakumara, D. G. J., & Nagaishi, M. (2013). City-to-city level cooperation for generating urban co-benefits: The case of technological cooperation in the waste sector between Surabaya (Indonesia) and Kitakyushu (Japan). *Journal of Cleaner Production*, *58*, 43–50. https://doi.org/10.1016/j.jclepro.2013.08.002

Lalander, C., Diener, S., Zurbrügg, C., & Vinnerås, B. (2019). Effects of feedstock on larval development and process efficiency in waste treatment with black soldier fly (*Hermetia illucens*). *Journal of Cleaner Production*, *208*, 211–219. https://doi.org/10.1016/j.jclepro.2018.10.017

Lazcano, C., & Domínguez, J. (2014). The use of vermicompost in sustainable agriculture: Impact on plant growth and soil fertility. *Soil Nutrients*, *10*(1-23), 187.

Lee, H. J., Kim, J. H., Ji, D. S., & Lee, C. H. (2019). Effects of heating time and temperature on functional properties of proteins of yellow mealworm larvae (*Tenebrio molitor* L.). *Food Science of Animal Resources*, *39*(2), 296–308. https://doi.org/10.5851/kosfa.2019.e24

Lekammudiyanse, L. M. M. U., & Gunatilake, S. K. (2008). Efficiency of the household compost bin as a waste management technique in Sri Lanka (a case study in Gampaha Municipal Council area). *International Journal of Basic and Applied Sciences*, *10*(1), 89–94.

Lih, T. M. (2015). *Production of fertilizer using food wastes of vegetables and fruits* [Unpublished Bachelor's dissertation]. Universiti Malaysia Sarawak.

Liu, Z., Minor, M., Morel, P. C. H., & Najar-Rodriguez, A. J. (2018). Bioconversion of three organic wastes by black soldier fly (Diptera: Stratiomyidae) larvae. *Environmental Entomology*, *47*(6), 1609–1617. https://doi.org/10.1093/ee/nvy141

López-Serrano, L., Calatayud, N., López-Galarza, S., Serrano, R., & Bueso, E. (2021). Uncovering salt tolerance mechanisms in pepper plants: A physiological and transcriptomic approach. *BMC Plant Biology*, *21*(1), 169. https://doi.org/10.1186/s12870-021-02938-2

Lü, C., Ren, H., Zhang, Y., & Shen, Y. (2010). Leaf area measurement based on image processing. In *2010 International Conference on Measuring Technology and Mechatronics Automation* (Vol. 3, pp. 580-582). IEEE Publishing. https://doi.org/10.1109/icmtma.2010.141

Ma, L., Duan, T., & Hu, J. (2020). Application of a universal soil extractant for determining the available NPK: A case study of crop planting zones in central China. *Science of the Total Environment*, *704*, 135253. https://doi.org/10.1016/j.scitotenv.2019.135253

Massri, M., & Labban, L. (2014). Comparison of different types of fertilizers on growth, yield and quality properties of watermelon (*Citrllus lanatus*). *Agricultural Sciences*, *5*(6), 475–482. https://doi.org/10.4236/as.2014.56048

Melikoglu, M., Lin, C. S. K., & Webb, C. (2013). Analyzing global food waste problem: Pinpointing the facts and estimating the energy content. *Central European Journal of Engineering*, *3*(2), 157–164. https://doi.org/10.2478/s13531-012-0058-5

Moonrungsee, N., Pencharee, S., & Jakmunee, J. (2015). Colorimetric analyzer based on mobile phone camera for determination of available phosphorus in soil. *Talanta*, *136*, 204–209. https://doi.org/10.1016/j.talanta.2015.01.024

Mu, D., Horowitz, N., Casey, M., & Jones, K. (2017). Environmental and economic analysis of an in-vessel food waste composting system at Kean University in the U. S. *Waste Management*, *59*, 476–486. https://doi.org/10.1016/j.wasman.2016.10.026

Muhammad Firdaus, A. R., Abu Samah, M. A., & Abd Hamid, K. B. (2018). CHNS analysis towards food waste in composting. *Journal CleanWAS*, *2*(1), 6–10. https://doi.org/10.26480/jcleanwas.01.2018.06.10

Muruga, M., Arumugam, V., & Heikal Ismail, M. (2021). Bio-compost behaviour as soil additive by food waste pretreatment on the growth of *Abelmoschus esculentus* L.: A systematic review. *Pertanika Tropical Agricultural Science*, *44*(4), 825-844. https://doi.org/10.47836/pjtas.44.4.07

Myers, H. M., Tomberlin, J. K., Lambert, B. D., & Kattes, D. (2008). Development of black soldier fly (Diptera: Stratiomyidae) larvae fed dairy manure. *Environmental Entomology*, *37*(1), 11–15. https://doi.org/10.1093/ee/37.1.11

Najar, I. A., Khan, A. B., & Hai, A. (2015). Effect of macrophyte vermicompost on growth and productivity of brinjal (*Solanum melongena*) under field conditions. *International Journal of Recycling of Organic Waste in Agriculture*, *4*(2), 73–83. https://doi.org/10.1007/s40093-015-0087-1

Oworu, O., Dada, O., & Majekodunmi, O. (2010). Influence of compost on growth, nutrient uptake and dry matter partitioning of grain amaranths (*Amaranthus hypochondriacus* L.). *Libyan Agriculture Research Center Journal International*, *1*(6), 375–383.

Palaniveloo, K., Amran, M. A., Norhashim, N. A., Mohamad-Fauzi, N., Peng-Hui, F., Hui-Wen, L., Kai-Lin, Y., Jiale, L., Chian-Yee, M. G., Jing-Yi, L., Gunasekaran, B., & Razak, S. A. (2020). Food waste composting and microbial community structure profiling. *Processes*, *8*(6), 723. https://doi.org/10.3390/pr8060723

Pandey, P. K., Vaddella, V., Cao, W., Biswas, S., Chiu, C., & Hunter, S. (2016). In-vessel composting system for converting food and green wastes into pathogen free soil amendment for sustainable agriculture. *Journal of Cleaner Production*, *139*, 407–415. https://doi.org/10.1016/j.jclepro.2016.08.034

Paritosh, K., Kushwaha, S. K., Yadav, M., Pareek, N., Chawade, A., & Vivekanand, V. (2017). Food waste to energy: An overview of sustainable approaches for food waste management and nutrient recycling. *BioMed Research International*, *2017*, 2370927. https://doi.org/10.1155/2017/2370927

Pastor, B., Velasquez, Y., Gobbi, P., & Rojo, S. (2015). Conversion of organic wastes into fly larval biomass: Bottlenecks and challenges. *Journal of Insects as Food and Feed*, *1*(3), 179–193. https://doi.org/10.3920/jiff2014.0024

Patil, S., & Bodhe, S. (2011). Betel leaf area measurement using image processing. *International Journal on Computer Science and Engineering*, *3*(7), 2656–2660.

Praveen S. (2009). *Analysis of micronutrients in soil by using AA 800 atomic absorption spectrophotometer*. https://resources.perkinelmer.com/lab-solutions/resources/docs/APP_MicronutrientsInSoilbyAA.pdf

Razaq, M., Zhang, P., Shen, H. L., & Salahuddin. (2017). Influence of nitrogen and phosphorous on the growth and root morphology of *Acer mono*. *PLOS One*, *12*(2), e0171321. https://doi.org/10.1371/journal.pone.0171321

Rich, N., & Bharti, A. (2015). Assessment of different types of in-vessel composters and its effect on stabilization of MSW compost. *International Research Journal of Engineering and Technology*, *2*(3), 805–813.

Risse, L. M., & Faucette, B. (2009). *Food waste composting: Institutional and industrial applications*. https://extension.uga.edu/publications/detail.html?number=B1189

Roe, N. E., Stoffella, P. J., & Bryan, H. H. (1993). Utilization of MSW compost and other organic mulches on commercial vegetable crops. *Compost*

Science and Utilization, *1*(3), 73–84. https://doi.org/10.1080/1065657X.1993.10757892

Saer, A., Lansing, S., Davitt, N. H., & Graves, R. E. (2013). Life cycle assessment of a food waste composting system: Environmental impact hotspots. *Journal of Cleaner Production*, *52*, 234–244. https://doi.org/10.1016/j.jclepro.2013.03.022

Shumo, M., Khamis, F., Tanga, C., Fiaboe, K., Subramanian, S., Ekesi, S., van Huis, A., & Borgemeister, C. (2019). Influence of temperature on selected life-history traits of black soldier fly (*Hermetia illucens*) reared on two common urban organic waste streams in Kenya. *Animals*, *9*(3), 79. https://doi.org/10.3390/ani9030079

Singh, A., & Kumari, K. (2019). An inclusive approach for organic waste treatment and valorization using black soldier fly larvae: A review. *Journal of Environmental Management*, *251*, 109569. https://doi.org/10.1016/j.jenvman.2019.109569

Singh, A. L., Jat, R. S., Chaudhari, V., Bariya, H., & Sharma, S. J. (2010). Toxicities and tolerance of mineral elements boron, cobalt, molybdenum and nickel in crop plants. *Plant Stress*, *4*(2), 31–56.

Singh, R. P., Singh, P., Ibrahim, M. H., & Hashim, R. (2011). Land application of sewage sludge: Physicochemical and microbial response. In D. Whitacre (Ed.), *Reviews of environmental contamination and toxicology* (Vol. 214, pp. 41–61). Springer. https://doi.org/10.1007/978-1-4614-0668-6_3

Shafique, I., Andleeb, S., Aftab, M. S., Naeem, F., Ali, S., Yahya, S., Ahmed, F., Tabasum, T., Sultan, T., Shahid, B., Khan, A. H., Islam, G. U., & Abbasi, W. A. (2021). Efficiency of cow dung based vermi-compost on seed germination and plant growth parameters of *Tagetes erectus* (Marigold). *Heliyon*, 7(1), e05895. https://doi.org/10.1016/j.heliyon.2020.e05895

St-Hilaire, S., Sheppard, C., Tomberlin, J. K., Irving, S., Newton, L., McGuire, M. A., Mosley, E. E., Hardy, R. W., & Sealey, W. (2007). Fly prepupae as a feedstuff for rainbow trout, *Oncorhynchus mykiss*. *Journal of the World Aquaculture Society*, *38*(1), 59–67. https://doi.org/10.1111/j.1749-7345.2006.00073.x

Thenabadu, M., Abeyweera, R., Jayasuriya, J., & Senanayake, N. S. (2015). Anaerobic digestion of food and market waste; waste characterization and biomethane potential: A case study in Sri Lanka. *SLEMA Journal*, *18*(2), 29-33. https://doi.org/10.4038/slemaj.v18i2.18

Tong, R. C., Whitehead, C. S., & Fawole, O. A. (2021). Effects of conventional and bokashi hydroponics on vegetative growth, yield and quality attributes of bell peppers. *Plants*, *10*(7), 1281. https://doi.org/10.3390/plants10071281

Toumi, R. (2017). *Design of a composting bin to convert aui ' s biomass to an organic feltilizer* [Unpublished Master's dissertation]. Al Khawayn University.

Ukoje, J., & Yusuf, R. (2013). Organic fertilizer: The underestimated component in agricultural transformation initiatives for sustainable small holder farming in Nigeria. *Ethiopian Journal of Environmental Studies and Management*, *6*(6), 794-801. https://doi.org/10.4314/ejesm.v6i6.10s

Vinoth Kumar, K., & Kasturi Bai, R. (2008). Solar greenhouse assisted biogas plant in hilly region - A field study. *Solar Energy*, *82*(10), 911–917. https://doi.org/10.1016/j.solener.2008.03.005

Waqas, M., Nizami, A. S., Aburiazaiza, A. S., Barakat, M. A., Rashid, M. I., & Ismail, I. M. I. (2018). Optimizing the process of food waste compost and valorizing its applications: A case study of Saudi Arabia. *Journal of Cleaner Production*, *176*, 426–438. https://doi.org/10.1016/j.jclepro.2017.12.165

Warman, P. R., Rodd, A. V., & Hicklenton, P. (2009). The effect of MSW compost and fertilizer on

extractable soil elements and the growth of winter squash in Nova Scotia. *Agriculture, Ecosystems and Environment*, *133*(1–2), 98–102. https://doi.org/10.1016/j.agee.2009.05.010

Xiao, Y., Zeng, G. M., Yang, Z. H., Shi, W. J., Huang, C., Fan, C. Z., & Xu, Z. Y. (2009). Continuous thermophilic composting (CTC) for rapid biodegradation and maturation of organic municipal solid waste. *Bioresource Technology*, *100*(20), 4807–4813. https://doi.org/10.1016/j.biortech.2009.05.013

Yang, L., Li, F., & Chu, H. (2014). Effects of food waste compost on soil microbial populations, tomato yield, and tomato quality. *Communications in Soil Science and Plant Analysis*, *45*(8), 1049–1058. https://doi.org/10.1080/00103624.2014.884103

Yoon, C. H., Jeon, S. H., Ha, Y. J., Kim, S. W., Bang, W. Y., Bang, K. H., Gal, S. W., Kim, I. S., & Cho, Y. S. (2020). Functional chemical components in *Protaetia brevitarsis* larvae: Impact of supplementary feeds. *Food Science of Animal Resources*, *40*(3), 461–473. https://doi.org/10.5851/kosfa.2020.e25

Zandonadi, D. B., Canellas, L. P., & Façanha, A. R. (2007). Indolacetic and humic acids induce lateral root development through a concerted plasmalemma and tonoplast H^+ pumps activation. *Planta*, *225*(6), 1583–1595. https://doi.org/10.1007/s00425-006-0454-2

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Effects of Three Rainfall Patterns on Soil Chemical Properties in Black Pepper Cultivation in a Hilly Topography

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ABSTRACT

This study was conducted to determine the effect of the rainfall pattern on cation nutrients in black pepper cultivation in a hilly topography. A field study was conducted in black pepper cultivation in a hilly topography around Bintulu, Sarawak, Malaysia, with a 26° slope during the Northeast monsoon in 2020. Six blocks were established on 462.56 m^2, with four subsequent soil samples (0-20 cm) collected per block after the rainfall. Soil samples were analysed using the standard pH, total organic carbon (TOC), soil texture, total nitrogen (TN), available phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), and manganese (Mn) in triplicate. Rainfall pattern (October < November > December) affects TN (300.31-1422.90 mg/kg) and K availability (13.54-166.68 mg/kg), especially during peak season in November 2020. Available P, Ca, Mg, Fe, and Mn exhibit minimum rainfall effect but are closely related to combined interaction with parent material and topography. Therefore, proper soil management, such as applying fertiliser using top dressing, foliar spray, and manure amendment, including growing cover crop, is recommended to improve nutrient availability.

Keywords: Black pepper, intensity, Northeast monsoon, rainfall pattern, slope, topography

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INTRODUCTION

Agricultural activity on >10^{o} is highly discouraged due to several reasons. First, farmers might have to bear high maintenance costs and encounter impracticability to sustain productivity while conducting cultivation activity at a steep area (Izzah & Wan Asrina, 2019; Nguyen & Pham, 2018; Paulus et al., 2011). Apart from that, cultivation activity on hilly topography with steep slopes faces the risk of soil loss, leading to infertility. Infertility problems in hilly areas, particularly among smallholders, are prone to happen since the farmers tend to neglect proper land establishment, such as constructing terraces, covering the soil surface with a cover crop, growing perennial with deep taproots to hold and stabilising soil particles. The neglect during farming might contribute to the soil system issues and reduce crop growth due to the lower amount of soil nutrients attributed to climatic conditions (Siswanto & Sule, 2019; Zhang et al., 2011).

Previous research conducted in Tikolod, Sabah confirmed that minimum conservation practices by the farmer on 30^{o} slope accelerate soil loss when cultivating ginger and hill paddy (Gregersen et al., 2003). A similar study conducted by Mohamad et al. (2018) also found that soil erosion has a significant correlation with slope conditions, as the bare soil surface with low vegetation coverage on steep slopes can speed up the erosion process. Next, slope orientation can affect the nutrient availability in the soil. It is reported that at least 23.60 mg/kg of P is available on tilt-up in the upper slope compared to the steeper area (Samndi & Mahmud, 2014). Several nutrient deficiency problems reported in previous literature involving N, K, and other essential elements eventually lead to lower yields (Izzah & Wan Asrina, 2019; Srinivasan et al., 2007). The effect of the insufficient nutrient is more prominent in a tropical country, especially East Malaysia, which receives higher rainfall up to 4,600 mm annually, and the circumstance facilitates the nutrient movement caused by a breakdown of soil aggregates (Sa'adi et al., 2017).

Rainfall can cause nutrients in soil colloids transported to another area, and this impact can be observed in coarse textural soil. Previous research emphasises that intense rainfall at 60 mm/h can affect N (Zanon et al., 2020), P, and K availability (Luo et al., 2013; Yaşar Korkanç & Dorum, 2019). Zanon et al. (2020) pointed out that increasing supplementation of N in liquid form from dairy manure in 0, 60, 120, and 180 m^{3}/ha/year sequence on sandy clay loam texture suffers N loss during field simulated rainfall study. A study by Luo et al. (2013) on a hillslope in China has documented a higher P loss when increasing slope gradient from 5^{o}, 10^{o}, and 15^{o} compared to normal practices when applying with inorganic fertiliser in 25-30 days before quantifying the loss with rainfall simulation. On another note, Yaşar Korkanç and Dorum (2019) highlighted a contradicting finding in which they stated that the lower rate of P loss during longer rainfall duration under simulated conditions is caused by the dilution effects with increasing runoff volume.

Meanwhile, it was found that K encounters the highest loss regardless of farmer practices due to higher ion mobility in soil. It was confirmed by Bertol et al. (2003) that K mobility is considered high due to ion affinity to soil colloidal activity, although the soil is covered with vegetation nearly 100%. Regardless of rainfall orientation, the impact can be more profound with the increasing intensity. For example, 60 mm/hr to 120 mm/hr might illustrate soil incapability to store or infiltrate excessive water, which causes rapid surface flow that carries soil particles, including nutrients. However, the nutrient loss is probably lower in clay compared to sandy particles, and it still depends on slope orientation, climatic, and environmental conditions. In several cases, the availability of ferrous ion (Fe^{2+}), aluminium ion (Al^{3+}), and hydrogen ion (H^{+}) increase significantly as the ammonium cation (NH_4^{+}) and potassium ion (K^{+}) are extensively removed (Meda et al., 2002; Mendes et al., 2016).

Black pepper needs a proper drainage system. Thus, growing the black pepper in a hilly topography is preferable, especially in East Malaysia, since it is surrounded by many hill areas previously left as secondary forest. The Malaysian Pepper Board (MPB) is a responsible agency that provides partially free training courses, workshops, and consultations at the field sites to raise farmer awareness in practising Good Agricultural Practices (GAP) in black pepper plantations. However, despite the effort from the agency, there are still some cases where a few farmers have suffered unsuccessful cultivation due to inappropriate farming practices and financial issues. For example, some farmers are still cultivating the black pepper using traditional practices such as using bare soil surface and non-living pole and constructing a minimum terrace (Izzah & Wan Asrina, 2018; Paulus et al., 2011). Subsequently, this might affect soil fertility and contributes to a prominent effect for the nutrient-demanding crop. Therefore, it is crucial to ensure sufficient nutrients during 24 months of growth for good canopy formation. Since black pepper is cultivated in hilly topography within tropical climatic conditions with minimum effort on soil management, lower nutrient availability during rainfall has been observed.

Hence, to understand the problem, the study was conducted to investigate the effect of rainfall patterns on cation nutrients in black pepper cultivation in a hilly topography.

MATERIALS AND METHODS

Study Area

This study was conducted during the Northeast monsoon season in 2020 with 4,600 mm of hilly topography annual precipitation with 26° in Bintulu, Sarawak, Malaysia (3° 0' N, 113° 1' E). This farm was a secondary forest converted into black pepper cultivation (2 ha) with invisible terracing or cover crops. However, for this specific study, only 0.06 ha was utilised. This site was cultivated with a mixed variety (132 vines) of *Uthirancotta*, locally known as *Indian*/*Thambi*, and a wild variety, namely *Rembai*, by the farmer on non-living

Table 1
Soil characteristics at the studied site

Variables	October	November	December
pH	4.44	4.49	4.36
Total organic carbon (%)	1.59	1.33	1.60
Texture		Sandy Loam	

poles from *Commersonia bartramia* bole with 2.1 m x 1.8 m spacing. The crop age was 16 months old and identified as at a young phase (<24 months) with the soil of Bekenu Series developed over mixed sedimentary rock with sand >76.00%, recognised as a sandy loam [United States Department of Agriculture (USDA) triangle, Table 1]. Soil mound was prepared for each vine and re-mounded yearly to restore soil loss, including amendment of 1 kg lime/mould as standard practices. The NPK green (15:15:15) was given at 100 g/vine twice in October and once in November and December. Due to several factors, such as market price instability and farm location in a remote area, the farmer has to limit agricultural inputs.

Soil Sampling and Data Processing

About six blocks were established with a size of 7.78 m x 9.80 m around 462.56 m^2 (23.60 m x 19.60 m), and four surface soil samples (0-20 cm) were collected per block after <12 hours of a rainfall event. An automatic rain gauge with a tipping bucket (Model WS2310CA, Misol, China) was installed at the top of the site. The samples were air-dried, grounded, and sieved through 2 mm mesh. Acid digestion procedure was performed using standard protocol by Food and Agriculture Organization (FAO) (2021) and analysed for total nitrogen (TN), while available phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), and manganese (Mn) extracted using Mehlich-1 method with a 1:5 ratio of weak double acid (Tan, 2005) were carried out in triplicate. The TN was determined using AutoAnalyser 3 (Model HR, SEAL Analytical, USA) by high-range working standard, while P using colourimetric, which was then determined through UV-Vis Spectrometer at 820 nm absorption (Model Lambda 25, Perkin Elmer, USA). Other samples were analysed using Atomic Absorption Spectrometer (Model AA800, Perkin Elmer, USA). Meanwhile, pH value from using water by one part of the soil and five parts of water (1:5), soil texture with hydrometer procedure, and total organic carbon (TOC) through dry combustion were investigated by following the method described by Tan (2010) and Sutherland (1998), respectively.

Data analysis was performed by calculating the mean value, including statistical analysis, using SAS ver. 9.4, and a significant difference was tested using Tukey's honest significant difference test (Tukey's HSD). The graph was plotted using SigmaPlot ver. 14.0 and arranged into three rainfall patterns (October, November, and December). Rainfall intensity was

calculated by dividing rainfall volume by duration.

RESULTS AND DISCUSSION

Figure 1 presents the data on TN in October 2020, which shows a decreasing trend until day nine (D9). The result escalated 2.5-folds on day 10 (D10) with a moderate decrease and gradually increased in November and December 2020, respectively. Lower intensity on day 22 (D22) displays an increasing TN (1104 mg/kg). Phosphorus availability exhibits unaffected (~3 mg/kg) throughout rainfall intensity, but it only indicates a higher concentration on day 19 (D19) (5.9 mg/kg). A sharp decrease in K was recorded on day 3 (D3) by almost 7-folds, and it slowly increased at the end of October ranged from 53 to 59 mg/kg. Potassium was continuously kept <34 mg/kg with increasing intensity from November until December. A similar trend was observed in Ca, where the declination of concentration has occurred from day 2 (D2) until day 22 (D22), although a slight increase was visible on day 3 (D3) (5 mg/kg), day 16 (D16) (4.4 mg/kg), and day 20 (D20) (4.2 mg/kg). Furthermore, Mg, Fe, and Mn exhibit a similar nutrient pattern apart from Fe and Mn availabilities were prominent in November on day 14 (D14) (400 mg/kg) and day 13 (D13) (3 mg/kg), respectively. From the analysis, rainfall intensity has a lower impact on Mg, Fe, and Mn than N and K. Meanwhile, Figure 2 shows nutrient availability according to rainfall patterns with significant value is presented in total N and available K. Meanwhile, available P, Ca, Mg, Fe, and Mn exhibit comparable value throughout three rainfall months.

This study revealed that TN in the black pepper farm was affected by constant rainfall and intensity between October and November (Figures 1 and 2). Total N decreased from day 1 (D1), day 9 (D9), day 11 (D11), and day 18 (D18), even when the cultivation was fertilised with NPK green on day 4 (D4), day 14 (D14), and day 18 (D18) at the rate of 100 g/vine. This situation underlines that most nutrients can be washed away when practising surface application, with a prominent effect shown in day 5 (D5) by 2-folds lower than day 4 (D4). A rapid TN movement is accelerated by steep topography (26^{o}) with a bare surface and is dominated by a sand particle (Arunrat et al., 2020). Moreover, high rainfall significantly lowers the TN availability in soil due to incapable soil particles holding or retaining the ion, leading to repetitive N application (two times a month). N in soil may be available to the crop in two different forms: nitrogen-nitrate ($N\text{-}NO_3^-$) and nitrogen-ammonium ($N\text{-}NH_4^+$). These two forms are easily lost in the soil system, especially in nitrate (NO_3) that is heavily available during the wet season. It is easily transported due to rapid nitrification (Gu & Riley, 2010; Hagedorn et al., 1997).

Moreover, a higher coarse particle may yield <80 cmol/kg of cation exchange capacity (CEC) compared to anion exchange capacity (AEC) for Bekenu soil, which is lower than the CEC value, and the effects can be seen on lower ion adsorption on soil colloidal. This continuous TN decrease

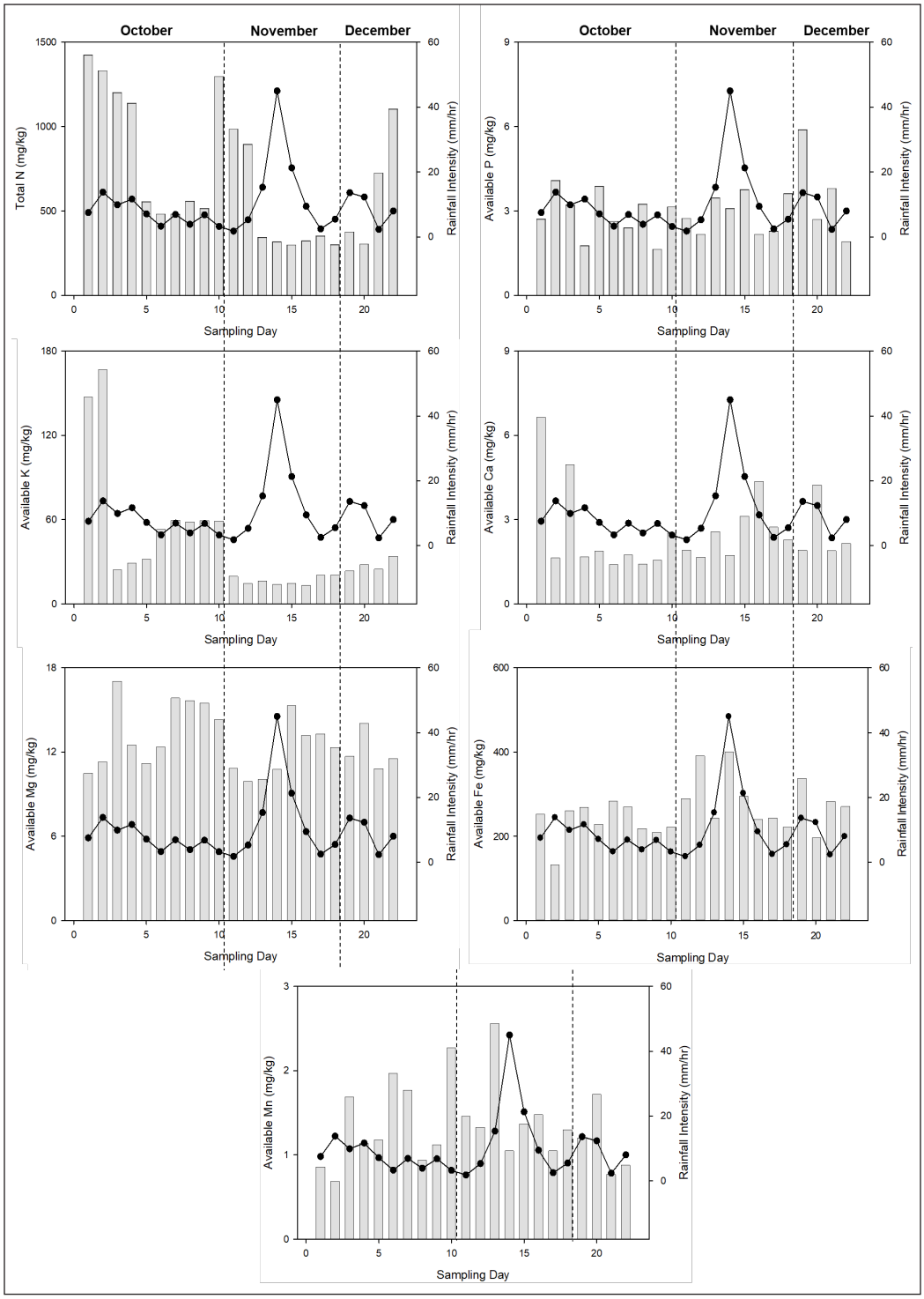

Figure 1. Nutrient concentration in the soil after three rainfall patterns (October, November, and December 2020) with 22 sampling days (D)

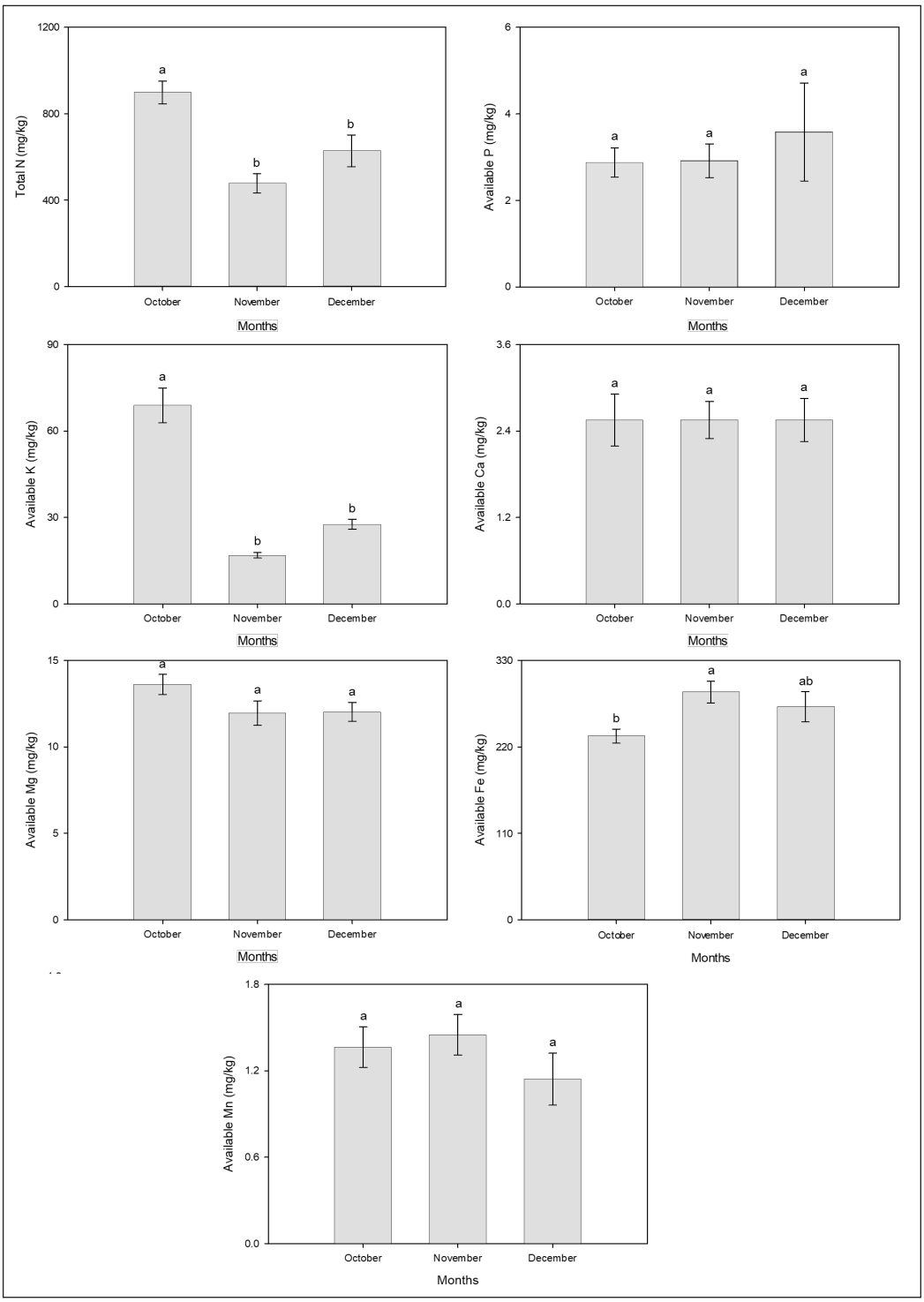

Figure 2. Average nutrient concentration in October, November, and December. Different letters indicate statistically significant differences at $p = 0.05$ using Tukey's HSD with standard error

can impact nutrient uptake in black pepper, which might endanger catkin growth and berries yield (Izzah & Wan Asrina, 2019; Sharangi, 2011). Accordingly, lower TN on black pepper affects yield, plant height, and weight (Sharangi, 2011; Sivaraman et al., 1999). These results offer compelling evidence in November by representing lower TN on peak rainfall than on early October and end of December rainfall events (Figure 2). In addition, the amendment of manure or leguminous crops can increase TN availability by enhancing the mineralisation rate naturally, and sustainability will protect the soil surface (Hua et al., 2020; Saka et al., 2017; Stagnari et al., 2017). Sharangi (2011) proved that a 25% farmyard manure mixture with 75% urea has a promising effect on the black pepper crop.

On the contrary, P continues to show lower availability regardless of rainfall pattern and after NPK fertilisation. The result is subjected to low inheritance by parent material developed in mixed sedimentary rocks (Bekenu Series) (Emmanuel et al., 2020; Yokoyama et al., 2018). Bekenu Series is well-drained soil and extensively used in agriculture with low fertility. Paramananthan (2000) emphasised that proper fertilisation and land soil conservation techniques can sustain the soil series for extensive cultivation. However, this soil series may exhibit fertiliser supplementation that can absorb aluminium/iron (Al/Fe) oxides and hydroxides in extremely acidic forms, forming various complexes. Under those circumstances, P will become unavailable for black pepper uptake, and the best way to improve P solubility is through the lime application (Opala, 2017; Penn & Camberato, 2019; Simonsson et al., 2018). Active P adsorption is common due to the fixation mentioned previously. Therefore, alternative management should enhance P availability, showing a promising outcome by using certain animal manure or selected compost (Shamshuddin et al., 2011). During this study, about 400 g chicken manure (average 8 g/kg total P) was placed three hours before rainfall on day 4 (D4) to increase adsorption sites effectively; however, no effect on P availability was observed. This outcome might be influenced by lower P application than the recommended concentration at 27.5 g/vine/year (Srinivasan et al., 2007). Moreover, lower P in this study could potentially appear through repetitive rainfall events. In addition, the hilly topography provides a minimum effect on P availability as it distributes continual through rainfall patterns.

Notably, K was affected by peak rainfall in November 2020 and exhibited lower availability attributed to solubility where excess water could displace ions to deeper soil layers or fate in a runoff (Figure 1 and Figure 2). Deficient K can contribute to nutrient hunger, and alternatively, the foliar spray was performed on day 13 (D13) and day 18 (D18). Unfortunately, the application failed to improve K uptake, which is vital for fruit development, and it remained depleted until the end of this study. Concerns have arisen on the amount of NPK fertiliser given in October, which is 200 g/vine (100

g/vine per application) because it could cause depletion of K resources since the actual amount recommended by MPB is 500 g/vine (Paulus et al., 2011). Previous studies summarised that declination on K concentration in this study could be subjected to the combined effects of conditions such as acidic soil, high coarse soil fraction, high rainfall intensity, steeper slope, and competition between cations for the adsorption sites, which less favourable for K ion compared to other divalent and trivalent cation nutrients (Izzah & Wan Asrina, 2018; Mendes et al., 2016; Tan, 2010).

Ca, Mg, Fe, and Mn was also observed according to the rainfall patterns in which Ca displayed increasing intensity in November, although 4 kg lime was applied on the soil surface on day 4 (D4). Liming process increased soil pH from 4.44 to 4.49. However, it decreased again to 4.36 in December, representing lower Ca availability due to the dilution effect replacing H^+. Acidic conditions, including frequent rainfall and intensity, will intensify leaching occurrence, and liming will displace aluminium ion (Al^{3+}) and manganese ion (Mn^{2+}) from soil colloids then precipitate in soil solution (Goulding, 2016; Hess et al., 2020; Yao et al., 2021). Additionally, liming on sandy soil has proven the low mobility, minimum reaction, and lack of soluble by-product (anions) of reaction with acidity (Meda et al., 2002; Nunes et al., 2019), evident by small pH increments.

Meanwhile, Mg showed persistent interveinal chlorosis, which could be noticeable on older leaves in this study, representing chlorophyll degradation. These results extend the knowledge of the possibility antagonism effect occurs in the sandy due to weakly bounded Mg that imply high mobility in soil, especially in the wet season in the steep areas (Senbayram et al., 2015; Yan & Hou, 2018). Besides, Fe was unaffected by rainfall patterns in this study. Nevertheless, its solubility was regulated by lower soil pH (4.36-4.49). Therefore, the result on Mn is probably influenced by the proper drainage system and sufficient aeration caused by the nature of the soil in the research site that inherits lower Mn caused by a weak bond with soil colloids (Siskawardani et al., 2016).

CONCLUSION

The findings of this study indicate that TN and K are highly affected by rainfall patterns, especially in November, which is during the peak monsoon season, compared to P, Ca, Mg, Fe, and Mn, with combined interaction on parent material and topography. Fertiliser amendment as a top dressing, foliar spray, manure supplementation, and growing legume cover crop is recommended to improve nutrient availability. This finding might not represent a well-established site with proper soil management, but it might provide a picture when minimum consideration is taken. This study will serve as a foundation for future studies to the extent of the research on the direct implication of cultivating crops on a steeper slope.

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REFERENCES

Arunrat, N., Kongsurakan, P., Sereenonchai, S., & Hatano, R. (2020). Soil organic carbon in sandy paddy fields of Northeast Thailand: A review. *Agronomy*, *10*(8), 1061. https://doi.org/10.3390/agronomy10081061

Bertol, I., Mello, E. L., Guadagnin, J. C., Zaparolli, A. L. V., & Carrafa, M. R. (2003). Nutrient losses by water erosion. *Scientia Agricola*, *60*(3), 581-586. https://doi.org/10.1590/S0103-90162003000300025

Emmanuel, H., Samuel, O. A., Kwame, A. F., Kofi, A., Thomas, A., Muhammed, A., Joshua, Y. A., & John, B. (2020). Phosphorus sorption in tropical soils. *AIMS Agriculture and Food*, *5*(4), 599-616. https://doi.org/10.3934/agrfood.2020.4.599

Food and Agriculture Organization. (2021). *Standard operating procedure for soil nitrogen - Kjeldahl method*. FAO.

Goulding, K. W. T. (2016). Soil acidification and the importance of liming agricultural soils with particular reference to the United Kingdom. *Soil Use and Management*, *32*(3), 390-399. https://doi.org/10.1111/sum.12270

Gregersen, B., Aalbæk, J., Lauridsen, P., Kaas, M., & Lopdrup, U. (2003). *Land use and soil erosion in Tikolod, Sabah, Malaysia*. https://www.researchgate.net/publication/253679071_LAND_USE_AND_SOIL_EROSION_IN_TIKOLOD_SABAH_MALAYSIA

Gu, C., & Riley, W. J. (2010). Combined effects of short term rainfall patterns and soil texture on soil nitrogen cycling — A modeling analysis. *Journal of Contaminant Hydrology*, *112*(1), 141-154. https://doi.org/10.1016/j.jconhyd.2009.12.003

Hagedorn, F., Steiner, K. G., Sekayange, L., & Zech, W. (1997). Effect of rainfall pattern on nitrogen mineralization and leaching in a green manure experiment in south Rwanda. *Plant and Soil*, *195*(2), 365-375. https://doi.org/10.1023/A:1004266205502

Hess, L. J. T., Hinckley, E. L. S., Robertson, G. P., & Matson, P. A. (2020). Rainfall intensification increases nitrate leaching from tilled but not no-till cropping systems in the U. S. Midwest. *Agriculture, Ecosystems and Environment*, *290*, 106747. https://doi.org/10.1016/j.agee.2019.106747

Hua, W., Luo, P., An, N., Cai, F., Zhang, S., Chen, K., Yang, J., & Han, X. (2020). Manure application increased crop yields by promoting nitrogen use efficiency in the soils of 40-year soybean-maize rotation. *Scientific Reports*, *10*(1), 14882. https://doi.org/10.1038/s41598-020-71932-9

Izzah, A. H., & Wan Asrina, W. Y. (2018). Spatial variability of nitrogen, phosphorus, and potassium using geospatial techniques on black pepper farms. *Azarian Journal of Agriculture*, *5*(3), 76-85.

Izzah, A. H., & Wan Asrina, W. Y. (2019). Black pepper in Malaysia: An overview and future prospects. *Agricultural Reviews*, *40*(4), 296-302. https://doi.org/10.18805/ag.R-129

Luo, C., Gao, Y., Zhu, B., & Wang, T. (2013). Sprinkler-based rainfall simulation experiments to assess nitrogen and phosphorus losses from a hillslope cropland of purple soil in China. *Sustainability of Water Quality and Ecology*, *1-2*, 40-47. https://doi.org/10.1016/j.swaqe.2014.03.001

Meda, R., Pavan, A., Cassiolato, E., & Miyazawa, M. (2002). Dolomite lime's reaction applied on the surface of a sandy soil of the Northwest Paraná, Brazil. *Brazilian Archives of Biology and Technology*, *45*(2), 219-222. https://doi.org/10.1590/S1516-89132002000200014

Mendes, W. C., Alves Júnior, J., Cunha, P. C. R., Silva, A. R., Evangelista, A. W. P., & Casaroli, D. (2016). Potassium leaching in different soils as a function of irrigation depths. *Revista Brasileira de Engenharia Agrícola e Ambiental*, *20*(11), 972-977. https://doi.org/10.1590/1807-1929/agriambi.v20n11p972-977

Mohamad, N., Jamal, M., Annammala, K., Yusop, Z., Alias, N., & Sugumaran, D. (2018). Impact of forest conversion to agricultural plantation on soil erosion. In *MATEC Web of Conferences* (Vol. 250, p. 04004). EDP Sciences. https://doi.org/10.1051/matecconf/201825004004

Nguyen, X. H., & Pham, A. H. (2018). Assessing soil erosion by agricultural and forestry production and proposing solutions to mitigate: A case study in Son La Province, Vietnam. *Applied and Environmental Soil Science*, *2018*, 2397265. https://doi.org/10.1155/2018/2397265

Nunes, M. R., Denardin, J. E., Vaz, C. M. P., Karlen, D. L., & Cambardella, C. A. (2019). Lime movement through highly weathered soil profiles. *Environmental Research Communications*, *1*(11), 115002. https://doi.org/10.1088/2515-7620/ab4eba

Opala, P. A. (2017). Influence of lime and phosphorus application rates on growth of maize in an acid soil. *Advances in Agriculture*, *2017*, 7083206. https://doi.org/10.1155/2017/7083206

Paramananthan, S. (2000). *Soils of Malaysia: Their characteristics and identification*. Academy of Sciences.

Paulus, A. D., Sim, S. L., Eng, L., Megir, G., & Rosmah, J. (2011). *Pepper production technology in Malaysia*. Malaysian Pepper Board and Department of Agriculture Sarawak.

Penn, C., & Camberato, J. (2019). A critical review on soil chemical processes that control how soil pH affects phosphorus availability to plants. *Agriculture*, *9*(6), 120. https://doi.org/10.3390/agriculture9060120

Sa'adi, Z., Shahid, S., Chung, E. S., & Ismail, T. (2017). Projection of spatial and temporal changes of rainfall in Sarawak of Borneo Island using statistical downscaling of CMIP5 models. *Atmospheric Research*, *197*, 446-460. https://doi.org/10.1016/j.atmosres.2017.08.002

Saka, H. A., Azeez, J. O., Odedina, J. N., & Akinsete, S. J. (2017). Dynamics of soil nitrogen availability indices in a sandy clay loam soil amended with animal manures. *International Journal of Recycling of Organic Waste in Agriculture*, *6*(2), 167-178. https://doi.org/10.1007/s40093-017-0165-7

Samndi, M. A., & Mahmud, A. T. (2014). Distribution of potassium forms along a hillslope positions of newer basalt on the Jos Plateau Nigeria. *International Journal of Soil Science*, *9*(3), 90-100. https://doi.org/10.3923/ijss.2014.90.100

Senbayram, M., Gransee, A., Wahle, V., & Thiel, H. (2015). Role of magnesium fertilisers in agriculture: Plant–soil continuum. *Crop and Pasture Science*, *66*(12), 1219-1229. https://doi.org/10.1071/CP15104

Shamshuddin, J., Anda, M., Ishak, C., & Omar, S. (2011). Growth of cocoa planted on highly weathered soil as affected by application of basalt and/or compost. *Communications in Soil Science and Plant Analysis*, *42*(22), 2751-2766. https://doi.org/10.1080/00103624.2011.622822

Sharangi, A. (2011). Performance of rooted cuttings of black pepper (*Piper nigrum* L.) with organic substitution of nitrogen. *International Journal of Agricultural Research*, *6*(9), 673-681. https://doi.org/10.3923/ijar.2011.673.681

Simonsson, M., Östlund, A., Renfjäll, L., Sigtryggsson, C., Börjesson, G., & Kätterer, T. (2018). Pools and solubility of soil phosphorus as affected by liming in long-term agricultural field experiments. *Geoderma*, *315*, 208-219. https://doi.org/10.1016/j.geoderma.2017.11.019

Siskawardani, D. D., Onthong, J., Khawmee, K., & Poonpakdee, C. (2016). Manganese status in upland and lowland rubber-growing soils in Songkhla province, southern Thailand. *Agriculture and Natural Resources*, *50*(4), 321-325. https://doi.org/10.1016/j.anres.2016.01.005

Siswanto, S. Y., & Sule, M. I. S. (2019). The impact of slope steepness and land use type on soil properties in Cirandu sub-sub catchment, Citarum Watershed. In *IOP Conference Series: Earth and Environmental Science* (Vol. 393, No. 1, p. 012059). IOP Publishing. https://doi.org/10.1088/1755-1315/393/1/012059

Sivaraman, K., Kandiannan, K., Peter, K. V., & Thankamani, C. K. (1999). Agronomy of black pepper (*Piper nigrum* L.) - A review. *Spices and Aromatic Crops*, *8*(1), 1-18.

Srinivasan, V., Dinesh, R., Hamza, S., & Parthasarathy, V. A. (2007). Nutrient management in black pepper (*Piper nigrum* L.). *Nutrition and Natural Resources*, *2*(62). https://doi.org/10.1079/PAVSNNR20072062

Stagnari, F., Maggio, A., Galieni, A., & Pisante, M. (2017). Multiple benefits of legumes for agriculture sustainability: An overview. *Chemical and Biological Technologies in Agriculture*, *4*(1), 2. https://doi.org/10.1186/s40538-016-0085-1

Sutherland, R. A. (1998). Loss-on-ignition estimates of organic matter and relationships to organic carbon in fluvial bed sediments. *Hydrobiologia*, *389*(1-3), 153-167. https://doi.org/10.1023/A:1003570219018

Tan, K. H. (2005). *Soil sampling, preparation, and analysis*. Taylor & Francis.

Tan, K. H. (2010). *Principles of soil chemistry*. Taylor & Francis.

Yan, B., & Hou, Y. (2018). Effect of soil magnesium on plants: A review. In *IOP Conference Series: Earth and Environmental Science* (Vol. 170, No. 2, p. 022168). IOP Publishing. https://doi.org/10.1088/1755-1315/170/2/022168

Yao, Y., Dai, Q., Gao, R., Gan, Y., & Yi, X. (2021). Effects of rainfall intensity on runoff and nutrient loss of gently sloping farmland in a karst area of SW China. *PLOS One*, *16*(3), e0246505. https://doi.org/10.1371/journal.pone.0246505

Yaşar Korkanç, S., & Dorum, G. (2019). The nutrient and carbon losses of soils from different land cover systems under simulated rainfall conditions. *CATENA*, *172*, 203-211. https://doi.org/10.1016/j.catena.2018.08.033

Yokoyama, D., Mori, T., Wagai, R., Hiradate, S., & Kitayama, K. (2018). Characteristics of phosphorus fractions in the soils derived from sedimentary and serpentinite rocks in lowland tropical rain forests, Borneo. *Soil Science and Plant Nutrition*, *64*(2), 218-221. https://doi.org/10.1080/00380768.2017.1421018

Zanon, J. A., Favaretto, N., Democh Goularte, G., Dieckow, J., & Barth, G. (2020). Manure application at long-term in no-till: Effects on runoff, sediment and nutrients losses in high rainfall events. *Agricultural Water Management*, *228*, 105908. https://doi.org/10.1016/j.agwat.2019.105908

Zhang, S., Zhang, X., Huffman, T., Liu, X., & Yang, J. (2011). Influence of topography and land management on soil nutrients variability in Northeast China. *Nutrient Cycling in Agroecosystems*, *89*, 427-438. https://doi.org/10.1007/s10705-010-9406-0

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Potential of Open-Air Hydroponic System in Producing Highly Nutritional Composition Maize Fodder for Goat Farming

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ABSTRACT

This study aimed to identify the chemical composition of hydroponic maize fodder (HMF) from two varieties of maize grains (popcorn and feed corn). A completely randomized design (CRD) with three replications was used in which popcorn was irrigated with clean tap water (T1) and nutrient solution (T2); feed corn irrigated with clean tap water (T3) and nutrient solution (T4). Seven-days green fodders were sampled for chemical analysis. The crude protein (CP) content was the highest at 7.48% in T4 compared to popcorn (P<0.05) and T3 (P>0.05). Treatment 3 showed the highest dry matter content as 94.42% (P>0.05) and organic matter content observed as 98.29% especially compared with T1 (P<0.05). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents of feed corn were insignificantly different, but T3 was observed as 67.66% and 41.55%, respectively, which were higher than T1 (P<0.05). Although feed corn had better nutritional value than popcorn, popcorn showed a 7% higher germination rate than

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feed corn (P<0.05). As a result, the total yield of 1.5 kg hydroponic maize fodder per kg feed corn was lower than popcorn (2.5 kg per kg grains). Hence, popcorn was used to grow HMF with the open-air hydroponic system. HMF showed better feed nutritive composition than conventionally planted Napier grass. A lower concentration of indigestible fiber (P<0.05) and a higher concentration of crude protein (12.28%) was observed in HMF compared to CP in Napier grass (7.22%) (P<0.05). In conclusion, the open-air hydroponics system can be an alternative method among smallholders by replacing conventionally planted fodder.

Keywords: Feed corn, Napier grass, nutrient solution, open-air hydroponic system, popcorn

INTRODUCTION

Most livestock farmers in Malaysia are smallholders prone to providing low quality and quantity of feed, which unavoidably leads to low productivity. There is less concern for nutritional management due to limited time, cost constraints, and labor issues (Ghani et al., 2017). The challenges faced by smallholder farmers include the reduced pasture area and unavailability of land for fodder cultivation, forcing them to rely more on concentrates than roughage, which increases production costs. Hence, a proper feeding regime is importantly provided and implemented for the requirement of goats to support optimal farm production and be economical.

Automated and mechanized intensive production systems such as hydroponics technology were recently introduced as future alternative growing fodder for livestock. The hydroponic technology embodies the concept of 'owner-operator', which means a smallholder typically runs the farm's day-to-day operations. Hence, much research has implemented the suggestion of growing different fodder crops through hydroponic technology, including barley (Fazaeli et al., 2012); oats, wheat (Kantale et al., 2017); sorghum, alfalfa, cowpea (Al-Karaki & Al-Hashimi, 2012); and maize (Naik et al., 2014). This system has been highlighted as a cost-effective method due to its ability to increase fodder production with minimal land or space, water, and labor (Al-Karaki & Al-Hashimi, 2012). In addition, high digestibility and crude protein contents make hydroponic fodder suitable for ruminants (Naik et al., 2014).

In Malaysia, a commercial hydroponic fodder system hailed as "landless fodder production" was introduced by a foreign company. The system's performance was conducted at Universiti Putra Malaysia (UPM) in 1996 to produce feed for animals that required high energy feed, such as horses and ruminants (Abdullah, 2001). The barley grass was hydroponically germinated and grown in this imported environmentally controlled cabin. However, the commercial marketability of the imported system is directed mainly at high-value animals, such as equines and livestock producing high-value products, including high producing

dairy cows, milk goats, and deer (Francis et al., 2018; Naik et al., 2015), resulting in the limited literature published that solely address hydroponic fodder for goat. Although research in hydroponic fodder has increased, further research is needed to develop low-cost devices for hydroponic fodder production using locally available materials on different livestock categories.

In this study, maize grains were the choice of hydroponics fodder production due to their easy availability, good biomass production, and quick growing habit. As the productivity of the livestock in a farm is highly dependent on the nutritive value of the fodder, this study was conducted to evaluate the nutritive value of maize grains to produce hydroponics fodder.

METHODOLOGY

Selection of Maize Grains for Hydroponic Maize Fodder

The preliminary study used twelve polyethylene trays sized 44 cm width x 34 cm length x 5 cm depth with holes at the base to allow excess water drainage from irrigation based on a completely randomized design. Two varieties of maize grains were purchased from different sources: popcorn from Kinghin Sdn. Bhd. (baking ingredients and packaging supplier company located at Melaka, Malaysia) and feed corn from Ng Sing Heng Sdn. Bhd. (store wholesaler located at Jalan Lorong Pandan, Melaka, Malaysia). Both grains were cleaned, treated, and sprouted into HMF described by Morgan et al. (as cited in Naik et al., 2015, p. 3).

There were two different irrigation treatments for each variety of maize grains. It led to the formation of four treatments groups: two groups were popcorn irrigated with tap water, which is free from additives (T1) and nutrient solution (T2); another two groups were feed corn irrigated with clean tap water (T3) and nutrient solution (T4). Treatment groups T2 and T4 were irrigated with tap water for the first three days, and the nutrient solution was used from day 4 to day 7 (Morgan et al. as cited in Naik et al., 2015, p. 3). The nutrient solution was prepared using the chemically based hydroponic solutions purchased from Nursery Petani Kota, Selangor, Malaysia. All maize grains were manually irrigated using a plastic sprayer (2 liters) hourly from 0700 to 1900. The growth of hydroponic maize fodders (HMF) sprouted from two varieties of maize grains was summarized in Table 1.

Table 1

The seven-day growth of popcorn and feed corn irrigated with clean water and nutrient solution

Growth period (Days)	Treatment groups			
	T1	T2	T3	T4
1				
2				
3				
4				
5				
6				
7				

Note. T1 = Popcorn irrigated with tap water; T2 = Popcorn irrigated with the nutrient solution; T3 = Feed corn irrigated with tap water; T4 = Feed corn irrigated with the nutrient solution

Germination Test. A paper towel was placed in a plastic tray sized 44 cm width x 34 cm length x 5 cm depth and moistened with water until it was thoroughly damp. A total of 100 seeds were placed in rows on the towel. Another paper towel was loosely placed onto the first paper towel and moistened, leaving the seeds sandwiched between the two towels. The substrates were kept closed and placed in an area out of direct sunlight that stays at room temperature (International Seed Testing Association [ISTA], 2016). After the seven-day germination period, the paper towel at the top was removed, and the seedlings that had shoots longer than 1½ inches (and at least one firm root) were counted as viable seeds in the germination rate. The germination rate was determined through the formula below:

$$\text{Germination rate (\%)} = \frac{\text{Number of germinating seeds}}{\text{Total number of seeds tested}} \times 100$$

Conversion Ratio. The biomass production conversion ratio was based on fresh fodder produced per unit of seed used (Peer & Leeson, 1985).

$$\text{Conversion ratio} = \frac{\text{Fresh weight of green fodder (kg)}}{\text{Fresh weight of seed used (kg)}}$$

Open-Air Hydroponic System

A used cattle pen-sized 35 ft width and 27 ft length were sheltered with polyethylene sunshade netting to protect green fodders from heat before installing the open-air hydroponic fodder growing system (Figure 1). The cattle pen was also fenced with galvanized welded iron wire mesh (bottom) and zinc sheet (top) as a protective measurement from rodents. Two water tanks were used as the water storage system: Tank 1 was fed by clean tap water, and Tank 2 was fed by water from an outlet at the bottom of Tank 1 (Figure 2). Two centrifugal pumps (Model CPM-158, AC 200 - 240 V ~50 Hz, Victa™, Malaysia) connected to Tank 2 pumped water to a filter and then to the water channels (Figure 3). Polyethylene pipes sized 25 mm were used as water-conducting networks from tanks, centrifugal pumps, filters, and water channels. The water channels drained water into eleven water ducts composed of polyethylene pipes sized 16 mm. The water flow of five ducts was drained from Pump 1 and another six ducts from Pump 2. The water ducts were spaced 2 ft apart, and plastic misting spray nozzles were placed at 2-ft intervals along these water ducts (Figure 4).

Polyethylene trays sized 52.0 cm length × 32.0 cm width × 2.5 cm height with holes at the base to allow excess water drainage

from irrigation was used. The HMF was grown for seven days, as shown in Figure 5. All grains were washed and soaked for four hours in tap water (Morgan et al., cited in Naik et al., 2015, p. 3). After 24 hours of germination covered with wet cloths, sprouted grains were spread on the hydroponic trays at a rate of 1 kg per tray. Seven days were considered to evaluate the trays produced quality hydroponic fodder (Naik et al., 2015). No supplemental light was used for this hydroponics system due to the high availability of sunlight that Malaysia experiences per year. Photosynthesis is not dominant for the seedling's metabolism until the end of day 5, when the chloroplasts are activated (Sneath & Mclntosh, 2003).

Figure 1. A used cattle pen was transformed into the open-air hydroponic green fodder growing system

Figure 2. A two-tanks system was applied to ensure a continuous supply of water

Figure 3. The water-conducting network from Tank 2, two centrifugal pumps and filters

Figure 4. Distance between water ducts, which were smaller diameter than water channels (left), and the placement of misting spray nozzles along the water ducts (right)

Figure 5. The hydroponic maize fodder, which was grown for seven days

Napier Grass

The conventional green fodder, Napier grass (*Penniseutm puprpureum*), was already planted at the farm and fertilized with goat manure. The grass was harvested daily from the pasture (8–10 weeks). Napier grass was harvested at about 1.0–1.5 m height and then chopped to a size of about 3–5 cm using a fuel-driven chopper machine.

Chemical and Statistical Analysis

The fresh fodders were weighed and sampled to measure the fresh yield and estimate the conversion ratio. The representative samples (200 g each) of each green fodder were oven-dried at 60°C for 48 hours, ground to pass a 1-mm mesh screen sieve, and stored for chemical analysis. The nutritive values were determined by near-infrared spectroscopy (NIRS) (Model DS2500, FOSS, Denmark) with additional calibration from the fodder samples analyzed using standard laboratory procedure. The amount of crude protein (CP) was measured and calculated (N x 6.25) (Association of Official Analytical Chemists [AOAC], 1990). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using FiberCap 2023 System (FOSS, Denmark) (International Organization for Standardization [ISO], 2008).

The chemical compositions between two types of green fodders were compared using the independent sample *t*-test. In addition, the nutritive value of hydroponically sprouted maize fodder from two varieties of maize grains (popcorn and feed corn) was statistically analyzed using a one-way analysis of variance (ANOVA) with a significant level $P<0.05$. Finally, the significant differences between the mean were determined using the least significant difference (LSD) test. All the values from the results were stated as mean ± standard error of means (SEM).

RESULTS AND DISCUSSION

Selection of Maize Grains for Hydroponic Maize Fodder

All HMF were harvested on day seven and chemically analyzed (Table 2). There was no statistical difference in dry matter (DM) content between treatment groups. The popcorn (T1) and feed corn (T3) irrigated with clean tap water showed slight elevation with no significant difference of DM content as compared to popcorn (T2) and feed corn (T4) irrigated with nutrient solution. However, the CP content of the sprouted feed corn was higher than that of popcorn, especially the feed corn irrigated with nutrient solution (7.48%). Meanwhile, the organic matter (OM) content ranged from the least (93.71%) in T1 to the highest (98.29%) in T3 ($P<0.05$). At the same time, the lowest fibers content indicated by NDF and ADF was recorded from feed corn (T4) irrigated with the nutrient solution with an average of 67.40% and 40.95%, respectively. A germination test (ISTA, 2016) of both maize grains using tap water irrigation (T1 and T3) was recorded in Table 3. Popcorn showed a higher germination rate for the seven-day germination period, which was 7% higher than feed corn (84.3 %) ($P<0.05$).

By the end of the seven-day germination cycle, the hydroponics green fodder had grown into a mat of 15–23 cm in height, with germinated seeds embedded in white roots and green shoots. For the same sprouting time, the forage mat had reached a height of 20 to 30 cm, depending on the grain types (Naik et al., 2015). The yields ranged from 3.5 to 6.0 folds on a fresh basis with DM content of 11–14% are expected for hydroponics maize fodder or hydroponically sprouted maize grains (Naik et al., 2014). Similarly, there are reports of a 3.7–4.5 times increase in the fresh weight with DM content of 19.26–19.7% in 6–7 days of hydroponics barley fodder (Dung et al., 2010; Fazaeli et al., 2011). Nevertheless, an earlier study obtained a ratio of 2.76 to 3 kg green fodder per kg of barley seed (Al-Ajmi et al., 2009). There are several affecting factors of the biomass production conversion ratio, including management, amount, and frequency of irrigation, usage of nutrient solution, climate (temperature and humidity), density, and position of lights, type, and quality of the grain, a load of seeds on each tray and growth period (Hubballi et al., 2010; Molla & Birhan, 2010; Trubey et al., 1969).

Table 2

Chemical composition of two varieties of maize grains irrigated with tap water and nutrient solutions

Parameters (% of DM)	Treatment groups			
	T1	T2	T3	T4
	n = 12	n = 12	n = 12	n = 12
DM	92.03 ± 0.13	91.27 ± 1.09	94.42 ± 0.13	91.59 ± 3.85
CP	7.10 ± 0.01^{a}	7.37 ± 0.01^{ab}	7.42 ± 0.00^{bc}	7.48 ± 0.01^{c}
OM	93.71 ± 0.99^{a}	96.69 ± 0.48^{ab}	98.29 ± 0.13^{b}	97.24 ± 0.78^{ab}
NDF	70.62 ± 0.08^{c}	69.70 ± 0.11^{bc}	67.66 ± 00.05^{ab}	67.40 ± 0.06^{a}
ADF	42.30 ± 0.04^{b}	41.56 ± 0.13^{a}	41.55 ± 0.04^{a}	40.95 ± 0.07^{a}

Note. T1 = Popcorn irrigated with tap water; T2 = Popcorn irrigated with the nutrient solution; T3 = Feed corn irrigated with tap water; T4 = Feed corn irrigated with the nutrient solution; DM = Dry matter; CP = Crude protein; OM = Organic matter; NDF = Neutral detergent fiber; ADF = Acid detergent fiber; n = Number of samples; All analyses are mean ± standard error of means (SEM); ^{a,b,c} = Means with different superscripts within a row are significantly different ($P<0.05$)

Table 3

Comparisons of germination rate (%) of popcorn and feed corn irrigated with tap water

Growth period (days)	Popcorn	Feed corn
	n = 100	n = 100
1	84.7 ± 0.88[a]	57.7 ± 1.20[b]
2	88.3 ± 0.88[a]	74.0 ± 1.15[b]
3	89.7 ± 0.67[a]	80.3 ± 0.88[b]
4	89.7 ± 1.20[a]	83.7 ± 0.33[b]
5	91.7 ± 0.67[a]	83.7 ± 1.20[b]
6	91.7 ± 0.88[a]	84.0 ± 0.58[b]
7	91.7 ± 0.33[a]	84.3 ± 0.88[b]

Note. n = Number of samples; All analyses are mean ± standard error of means (SEM); Means with different superscript letters in a row are significantly different ($P< 0.05$)

The popcorn (T1) and feed corn (T3) irrigated with tap water expressed an insignificant higher DM content compared to the other HMF irrigated with nutrient solution (T2 and T4). Dung et al. (2010) reported a similar finding that the use of nutrient solution lowers the DM loss, which may be due to the absorption of minerals, thus increasing the ash content and the final weight of the hydroponics fodder. Besides, the DM loss could be due to the significant water uptake during germination and vegetation (Naik et al., 2015). The more significant dry weight losses and increasing trend in protein content could be attributed to the loss of dry weight, particularly carbohydrates, via respiration during germination. In a seven-day sprout, scarcity of time for significant DM accumulation due to photosynthesis begins around day 5 when the chloroplasts are activated (Dung et al., 2010). Thus, the increased structural carbohydrate in sprout green forage would change the quantity of the other nutrients such as protein in a higher percentage.

However, the present study observed that the CP content of treatment groups T2 and T4 is significantly higher than the other HMF irrigated with tap water (T1 and T3). It may be due to the absorption of nitrates of nutrient solution correlates with the metabolism of nitrogenous compounds from storage carbohydrates, leading to increasing CP content. It is because the radicle (root) extends from day 4, allowing for mineral absorption, resulting in rapid changes in ash and protein contents (Morgan et al. as cited in Naik et al., 2015, p. 4). The highest detergent fibers (NDF and ADF) content was found in popcorn irrigated with clean tap water (T1). NDF in forages represents the indigestible and slowly digestible components in the plant cell wall. Cellulose and hemicellulose are quantitatively the most prominent, essentially indigestible, and resistant to microbial infection in the rumen of ruminants due to their complex chemical structure. Lignin and cutin, the

other main components of NDF, are nearly indigestible in both the rumen and the lower intestines. ADF is similar to NDF but without hemicellulose, containing cellulose, lignin, and cutin.

Generally, there were significant differences ($P<0.05$) in the chemical content of hydroponics fodder maize germinated from feed corn (T4) irrigated with nutrient solution compared to the popcorn irrigated with tap water (T1) and nutrient solution (T2). However, Agius et al. (2019) emphasized that hydroponic fodders used the resources and energy of the seeds, no additional nutrients were needed for the production. Some reports on a slight increase in the nutrient content of the sprouts, but this does not rationalize the additional cost of using a nutrient solution instead of freshwater (Dung et al., 2010; Sneath & Mclntosh, 2003). On top of that, higher urea concentrations did not have a beneficial effect on growth and yield; instead, an adverse effect was observed on hydroponic maize production (Aruna et al., 2018). Thus, using tap water irrigation might save money in the long run for smallholder livestock farmers.

Although feed corn had better nutritional value than popcorn, the root mat of treatment groups T3 and T4 was moldy, not detected in treatment groups T1 and T2. The mold growth might be related to the quality of the grain because all grains were managed with a standardized method. The moldy sprouts have been shown to reduce growth performance and are a known cause of animal death (Kumar et al., 2018). In addition, the fresh yield of the crops is essential for successful hydroponic fodder production. Germination of 1 kg popcorn would produce approximately 2.5 kg of fresh fodder (T1 and T2) compared to 1 kg of feed corn that grew about 1.5 kg HMF sprouted from popcorn had a thicker mat of roots and longer than the feed corn. Thus, popcorn with a higher conversion ratio and the germination rate was used to grow HMF with the open-air hydroponic system.

Chemical Composition between Conventional Fodder and Hydroponic Maize Fodder

The total percentage of the chemical composition in green fodders was summarized in Table 4. The result showed a higher DM composition in Napier grass ($P<0.05$). The CP content presently denoted in HMF was 12.28% higher than the Napier grass ($P<0.05$). Napier grass showed significantly higher OM content, 90.99% compared to 88.85% in HMF. Besides, higher NDF was observed in Napier grass ($P<0.05$). In addition, ADF and ADL composition were significantly higher in Napier grass, which possessed approximately 3 and 14 times higher than HMF, respectively.

DM is a primary indicator of the number of nutrients available to the animal in a feed. Livestock voluntarily consume a certain amount of DM per day (measured in kg/day) to maintain health, growth, and production (Naik et al., 2014). The values of DM reported by Lounglawan et al. (2014) as 18.93% in Napier grass harvested at

10 cm height on the 60th day, Ghani et al. (2017) as 13.04% in which Napier grass was harvested manually from pasture at the age of 8 weeks, Rahman et al. (2014) as 20.79% were lower than the present finding, 26.01%. In the present investigation, the DM content in HMF was 24.75%, lower than Napier grass because of high moisture content. However, this value was higher than the results reported by Naik et al. (2014) and Gebremedhin (2015) in HMF as 18.30% and 18.48%, respectively. Higher DM content in HMF was reported if harvested at an older age and cultivated in a nutrient solution. A study conducted by Thadchanamoorthy and Pramalal (2012) reported higher DM content as 26.07% in 10 days old HMF, while Adebiyi et al. (2018) noted 25.00% DM content in 7 days old HMF, which was cultivated with nutrient solution.

Table 4

Chemical composition of green fodder

Parameters (% of DM)	Conventional Napier green fodder	Hydroponic maize fodder
	n = 32	n = 32
DM	26.01 ± 0.38[b]	24.75 ± 0.20[a]
CP	7.22 ± 0.09[a]	12.28 ± 0.25[b]
OM	90.99 ± 0.10[b]	88.85 ± 0.35[a]
NDF	75.66 ± 0.24[b]	64.02 ± 0.45[a]
ADF	48.92 ± 0.38[b]	17.90 ± 0.51[a]
ADL	43.55 ± 0.32[b]	3.49 ± 0.40[a]
Hemicellulose	26.80 ± 0.60[a]	46.09 ± 0.92[b]
Cellulose	5.38 ± 0.66[a]	14.41 ± 0.70[b]
Ash	8.93 ± 0.15[a]	11.22 ± 0.38[b]

Note. DM = Dry matter; CP = Crude protein; OM = Organic matter; NDF = Neutral detergent fiber; ADF = Acid detergent fiber; ADL = Acid detergent lignin; n = Number of samples; All analyses are mean ± standard error of means (SEM); Means with different superscript letters in a row are significantly different (P<0.05)

The CP content presently denoted in HMF was 12.28% higher than the Napier grass (P<0.05). CP in feeding stuff includes the true protein containing amino acids and non-protein nitrogenous compounds such as amides. HMF was preferred as a source of quality forage for livestock because it has a high protein (Ndaru et al., 2020). It is encouraging to compare the findings of this study with the results by Jemimah et al. (2018), who recorded 10.55% CP content of HMF with an 8-days growth period but lower than the CP content reported by several studies as ranged from 13.30% to 16.54% (Adebiyi et al., 2018; Kide et al., 2015; Naik et al., 2012, 2013, 2014; Thadchanamoorthy

& Pramalal, 2012). The minor differences in minor results are probably due to the variety and quality of seed used, light intensity, quality of the irrigation water, and germination time (Kaouche-Adjlanea et al., 2016). A study showed that HMF would have higher CP content (24.07 g/100g) when harvested on the eighth day (Islam et al., 2016). However, the current study produced outdoor HMF exposed to high humidity, unlike those grown in a greenhouse. High humidity is one of the promoting factors of mold growth at the mat of roots when the length of the growth period increases. Generally, a concentration of 6–8% CP in the basal forage is a threshold for a response by ruminant livestock to N supplements (Mathis et al., 2000). The CP content of Napier grass investigated in the present study was 7.22%. The value was higher than the findings obtained by Zailan et al. (2016) in common Napier grass harvested at an interval of 8 weeks as 6.44% and Ghani et al. (2017) as 3.88%. The higher value of CP was reported by Rambau et al. (2016) in Napier grass leaves harvested at intermediate stage (8 weeks) as 140.4 g/kg and Halim et al. (2013) in common Napier grass as 9.79%, Lounglawan et al. (2014) as 8.87%, Bayble et al. (2007) as 14.13% in Napier grass. The increment in enzymatic activities of nutrients can improve the CP content in HMF (Naik et al., 2013).

Napier grass showed significantly higher OM (90.99%) content due to the application of fertilizer after each cutting (P<0.05). The value of total ash (11.22%) observed in the HMF of the present study is higher than the results reported by Naik et al. (2013) as a range of 1.75-3.80%. The total ash content rises on account of the mineral absorption by roots throughout the sprouting phase. HMF had higher palatability due to the younger harvesting age that showed a decrease in the number and size of cell walls for the synthesis of structural carbohydrates (Bayble et al., 2007). The comparable crude fiber content was reported by a few studies in HMF as a range of 9.33-14.10% (Gebremedhin, 2015; Kide et al., 2015; Naik et al., 2013). The higher value of crude fiber was reported by Adebiyi et al. (2018) as 14.77%, and lower values were also reported by Thadchanamoorthy and Pramalal (2012) as 8.21% and Naik et al. (2014) as 6.37%.

Although Napier grass showed a higher DM composition, the lower value of the CP justifies the need for HMF as alternative green fodders. In small ruminants, the amount of protein is more important than the quality of protein (Valente, 2016). In addition, the significantly lower NDF, ADF, and ADL in HMF represent lower indigestible fibers, which predict HMF as more acceptable green fodder to animals.

CONCLUSION

The seven-days hydroponic maize fodder can be produced by using an open-air hydroponic system. The usage of nutrient solution is unnecessary to produce a similar fresh weight of fodder corresponding with sprouts irrigated with clean tap water show sizeable increment in nutrients such as crude protein, dry matter, and organic matter. It could be suggested that the outdoor-grown

HMF is highly palatable due to its tenderness and younger harvesting age possess lower indigestible fibers (including NDF and ADF) compared to the conventional fodder. The highly nutritious HMF is fed as alternative green fodder because of the improved crude protein content. Although the dry matter of HMF was significantly lower than Napier grass, this shortcoming could be overcome by adding dry fodder or concentrate to the livestock diet.

From the agricultural economic aspect, it could be suggested that the outdoor-grown hydroponic maize fodder produced in this study might be an effective feeding solution to the current feeding management problem, which is the conventional labor-intensive cut-and-carry system faced by smallholders of the goat industry in Malaysia. This low-cost method can attract smallholders because it is less laborious, reuses the unutilized space, and does not require a greenhouse for the growth of HMF. Furthermore, there was no usage of chemical fertilizer and pesticide throughout the production of HMF results in a safe and contamination-free ruminant feed.

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REFERENCES

Abdullah, A. (2001). *Nutritive value of barley fodder grown in a hydroponics system* [Doctoral dissertation, Universiti Putra Malaysia]. Universiti Putra Malaysia Institutional Repository. http://psasir.upm.edu.my/id/eprint/10568/1/FP_2001_24_A.pdf

Adebiyi, O. A., Adeola, A. T., Osinowo, O. A., Brown, D., & Ng'Ambi, J. W. (2018). Effects of feeding hydroponics maize fodder on performance and nutrient digestibility of weaned pigs. *Applied Ecology and Environmental Research*, *16*(3), 2415-2422. https://doi.org/10.15666/aeer/1603_24152422

Agius, A., Pastorelli, G., & Attard, E. (2019). Cows fed hydroponic fodder and conventional diet: Effects on milk quality. *Archives Animal Breeding*, *62*(2), 517-525. https://doi.org/10.5194/aab-62-517-2019

Al-Ajmi, A., Salih, A., Kadhim, I., & Othman Y. (2009). Yield and water use efficiency of barley fodder produced under hydroponic system in GCC countries using tertiary treated sewage effluents. *Journal of Phytology*, *1*(5), 342-348.

Al-Karaki, G. N., & Al-Hashimi, M. (2012). Green fodder production and water use efficiency of some forage crops under hydroponic conditions. *International Scholarly Research Notices*, *2012*, 924672. https://doi.org/10.5402/2012/924672

Aruna, P., Chakravarthi, M. K., Murthi, A. K., Lavanya, R., & Siva, S. (2018). Effect of graded levels of urea fertilizer on growth and biomass yield of maize under low-cost hydroponic fodder production system. *Forage Research*, *43*(4), 283-286.

Association of Official Analytical Chemists. (1990). *Official methods of analysis* (15th ed.). AOAC.

Bayble, T., Melaku, S., & Prasad, N. K. (2007). Effects of cutting dates on nutritive value

of Napier (*Pennisetum purpureum*) grass planted sole and in association with Desmodium (*Desmodium intortum*) or Lablab (*Lablab purpureus*). *Livestock Research for Rural Development*, *19*(1), 120-136.

Dung, D. D., Godwin, I. R., & Nolan, J. V. (2010). Nutrient content and *in sacco* degradation of hydroponic barley sprouts grown using nutrient solution or tap water. *Journal of Animal and Veterinary Advances*, *9*(18), 2432-2435. https://doi.org/10.3923/javaa.2010.2432.2436

Fazaeli, H., Golmohammadi, H. A., Shoayee, A. A., Montajebi, N. & Mosharraf, S. (2011). Performance of feedlot calves fed hydroponics fodder barley. *Journal Agricultural Science Technology*, *13*(3), 367-375.

Fazaeli, H., Golmohammadi, H. A., Tabatabayee, S. N., & Asghari-Tabrizi, M. (2012). Productivity and nutritive value of barley green fodder yield in hydroponic system. *World Applied Sciences Journal*, *16*(4), 531-539.

Francis, J. M., Apgar, G. A., Crandell, K. G., Handlos, G. C., & Perry, E. B. (2018). The effects of hydroponic wheat fodder on fecal metabolites in equines. *Journal of Equine Veterinary Science*, *70*, 84-90. https://doi.org/10.1016/j.jevs.2018.05.212

Gebremedhin, W. K. (2015). Nutritional benefit and economic value of feeding hydroponically grown maize and barley fodder for Konkan Kanyal goats. *IOSR Journal of Agriculture and Veterinary Science*, *8*(7), 24-30.

Ghani, A. A. A., Shahudin, M. S., Zamri-Saad, M., Zuki, A. B., Wahid, H., Kasim, A., Salisi, M. S., & Hassim, H. A. (2017). Enhancing the growth performance of replacement female breeder goats through modification of feeding program. *Veterinary World*, *10*(6), 630-635. https://doi.org/10.14202/vetworld.2017.630-635

Halim, R. A., Shampazuraini, S., & Idris, A. B. (2013). Yield and nutritive quality of nine Napier grass varieties in Malaysia. *Malaysian Journal of Animal Science*, *16*(2), 37-44.

Hubballi, M., Nakkeeran, S., Raguchander, T., Anand, T., & Samiyappan, R. (2010). Effect of environmental conditions on growth of *Alternaria alternata* causing leaf blight of noni. *World Journal of Agricultural Sciences*, *6*(2), 171-177.

International Organization for Standardization. (2008). *ISO 13906:2008: Animal feeding stuffs - Determination of acid detergent fibre (ADF) and acid detergent lignin (ADL) contents*. https://www.iso.org/standard/43032.html

International Seed Testing Association. (2016). *International rules for seed testing*. ISTA.

Islam, R., Jalal, N., & Akbar, M. A. (2016). Effect of seed rate and water level on production and chemical analysis of hydroponic fodder. *European Academic Research*, *4*(8), 6724-6753.

Jemimah, E. R., Gnanaraj, P. T., Muthuramalingam, T., Devi, T., & Vennila, C. (2018). Productivity, nutritive value, growth rate, biomass yield and economics of different hydroponic green fodders for livestock. *International Journal of Livestock Research*, *8*(9), 261-270. https://doi.org/10.5455/ijlr.20171013104959

Kantale, R. A., Halburge, M. A., Deshmukh, A. D., Dhok, A. P., Raghuwanshi, D. S., & Lende, S. R. (2017). Nutrient changes with the growth of hydroponics wheat fodder. *International Journal of Science, Environment and Technology*, *6*(3), 1800-1803.

Kaouche-Adjlanea, S., Ahmed Serir, A., Bafdel, M., & Benhacine, R. (2016). Techno-economic approach to hydroponic forage crops: Use for feeding dairy cattle herd. *Journal of Applied Environmental and Biological Sciences*, *6*(3), 83-87.

Kide, W., Desai, B. G., & Kumar, S. (2015). Nutritional improvement and economic value

of hydroponically sprouted maize fodder. *Life Sciences - International Research Journal*, *2*(2), 76-79.

Kumar, R., Mathur, M., Karnani, M., & Dutt, S. (2018). Hydroponics: An alternative to cultivated green fodder: A review. *Journal of Entomology and Zoology Studies*, *6*(6), 791-795.

Lounglawan, P., Lounglawan, W., & Suksombat, W. (2014). Effect of cutting interval and cutting height on yield and chemical composition of King Napier grass (*Pennisetum purpureum* x *Pennisetum americanum*). *APCBEE Procedia*, *8*, 27-31. https://doi.org/10.1016/j.apcbee.2014.01.075

Mathis, C. P., Cochran, R. C., Heldt, J. S., Woods, B. C., Abdelgadir, I. E. O., Olson, K. C., Titgemeyer, E. C., & Vanzant, E. S. (2000). Effects of supplemental degradable intake protein on utilization of medium-to low-quality forages. *Journal of Animal Science*, *78*(1), 224-232. https://doi.org/10.2527/2000.781224x

Molla, A., & Sharaiha, R. K. (2010). Competition and resource utilization in mixed cropping of barley and durum wheat under different moisture stress levels. *World Journal of Agricultural Sciences*, *6*(6), 713-719.

Naik, P. K., Dhuri, R. B., Karunakaran, M., Swain, B. K., & Singh, N. P. (2013). Hydroponics technology for green fodder production. *Indian Dairyman*, *65*(3), 54-58.

Naik, P. K., Dhuri, R. B., Karunakaran, M., Swain, B. K., & Singh, N. P. (2014). Effect of feeding hydroponics maize fodder on digestibility of nutrients and milk production in lactating cows. *The Indian Journal of Animal Sciences*, *84*(8), 880-883.

Naik, P. K., Dhuri, R. B., Swain, B. K., & Singh, N. P. (2012). Nutrient changes with the growth of hydroponics fodder maize. *Indian Journal of Animal Nutrition*, *29*(2), 161-163.

Naik, P. K., Swain, B. K., & Singh, N. P. (2015). Production and utilisation of hydroponics fodder. *Indian Journal of Animal Nutrition*, *32*(1), 1-9.

Ndaru, P. H., Huda, A. N., Prasetyo, R. D., Shofiatun, U., Nuningtyas, Y. F., & Ndaru, R. K. (2020). Providing high quality forages with hydroponic fodder system. In *IOP Conference Series: Earth and Environmental Science* (Vol. 478, No. 1, p. 012054). IOP Publishing. https://doi.org/10.1088/1755-1315/478/1/012054

Peer, D. J., & Leeson, S. (1985). Feeding value of hydroponically sprouted barley for poultry and pigs. *Animal Feed Science and Technology*, *13*(3-4), 183-190. https://doi.org/10.1016/0377-8401(85)90021-5

Rahman, M. M., Abdullah, R. B., Wan Khadijah, W. E., Nakagawa, T., & Akashi, R. (2014). Feed intake and growth performance of goats offered Napier grass (*Pennisetum purpureum*) supplemented with concentrate pellet and soya waste. *Sains Malaysiana*, *43*(7), 967-971.

Rambau, M. D., Fushai, F., & Baloyi, J. J. (2016). Productivity, chemical composition and ruminal degradability of irrigated Napier grass leaves harvested at three stages of maturity. *South African Journal of Animal Science*, *46*(4), 398-408. https://doi.org/10.4314/sajas.v46i4.8

Sneath, R., & McIntosh, F. (2003). *Review of hydroponic fodder production for beef cattle*. https://cpb-us-east-1-juc1ugur1qwqqqo4.stackpathdns.com/blogs.cornell.edu/dist/e/4211/files/2014/05/Hydroponicfodder-article-11wpnm0.pdf

Thadchanamoorthy, S., & Pramalal, V. J. C. (2012). *Evaluation of hydroponically grown maize as a feed source for rabbits*. http://agri.pdn.ac.lk/ext_staff/upload/publications/publication_249_dept_5_fbe225af2b33ebfaab9e85855d643937.pdf#page=17

Trubey, C. R., Rhykerd, C. L., Noller, C. H., Ford, D. R., & George, J. R. (1969). Effect of light, culture solution, and growth period on growth and chemical composition of hydroponically produced oat seedlings. *Agronomy Journal*, *61*(5), 663-665. https://doi.org/10.2134/agronj1969.00021962006100050003x

Valente, T. N. P., da Silva Lima, E., dos Santos, W. B. R., Cesario, A. E. S., Tavares, C. A. J., & de Freitas, M. A. M. (2016). Ruminal microorganism consideration and protein used in the metabolism of the ruminants: A review. *African Journal of Microbiology Research*, *10*(14), 456-464. https://doi.org/10.5897/AJMR2016.7627

Zailan, M. Z., Yaakub, H., & Jusoh, S. (2016). Yield and nutritive value of four Napier (*Pennisetum purpureum*) cultivars at different harvesting ages. *Agriculture and Biology Journal of North America*, *7*(5), 213-219. https://doi.org/10.5251/abjna.2016.7.5.213.219

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Environmental Impact on Duration of Flowering and Fruiting of Rattan (*Calamus castaneus)* in Peninsular Malaysia

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ABSTRACT

The phenological events of rattan were seldomly studied due to its nature of survival by climbing on other trees (i.e., liana). However, some rattans are non-climbing and found to produce fruits throughout the year. Therefore, it aimed to record *Calamus castaneus* duration of flowering and fruiting over a year and identify which environmental variables affect the duration. This study was conducted for 12 months in three forest reserves, namely Bukit Mertajam Forest Eco-Park (BMFEP), Teluk Bahang Forest Reserve (TBFR), and Segari Melintang Forest Reserve (SMFR). Five study plots (10 m x 10 m each) were established, and overall, 53 *C. castaneus* individual palm in the plots were monitored. Findings have revealed that female inflorescences bloomed shorter than males. Also, *C. castaneus* fruits take about three to four months to get matured. The canonical correspondence analysis (CCA) highly regulated the event by microclimate and locality. Thus, the flowering and fruiting duration may not directly be affected by a very specific environmental factor.

Keywords: *Calamus castaneus*, duration, environment, flowering, fruiting, rattan

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INTRODUCTION

Calamus was among the 13 genera of rattan with the highest economic value. This genera comprises 370 species, recorded international rattan trade with over USD 6.5 billion a year (Ali & Barizan, 2001; Dransfield, 1979; Wan Ariffin et al., 2018). Rattan, in general, is a spiny, climbing palm that uses its climbing organ (flagella or cirrus) to hook other trees to reach for

sunlight (Dransfield, 2001). Thus, the interaction with its immediate surrounding is interesting and crucial both biologically and ecologically. Working in a natural forest is extremely tough and time-consuming. Therefore, we decided to study *Calamus castaneus* (Figure 1), an acaulescent, dioecious rattan, to avoid difficulty in observing its reproductive biology. Unlike other rattan species, this species does not possess a climbing organ, and it also produces flowers and fruit throughout the year. *Calamus castaneus* was chosen as a study subject due to the low exploitation of its cane, therefore making its natural population unaffected (Kidyoo & McKey, 2012). The flowering and fruiting events continue throughout the year, making this species an important food source for wildlife within its surrounding.

Despite low commercial value as cane, this species provides flowers and fruit for animals and insects as a reward for pollination and seed dispersals (Watanabe & Suzuki, 2008). The flower's crimson red colour would attract insects, helping pollination between male and female plants. Among the admirer of these sweet yet acidic fruits are primates such as macaques (Ruppert et al., 2016). In addition, the leaf sheath makes an excellent roofing material, and the immature fruit was consumed by aboriginal people to cure cough (Dransfield, 1979).

Figure 1. *Calamus castaneus* Griff. is an acaulescent, dioecious rattan

In terms of ecology, this species plays a major role in symbiosis with ants (Dransfield, 1979). Based on observation, the broad, lush leaf sheath holds the fallen dry leaves from the canopy into the crown, hence creating a home for the ants. In return, the ants supply nutrients for the host and nourish the soil around them. This process was crucial to maintaining the forest ecology. Therefore, this study was planned to know the duration

of flowering and fruiting and environmental factors affecting them.

MATERIALS AND METHODS

Study Sites

Sampling was conducted in three forest reserves in the northern region of Peninsular Malaysia (Figure 2), namely: Segari Melintang Forest Reserve (SMFR; 04° 19' 34" N, 100° 34' 57" E) in Perak, Teluk Bahang Forest Reserve (TBFR; 05° 26' 34" N, 100° 13' 14" E), and Bukit Mertajam Forest Eco-Park (BMFEP; 05° 21' 57" N, 100° 28' 58" E) in Pulau Pinang. SMFR was dominated by trees of the Dipterocarpaceae family with patches of alluvial freshwater swamp along the forest edge. The reserve comprises 408 hectares (ha) Virgin Jungle Reserve (VJR) that was strictly protected (Ruppert et al., 2016). TBFR is a lowland and hilly Dipterocarp Forest. One hundred and seventeenth hectares of TBFR were protected as VJR; meanwhile, BMFEP comprises a hilly dipterocarp forest covering 37 ha. This study started in March 2017 and was conducted for a year.

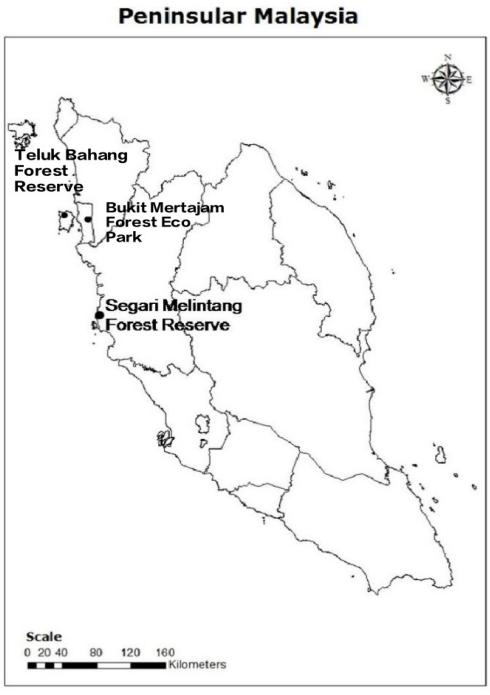

Figure 2. The map shows the location of the three study sites in Peninsular Malaysia

Study Species

Calamus castaneus is a dioecious, clustering acaulescent palm with no climbing organ (i.e., cirrus and/or flagella). Its leaves can grow up to 4 m long with a petiole length of 1.5 m. One of the striking characteristics of *C. castaneus* is its grey spines with yellowish based and the presence of glaucous or dull dirty grey indumentose under the leaves. Female inflorescences have fewer branching than male inflorescences, with 15 rather stiff packed rachillas enclosed with compact bracts in fruit extending. Fruit is a scaly and rich chestnut brown coloured, 25 mm long and 18 mm wide when ripe (Dransfield, 1979). This species grew throughout Peninsular Malaysia except for Perlis (Dransfield, 1979) and was categorised as Not Evaluated (NE) under Malaysia Plant Red List.

Population Structure and Sex Determination

Five study plots (each 10 m x 10 m, the distance between each plot is at least 50 m apart from each other) were established for a long-term rattan survey in the three sites.

The cluster of rattan within the sampling plots was randomly distributed. Since *C. castaneus* is a common palm, the plants can be easily found alongside a small stream. Identification of the studied species was given by Dransfield (1979). The plants within the study plot were marked with numbered plastic tags. Only the plant that had borne inflorescences or infructescences were chosen. The distance between plots was made sure less than 50 m from each other to ensure that sampling for whole the population could be finished in a day. At each location, the population is comprised of 53 plants (28 males, 25 females). Observations of flowering determined the sex of each plant and fruiting from previous seasons noted that only female plants would bore infructescences. A complete sample of *C. castaneus* was collected from the field and deposited at the USM Herbarium with the catalogued number of USM Herbarium 11783.

Monitoring Flowering and Fruiting

The number of inflorescences was selected in each plot, and the number of female and male flowers in each inflorescence was recorded. Each individual's number of flowers produced was estimated by comparing the number of inflorescences produced by each flowering individual and the mean number of flowers per male and female inflorescences (Kidyoo & McKey, 2012). On the other hand, the fruits per rachilla in infructescence were recorded. Through some modification from Kidyoo and McKey (2012), the number of fruits borne by each infructescence was estimated by the number of fruits per rachilla. The fruit width and length were measured each month using a ruler. Several fruits were taken back as a sample for a cross-section to observe the growth stages inside the fruit.

Pollinators and Seed Dispersers

Insect pollinators that regularly visited flowers of both sexes were observed and recorded. The visitation behaviour of insect visitors such as pollen collection, nectar collection, and contact with anthers of staminate flowers or stigma of pistillate flowers were recorded (Kidyoo & McKey, 2012). The pollinators were captured by net using the caught-and-release method. Photographs were taken to identify the species. In addition, possible seed dispersers within the plot were examined by the track they had left, such as footprints or leftover fruits and seeds.

Microclimate Sampling and Soil Properties Analysis

Microclimate readings (i.e., light intensity, relative humidity, air temperature, soil temperature, percentage of gap opening, and level of disturbance) were noted between 10 a.m. until 12 p.m. every month (Hardwick et al., 2015). Light intensity was measured using a portable luxmeter (Model HI 97500 portable lux meter, Hanna Instruments, USA). In contrast, air temperature and relative humidity were measured using a portable thermo-hygrometer (Model HI 9564 portable, water-resistant thermo-hygrometers, Hanna Instruments, USA).

The readings were taken 1.5 metres above ground within the plot near the cluster of *C. castaneus*. Soil temperature was measured using a soil thermometer by shoving the probe about 10 cm deep in the soil. Soil samples were collected near the cluster of studied species within the plot and brought back to the laboratory to analyse soil bulk density, soil pH, soil moisture content, and soil texture analysis. Three soil samples were collected in each site's plot and were secured in a zip-lock plastic bag. Soil pH was determined with a soil pH meter using the 1:5 ratio of soil and water, respectively. Soil texture was determined with the mechanical method to find out the sand, silt, and clay. The determination of soil texture as in percentage of sand, silt and clay were referred to as United States Department of Agriculture (USDA) textural triangle (Brady & Weil, 2014).

Canopy Gap Opening

The percentage of canopy gap opening status (Table 1) and disturbance index (DI) (Table 2) were referred to by Mansor (2001).

Table 1
Percentage range and canopy gap opening status

Percentage range (%)	Gap opening status
0–25	Closed area
26–40	Partly closed area
41–60	Slightly opened area
61–80	Partly opened area
81–100	Highly opened area

Disturbance Index

Level of disturbance considering certain factors such as socioeconomic, ecology, and infrastructure were modified from Mansor (2001) by Rozali (2014) using DI. The index was scaled from 1 to 5, with one represented as the lowest disturbed and five as the highest disturbed (Table 2).

Table 2
Characterisation for disturbance criteria

Feature	Disturbance scale
	Trails (g_1)
Infrastructure:	
Unexplored forest overgrown by big trees	1
Unexplored forest and filled with bushes	2

Table 2 (*Continue*)

Feature	Disturbance scale
	Trails (g_1)
Infrastructure:	
Explored forest with small trails <50 cm width (rarely used by visitors)	3
Explored forest with bigger trails >50 cm width (always used by visitors)	4
Paved roads	5
	Visitors per day (g_2)
Socioeconomics:	
1–15 visitors	1
16–25 visitors	2
26–35 visitors	3
36–45 visitors	4
> 45 visitors	5
	Land use (g_3)
Ecology:	
Virgin forest	1
Secondary forest	2
Reserve forest	3
Agriculture	4
Clearing's forest	5
	Water resource (g_4)
Big streams or waterfalls	1
Watercourse	2
Recreational ponds	3
Damp	4
Dried area, no watercourse in the forest	5
	Canopy opening (g_5)
0–25 %	1
26–40 %	2
41–60 %	3
61–80 %	4
81–100 %	5

The percentage of DI was calculated with the following formula:

$$DI = g_1 + g_2 + g_3 + g_4 + g_5$$

where:
DI = Disturbance index
g_1 = Trails
g_2 = Number of visitors
g_3 = Land use
g_4 = Water supply
g_5 = Gap forest cover

$$\text{Percentage of disturbance} = [(g_1 + g_2 + g_3 + g_4 + g_5) / (25)] \times 100$$

The percentage of disturbance index (range) based on total disturbance index recorded from each plot, given in Table 3.

Table 3
Percentage range and disturbance status

Percentage range (%)	Disturbance status
0–25	Low disturbed area
25–50	Moderately low disturbed area
50–75	Moderately high disturbed area
75–100	Highly disturbed area

Data Analysis

The flowering and fruiting parameters of *C. castaneus* at all sites were recorded, and the duration of these was plotted in the graph. Microclimate data and soil properties analysis of each site were compiled and analysed using one-way analysis of variance (ANOVA) with post-hoc Tukey's test. The influence of microclimate and soil properties on the flowering and fruiting of *C. castaneus* were noted using canonical correspondence analysis (CCA) of CANOCO version 4.5 (Ter Braak, 1988).

RESULTS AND DISCUSSION

Population Structure and Sex Determination

Observations have been made on 53 studied plants (28 males, 25 females) in all sites for a year. All mature plants within the plots were observed monthly. From Table 4, SMFR denoted the highest number of males (16 individuals) and females (16 individuals), making it the highest number (32 individuals) of *C. castaneus* individual palm among all sites. Based on a year sampling, there was no existence of female plants in TBFR plots. A site survey during late 2016 on TBFR shows a limited number of female plants (personal observation). However, plots were not established in the presence of female plants due to safety precautions. Hence, a comparing the flowering duration of female plants can only be carried out between SMFR and BMFEP. It also applies to the duration of fruiting as only female plants would bear fruits.

Table 4

Abundance of Calamus castaneus *individuals by sexes in all investigated forests*

Sexes	Teluk Bahang Forest Reserve	Segari Melintang Forest Reserve	Bukit Mertajam Forest Eco Park
Male	6	16	6
Female	-	16	9
Total	6	32	15

Duration of Anthesis in Each Site

The flowering structure of male and female inflorescences can be seen clearly as in Figures 3 and 4. Generally, male inflorescences bloomed for a more extended period and were also more highly branched, laxer, and sinuose than female inflorescences.

Figure 3. Male inflorescences were highly branched

Figure 4. Female inflorescences with stiff crowded rachillae

There were six individuals of male plants recorded in the TBFR site (Figure 5). Among the six plants, only two individuals displayed blooming throughout the year. The blooming was very short, ranging from one month to the longest four months. At the end of the anthesis, the flower shed and both types of plants stopped producing flowers from October 2017 until the end of the sampling period.

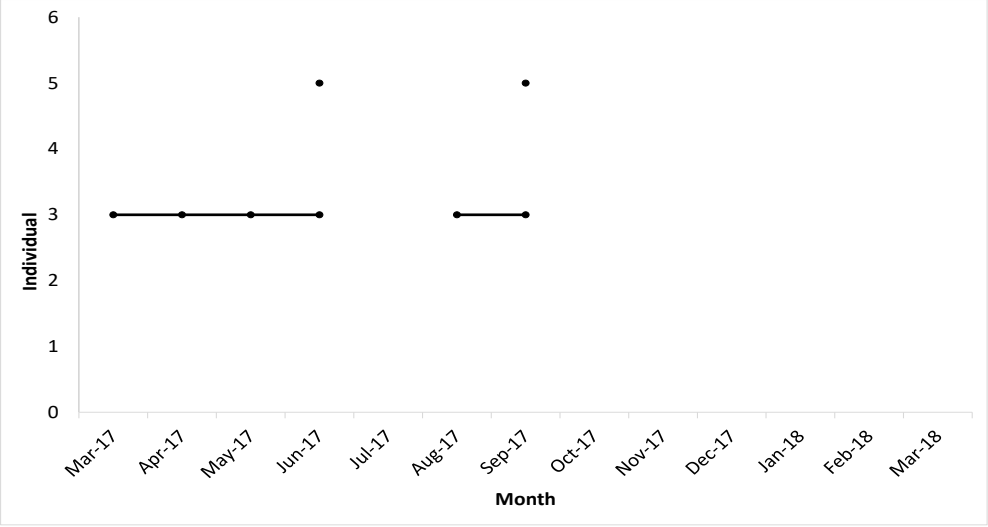

Figure 5. Duration of anthesis in *Calamus castaneus* male inflorescences in Teluk Bahang Forest Reserve

Plants of both sexes are flowered throughout the year. Based on Figure 6, the pattern of duration between male and female inflorescences was different. Male plants produced larger inflorescences with a greater degree of branching. Thus, male plants produced a more significant number of flowers compared with female plants (Kidyoo & McKey, 2012). Almost all male inflorescences in SMFR flowered throughout the sampling period. Flowering time in male inflorescences occurred continuously. The largest gap for an individual not producing any flowers was six months (n = 15), and the smallest gap was a month (n = 6, 9, 11, 12, 13, and 14). Meanwhile, only one individual of female inflorescences bloomed for a year (n = 22). The shortest flowering time was in December 2017, when the female individual bloomed only for a month (n = 24).

Unlike SMFR, the flowering time of male and female inflorescences in BMFEP (Figure 7) were comparatively shorter. The longest blooming of male inflorescences was six months (n = 4) which started in May 2017 and ended in October 2017. Based on observation, two (2) individuals (n = 3 and n = 7) do not produce any inflorescences at all. Most female inflorescences bloomed about two to three months on this site — most of them producing flowers in August 2017. However, after September 2017, all-

female inflorescences in BMFEP stopped producing a flower. All forest reserves and national parks in Pulau Pinang, including TBFR and BMFEP, were closed for visitors from November 2017 until January 2018 due to landslides and tree fall caused by the monsoon. Natural disasters and disturbances have had a destructive impact on the flowering of male inflorescences since there were no signs of blooming in TBFR and BMFEP during the period.

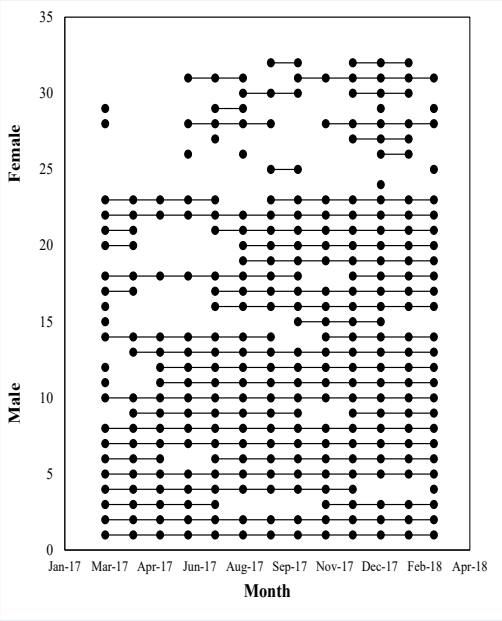

Figure 6. Duration of anthesis in *Calamus castaneus* male (n = 1–16) and female (n = 17–32) inflorescences in Segari Melintang Forest Reserve

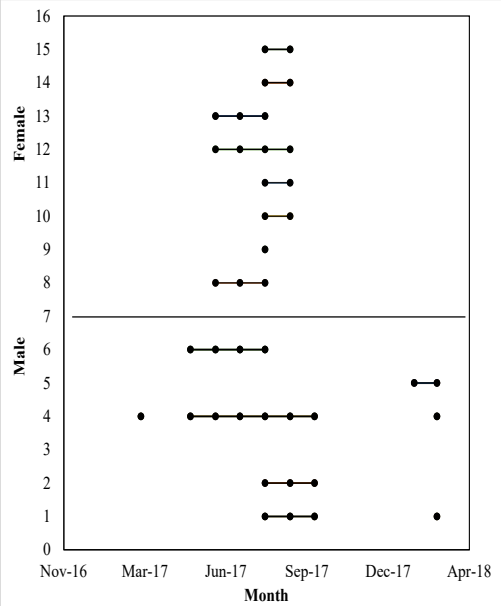

Figure 7. Duration of anthesis in *Calamus castaneus* male (n = 1–6) and female (n = 7–15) inflorescences in Bukit Mertajam Forest Eco Park

Duration of Fruiting

Ripe fruit can grow to 25 mm long and 18 mm wide. The pronounced beak and rich chestnut colour can be seen as in Figure 8.

In this study, the duration of fruiting can only be compared in SMFR (16 individuals) and BMFEP (nine individuals) due to the absence of female plants of *C. castaneus*

Figure 8. Cross-section of *Calamus castaneus* fruit

inside the plot established in TBFR. Based on Figure 9, there were no female plants monitored to fruit throughout the year in BMFEP, whereas five individuals were observed to produce fruits throughout the year (n = 12,1 3, 14, 15, 16) in SMFR. From observation, it took about 3 to 4 months for the fruits to reach their maturation. Nevertheless, not all fruits from one infructescence would reach maturity. Before they were ripe (personal observation), half of it was attacked by beetles, eaten by predators or infected by pyrenomycete fungus that filled the entire fruit. As a result, the scales appear to be embedded in black powder (Dransfield, 1979).

One individual of *C. castaneus* would exhibit different fruiting stages (ripe, unripe, budding, and flowering) simultaneously (Ruppert et al., 2014). However, it was assumed that all the flowers had already developed into fruits, and the flowering cycle could be halted until all the fruit was shed. Choong and Wickneswari (2016) discussed that dioecy in rattan had limited its breeding and cultivation. Besides that, gender in *Calamus* species can only be identified after the first flowering. Dransfield (1974) reported that rattan seeds would need one to six months to germinate. Female plants only produce the fruits and progeny, and the reducing number of mature fruits per inflorescences would affect the distribution and ratio of sexes in any locality (Manokaran, 1985: Renuka & Rugmini, 2007). Hence, due to the dioecious nature of nearly all rattan, cross-pollination must occur. It was uncommon to find solitary female individuals bearing well-developed fruits but male plants being nowhere to be found as fruits could not be formed without pollen from male plants (Dransfield, 1979; Manokaran, 1985).

From Figure 9, *C. castaneus* fruits longer in SMFR than BMFEP. The average duration of fruiting in BMFEP is three months, and the most extended period of fruiting could reach about six months. However, *C. castaneus* in SMFR displayed various fruiting duration from one month to continuously fruiting for one year. The average period of fruiting recorded was three to four months. In addition, one same individual could fruit a year thrice after the fruit had already been shed. Although the pattern was quite non-uniformed, a single *C. castaneus* individual in this site could fruit up to four times in a year. Hence, the continuous production of *C. castaneus* fruit has been a great food source for the animal inside the forest. If animals do not eat it, the fruit will rot and fall on the forest floor, making a chance to develop a new seedling.

Relationship between Environmental Variables with the Duration of Flowering and Fruiting of *Calamus castaneus*

This study collected the data on environmental variables (i.e., microclimate parameters and soil properties) twice a month for a year. One-way ANOVA with post-hoc Tukey's test at $p < 0.05$ was used to analyse the significant difference between each site. The significant differences were displayed in superscripts a, b, and c, as in Table 5. In Table 5, relative humidity, soil

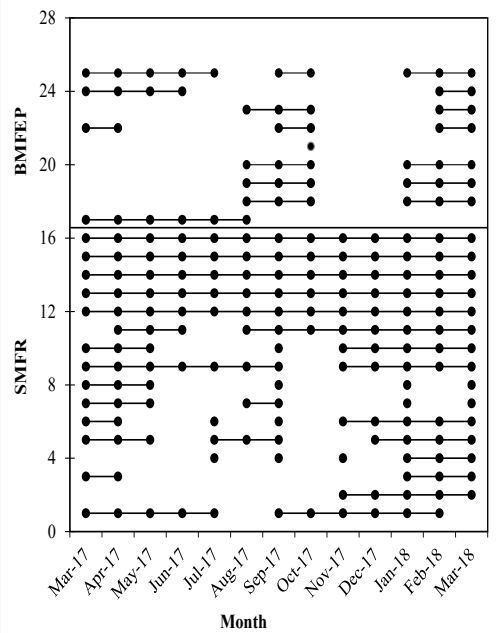

Figure 9. Duration of fruiting of Calamus castaneus in Segari Melintang Forest Reserve (n = 1–16) and Bukit Mertajam Forest Eco Park (n = 17–25)

temperature, disturbance index level, soil pH, and soil moisture content showed a significant difference between each site. Lara et al. (2017) added that different environmental variables, i.e., relative humidity and cloudiness, may influence the flowering pattern observed. However, the rest of the environmental variables shows no significant differences. Aminuddin (1985), Manokaran (1985), and Nainggolan (1985) stated that environmental parameters such as listed in Table 5 could affect rattan growth and survival in the forest. However, the interaction between rattan flora and soil type is still not clear.

Table 5

Environmental variables parameter (Mean ± SE) measured from March 2017 until March 2018 summarised in table showed

Parameter	TBFR	SMFR	BMFEP
Relative humidity (%)	83.70 ± 1.34^{b}	71.59 ± 2.21^{a}	81.14 ± 2.18^{b}
Air temperature (°C)	25.0 ± 2.14^{a}	28.50 ± 1.01^{a}	25.35 ± 0.40^{a}
Light intensity (kLux)	1.00 ± 0.18^{a}	1.11 ± 0.18^{a}	1.10 ± 0.18^{a}
Soil temperature (°C)	26.15 ± 0.10^{b}	26.81 ± 0.15^{c}	24.28 ± 0.14^{a}
Canopy gap opening (%)	25.5 ± 4.77^{a}	46.7 ± 8.47^{a}	36.4 ± 5.41^{a}
Disturbance index (%)	37.6 ± 0.98^{b}	30.8 ± 2.15^{a}	60.8 ± 1.5^{c}
Soil pH	5.74 ± 0.15^{b}	6.09 ± 0.33^{b}	4.82 ± 0.05^{a}
Soil moisture content (%)	24.82 ± 0.65^{a}	32.26 ± 3.30^{a}	45.26 ± 3.70^{b}
Soil bulk density (gcm^{-3})	0.97 ± 0.12^{a}	0.91 ± 0.14^{a}	0.76 ± 0.09^{a}
Soil texture analysis	Sand	Sand	Loamy sand

Note. Superscript a, b, and c indicates the significant difference at $p < 0.05$ by post-hoc Tukey's test in each parameter between sites. TBFR = Teluk Bahang Forest Reserve; SMFR = Segari Melintang Forest Reserve; BMFEP = Bukit Mertajam Forest Eco-Park

The CCA of plant production and environmental variables, as summarised in Table 6, shows that the eigenvalue was less than 0.5, which indicate that species-environment relation was low. The cumulative variance explained by the first three-axis of the species-environment relationship in the CCA of TBFR, SMFR, and BMFEP were all 0% (Tables 6, 7, and 8). The CCA ordination plot was displayed as in Figures 10, 11, and 12. The direction and length of the arrow that extends from the centre of the ordination diagram displayed the strength between plant production and environmental variables.

Table 6

Summary of the canonical correspondence analysis (CCA) of the Calamus castaneus *plant production and environmental data in Teluk Bahang Forest Reserve*

Axes	1	2	3	4	Total inertia
Eigenvalues	0.002	0.058	0	0	0.061
Species-environment correlations	0.201	0	0	0	
Cumulative percentage variance					
of species data	4.1	100	0	0	
of species-environment relation	100	0	0	0	
Sum of all eigenvalues					0.061
Sum of all canonical eigenvalues					0.002

Figure 10 displays that all ten (10) environmental variables listed in this study do not affect flower production in TBFR. The arrow of environmental variables lies on the horizontal axis, which means the was no relationship of microclimate parameters and soil properties on the generation of *C. castaneus* flower in TBFR.

SMFR has displayed the longest and most frequent flowering duration over the year. Hence, Figure 11 represents the environmental variables that influence the duration of its flowering. Based on Eigenvalues, high soil pH value, and increased soil bulk density will reduce the duration of flowering and fruiting of *C. castaneus*. Although some rattan species need high soil pH to grow, others demand low soil pH. However, too acidic soil will affect rattan development and eventually will retard the plant. In addition, soil pH will affect mineral nutrient availability on rattan growth as some nutrients become inaccessible at certain pH levels (Lilly, 2010). Moreover, compacted soil will inhibit the growth of roots, reduce the movement of gaseous exchange in the root zone and may also decrease water diffusion (Brady & Weil, 2014; Lilly, 2010).

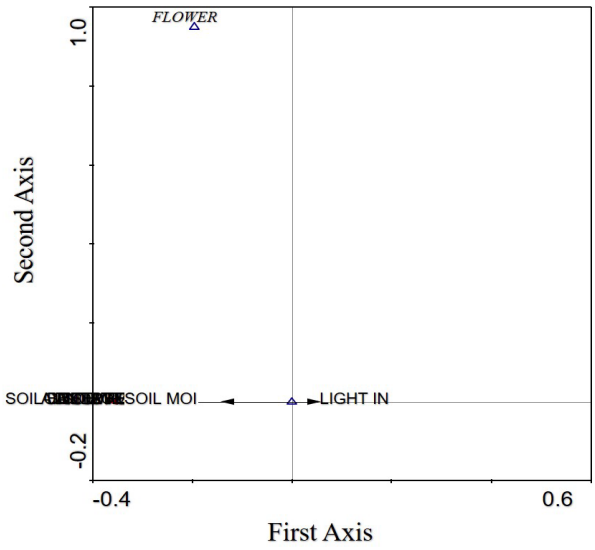

Figure 10. Ordination plot of canonical correspondence analysis (CCA) between environmental variables (arrows) with flower (triangles) in Teluk Bahang Forest Reserve

Note. SBD = Soil bulk density; DI = Disturbance index; RH = Relative humidity; SOIL TEMP = Soil temperature; AIR TEMP = Air temperature; PH = Soil pH; SOIL MOI = Soil moisture content; LIGHT IN = Light intensity; GAP = Percentage of gap opening

Table 7

Summary of the canonical correspondence analysis (CCA) of the Calamus castaneus *plant production and environmental data in Segari Melintang Forest Reserve*

Axes	1	2	3	4	Total inertia
Eigenvalues	0.012	0.002	0.085	0.038	0.137
Species-environment correlations	0.35	0.24	0	0	
Cumulative percentage variance					
of species data	8.6	10.3	72.1	100	
of species-environment relation	83.1	100	0	0	
Sum of all eigenvalues					0.137
Sum of all canonical eigenvalues					0.014

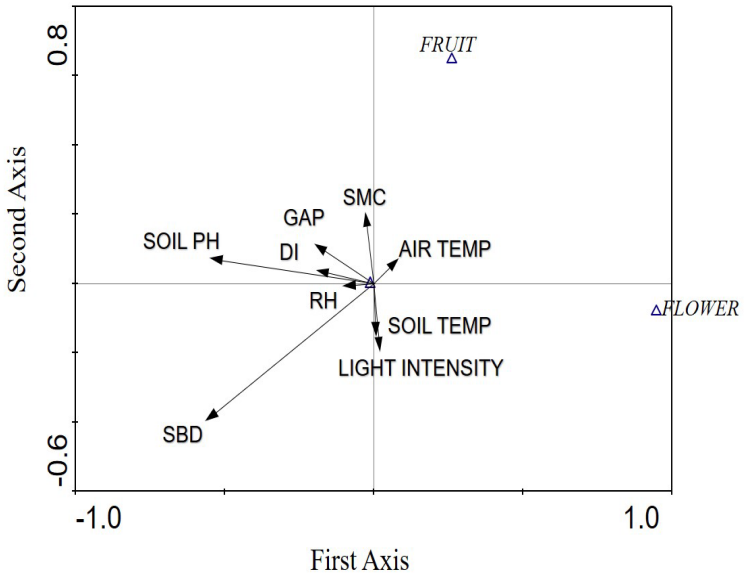

Figure 11. Ordination plot of canonical correspondence analysis (CCA) between environmental variables (arrows) with flower and fruit (triangles) in Segari Melintang Forest Reserve

Note. SBD = Soil bulk density; DI = Disturbance index; RH = Relative humidity; SOIL TEMP = Soil temperature; AIR TEMP = Air temperature; SMC = Soil moisture content; GAP = Percentage of gap opening

Table 8

Summary of the canonical correspondence analysis (CCA) *of the* Calamus castaneus *plant production and environmental data in Bukit Mertajam Forest Eco-Park*

Axes	1	2	3	4	Total inertia
Eigenvalues	0.02	0.00	0.13	0.11	0.27
Species-environment correlations	0.38	0.17	0.00	0.00	
Cumulative percentage variance					
of species data	7.8	9.2	58.4	100	
of species-environment relation	84.9	100	0	0	
Sum of all eigenvalues					0.269
Sum of all canonical eigenvalues					0.025

The CCA graph in Figure 12 and eigenvalue in Table 8 show high relative humidity, percentage of gap opening, soil temperature, disturbance index level, soil pH, and air temperature would give positive feedback on flowers and fruits production. Manokaran (1989) stated that a period of high rainfall after dry seasons and high temperatures would trigger flowering in rattan. An increase in soil moisture content would decrease fruiting but increase flowering. In the meantime, the low light intensity would stimulate flowering and promotes fruiting. Some rattan species were able to grow under the lower light condition with well-established canopies and soil with good moisture (Powling, 2004).

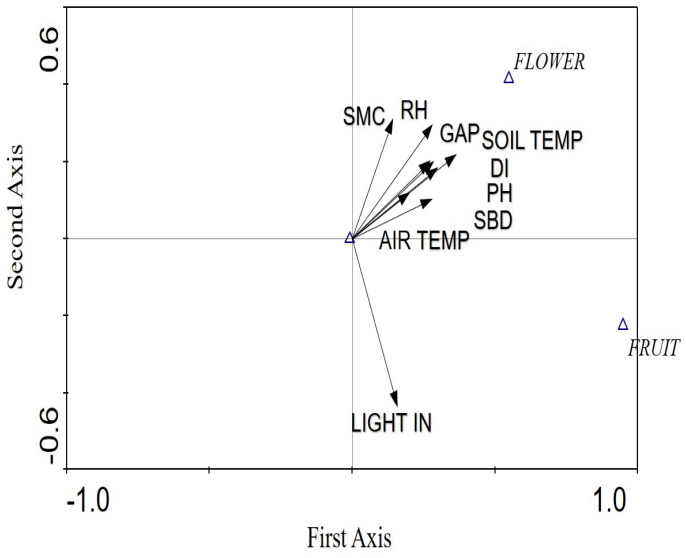

Figure 12. Ordination plot of canonical correspondence analysis (CCA) between environmental variables (arrows) with flower and fruit (triangles) in Bukit Mertajam Forest Eco-Park

Note. SMC = Soil moisture content; RH = Relative humidity; GAP = Percentage of gap opening; SOIL TEMP = Soil temperature; DI = Disturbance index; PH = Soil pH; SBD = Soil bulk density; AIR TEMP = Air temperature; LIGHT IN = Light intensity

New Shoot from Female Inflorescences of *Calamus castaneus*

During the sampling period in SMFR, we record a female individual that produces a new vegetative shoot from the apex of the inflorescences as in Figure 13 (GPS: N 04° 19' 34.7" E 100° 34' 57.9"). The *C. castaneus* individual palm was found near a small stream. This individual spotted a growing shoot in early October 2017 and slowly dried at the end of November 2017. It was then eventually turning brown completely and died in March 2018. Throughout the observation period, the shoot does not produce any roots. Besides that, fresh flowers and edible fruit were present on the same plants. The same event occurred in SMFR as Ruppert et al. (2012) recorded but on male inflorescence. Until today, we still do not know whether this phenomenon is another form of sexual reproduction or just another mutant.

Figure 13. New vegetative shoot from female inflorescence apex

CONCLUSION

Overall, female inflorescences bloomed shorter than males. On average, female inflorescences would bloom for two to three months while male inflorescences for six months. However, the fruit of *Calamus casteneus* mature in three to four months. It has been observed that flowering and fruiting are highly regulated by microclimate and locality but did not significantly affect by a very specific environmental factor.

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REFERENCES

Ali, A. R. M., & Barizan, R. S. R. (2001). *Important rattan species of Malaysia*. http://www.fao.org/3/x9923e/x9923e07.htm

Aminuddin, M. (1985). *Performances of some rattan species in growth trials in Peninsular Malaysia*. https://www.inbar.int/wp-content/uploads/2020/05/1493102176.pdf#page=43

Brady, N. C., & Weil, R. R. (2014). *The nature and properties of soils* (14th ed.). Pearson Education Limited.

Choong, C. Y., & Wickneswari, R. (2016). Sexing in rattans. *Malaysian Applied Biology*, *45*(2), 1–10.

Dransfield, J. (1974). *A short guide to rattan*. Regional Centre for Tropical Biology.

Dransfield, J. (1979). *A manual of the rattans of the Malay Peninsula*. Forest Department, Ministry of Primary Industries Malaysia.

Dransfield, J. (2001). Taxonomy, biology and ecology of rattan. *Unasylva*, *52*(205), 11-13.

Hardwick, S. R., Toumi, R., Pfeifer, M., Turner, E. C., Nilus, R., & Ewers, R. M. (2015). The relationship between leaf area index and microclimate in tropical forest and oil palm plantation: Forest disturbance drives changes in microclimate. *Agricultural and Forest Meteorology*, *201*, 187–195. https://doi.org/10.1016/j.agrformet.2014.11.010

Kidyoo, A. M., & McKey, D. (2012). Flowering phenology and mimicry of the rattan *Calamus castaneus* (Arecaceae) in southern Thailand. *Botany*, *90*(9), 856-865. https://doi.org/10.1139/b2012-058

Lara, C. E., Díez, M. C., Restrepo, Z., Núñez, L. A., & Moreno, F. (2017). Flowering phenology and flower visitors of the Macana palm *Wettinia kalbreyeri* (Arecaceae) in an Andean montane forest. *Revista Mexicana de Biodiversidad*, *88*(1), 106–112. https://doi.org/10.1016/j.rmb.2017.01.001

Lilly, S. J. (2010). *Arborists' certification guide*. Internal Society of Arboriculture.

Manokaran, N. (1985). Biological and ecological considerations pertinent to the silviculture of rattans. In K. M. Wong & N. Manokaran (Eds.), *Proceedings of Rattan Seminar* (pp. 95–105). The Rattan Information Centre.

Manokaran, N. (1989). Flowering and fruiting patterns of *Calamus caesius* Bl. In A. N. Rao & I. Vongkaluang (Eds.), *Recent Research on Rattan: Proceedings of the International Rattan Seminar* (pp. 122-129). Kasetsart University.

Mansor, A. (2001). *Kajian kepelbagaian dan ekologi rumpai di kawasan hutan paya Semenanjung Malaysia* [Study of the diversity and ecology of weeds in the swamp forest area of Peninsular Malaysia] [Unpublished Doctoral thesis]. Universiti Sains Malaysia.

Nainggolan, P. H. J. (1985). Preliminary observations on the effect of different canopy and soil moisture conditions on the growth of *Calamus Manan* (Manau). In K. M. Wong & N. Manokaran (Eds.), *Proceedings of Rattan Seminar* (pp. 72–76). The Rattan Information Centre.

Powling, A. (2004). *Rattans : Taxonomy and ecology (LIPI report 2004)*. https://www.opwall.com/uploads/2017/11/Opwall-Indonesia-Buton-Rattan-Report-2004.pdf

Renuka, C., & Rugmini, P. (2007). *Development of conservation strategies for selected, endangered rattan species of the Western Ghats*. http://docs.kfri.res.in/KFRI-RR/KFRI-RR295.pdf

Rozali, W. N. F. Z. W. (2014). *Composition of rattan communities (Arecaceae, subfamily Calamoideae) in forest reserves of Penang* [Unpublished Master's thesis]. Universiti Sains Malaysia.

Ruppert, N., Mansor, A., & Anuar Mohd Sah, S. (2012). New shoot from inflorescences in *Calamus castaneus* in Peninsular Malaysia. *Palms*, *56*(1), 36-40.

Ruppert, N., Mansor, A., & Anuar Mohd Sah, S. (2014). A key role of the Southern pig-tailed macaque *Macaca nemestrina* (Linnaeus) in seed dispersal of non-climbing rattans in Peninsular Malaysia. *Asian Primates Journal*, *4*(2), 42–51.

Ruppert, N., Mansor, A., & Anuar Mohd Sah, S. (2016). Rattan (Calamoideae) abundance and above-ground biomass at a primary rainforest of Peninsular Malaysia. *Plant Ecology and Diversity*, *9*(1), 63–67. https://doi.org/10.1080/17550874.2015.1081650

Ter Braak, C. J. F. (1988). *CANOCO - A FORTRAN program for canonocal community ordination by (partial) (detrended) (canonical) correspondence analysis, pincipal components analysis and redundancy analysis* (version 2.1). Agricultural Mathematics Group.

Wan Ariffin, W. T., Rene, K., Muralidharan, E. M., Sreekumar, V. B., Chowdhary, C., Sheng, L. R., Viet, T. L., Sunderland, T., Haider, R., Tekpetey, S., Olorunnisola, A. O., Achdiawan, R., & Hourt, H. E. (2018). *Rattan terminologies*. International Bamboo and Rattan Organisation (INBAR).

Watanabe, N. M., & Suzuki, E. (2008). Species diversity, abundance, and vertical size structure of rattans in Borneo and Java. *Biodiversity and Conservation*, *17*(3), 523-538. https://doi.org/10.1007/s10531-007-9268-1

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Complementarity in Rubber-Salacca Intercropping System Under Integrated Fertilization Mixed with Organic Soil Amendments

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ABSTRACT

The replanting practice of rubber monocropping in Southern Thailand has depleted soil fertility. Most rubber planted areas in the region were under intensive chemical fertilization resulting in less soil organic matters and root proliferation. With the instability of rubber prices, some rubber farmers converted from monocropping into intercropping. Integrated fertilization in which mixed organic-inorganic fertilizers are combined with organic soil amendments could be considered in a rubber-based intercropping system to increase land productivity with cost-saving fertilization by rehabilitating soil properties. A study was conducted at a rubber-salacca intercropping farm comprised of 14-year-old mature rubber trees associated with eight-year-old salacca palms to identify the consequences of the integrated fertilization combined with two organic soil amendments: humic acid (HSA); chitosan (CSA). Changes in soil organic matter (SOM), leaf area index (LAI), fine root traits, tree physiological status, and crop productions under the two integrated fertilization were compared against the controlled application of conventional chemical fertilizer. The CSA application increased the SOM in the topsoil layer by 80%. In the 21-40 cm soil depth, the rubber roots treated with HSA and the salacca palm roots treated with CSA showed greater fine root length density (FRLD). Under CSA, the physiological status of the rubber trees showed less stress. The treatments of HSA and CSA showed 145% and 72%, respectively, higher in total production of salacca palm than that of the

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chemical fertilization. Improvements in the soil fertility, the root's function, the crops' yields, and the tree physiological status were consequences as complementarity in the system under the integrated fertilizations.

Keywords: Chitosan, *Hevea brasiliensis*, humic acid, intercropping, integrated fertilization, soil amendment

INTRODUCTION

Most natural rubber (*Hevea brasiliensis*) growing areas in Southern Thailand are currently in the second or third replanting cycle of rubber monocropping. This replanting practice of the same perennial monocrop has depleted soil fertility substantially (Umami et al., 2019; Vrignon-Brenas et al., 2019). Besides, about 67% of the region's rubber growing area was under intensive application of chemical fertilizer (National Statistical Office, 2013) to meet targeted immature period and economic yield. Its long-term application accumulated adverse effects on soil structure, such as soil acidification, soil water pollution, and soil organic matter shortage — consequently, root functions like less root proliferation and nutrient-uptake activities.

In the last two decades, due to the instability of rubber prices, some rubber farmers in the area started converting into intercropping to widen the on-farm income sources and increase land productivity (Hougni et al., 2018; Romyen et al., 2018). In the area, most rubber-based intercropping farms were transformed from mature monocropping rubber farms and mostly intercropped with perennial cash crops like bamboo, coffee, cacao, ginger, and salacca anticipation long-term economic benefits (Jongrungrot et al., 2014). However, some combinations of rubber-based intercropping experienced adverse effects on the growth and yield of the crops due to some competitions in root interactions and nutrient uptakes (Langenberger et al., 2017). Thus, in these types of permanent rubber-based intercropping, complementarity interactions in the system are the main consideration in which the crops and other components are facilitative complements each other to achieve ecological benefits together with healthy physiological status of the crops and vegetative growth, ensuring sustainable crop yields for long-term economic benefits (Bybee-Finley & Matthew, 2018).

Since rubber tree transforms sucrose into natural rubber, cis polyisoprene, as a product of the tree's defense mechanism in response to human interventions (latex harvesting) and environmental conditions, the healthy physiological status of the tree plays a crucial role in natural rubber production (Adou et al., 2017; Obouayeba et al., 2011). Biochemical compositions, mainly sucrose (Suc), inorganic phosphorus (Pi), and reduced thiols (R-SH) contents, are analyzed to evaluate the physiological status and yield potential of rubber trees (Christophe et al., 2018). As the sucrose are transformed into rubber molecules in the laticiferous system, high Suc content in the rubber latex indicates less sucrose utilization in the defense mechanism. Overexploitation in latex harvesting significantly reduces

the Suc content in the latex, reflecting the high stress of the physiological status of the tree (Doungmusik & Sdoodee, 2012). The Pi represents the main constituent of the energy metabolism in the laticiferous system and exhibits the level of sucrose utilization and intensity of biosynthetic activity. Atsin et al. (2016) reported that Pi content was positively associated with the active metabolism; thus, a higher Pi indicated a significant yield potential under healthy rubber trees. The reduced thiols are important antioxidants to protect the laticiferous cells in the defense mechanism and reduce oxidative stresses mainly caused by latex harvesting (Purwaningrum et al., 2019). Low R-SH content in the latex indicates high physiological stress of the laticiferous system.

According to the principle of integrated nutrient management, harmonious utilization of farm nutrient sources such as organic manure and farm wastes, mixed with inorganic fertilizers could be considered an integrated fertilizer (Food and Agriculture Organization of the United Nations [FAO], 2016) in the rubber-based intercropping system to increase land productivity with cost-saving fertilization through improvement or rehabilitation of soil properties.

One of the integrated usages of available farm wastes, humic acid extracted from vermicompost of biodegradable farm wastes like animal manures, green manures, and crop residues, has been widely applied as an organic soil amendment (Selladurai & Purakayastha, 2016). It enhances microbial activities and a population that transform insoluble mineral nutrients into available nutrient form for plant in the soil, thus higher soil nutrient content (Li et al., 2019). In humic acid-treated soil, pH buffering capacity, organic matter, and cation exchange capacity were improved with more significant soil physical properties resulting in enhanced root performances like fine root proliferation and nutrient uptake (Buyukkeskin et al., 2015; Cahyo et al., 2014). It was reported that the growth rates of nursery and immature rubber plants were enhanced by reducing chemical fertilizer usages and supplementing a humic acid application (Dharmakeerthi et al., 2013). Likewise, chitin and chitosan processed from chitin-containing wastes from the fishery industry, available in the area, have been widely applied as a natural plant elicitor. Chitosan-treated plants improved pathogen resistance because of their antimicrobial properties and defense mechanism (Sharp, 2013). With improved plant metabolism, vegetative growth of plant and crop yield were significant under chitosan application in combination with chemical fertilizer (Y. C. Chen et al., 2016).

Although the sources for these organic soil amendments are available in the area, their usages have not been found yet in the rubber farms and rubber-based intercropping. Furthermore, studies related to the integrated fertilizations in rubber-based intercropping systems are also limited in the scientific literature. Thus, an experiment was conducted at a mature rubber-intercrop farm to investigate

the consequences of the agroecosystem components' interactions under integrated fertilizer applications combined with different organic soil amendments compared to conventional chemical fertilization.

MATERIALS AND METHODS

A mature rubber farm intercropped with salacca palm (*Salacca zalacca*) situated at 6°59'46.9"N, 100°34'58.6"E in Na Mom district, Songkhla province, Southern Thailand, was selected for the experimental study. The area receives an annual rainfall of about 2,000 mm distributed from June to December. In general, monthly rainfall precipitates less than 200 mm from June to September, around 300 mm in October and November, and peaks in December with about 500 mm.

The farm was started as a monocrop rubber replanting with RRIM 600 cultivar planted in a spacing of 6 m x 3 m on flat land in 2002. The rubber trees have been harvested, applying a tapping system of one-third spiral of tapping cut length and two-day tapping in three days since 2008. The heights of the rubber tree were around 18 m, and the stem girths were average at 79 cm at the height of 170 cm from the ground. The associated plant, salacca palm, was intercropped in 2008 between the rubber rows with the same spacing as the rubber planting. As a result, the palm's growths were uniform, with the average height and width of their canopies of 3.6 m and 4.5 m, respectively.

The experiment was designed in a randomized complete block design comprised of three fertilization treatments with three replications. Each replicated plot covered one row of ten rubber trees and adjacent two rows of the salacca palms. The treatments were formulated to compare the applications of two different organic soil amendments combined with mixed organic-inorganic fertilizer against the controlled application of conventional chemical fertilizer (Table 1).

Table 1

Summary of the three treatments of fertilizations

Treatments	Chemical fertilizer		Organic fertilizer		Organic soil amendment	
	Types	Application rate	Types	Application rate	Types	Application rate
T1	Compound fertilizer (30-5-18)	1 kg tree^{-1} y^{-1} (3 times)	-	-	-	-
T2	Compound fertilizer (30-5-18)	0.5 kg tree^{-1} y^{-1} (3 times)	Composted cow manure	10 kg (3 times)	Humic acid	100 mL 20 L^{-1} water (3 times)
T3	Compound fertilizer (30-5-18)	0.5 kg tree^{-1} y^{-1} (3 times)	Composted cow manure	10 kg (3 times)	Chitosan	100 mL 20 L^{-1} water (3 times)

In the control treatment (T1), chemical compound fertilizer 30-5-18 nitrogen-phosphorus-potassium (N-P-K) was broadcasted at a rate of one kilogram per rubber tree per year between the rows of the rubber trees and the salacca palms in March, July, and November 2016. In the other treatments (T2 and T3), the chemical fertilizer mixed at a rate of 0.5 kg per rubber tree with 10 kilograms of composted cow manure was applied from April. Then, humic-acid soil amendment (HSA) prepared by mixing 100 mL of vermicompost-derived humic acid (pH 6.5, 5% humic acid, 50% organic matter, 5% total nitrogen, 2.5% total potassium, 0.06% total phosphorus, 0.25% calcium) with 20 L of water and sprayed on the soil between the rubber trees and the palms in T2 from May (Ruangkhanab & Lim, 2005). Then, with the same application rate as the HSA treatment, 100 mL of the chitosan (pH 5.5~6, 6.5% organic carbon, 0.05% nitrogen, 0.01% phosphorus oxide, 0.01% potassium) mixed with 20 L of water was applied as the chitosan soil amendment (CSA) in T3 from May. All these fertilizations were applied three times with a third-monthly interval during the study period.

Soil organic matters (SOM) from soil depths of 0–20 cm and 21–40 cm of each plot were determined using Walkley-Black's titration method (FAO, 2020) in February 2016 and February 2017 to compare the SOM contents before and after treatments.

Changes in leaf area index (LAI) at the farm were monitored monthly by the hemispherical photography method from June to December 2016. The hemispherical photos were taken vertically upward from 1.2 m above the ground at three different points in the inter-row between the rubber trees and the salacca palms at every treatment plot by using Nikon Coolpix 8400 camera (Nikon, Japan) with a fish-eye lens (Bianchi et al., 2017). The Gap Light Analyzer (GLA) software version 2.0 was used to analyze the fish-eye captured images.

Changes in fine root traits, notably fine root diameter (FRD) and fine root length density (FRLD) of both crops, were monitored in two layers of soil depths (0–20 cm and 21–40 cm) by using the Prince of Songkla University (PSU) minirhizotron root scanner through 10 cm in diameter with 100 cm long of two acrylic access tubes per treatment plot installed with 45° angle of slope in the soil (Saelim et al., 2019; Vamerali et al. 2011) between the rubber tree and the palm. Two months after installing the acrylic tubes, the root images were scanned monthly from June to December 2016. The scanned images were analyzed using the Rootfly software (version 2.0.2).

Latex samples were collected monthly from each plot to analyze the latex production expressed in dry rubber weight per tapping per tree (g tap^{-1} $tree^{-1}$). The collected samples were coagulated using formic acid and then dried at 70 °C for 16 h to calculate the dry weight of rubber content in the latex as recommended by ISO 126:2005. Productions of the salacca palms in yield per cluster and total yield per palm were recorded collectively at the end of the study period from randomly selected seven palms from each plot.

The biochemical parameters of latex, namely sucrose (Suc) content, inorganic phosphorus (Pi) content, and reduced thiols (R-SH) content, were measured monthly from latex samples taken from selected rubber trees of each treatment plot by following the latex micro-diagnosis method of the French Agricultural Research Centre for International Development (CIRAD). (Chantuma et al., 2011).

Data collected were analyzed with the R software (version 3.6.2) using a one-way analysis of variance (ANOVA). In addition, Duncan's multiple range tests were performed at $p \leq 0.05$ to compare the data pairs, and Pearson's linear correlation (r) at $p \leq 0.05$ was applied in correlation analysis.

RESULTS

Comparisons of SOM

The higher content of SOM was found in the topsoil layer (0–20 cm depth). In comparison, the deeper soil layers had relatively lower organic matter content under all treatments after the experiment (Figure 1). Although all treatments increased the SOM in all layers of soil depth, the top layers under T1 and T3 showed remarkably higher soil organic matter contents. T3 increased the SOM in the topsoil layer by 80%, followed by T1, with an increase of 38% after the experiment.

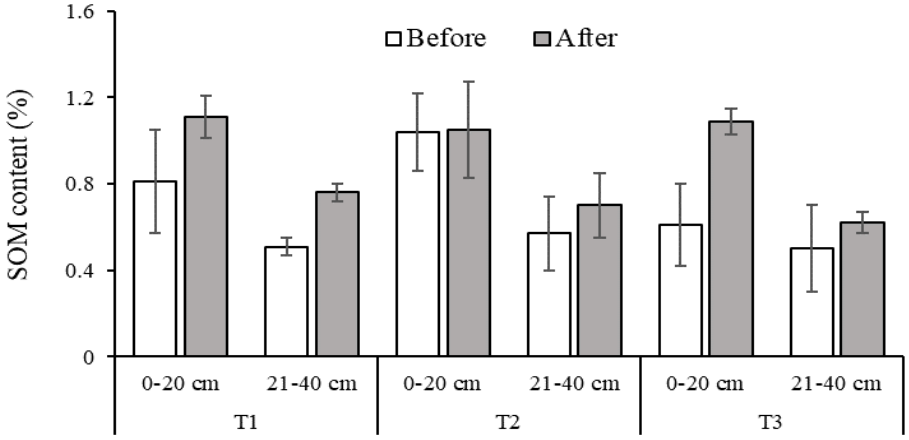

Figure 1. Comparison of soil organic matter (SOM) among the treatments before and after the experiment

LAI of the Farm

Although there were no significant differences in the LAIs among the treatments during the study, the changes followed a similar trend (Figure 2). The LAIs of the farm started increasing in July with just over 1.10 and reached their maximum values ranging between 1.50 and 1.71 in September. Then they decreased to their lowest values between 1.00 and 1.20 in October and

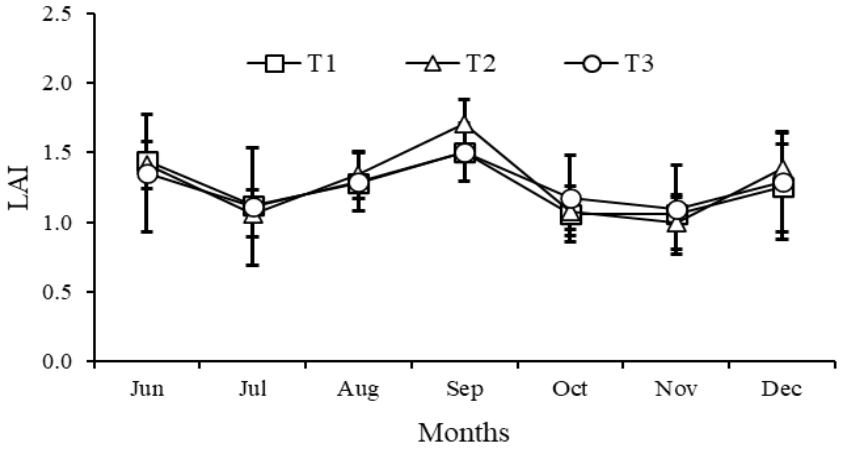

Figure 2. Changes in leaf area index (LAI) of the farm under the three treatments (from June to December 2016)

November, respectively. However, the LAI values of the farm increased back in the range of 1.29 and 1.39 in December.

Fine Root Traits of the Rubber Tree

FRDs of the rubber trees under T1 were found as the largest over those of the other treatments from June to September in both soil layers (0–20 cm and 21–40 cm) (Figure 3). In the soil depth of 21–40 cm, the average size of the FRD under T1 was higher than that of T2 and T3 by 27% and 28%, respectively (Figure 3B).

In terms of changes in FRLD (Figure 3 C), all treatments resulted in a stable trend ranging between 0.34 and 0.70 cm cm^{-2} in the topsoil layer during the study period. In the soil depth of 21–40 cm (Figure 3 D), the rubber trees under T2 were observed with the highest FRLD at over 1.44 cm cm^{-2} between July and October. After October, however, it decreased slightly with the densities of 1.46 and 1.09 cm cm^{-2} in November and December, respectively.

Fine Root Traits of the Salacca Palm

The fine roots of the salacca palm in the soil depth of 0-20 cm (Figure 4A) under T1 showed the largest diameter sizes ranged between 0.82 to 1.23 cm, while the other treatments resulted in smaller sizes of the FRDs ranging between 0.67 and 0.95 cm. In the soil depth of 21–40 cm, the sizes of FRD under T1 were also larger than those under other treatments in July, August, September, and October (Figure 4 B).

Monthly changes of the FRLD of the salacca palm (Figure 4 C) in the soil depth of 0-20 cm were stable between 0.20 and 0.38 cm cm^{-2} and did not show a significant difference during the study period. However, in 21–40 cm soil depth, T3 resulted in the highest FRLD in July, October, November, and December with 0.60, 0.64, 0.46, and 0.40 cm, respectively (Figure 4 D).

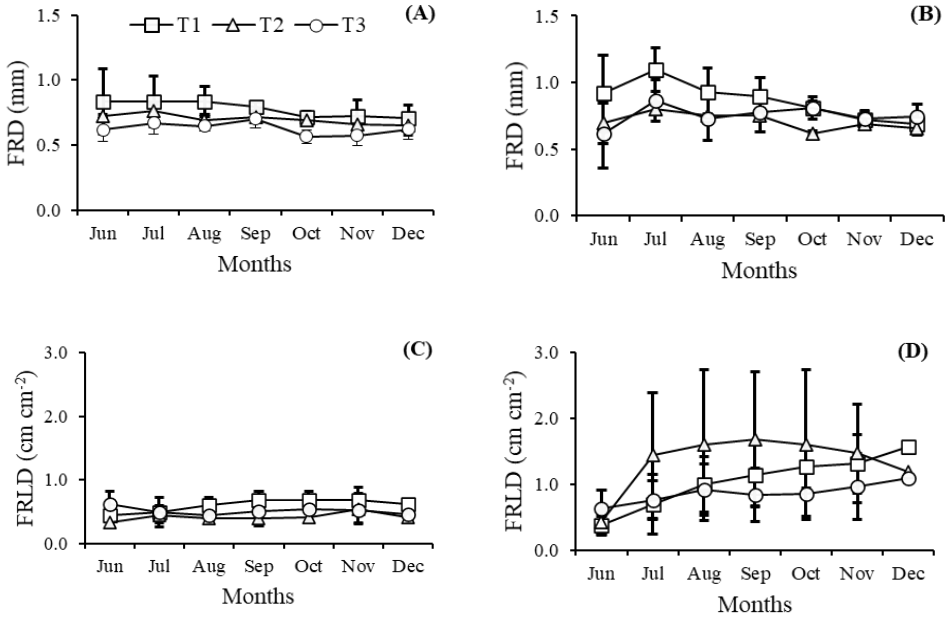

Figure 3. Monthly changes in fine root traits of the rubber tree: fine root diameter (FRD) at the soil depths of (A) 0-20 cm and (B) 21-40 cm; fine root length density (FRLD) at the soil depth of (C) 0-20 cm and (D) 21-40 cm (from June to December 2016)

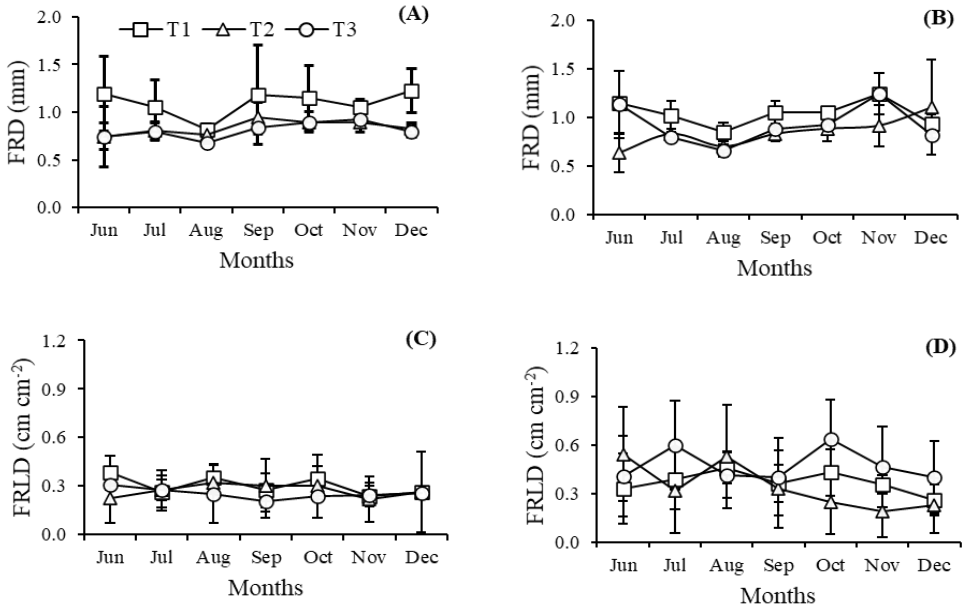

Figure 4. Monthly changes in fine root traits of the salacca plam: fine root diameter (FRD) at the soil depths of (A) 0-20 cm and (B) 21-40 cm; fine root length density (FRLD) at the soil depth of (C) 0-20 cm and (D) 21-40 cm (from June to December 2016)

Latex Production

Although there were no significant differences among the latex productions under the different treatments, the latex productions varied with different seasons (Figure 5). At the beginning of the rainy season, the productions under all treatments dropped their yields from about 60 g tap^{-1} tree^{-1} in June to less than 40 g tap^{-1} tree^{-1} in July. Then, the production increased to the highest level between 73 and 80 g tap^{-1} tree^{-1} in September. However, all treatments showed less production with around 30 g tap^{-1} tree^{-1} in November. Finally, in December, the productions under T1, T2, and T3 surged back, respectively, with 80, 65, and 50 g tap^{-1} tree^{-1}. The result of Pearson's linear correlation (r = + 0.6024) at $p \leq 0.05$ confirmed a positive correlation between the monthly changes of the LAIs and the latex production under all treatments (Figure 6).

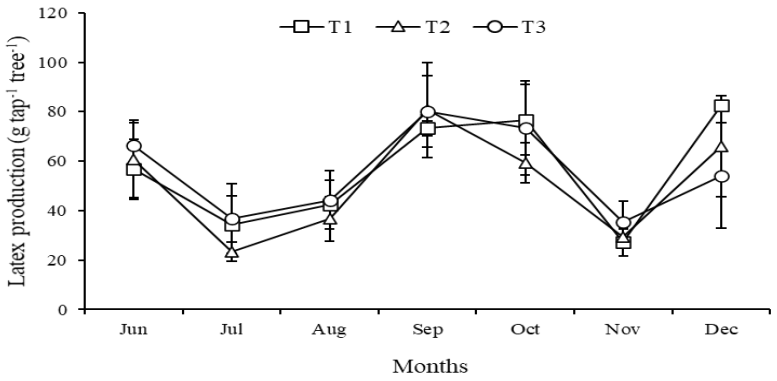

Figure 5. Monthly changes in average daily production of latex (g tap^{-1} tree^{-1}) under the treatments (from June to December 2016)

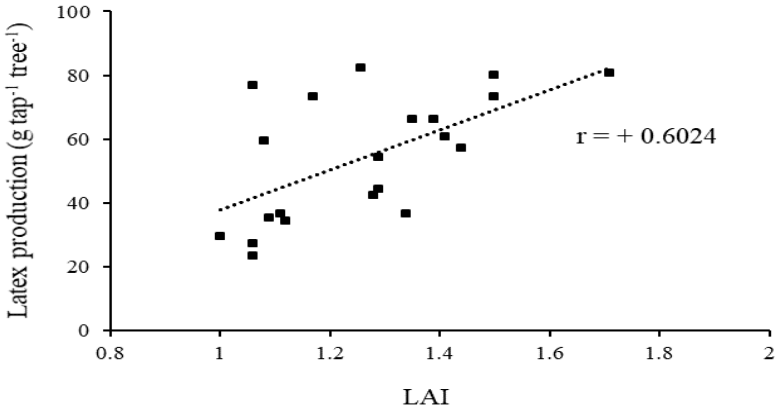

Figure 6. Relationship between the changes of LAI and latex productions

Latex Biochemical Composition

Suc contents of all treatments decreased gradually between July and October, except that of T2 showed a peak at 13.66 mM in August (Figure 7 A). The Suc contents of T1 and T2 reached their minimum levels of 1.79 and 2.43 mM, respectively, in November. However, T3 showed an upward trend in November after its lowest level of 4.65 mM in October. In December, the Suc content under T3 reached 9.77 mM as the highest level in that month, followed by T2 and T1 with 6.76 and 3.53 mM, respectively.

Pi content under T2 decreased from 21.33 mM in June to 10.52 mM in July (Figure 7 B). The contents under T1 and T3, however, were stable between 10.54 and 12.61 mM from June to September. Between September and November, the Pi contents of all treatments increased, and that of T3 was the highest with 30.59 mM followed by that of T2 and T1, respectively, in November. Then, the Pi contents under all treatments decreased again in December.

R-SH levels of the treatments were different in June as that of T3 was at 0.43 mM as the highest, followed by T1 and T2 with 0.30 mM and 0.15 mM, respectively (Figure 7C). After July, however, all treatments increased slightly until November, and the R-SH level under T3 was the highest in November. Then in December, the R-SH level of all treatments declined under 0.30 mM.

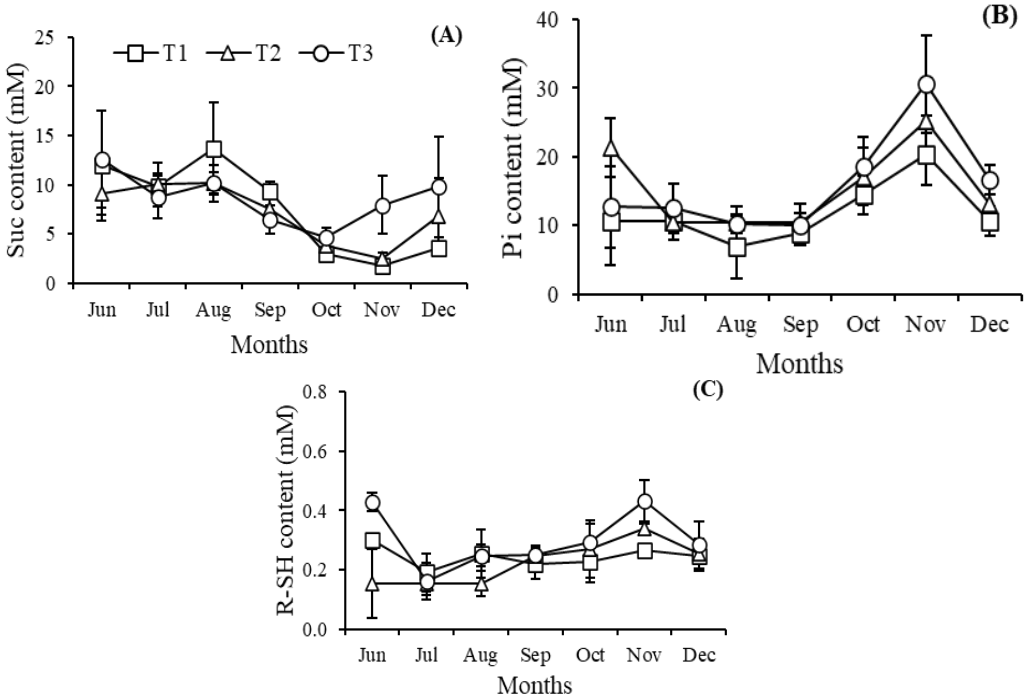

Figure 7. Monthly changes in biochemical composition (A) sucrose – Suc content; (B) inorganic phosphorus – Pi content; (C) reduced thiols – R-SH content of latex under the treatments (from June to December 2016)

Salacca Palm Production

The salacca productions were significantly different among the treatments in yield per cluster, and total yield per palm (Table 2) as T2 delivered the highest weight with 1.60 kg cluster^{-1} followed by T3 with 1.33 kg cluster^{-1} while that of T1 was the lowest at 0.77 kg cluster^{-1}. Likewise, the total yields (kg palm^{-1}) of T2 and T3 were 145% and 72%, respectively, higher than T1.

Table 2

Production of the salacca palms among the treatments

Treatment	Yields of the salacca palm	
	kg cluster^{-1}	kg palm^{-1}
T1	0.77 ± 0.05^{c}	2.50 ± 0.89^{c}
T2	1.60 ± 0.09^{a}	6.13 ± 1.10^{a}
T3	1.33 ± 0.21^{ab}	4.38 ± 1.50^{b}

Note. Different lower-case letters in the same column are significantly different at $p \leq 0.05$ by Duncan's multiple range test

DISCUSSION

Soil Fertility Improvement

The study observed that the plot amended with CSA had a maximum level of SOM content in the topsoil layer. The result was likely due to the enzymatic soil microbial activities improved by CSA, enhancing the decomposition process of organic materials in the topsoil layer (Sawaguchi et al., 2015). Besides, the soil microbial population increased and decomposed themselves, resulting in a higher level of organic matter in the soil. The higher content of SOM is an indicator of healthy soil with efficient infiltration and water-holding capacity, thus higher nutrient availability (C. Chen et al., 2017; Nannipieri et al., 2017).

Development of the Fine Root Traits

It was noticed that the FRD of both crops under T1 showed a larger size in both soil layers in general. It signaled high limitation in the movements of water and nutrients from the soil to the roots resulting in low vegetative growth and productivity (Comas et al., 2013). Conversely, roots with smaller diameters have greater hydraulic conductivity and tolerate drought conditions (Henry et al., 2012). The small diameters of the fine roots under T2 and T3 reflected the better performance of the root functions because of the higher availability of nutrients and water in the soil under the organic soil amendment application (du Jardin, 2015).

In all treatments, the FRLD of rubber trees in the soil depth of 21–40 cm showed upward trends once the rainy season began but in the soil depth of 0-20 cm. It indicated that the development of rubber fine roots in the soil depth of 21–40 cm was more

responsive to the rainfall than the topsoil layer. A study conducted in the same province by Saelim et al. (2019) also found that the fine roots of the 16-year-old rubber, particularly in the soil depth 20–30 cm developed at a higher rate in the rainy season. The result was consistent with Maeght et al.'s (2015) finding in north-eastern Thailand that the fine rubber roots within the soil depth of 2 m exhibited higher root emergences during the rainy season. Among the treatments, the rubber trees treated with the HSA showed higher FRLD in 21–40 cm soil depth from July to October. Wasson et al. (2012) remarked that a root system with greater FRLD in deeper soil could uptake water and nutrients at high efficiency. Cahyo et al. (2014) reported that root growth and performance were more obvious than other vegetative parts under the HSA. It could serve as auxin and promote cell enlargement by stimulating the cell wall loosening leading to greater vegetative growth (Jindo et al., 2012). However, it was noticed that the FRLDs of the salacca palm were higher under the CSA in the soil depth of 21–40 cm. CSA could enhance cation properties and water holding capacity in the soil, thereby more significant development of fine roots resulting in better nutrient uptakes and improved crop yield (Sharp, 2013).

The Vegetative Growth and Production of the Crops

The study confirmed a positive relationship between the LAIs and latex production under all treatments. At the beginning of the rainy season, in July and August, latex harvest (tapping) activities could not be carried out regularly due to the disturbance of uneven raining patterns resulted in yield drops in all treatments. The latex productions under all treatments were at maximum levels in September, while leaves in the rubber canopy reached the ultimate growth stage. Since the planted cultivar, RRIM 600 clone, is susceptible to phytophthora leaf fall disease (Krishnan et al., 2019), which occurs typically during the rainy season, the rubber trees in the farm were attacked by the disease, thus fewer values of LAI in November. In the meantime, it was observed that the latex yields under all treatments dropped from their maximum yields. Leaf area is a functional part of a tree's photosynthesis and determines photosynthetic efficiency, reflecting sucrose synthesis (Weraduwage et al., 2015). Since natural rubber is a photosynthesis product of *H. brasiliensis* through sucrose synthesis in non-photosynthesis laticiferous tissue, the leaf area of the rubber tree influences latex yield and dry mass production of rubber (Zhu et al., 2018).

Regarding salacca production, the treatments of the integrated fertilizations delivered significantly higher yields compared to that of the chemical fertilization. It was contributed by the beneficial effects of the integrated fertilization that organic fertilizer and organic soil amendments could promote inorganic fertilization effectiveness, thereby more extended availability of nutrients in the soil (Wu et al., 2020). In addition, it could improve the soil's physical

properties such as cation exchange capacity and water holding capacity, enhancing root proliferation and the root system's nutrient uptake functions, resulting in higher crop yield (Sharp, 2013).

In addition, it was noticed that yields per cluster in all treatments were apparently higher than the average yield of around 0.6 kg per cluster of conventional salacca-fruit intercropping (Sumantra & Martiningsih, 2018). In rubber-based intercropping, the canopy of mature rubber trees reduces extreme temperature and intense irradiance, improving the adaptability of understorey plants especially shade-required species like salacca palm (Montagnini, 2011; Rappaport & Montagnini, 2014). Along with the favorable weather conditions, the co-existence of the different canopy architectures, like the combination of rubber trees and salacca palms, enhancing light interception and distribution in the farm contributes to a greater photosynthetic rate resulting in yield improvement of the crops (Sumantra et al., 2012; Tang et al., 2019; Xianhai et al., 2012).

Less Physiological Stress of the Rubber Tree

It was observed that all treatments showed higher Suc content, lower Pi content, and lower yields at the beginning of the rainy season after the dry season. It reflected low metabolic utilization or insufficient conversion of sucrose into cis-isoprene rubber molecules in the latex resulting in higher Suc content remaining and fewer rubber particles in the latex (Purwaningrum et al., 2015). Then, in September and October, the yields of all treatments were at a high level with an elevation of the Pi contents. It indicated the high metabolism of the laticiferous contributed by the regular tapping activity (Atsin et al., 2016). However, in November, the Suc contents under T1 and T2 declined to the lowest level, and their productions also plunged to less than 30 g tap^{-1} $tree^{-1}$ at that month, reflecting that the rubber trees were exhausted with the shortage of sucrose supply because of the effects of the high-frequency latex harvest practice (overexploitation) and the occurrences of the abnormal leaf fall disease. A study by Obouayeba et al. (2011) indicated that low sucrose content less than around 3-4 mM associated with yield drops reflected the initial symptom of the tree stress with physiological disorders in the laticiferous system leading to tapping panel dryness. The intensity of physiological stress could vary between rubber clones due to their different sugar loading capacities (Gohet et al., 2015). In addition, the abnormal leaf fall disease destructed the photosynthesis functions, thereby reducing the Suc's sufficient supply, resulting in the yield drop. However, the Suc content, the Pi content, and the R-SH content under T3 was at a high level, and the yield in T3 remained over 30 g tap^{-1} $tree^{-1}$ and was not as low as that of the others. These physiological responses reflected less physiological stress of the laticiferous system (Sainoi et al., 2017) and the lesser effect of the phytophthora attack under T3 compared to those of the other treatments. It was likely to be the CSA's antimicrobial

effect since its application restrained and slowed down the growth of the pathogen by enhancing the response of the plant's immune system (Sunpapao & Pornsuriya, 2014).

CONCLUSION

The study observed that both HSA and CSA treatments improved the fine root trait developments of the crops, particularly in the soil depths of 21–40 cm. The fine rubber roots were responsive under the HSA, while the fine root growths of the salacca showed more significance under the CSA. It was found that a positive correlation between the average yields of rubber and the LAI in the farm. The study highlighted that the advantages of CSA on rubber trees that its application improved the tree physiological status. Thus, the latex biochemical composition levels and the daily yield were maintained under the CSA application during the intensive latex harvest practices and the phytophthora leaf disease attack. A significant increase in soil organic matter under the CSA treatment was also advantageous.

The higher yields per cluster of salacca trees in all treatments compared to other conventional salacca farms indicated the beneficial effect of the rubber-salacca combination. In addition, the significantly higher yields of salacca under the HSA and CSA further approved the effect of the integrated fertilizations.

The study highlighted the complementarity effect resulting from harmonious interactions between the integrated fertilization and agroecosystem components of the rubber-salacca intercropping. Therefore, it is suggested that the mixed organic-inorganic fertilization with organic soil amendments could be utilized in rubber-based intercropping as effectively integrated fertilization to reduce the usage of chemical fertilizer without affecting the crop yields.

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REFERENCES

Adou, C. Y. B., Atsin, O. J. G., Essehi, J. L., Ballo, K. E., Soumahin, F. E., Obouayeba, P. A., Kouakou, H. T., & Obouayeba, S. (2017). Latex micro diagnosis, modern management tool of rubber plantation of clones with moderate metabolism GT 1, RRIC 100 and BPM 24. *Journal of Applied Biosciences*, *121*, 12098-12109. https://doi.org/10.4314/jab.v121i1.1

Atsin, G. J. O., Soumahin, E. F., Kouakou, T. H., Elabo, A. E. A., Okoma, K. M., & Obouayeba, S. (2016). Agronomic potential of some rubber tree clones (*Hevea brasiliensis*) of the fast metabolic activity class in the absence of hormonal stimulation in southwestern of Côte d'Ivoire. *American Journal of Experimental Agriculture*, *13*(4), 1-13. https://doi.org/10.9734/AJEA/2016/26930

Bianchi, S., Cahalan, C., Hale, S., & Gibbons, J. M. (2017). Rapid assessment of forest canopy and light regime using smartphone hemispherical photography. *Ecology and Evolution*, *7*(24), 10556-10566. https://doi.org/10.1002/ece3.3567

Buyukkeskin, T., Akinci, S., & Eroglu, A. E. (2015). Effects of humic acid on root development and nutrient uptake of *Vicia faba* L. (broad bean) seedlings grown under aluminum toxicity. *Communications in Soil Science and Plant Analysis*, *46*(3), 277-292. https://doi.org/10.1080/00103624.2014.969402

Bybee-Finley, K. A., & Matthew, R. R. (2018). Advancing intercropping research and practices in industrialized agricultural landscapes. *Agriculture*, *8*(6), 80. https://doi.org/10.3390/agriculture8060080

Cahyo, A. N., Ardika, R., Saputra, J., & Wijaya, T. (2014). Acceleration on the growth of rubber planting materials by using folia application of humic acid. *Agrivita*, *36*(2), 112-119. https://doi.org/10.17503/agrivita.v36i2.397

Chantuma, P., Lacote, R., Leconte, A., & Gohet, E. (2011). An innovative tapping system, the double cut alternative, to improve the yield of *Hevea brasiliensis* in Thai rubber plantations. *Field Crops Research*, *121*(3), 416-422. https://doi.org/10.1016/j.fcr.2011.01.013

Chen, C., Liu, W., Jiang, X., & Wu, J. (2017). Effects of rubber-based agroforestry systems on soil aggregation and associated soil organic carbon: Implication for land use. *Geoderma*, *299*, 13-24. https://doi.org/10.1016/j.geoderma.2017.03.021

Chen, Y. C., Yuan, S., Liu, H., Chen, Z., Zhang, Y., & Zhang, H. (2016). A combination of chitosan and chemical fertilizers improves growth and disease resistance in *Begonia hiemalis* Fotsch. *Horticulture, Environment, and Biotechnology*, *57*, 1-10. https://doi.org/10.1007/s13580-016-0119-4

Christophe, A. B. Y., Mathurin, O. K., Pacome, O. A., Olivier, A. G., Kouadio, B. E., Jean-Lopez, E., Jean, W. P., Francis, S. E., Hilaire, K. T., & Samuel, O. (2018). Latex micro diagnosis, modern management tool of rubber plantations of clones with active or rapid metabolism IRCA 18, IRCA 130, PB 235, PB 260 and PB 330. *European Scientific Journal*, *14*(3), 385. https://doi.org/10.19044/esj.2018.v14n3p385

Comas, L. H., Becker, S. R., Cruz, V. M., Byrne, P. F., & Dierig, D. A. (2013). Root traits contributing to plant productivity under drought. *Frontiers in Plant Science*, *4*, 442. https://doi.org/10.3389/fpls.2013.00442

Dharmakeerthi, R. S., Chandrasiri, J. A. S., & Edirimanne, V. U. (2013). Humic acid based liquid organic fertilizer improved the growth of nursery and immature rubber plants grown in Boralu soil series. *Journal of the Rubber Research Institute of Sri Lanka*, *93*, 1-15. http://doi.org/10.4038/jrrisl.v93i0.1863

Doungmusik, A., & Sdoodee, S. (2012). Enhancing the latex productivity of *Hevea brasiliensis* clone RRIM 600 using ethylene stimulation. *Journal of Agricultural Technology*, *8*(6), 2033-2042.

du Jardin, P. (2015). Plant biostimulants: Definition, concept, main categories and regulation. *Scientia Horticulturae*, *196*, 3-14. https://doi.org/10.1016/j.scienta.2015.09.021

Food and Agriculture Organization of the United Nations. (2016). Guidelines for the management of plant nutrients and their sources. In R. N. Roy, A. Finck, G. J. Glair, & H. L. S. Tandon (Eds.), *Plant nutrition for food security: A guide for integrated nutrient management* (pp. 141-192). FAO.

Food and Agriculture Organization of the United Nations. (2020). *Standard operating procedure for soil organic carbon. Walkley-Black method: Titration and colorimetric method*. FAO.

Gohet, E., Cavaloc, E., Cardoso, S., Cairo, I., Garcia, D., Rivano, F., Lacote, R., & Lesturgez, G. (2015). A first physiological assessment of latex clonal metabolic typology and rubber yield potential of "CMS" rubber tree clones. In *Proceedings of International Rubber Conference* (pp. 287-294). Agriculture Publishing House. https://doi.org/10.13140/RG.2.1.3007.4328

Henry, A., Cal, A. J., Batoto, T. C., Torres, R. O., & Serraj, R. (2012). Root attributes affecting water uptake of rice (*Oryza sativa*) under drought. *Journal of Experimental Botany*, *63*(13), 4751-4763. https://doi.org/10.1093/jxb/ers150

Hougni, D. J. M., Chambon, B., Penot, E., & Promkhambut, A. (2018). The household economics of rubber intercropping during the immature period in Northeast Thailand. *Journal of Sustainable Forestry*, *37*(8), 787-803. https://doi.org/10.1080/10549811.2018.1486716

Jindo, K., Aparecida, M., Navarro, E. C., Perez-Alfocea, F., Hernandez, T., Garcia, C., Aguiar, N. O., & Canellas, L. P. (2012). Root growth promotion by humic acids from composted and non-composted urban organic wastes. *Plant Soil*, *353*, 209-220. https://doi.org/10.1007/s11104-011-1024-3

Jongrungrot, V., Thungwa, S., & Snoeck, D. (2014). Tree-crop diversification in rubber plantations to diversify sources of income for small-scale rubber farmers in Southern Thailand. *Bois et Forets des Tropiques*, *68*(321), 21-32. https://doi.org/10.19182/bft2014.321.a31214

Krishnan, A., Joseph, L., & Roy, C. B. (2019). An insight into *Hevea – Phytophthora* interaction: The story of *Hevea* defense and *Phytophthora* counter defense mediated through molecular signalling. *Current Plant Biology*, *17*, 33-41. https://doi.org/10.1016/j.cpb.2018.11.009

Langenberger, G., Cadish, G., Martin, K., Min, S., & Waibel, H. (2017). Rubber intercropping: A viable concept for the 21st century?. *Agroforestry Systems*, *91*, 577-596. https://doi.org/10.1007/s10457-016-9961-8

Li, Y., Fang, F., Wei, J., Wu, X., Cui, R., Li, G., Zheng, F., & Tan, D. (2019). Humic acid fertilizer improved soil properties and soil microbial diversity of continuous cropping peanut: A three-year experiment. *Scientific Reports*, *9*, 12014. https://doi.org/10.1038/s41598-019-48620-4

Maeght, J. L., Gonkhamdee, S., Clement, C., Isarangkool Na Ayutthaya, S., Stokes, A., & Pierret, A. (2015). Seasonal patterns of fine root production and turnover in a mature rubber tree (*Hevea brasiliensis* Mull. Arg.) stand-differentiation with soil depth and implications for soil carbon stocks. *Frontiers in Plant Science*, *6*, 1022. https://doi.org/10.3389/fpls.2015.01022

Montagnini, F. (2011). Can tree plantations serve as catalysts of secondary forest succession?. In F. Montagnini & C. Finney (Eds.), *Restoring degraded landscapes with native species in Latin America* (pp. 3-28). Nova Science Publishers.

Nannipieri, P., Ascher, J., Ceccherini, M. T., Landi, L., Pietramellara, G., & Renella, G. (2017). Microbial diversity and soil functions. *European Journal of Soil Science*, *68*(1), 12-26. https://doi.org/10.1111/ejss.4_12398

National Statistical Office. (2013). *Executive summary: 2013 Agricultural census, southern region*. National Statistical Office.

Obouayeba, S., Soumahin, E. F., Okoma, K. M., Boko, A. M. C. K., Dick, K. E., & Lacote, R. (2011). Relationship between tapping intensity and tapping panel dryness susceptibility of some clones of *Hevea brasiliensis* in Southwestern Côte d'Ivoire. *Agriculture and Biology Journal of North America*, *2*(8), 1151-1159. https://doi.org/10.5251/abjna.2011.2.8.1151.1159

Purwaningrum, Y., Asbur, Y., & Junaidi. (2019). Latex quality and yield parameters of *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg. clone PB 260 for different tapping and stimulant

application frequencies. *Chilean Journal of Agricultural Research*, *79*(3), 347-355. https://doi.org/10.4067/S0718-58392019000300347

Purwaningrum, Y., Napitupulu, J. A., Siregar, T. H. S., & Hanum, C. (2015). Histology and physiology of BPM 1 clones with different exploitation systems. *International Journal of Sciences: Basic and Applied Research*, *21*, 138-148.

Rappaport, D., & Montagnini, F. (2014). Tree species growth under a rubber (*Hevea brasiliensis*) plantation: Native restoration via enrichment planting in southern Bahia, Brazil. *New Forests*, *45*, 715-732. https://doi.org/10.1007/s11056-014-9433-9

Romyen, A., Sausue, P., & Charenjiratragul, S. (2018). Investigation of rubber-based intercropping system in Southern Thailand. *Kasetsart Journal of Social Sciences*, *39*(1), 135-142. https://doi.org/10.1016/j.kjss.2017.12.002

Ruangkhanab, M., & Lim, M. (2005). Effect of chemical application on vegetative growth of longkong. *Songklanakarin Journal of Science and Technology*, *27*(3), 629-868.

Saelim, S., Sdoodee, S., & Chiarawipa, R. (2019). Monitoring seasonal fine root dynamics of *Hevea brasiliensis* clone RRIM 600 in Southern Thailand using minirhizotron technique. *Songklanakarin Journal of Science and Technology*, *41*(2), 341-348. https://doi.org/10.14456/sjst-psu.2019.43

Sainoi, T., Sdoodee, S., Lacote, R., & Gohet, E. (2017). Low frequency tapping systems applied to young-tapped trees of *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg. in Southern Thailand. *Agriculture and Natural Resources*, *51*(4), 268-272. https://doi.org/10.1016/j.anres.2017.03.001

Sawaguchi, A., Ono, S., Oomura, M., Inami, K., Kumeta, Y., Honda, K., Sameshima-Saito, R., Sakamoto, K., Ando, K., & Saito, A. (2015). Chitosan degradation and associated changes in bacterial community structures in two contrasting soils. *Soil Science and Plant Nutrition*, *61*(3), 471-480. https://doi.org/10.1080/00380768.2014.1003965

Selladurai, R., & Purakayastha, T. J. (2016). Effect of humic acid multinutrient fertilizers on yield and nutrient use efficiency of potato. *Journal of Plant Nutrition*, *39*(7), 949-958. http://doi.org/10.1080/01904167.2015.1109106

Sharp, R. G. (2013). A review of the applications of chitin and its derivatives in agriculture to modify plant microbial interactions and improve crop yields. *Agronomy*, *3*(4), 757-793. https://doi.org/10.3390/agronomy3040757

Sumantra, I. K., & Martiningsih, N. G. E. (2018). *The agroecosystem of Salak Gulapasir (*Salacca zalacca *var. Amboinensis) in new development areas in Bali*. https://www.researchgate.net/publication/342923683_The_Agroecosystem_of_Salak_Gulapasir_Salacca_zalacca_var_amboinensis_in_New_Development_Areas_in_Bali

Sumantra, I. K., Ashari, S., Wardiyati, T., & Suryanto, A. (2012). Diversity of shade trees and their influence on the microclimate of agro-ecosystem and fruit production of Gulapasir salak (*Salacca zalacca var. Amboinensis*) fruit. *International Journal of Basic and Applied Sciences*, *12*(6), 214-221.

Sunpapao, A., & Pornsuriys, C. (2014). Effects of chitosan treatments on para rubber leaf fall disease caused by *Phytophthora palmivora* Butler – A laboratory study. *Songklanakarin Journal of Science and Technology*, *36*(5), 507-512.

Tang, L., Yin, D., Chen, C., Yu, D., & Han, W. (2019). Optimal design of plant canopy based on light interception: A case study with loquat. *Frontiers in Plant Science*, *10*, 364. https://doi.org/10.3389/fpls.2019.00364

Umami, I. M., Kamarudin, K. N., Hermansah, & Abe, S. S. (2019). Does soil fertility decline under smallholder rubber farming? The case of a West Sumatran lowland in Indonesia. *Japan Agricultural Research Quarterly*, *3*(4), 279-297. https://doi.org/10.6090/jarq.53.279

Vamerali, T., Bandiera, M., & Mosca, G. (2011). Minirhizotrons in modern root studies. In S. Mancuso (Ed.), *Measuring roots as update approach* (pp. 341-361). Springer. https://doi.org/10.1007/978-3-642-22067-8_17

Vrignon-Brenas, S., Gay, F., Ricard, S., Snoeck, D., Perron, T., Mareschal, L., Laclau, J., Gohet, E., & Malagoli, P. (2019). Nutrient management of immature rubber plantations. A review. *Agronomy for Sustainable Development*, *39*, 11. https://doi.org/10.1007/s13593-019-0554-6

Wasson, A. P., Richards, R. A., Chatrath, R., Misra, S. C., Prasad, S. V. S., Regetzke, G. J., Kirkegaard, J. A., Christopher, J., & Watt, M. (2012). Traits and selection strategies to improve root systems and water uptake in water-limited wheat crops. *Journal of Experimental Botany*, *63*(9), 3485-3498. https://doi.org/10.1093/jxb/ers111

Weraduwage, S. M., Chen, J., Anozie, F. C., Morales, A., Weise, S. E., & Sharkey, T. D. (2015). The relationship between leaf area growth and biomass accumulation in *Arabidopsis thaliana*. *Frontiers in Plant Science*, *6*, 167. https://doi.org/10.3389/fpls.2015.00167

Wu, L., Jiang, Y., Zhao, F., He, X., Liu, H., & Yu, K. (2020). Increased organic fertilizer application and reduced chemical fertilizer application affect the soil properties and bacterial communities of grape rhizosphere soil. *Scientific Reports*, *10*, 9568. https://doi.org/10.1038/s41598-020-66648-9

Xianhai, Z., Mingdao, C., & Weifu, L. (2012). Improving planting pattern for intercropping in the whole production span of rubber tree. *African Journal of Biotechnology*, *11*(34), 8484-8490. https://doi.org/10.5897/AJB11.3811

Zhu, J., Qi, J., Fang, Y., Xiao, X., Li, J., Lan, J., & Tang, C. (2018). Characterization of sugar contents and sucrose metabolizing enzymes in developing leaves of *Hevea brasiliensis*. *Frontiers in Plant Science*, *9*, 58. https://doi.org/10.3389/fpls.2018.00058

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Detection of *PirA/B* Toxin Genes for Acute Hepatopancreatic Necrosis Disease (AHPND) and *Vibrio parahaemolyticus* in *Penaeus vannamei* Culture from Major White Shrimp Producing Farms in Malaysia

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ABSTRACT

The acute hepatopancreatic necrosis disease (AHPND) epidemic from 2010 to 2013 significantly affected white shrimp (*Penaeus vannamei*) production in Malaysia. This study aims to determine the status of AHPND in *P. vannamei* culture from detecting *PirA/B* toxin genes in hepatopancreas tissues and isolation of *Vibrio parahaemolyticus* for identification of pathogenic strain from major white shrimp producing farms in Malaysia. Bacteria from the hepatopancreas organ were cultured on tryptic soy agar and identified using API® 20 NE (bioMérieux, France) for *Vibrio* species. Confirmation of *PirA/B* toxin genes in hepatopancreas and *V. parahaemolyticus* isolates were determined by polymerase chain reaction (PCR). Twenty-three *V. parahaemolyticus* isolates were identified from 7.7% of the analysed samples. Fourteen (14) (4.7%) were detected with *PirA/B* toxin genes from districts of Johor such as Batu Pahat (1) and Kota Tinggi (8), Alor Setar, Kedah (3), Tawau, Sabah (1), and Kuching, Sarawak (1). In contrast, the other nine isolates (3%) contained only the plasmid. Genomic and phylogenetic tree analysis of four *V. parahaemolyticus* isolates carrying *PirA/B* toxin genes from this study showed that only one strain (Vp14) harbours

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the *PirA/B* complete genes in addition to displaying full sequence homology and coverage to the pVA1 plasmid. In contrast, other strains (AAT22, IKK3, and PK3) displayed partial sequence homology of plasmid harbouring key genes associated with conjugative transfer function but not the plasmid segments containing *PirA/B* toxin genes. Hence, this study showed that six farms were negative from AHPND. In contrast, four farms were positive with *PirA/B* toxin genes in juveniles from Pekan, Pahang (26.7%), Kuching and Sarikei, Sarawak (10% respectively), and Alor Setar, Kedah (3.3%).

Keywords: AHPND, *P. vannamei*, *PirA/B* toxin genes, prevalence, *V. parahaemolyticus*

INTRODUCTION

Acute hepatopancreatic necrosis disease (AHPND) or previously known as early mortality syndrome (EMS), is a bacterial disease caused by a unique strain of *Vibrio* spp., including *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Vibrio owensii*, *Vibrio campbelli*, *Vibrio punensis*, and other possible bacteria that contain ~70-kbp plasmid genes which encode homologous of the *Photorhabdus* insect-related toxins, *PirA/B* (Devadas et al., 2019; Lee et al., 2015; Yang et al., 2014). Nucleotide content [guanine-cytosine (GC)] of these two genes is only 38.2% and is substantially lower than the rest of the plasmid, which suggests that these genes are acquired (Feng et al., 2017; Han et al., 2015). In addition, a plasmid that contains *PirA/B* toxin genes was found in the pathogenic AHPND strain of *V. parahaemolyticus* (*VPAHPND*) but was absent in non-pathogenic strain, suggesting *PirA/B* toxin genes as the causative agent for AHPND. These *PirA/B* toxin genes were also found in *Photorhabdus* spp., which are gram-negative, luminescent, rod-shaped bacteria members of the family Enterobacteriaceae. In nature, *Photorhabdus* spp. establish an obligate, symbiotic relationship with entomopathogenic nematode *Heterorhabditis* spp., which are parasites of insect larvae that have a wide geographic distribution. The first detection of AHPND was reported in China in 2009, Malaysia and Vietnam in 2011, Thailand in 2012, and the Philippines in 2015 (Dabu et al., 2015; Food and Agriculture Organization of the United Nations [FAO], 2013, 2016).

Malaysia produced significant quantities of *Penaeus monodon* in the early 2000s but then switched largely to *P. vannamei* until the AHPND epidemic hit the country between 2011 and 2013. Sentinel surveillance based on reports of mortality cases in white shrimp farms from Malaysia showed an increasing number of cases since 2011, with the first report on the east coast of Johor and subsequently in Pahang, Perak, and Penang (Kua et al., 2016). The prevalence of AHPND was 50% and 26% in 2011 and 2012, respectively. Confirmation of AHPND was based on observation of clinical signs and the characteristic pathology of acute and terminal stages of AHPND in hepatopancreas organs (Kua et al., 2016). Pale discolouration and atrophy of hepatopancreas accompanied by loose

shells, empty stomach or discontinuous midgut, and corkscrew swimming behaviour were reported. In addition, mixed bacteria of *Vibrio parahaemolyticus*, *Vibrio fluvialis*, *Vibrio alginolyticus*, *Vibrio cholerae*, *Aeromonas hydrophila*, *Enterobacter cloacae*, *Pseudomonas* spp., and *Photobacterium damselae* were isolated from the hepatopancreas.

About 67% of shrimp production in Malaysia comes from white shrimps (Harkell, 2018). AHPND had caused a significant drop in *P. vannamei* production from 87,000 metric tonnes in 2010 to 67,000 metric tonnes in 2011. A continuous drop in *P. vannamei* production was recorded in 2012 with 48,991.81 metric tonnes (RM61.59 million) to 45, 473.74 metric tonnes (RM86.72 million) in 2013 (Department of Fisheries Malaysia [DOF], 2010, 2011, 2012, 2013). Statistical data in 2018 recorded *P. vannamei* production of 36,007.25 metric tonnes (RM79.8 million) (DOF, 2018). Despite increasing aquaculture areas and shrimp culture farms, diseases and mortalities have been identified as major obstacles to sustainable production. AHPND and hepatopancreatic microsporidians caused by *Enterocytozoon hepatopenaei* (EHP) have been reported as two emerging diseases from 2010 to 2015 that are usually occurred concurrently and have significantly affected shrimp production due to high mortalities (AHPND) and/or stunted growth (EHP) (FAO, 2017).

Giant tiger shrimp (*Penaeus monodon*), white leg shrimp (*Penaeus vannamei*), and oriental/Chinese white shrimp (*Penaeus chinensis*) are known to be infected with AHPND that is characterised by mass mortalities between 40% and 100% in 20–30 days of post-stocking in grow-out ponds (Lightner et al., 2012). Management practices in farms, including pond management and maintenance of good water quality, are known to prevent or avoid stressing shrimp and making them more susceptible to disease. However, low compliance with standards in good biosecurity and good aquaculture practices at farm and hatchery facilities have been identified as major factors favouring the spread of disease from one farm to the other (FAO, 2016). Therefore, shrimp aquaculture needs to continuously develop a systematic approach that implements responsible and science-based farming practices. Hence, the objective of this study is to determine the status of AHPND in *P. vannamei* culture from detection of *PirA/B* toxin genes and bacteria *V. parahaemolyticus* from hepatopancreas organ in relation to several routine management practices of pond culture.

MATERIALS AND METHODS

Sampling Size and Locations

A cross-sectional study on the status of AHPND was carried out from major shrimp producing areas in Malaysia, involving ten shrimp farms from 10 districts in Kedah, Penang, Terengganu, Pahang, Johor, Sarawak, and Sabah that started from January to November in 2019. A minimum of 30 pieces of juvenile shrimps/day of culture (DOC) aged less than 30 or between

31–45 DOC were collected randomly from various pond cultures (maximum 5 to 6 culture ponds/30 pieces). A total of 300 pieces of juveniles were sampled and analysed individually. The sampling sites, states, districts, months of sampling, and the number of samples are shown in Table 1.

Table 1

A sampling of Penaeus vannamei *at the day of culture (DOC) less than 30 days of age, or juveniles between 31–45 days old from 10 locations that show states, districts, months, and number of samples*

States	District	Month (2019)	Number of samples
Kedah	Alor Setar	April	30
Penang	Bkt. Tambun	February	30
Terengganu	Setiu	March	30
Sarawak	Sarikei	May	30
	Kuching	May	30
Johor	Batu Pahat	August	30
	Kota Tinggi	August	30
Pahang	Pekan	October	30
Sabah	Kudat	November	30
	Tawau	November	30
		TOTAL	300

Bacterial Culture, Isolation, and Identification

About 210 pieces of juvenile white shrimps aged less than 30 days old in pond culture (Kedah, Terengganu, Johor, Pahang, and Sabah) and 60 pieces of white shrimps (Sarawak) aged between 30–45 days old were tested to detect the presence of bacteria in the hepatopancreas organ. Bacteria were aseptically inoculated via direct streaking onto tryptic soy agar (TSA), which was incorporated with 1.5% sodium chloride (NaCl) and incubated at 30 °C for 18 to 24 hours, followed by sub-culture until pure isolate was obtained. *Vibrio parahaemolyticus* bacterial was identified using Gram staining, oxidase test, sensitivity to vibrio static 0129-disc agent (2, 4-Diamino-6, 7-di-iso-propylpteridine phosphate) (150/10 µg) and observation of the colour colony on thiosulfate citrate bile salt (TCBS) agar (Austin et al., 1997). A biochemical test for confirmation of *Vibrio* species was carried out using API® 20 NE Kit (bioMérieux, France). The *V. parahaemolyticus* isolates identified with 98% to 99.9% identical to the reference strain in the analytical profile index (API) software database was further tested for the presence of *PirA/B* toxin genes using a PCR

method. An isolate of *V. alginolyticus* was used as rooting for phylogenetic analysis.

DNA Extraction of Hepatopancreas Tissues and Bacteria Cells

A total of 300 hepatopancreas tissues samples in 95% alcohol fixation were tested for *PirA/B* toxin genes using a PCR method. DNA extraction was carried out using the cetyl trimethyl ammonium bromide (CTAB) and dodecyle trimethyl ammonium bromide (DTAB) method (IQ2000™, GeneReach Biotechnology Corp., Taiwan). In contrast, bacteria *V. parahaemolyticus* was extracted using G-Spin™ Genomic Bacteria Extraction Kit (iNtRON Biotechnology, Korea). About 30 mg of hepatopancreas tissues fixed in 95% alcohol was placed into a 1.5 mL tube containing 600 µL DTAB solution. Tissues were ground with a sterile disposable grinder until they were completely dissolved into DTAB solution. After that, they were incubated at 75 °C for 5 minutes and cooled down to room temperature. The mixture was vortexed and spun down briefly and was then added with 700 µL of chloroform and centrifuged at 12,000 x *g* for 5 minutes. Next, the upper aqueous phase was transferred into a new 1.5 mL tube and added 100 µL of CTAB solution and 900 µL of deionised water. It was then vortexed briefly, incubated at 75 °C for 5 minutes and centrifuged at 12,000 x *g* for 5 minutes. Next, the pellet was re-suspended with 150 µL dissolving solution, incubated at 75 °C for 5 minutes and spun at 12,000 x *g* for 5 minutes. Finally, the supernatant was transferred into a new 1.5 mL tube containing 300 µL of 95% ethanol. This procedure was repeated twice, whereby the pellet was washed with 75% ethanol in the last procedure. The final pellet was dried and dissolved in deionised water or Tris ethylenediaminetetraacetic acid (TE) buffer. Dissolving DNA was stored at -20 °C until used for polymerase chain reaction (PCR).

Total genomic DNA from *V. parahaemolyticus* isolate was extracted using G-spin™ Kit (iNtRON Biotechnology, Korea). About 1 mL of bacteria cells was harvested from an overnight culture (18–24 hours) at 30 °C in tryptic soy broth incorporated with 1.5% NaCl (OD_{600} 0.8–1.0) by centrifugation at 13,000 x *g* for 1 minute. The supernatant was removed, and cells were re-suspended by vortex and tapping. Bacteria cells pellet was extracted according to the manufacturer's instructions. The final collected DNA in a 1.5 mL tube was measured using a NanoDrop™ spectrophotometer (DS-11 Series, DeNovix, USA).

PCR Reaction Conditions

Nested PCR using AP4 primers was followed with some optimisation of annealing temperature using Maxime PreMix *i-Taq* (iNtRON Biotechnology, Korea) for detecting *PirA/B* toxin genes at 230 bp portion of a sequence, which includes 209 bp of the *ToxA* or *PirA* gene sequence plus 12 bp spacer sequence plus 9 bp of succeeding *ToxB* or *PirB* gene sequence (Dangtip et al., 2015). First-step PCR was performed using primer AP4-F1 with the sequence: 5'-ATGAGTAACAATATAAAACAT

GAAAC-3' and AP4-R1: 5'-ACGATTTCGACGTTCCCCAA-3', followed by nested PCR using AP4-F2 primer: 5'-TTGAGAATACGGGACGTGGG-3' and AP4-R2: 5'-GTTAGTCATGTGAGCACCTTC-3'. In the first PCR reaction, 20 µL of the total volume reaction mixture was prepared, which consisted of DNA template 2 µL, AP4-F1 primer (0.4 µL 100 pmol/µL), AP-R1 primer (0.4 µL 100 pmol/µL), and deionised water (17.2 µL). Then, amplification was performed with the following parameters: initiation denaturation at 94 °C for 2 minutes, followed by 35 cycles of 94 °C for 30 seconds, 53 °C for 30 seconds, and 72 °C for 90 seconds, and a final extension at 72 °C for 2 minutes. For the second step (nested) PCR reaction, 1 µL of the final solution from the first-step PCR was diluted with deionised water at the ratio of 1 to 100, 2 µL of the diluted solution was used with nested primers AP4-F2 (0.4 µL 100 pmol/µL), AP4-R2 (0.4 µL 100 pmol/µL), and deionised water (17.2 µL) were used to make up for 20 µL of total volume. Then, amplification was performed with initiation denaturation at 94 °C for 2 minutes, followed by 25 cycles of 94 °C for 30 seconds, 53 °C for 30 seconds, 72 °C for 30 seconds, and a final extension of 72 °C for 2 minutes. Following PCR, an aliquot of PCR products was analysed in a 1.5% gel containing green fluorescent dye nucleic acid staining solution (RedSafe™, iNtRON Biotechnology, Korea).

Sample Preparation for Sequencing and Phylogenomic Tree

Genomic deoxyribonucleic acid (gDNA) of five *Vibrio* spp., identified as IKK3, AAT22, PK3, Vp14, and SK6, were sent for sequencing via the iSeq100 Next Generation Sequencing System (GeneSEQ, Malaysia). Genome completeness analysis via BUSCO v4 was assessed for each assembled genome. In addition, protein-coding genes were briefly predicted from the assembly and were assessed for the presence of 1445 conserved genes found in *Vibrio* (vibrio_odb10). As expected from the high-quality assembly, each genome assembly exhibited genome completeness of >99.9%. The gDNA was fragmented using a Covaris ultra sonicator to approximately 350 bp. The fragmented DNA was end-repaired, adapter-ligated, and PCR-enriched using the NEBNext® Ultra™ II DNA Library Preparation Kit (New England Biolabs, USA) according to the manufacturer's instructions. The constructed libraries were normalised and sequenced on NovaSeq 6000 System (Illumina, USA) using a 2 x 150 bp read configuration (Simão et al., 2015).

Vibrio parahaemolyticus genome assemblies in National Center for Biotechnology Information (NCBI) that originated from Malaysia were used to infer a phylogenomic tree of five isolates (IKK3, AAT22, PK3, Vp14, and SK6) obtained from this study. The complete genome sequence of *V. parahaemolyticus* strain MVP1 was used as the reference genome. Each genome was subsequently aligned to

this reference genome to identify single nucleotide polymorphisms (SNPs) and generate a core SNP alignment. The Snippy v4.6.0 pipeline was used to perform these tasks. First, a maximum-likelihood tree was constructed using the FastTree2 setting, followed by visualisation and annotation in Figtree v1.4.1 (Price et al., 2010).

RESULTS

Biochemical Confirmation of Bacteria Isolates

Twenty-three isolates of *Vibrio* spp. were subjected to a biochemical test to confirm the species using API® 20 NE (bioMérieux, France). Isolates Vp14, IKK3, AAT22, and PK3 were identified as *V. parahaemolyticus* with 90% to 99.1% identical to the reference strain in the API system. They were Gram-negative halophilic bacteria, which produced green colony growth on thiosulfate citrate bile salt sucrose (TCBS) agar, was sensitive to vibrio static 0129-disc agent (150/10 µg) and exhibited cytochrome oxidase with catalase activity. Enzymatic assays showed that they were nitrate (NO_3) reductase, tryptophanase, glucose fermentation, gelatinase, and β-galactosidase but produced a negative reaction to arginine dihydrolase, urease, and esculin hydrolase. Carbohydrate assimilation showed a positive reaction to D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, maltose, D-gluconate, and L-malate but a negative reaction to caprate, adipate, citrate, and phenylacetate. *Vibrio alginolyticus* (SK6) produced similar results as *V. parahaemolyticus* isolates, except that it did not produce acid from glucose or decarboxylate β-galactosidase and did not break up arabinose.

Prevalence of AHPND from Detection of *PirA/B* Toxin Genes in Hepatopancreas of *Penaeus vannamei*

The highest prevalence of AHPND was found in juveniles/day of culture (DOC) of white shrimps from Pekan (8, 26.7%), Pahang. In contrast, the low prevalence was recorded in juvenile shrimps from Sarikei (3, 10%), Kuching (3, 10%), Sarawak, and Alor Setar (1, 3.3%), Kedah. The 7.3% of samples (22) were tested positive with *PirA/B* toxin genes with an overall mean prevalence of 5%. Results are shown in Table 2. AHPND prevalence was diagnosed by detecting *PirA/B* toxin genes from hepatopancreas tissues of white shrimps fixed in 95% alcohol and supported with or without the isolation of *V. parahaemolyticus* isolate that carries *PirA/B* toxin genes. AHPND was not detected in hepatopancreas tissues of juvenile white shrimps from Johor (60), Sabah (60), Terengganu (30), and Penang (30). However, culture isolates of *V. parahaemolyticus* obtained from white shrimp hepatopancreas at Kota Tinggi (8, 26.7%) and Batu Pahat (1, 3%) showed that these strains are pathogenic which carry *PirA/B* toxin genes.

Table 2

Detection of AHPND with PirA/B *toxin genes from the hepatopancreas of juvenile/DOC white shrimps*

States	District	Sample number (n)	Positive sample for *PirA/B* toxin genes (Prevalence, %)
Kedah	Alor Setar	30	1(3.3)
Penang	Bukit Tambun	30	-
Terengganu	Setiu	30	-
Johor	Batu Pahat	30	-
	Kota Tinggi	30	-
Pahang	Pekan	30	8(26.7)
Sarawak	Kuching	30	3(10)
	Sarikei	30	3(10)
Sabah	Kudat	30	-
	Tawau	30	-
Total		300	15(50)
Mean (%)			5.0

Note. Mean (%): (Number of the sample with *PirA/B* toxin genes/Total samples) x 100%

Prevalence of *Vibrio parahaemolyticus* with *PirA/B* Toxin Genes

Twenty-three (23) *V. parahaemolyticus* isolates were obtained from 270 samples of juvenile white shrimps, whereby 14 isolates were detected with *PirA/B* toxin genes. AHPND isolates were found in samples from Kota Tinggi (8, 26.7%) and Batu Pahat (1, 3.3%), Johor, Alor Setar, Kedah (3, 10%), and Kudat, Sabah (1, 3%). Although *V. parahaemolyticus* bacteria from culture media isolation were detected to have *PirA/B* toxin genes, direct PCR analysis of hepatopancreas tissues fixation (95% alcohol) of the similar samples failed to detect *PirA/B* toxin genes from these tissue samples. Virulence of AHPND-causing *V. parahaemolyticus* depends on the amount of *PirA/B* toxin released and caused cellular damage to hepatopancreas when *V. parahaemolyticus* bacteria initially colonise in the shrimp stomach and eventually reach the hepatopancreas (Han et al., 2015). In this situation, *V. parahaemolyticus* was considered a relatively non-virulent bacterium until it released a potent toxin (*PirA/B*) in the host tissues/hepatopancreas organ or induced the clinical disease condition and mortality in white shrimps. PCR analysis of hepatopancreas tissue of white shrimp sample was negative from Johor (60) and Sabah (60). The detection of *PirA/B* toxin genes from *V. parahaemolyticus* isolates obtained through the propagation of bacteria cells in the laboratory was not considered a positive case

for AHPND in this surveillance study but rather for identifying the pathogenic strain. PCR analysis of the cultured cells showed that 13 *V. parahaemolyticus* AHPND strains were identified from Kota Tinggi (8) and Batu Pahat (1), Johor; Alor Setar (3), Kedah, and Kudat (1), Sabah. Another study has shown that infection of AHPND depends on sufficient bacteria cells count to secrete or release *PirA/B* toxins in the shrimp tissues rather than the number of copies of toxin genes (Tinwongger et al., 2016).

Genomic Sequence of *PirA/B* Toxin Genes and pVA1 Plasmid

Five isolates of *V. parahaemolyticus* from Sarawak (Vp14), Kedah (IKK3), Johor (PK3, AAT22), and *V. alginolyticus* from Sabah (SK6) were subjected to whole-genomic sequences to determine the virulence of local strains obtained from this study. The whole-genome sequences were aligned to the pVA1 plasmid using blastN with an E-value of $1e^{-50}$ and subsequently visualised in Blast Ring Image Generator (Alikhan et al., 2011; Dong et al., 2019). Being consistent with the initial PCR screening result, strain Vp14 (Sarawak) was the only strain that harboured the *PirA/B* complete genes in addition to displaying full sequence homology and coverage to the pVA1 plasmid. In contrast, strain AAT22 (Johor) displayed significant sequence homology with at least 50% length of the plasmid harbouring key genes associated with conjugative transfer function but not the plasmid segment containing the *PirA/B* genes (Figure 1).

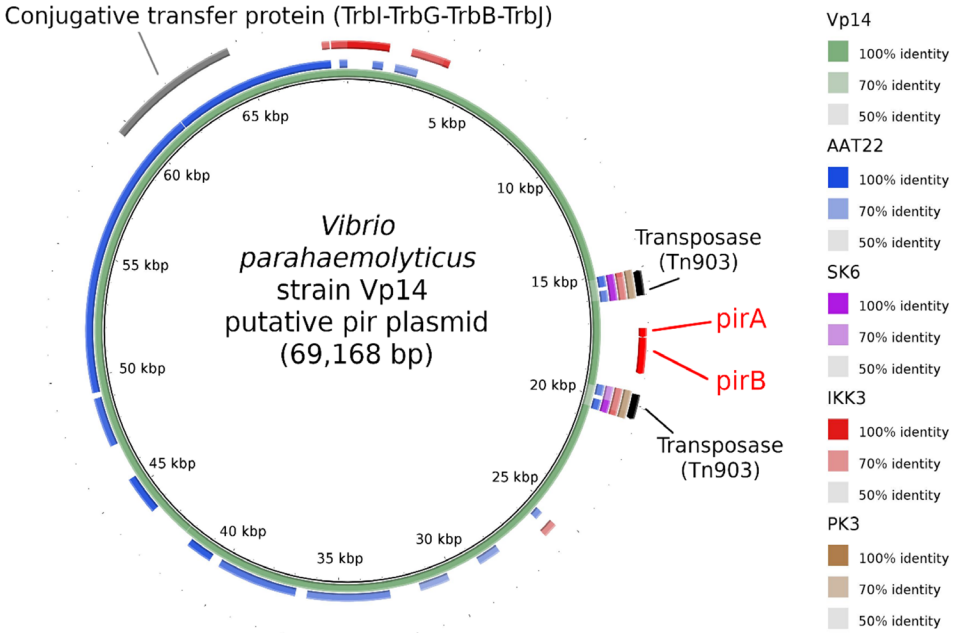

Figure 1. Circular visualization of the pVA1 plasmid and selected genes. Coloured rings indicate a genomic region with significant homology to the strain of interest (Vp14, AAT22, SK6, IKK3, and PK3)

Phylogenomic Tree of *Vibrio parahaemolyticus* Associated with Genome Assemblies in NCBI from Aquaculture in Malaysia

Four *V. parahaemolyticus* isolates, which were identified as Vp14 (Sarawak), AAT22 and PK3 (Johor), IKK 3 (Kedah), and SK6 (Sabah), were identified as *V. alginolyticus* that were subjected to genomic and phylogenetic analysis. The assembled genomes were compared to *Vibrio* spp. type strains to confirm their taxonomic assignment. Strains AAT22, IKK3, PK3, and Vp14, showed a pairwise average nucleotide identity of more than 98% to *V. parahaemolyticus*. In contrast, strain SK6 showed an average nucleotide identity of 98.5% to *V. alginolyticus* and less than 90% to other tested *Vibrio* species. Strain SK6 (Sabah) expectedly displayed a long branch length and was thus chosen as the outgroup for rooting, given its taxonomic assignment as *V. alginolyticus*. Strain Vp14 harbours the *PirA/B* toxin genes formed a monophyletic cluster with high SH-like support of a few MVP strains isolated from a shrimp pond water sample located in Negeri Sembilan in 2016. In contrast, strains IKK3 (Kedah), AAT22 and PK3 (Johor) only shared a relative distance ancestor with some of the publicly available strains, as evidenced by their relatively longer branch length (Figure 2). It suggested that they could be novel genomic lineages of *V. parahaemolyticus,* which was not reported previously in Malaysia.

DISCUSSION

This study showed that grow-out or juvenile white shrimps aged less than 30 days of culture in pond and post-larvae appeared most susceptible to AHPND infection with an overall mean prevalence of 5% and 4.7%, respectively. AHPND often occurs within 20–30 days of post stocking in grow-out ponds and causes mass mortalities in post-larvae shrimps (De Schryver et al., 2014). Therefore, most farms practise routine screening for major shrimp diseases that are known in aquacultures, such as white spot disease that is caused by white spot syndrome virus (WSD/WSSV), yellow head virus (YHV), taura syndrome virus (TSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV), AHPND, and EHP as parts of prevention programmes in their farms whereby the infected stocks will be destroyed.

This study identified 23 *V. parahaemolyticus* isolates from 7.7% of total samples. Fourteen (14) strains of *V. parahaemolyticus* were found to have *PirA/B* toxin genes, whereas nine isolates were detected with plasmid. Gross observation during sampling showed only mild changes in hepatopancreas organs with pale coloured, atrophied, empty midgut, and soft shell in several samples, suggesting that subclinical infection may go unnoticed. However, AHPND infection caused by *V. parahaemolyticus* that produce *PirA/B* toxin genes will usually cause severe pathological changes to hepatopancreas organ, which are shown via degeneration and massive sloughing of hepatopancreas cells, followed

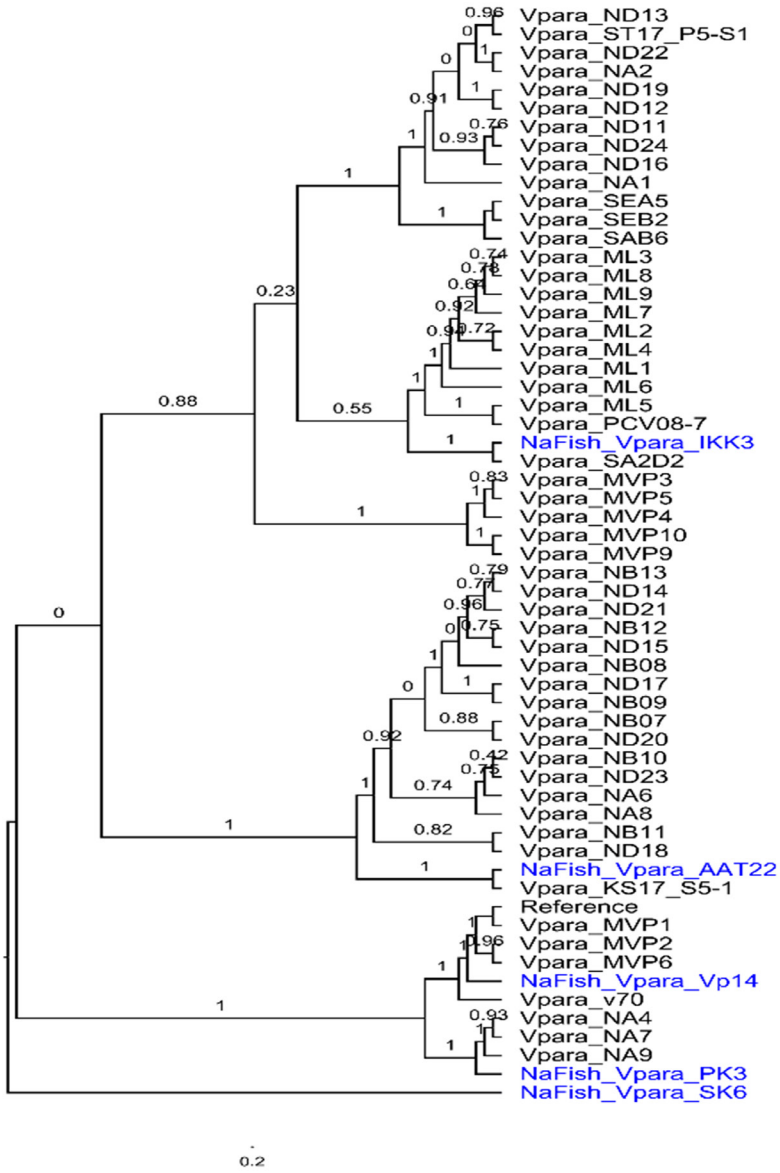

Figure 2. Maximum likelihood tree constructed based on the core SNP alignment of publicly available Malaysian *Vibrio parahaemolyticus* genomes and NaFish *Vibrio* genomes (blue labels). Strain SK6 was rooted as the outgroup, given its taxonomic classification as *Vibrio alginolyticus*. The branch lengths indicate the number of substitutions per site, while the SH-like bootstrap supports values that were indicated by the colour of the nodes.

by high mortalities in disease outbreaks (Lightner et al., 2012; Nunan et al., 2014). However, typical gross pathological changes associated with clinical disease of AHPND was not observed on-site, and farmers have not reported high mortalities. Nevertheless, the occurrence of *V. parahaemolyticus* bacteria with *PirA/B* toxin genes in the hepatopancreas of shrimp will increase their risk to AHPND under stressful conditions. Routine management practices applied in farms such as regular health screening of stocks, strict biosecurity measures, hygienic practices at the entrance and within farms culture area, and pond management and its water quality are among many factors that impact the management of disease-free culture.

The pVA1 plasmid is the source of the AHPND-causing toxin, whereas *PirA/B genes*, irrespective of other plasmidic factors of pVA1, are sufficient to produce symptoms associated with AHPND (Lee et al., 2015). Culturable cells of *V. parahaemolyticus* local strains were recovered from -20 ^{o}C storage. Genomic sequencing of four local strains of *V. parahaemolyticus* carrying *PirA/B* toxin genes showed that only one strain (Vp14) harboured the *PirA/B* complete genes, which displayed a full sequence of pVA1 virulent plasmid. The risk of AHPND outbreak can be reduced by controlling the *Vibrio* spp. activities, in particular, *V. parahaemolyticus* cells count. The bacterial concentration ranging ($5 \times 10^4 – 5 \times 10^5$ cfu/ml) from AHPND strain has been proven to be able to cause significant mortalities to *P. vannamei* from 60% to 100% within 3 to 6 days of post infections from the immersion challenge test (Tinwongger et al., 2016). Hence, the virulence of AHPND depends on sufficient bacterial count to release or secrete toxin rather than the number of copies of toxin genes (Tinwongger et al., 2016). Other studies showed that phytoplankton/green water and nutrient enrichment affect the microbial community in the ecosystem, especially bacterial load and interaction with the shrimp immune responses (De Schryver et al., 2014). Probiotic and/or algae-rich green waters are known to be able to create microbially matured water systems, whereby environments that are primarily colonised by slow-growing harmless bacteria may best guarantee the prevention of AHPND outbreaks (De Schryver et al., 2014). Probiotics and molasses help to increase the diversity of heterotrophic bacteria, including *V. parahaemolyticus* thus, effectively inhibiting pathogens (Bhatnagar & Pooja, 2013; Hu et al., 2016). Molasses are known to improve water quality, and it is suggested to be used during the nursery and grow-out phase of *P. vannamei* under limited water discharge or close system (Tzachi et al., 2007).

CONCLUSION

The health status of AHPND from grow-out/juvenile white shrimps aged less than 30 days of culture was determined through a cross-sectional study using a random sampling method. AHPND with the detection of *PirA/B* toxin genes and identification of *V. parahaemolyticus* with *PirA/B* toxin genes were determined from 10 major shrimp

producing areas in Malaysia. AHPND with *PirA/B* toxin genes detection from the hepatopancreas of white shrimps was found in Kuching and Sarikei, Sarawak at 10% prevalence, respectively, followed by 3.3% in Alor Setar, Kedah, and 26.7% from Pekan, Pahang. *PirA/B* toxin genes of AHPND were not detected in white shrimps from farms in Penang, Johor, Terengganu, and Sabah. Genomic and phylogenetic tree analysis of four *V. parahaemolyticus* isolates carrying *PirA/B* toxin genes from this study showed that only one strain (Vp14) harboured the *PirA/B* complete genes in addition to displaying full sequence homology and coverage to the pVA1 plasmid. In contrast, other strains (AAT22, IKK3, and PK3) displayed partial sequence homology of plasmid harbouring key genes associated with conjugative transfer function but not the plasmid segments containing the *PirA/B* toxin genes.

Many factors are known to influence the clinical manifestation of AHPND, such as the presence of *PirA/B* toxin genes and *V. parahaemolyticus* bacteria that carry *PirA/B* toxin genes in hepatopancreas tissues, as well as water quality and bacterial cell counts in pond culture. Health screening through regular observation of shrimp behaviour and health checks for important diseases in shrimp culture provides the best solution for early detection and management of health problems. The risk of spreading the disease to other farms can be prevented through removal and safe disposal of sick or dead shrimps, as well as emergency harvesting if necessary, or destruction of infected stocks as appropriate. Strict biosecurity measures and disinfection have been strictly implemented in almost all farms that the researchers visited or surveyed. This practice is believed to contribute significantly to controlling and preventing AHPND.

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REFERENCES

Alikhan, N. F., Petty, N. K., Ben-Zakour, N. L., & Beatson, S. A. (2011). BLAST Ring Image Generator (BRIG): Simple prokaryote genome comparisons. *BMC Genomics*, *12*(1), 402. https://doi.org/10.1186/1471-2164-12-402

Austin, B., Austin, D. A., Blanch, A. R., Cerdà, M., Grimont, F., Grimont, P. A. D., Jofre, J., Koblavi, S., Larsen, J. L., Pedersen, K., Tiainen, T., Verdonck, L., & Swings, J. (1997). A comparison of methods for the typing of

fish-pathogenic *Vibrio* spp. *Systematic and Applied Microbiology, 20*(1), 89-101. https://doi.org/10.1016/S0723-2020(97)80053-7

Bhatnagar, A., & Pooja, D. (2013). Water quality guidelines for the management of pond fish culture. *International Journal Environmental Science*, *3*(6), 5-30. https://doi.org/10.6088/ijes.2013030600019

Dabu, 1. M., Lim, J. J., Arabit, P. M. T., Orense, S. J. A. B., Tarbadillo, J. A., & Corre, V. L. (2015). The first record of acute hepatopancreatic necrosis disease in Philippines. *Aquaculture Research*, *48*(3), 792-799. https://doi.org/10.1111/are.12923

Dangtip, S., Sirikharin, P., Sanguanrut, S., Thitamadee, K., Sritunyalucksana, S., Taengchaiyaphum, R., Mavichak, P., Proespraiwong, P., & Flegel, T. W. (2015). AP4 method for two-tube nested PCR detection of AHPND isolates of *Vibrio parahaemolyticus*. *Aquaculture Representative*, *2*, 158-162. https://doi.org/10.1016/j.aqrep.2015.10.002

De Schryver, P., Defoirdt, T., & Sorgeloos, P. (2014). Early mortality syndrome outbreak: A microbial management issue in shrimp farming? *PLOS Path*, *10*(4), e1003919. https://doi.org/10.1371/journal.ppat.1003919

Department of Fisheries Malaysia. (2010). Perangkaan Perikanan Tahunan 2010 [Annual fisheries statistics 2010]. DOF. https://www.dof.gov.my/en/resources/fisheries-statistics-i/

Department of Fisheries Malaysia. (2011). Perangkaan Perikanan Tahunan 2011 [Annual fisheries statistics 2011]. DOF. https://www.dof.gov.my/en/resources/fisheries-statistics-i/

Department of Fisheries Malaysia. (2012). Perangkaan Perikanan Tahunan 2012 [Annual fisheries statistics 2012]. DOF. https://www.dof.gov.my/en/resources/fisheries-statistics-i/

Department of Fisheries Malaysia. (2013). *Perangkaan perikanan tahunan 2013* [Annual fisheries statistics 2013]. DOF. https://www.dof.gov.my/en/resources/fisheries-statistics-i/-

Department of Fisheries Malaysia. (2018). *Perangkaan tahunan perikanan 2018 jilid 1* [Annual fisheries statistics 2018 volume 1]. DOF. https://www.dof.gov.my/en/resources/i-extension-en/annual-statistics/

Devadas, S., Banerjee, S., Yusoff, S. F. M., Bhassu, S., & Shariff, M. (2019). Review: Experimental methodologies and diagnostic procedures for acute hepatopancreatic necrosis disease (AHPND). *Aquaculture*, *499*, 389-400. https://doi.org/10.1016/j.aquaculture.2018.06.042

Dong, X., Song, J., Chen, J., Bi, D., Wang, W., Ren, Y., Wang, H., Wang, G., Tang, K. F. J., & Wang, X. *(2019). Conjugative transfer of the pVA1-type plasmid carrying the PirAB ^{vp} genes results in the formation of new AHPND-causing Vibrio. Frontiers in Cellular and Infection Microbiology*, *195*(9), 1-11. https://doi.org/10.3389/fcimb.2019.00195

Feng, B., Liu, H., Wang, M., Sun, X. H., Pan, Y., & Zhao, Y. (2017). Diversity analysis of acute hepatopancreatic necrosis disease-positive *Vibrio parahaemolyticus* strains. *Aquaculture and Fisheries*, *2*(6), 278-285. https://doi.org/10.1016/j.aaf.2017.10.001

Food and Agriculture Organization of the United Nations. (2013). *FAO/MARD technical workshop on early mortality syndrome (EMS) or acute hepatopancreatic necrosis syndrome (AHPNS) of cultured shrimp*. FAO. https://www.fao.org/3/i3422e/i3422e00.htm

Food and Agriculture Organization of the United Nations. (2016). *Second international technical seminar/workshop on acute hepatopancreatic necrosis disease (AHPND) there is a way forward!*. FAO. https://www.fao.org/documents/card/en/c/28b6bd62-5433-4fad-b5a1-8ac61eb671b1/

Food and Agriculture Organization of the United Nations. (2017). *FishStatJ – Software for fishery and aquaculture statistical time series*. FAO.

Han, J. E., Tang, Kathy F. J., Tran, Loc H., & Lightner, D. V. (2015). Photorhabdus insect-related (Pir) toxin-like genes in a plasmid of Vibrio parahaemolyticus, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. *Disease Aquatic Organisms*, *113, 3*3-40. https://doi.org/10.3354/dao02830

Harkell, L. (2018). *Half of shrimp farmers in key Malaysia region switch to black tiger: Seafood business news from beneath the surface*. https://www.undercurrentnews.com/2018/08/27/half-of-malaysian-shrimp-farmers-in-key-region-switch-to-black-tiger/

Hu, X. J., Cao, Y. C., Wen, G. L., Zhang, X. Y., Xu, Y., Xu, W., & Li, Z. J. (2016). Effect of combined use of *Bacillus* and molasses on microbial communities in shrimp cultural enclosure systems. *Aquaculture Research*, *48*(6), 2691-2705. https://doi.org/10.1111/are.13101

Kua, B. C., Ahmad, I. A. R., Siti-Zahrah, A., Irene, J., Norazila, J., Nik-Haiha, N. Y., Fadzilah, Y., Mohammed, M., Siti-Rokhaiya, B., Omar, M., & Teoh, T. P. (2016). Current status of acute hepatopancreatic necrosis disease (AHPND) of farmed shrimp in Malaysia. In *Addressing Acute Hepatopancreatic Necrosis Disease (AHPND) and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia: Proceedings of the ASEAN Regional Technical Consultation on EMS/AHPND and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia* (pp. 55-59). Southeast Asian Fisheries Development Center.

Lee, C. T., Chen, I. T., Yang, Y. T., Ko, T. P., Huang, Y. T., Huang, J. Y., Huang, M. F., Lin, S. J., Chen, C. Y., Lin, S. S., Lightner, D. V., Wang, H. C., Wang, A. H., Hor, L. I., & Lo, C. F. (2015). The opportunistic marine pathogen Vibrio parahaemolyticus becomes virulent by acquiring a plasmid that expresses a deadly toxin. Proceedings of the National Academy of Sciences, *112*(34), 10798-10803. https://doi.org/10.1073/pnas.1503129112

Lightner, D. V., Redman, R. M., Pantoja, C. R., Noble, B. I., & Tran, Loc H. (2012). Early mortality syndrome affects shrimp in Asia. *Global Aqua Advocate*, *15*(1), 40.

Nunan, L., Lightner, D. V., Pantoja, C., & Gomez-Jimenez, S. (2014). Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. *Disease Aquatic Organisms*, *111*, 81- 86. https://doi.org/10.3354/dao02776

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2 – Approximately maximum-likelihood trees for large alignments. *PLOS One*, *5*(3), e9490. https://doi.org/10.1371/journal.pone.0009490

Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., & Zdobnov, E. M. (2015). BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*, *31*(19), 3210-3212. https://doi.org/10.1093/bioinformatics/btv351

Tinwongger, S., Nochiri, Y., Thawonsuwan, J., Nozaki, R., Kondo, H., Awasthi, S. P., Hinenoya, A., Yamasaki, S., & Hirono, I. (2016). Virulence of acute hepatopancreatic necrosis disease *PirAB*-like relies on secreted proteins not on gene copy number. *Journal Applied Microbiology*, *121*(6), 1755-1765. https://doi.org/10.1111/jam.13256

Tzachi, M. S., Susmitas, P., Mike, S., Abduk-Mehdi, A., Josh, M. B., Rodrigo, V. A., Zarrein, A., Margasanto, H., Ami, H., & David, L. B. (2007). Use of molasses as carbon source in limited discharge nursery and grow-out systems for *Litopenaeus vannamei*. *Aquacultural Engineering*, *36*(2), 184-191. https://doi.org/10.1016/j.aquaeng.2006.10.004

Yang, Y. T., Chen, I. T., Lee, C. T., Chen, C. Y., Lin, S. S., Hor, L. I., Tseng, T. C., Huang Y. T., Sritunyalucksana, K., Thitamadee, S., Wang, H. C., & Lo, C. F. (2014). Draft genome sequences of four strains of *Vibrio parahaemolyticus* three of which cause early mortality syndrome/acute hepatopancreatic necrosis disease in shrimp in China and Thailand. *Genome Announcement*, *2*(5), e00816-14. https://doi.org/10.1128/genomeA.00816-14

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Isolation and Molecular Identification of a Siderophore Producing Bacterium and its Antagonistic Effect Against *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4

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ABSTRACT

Bananas are one of the world's most consumed fruits. Developing countries in the Global South depend on bananas for food security and livelihoods. Still, the banana industry also drives a multinational trade worth billions of US dollars. In addition, banana plants also hold cultural and religious significances in many Asian countries. However, banana production faces several challenges, and one of the major issues is the Fusarium wilt disease caused by the fungus *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4). This disease threatens numerous banana cultivars, including the Cavendish, the most traded banana cultivar. Therefore, the objective of this study was to find effective measures to control the spread of this disease through antagonistic soil bacteria. This study isolated 14 fluorescent, siderophore-producing bacteria with *in vitro* inhibition rates of 21.73-50.38% against Foc TR4 from the soil surrounding banana plants. Most of the isolates were identified as *Pseudomonas* spp. via 16S rRNA sequencing, and phylogenetic analysis based on 16s rRNA sequences showed clustering of isolates congruent with the results of similarity searches. Three isolates that exhibited relatively higher antagonistic activity against *Fusarium oxysporum* f. sp. *cubense* compared to other isolates were JBAA132 (50.38%), K2B131 (46.28%), and KTP231 (45.38%). Isolate JBAA132 displayed differences in biochemical characteristics compared to its closest match (*Pseudomonas aeruginosa*

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type strain DSM50071). It also demonstrated nitrogen-fixing and phosphate solubilising properties common to plant growth promoters. Therefore, isolate JBAA132 may be further explored as a potential biocontrol agent in curbing the spread of Foc TR4.

Keywords: Biological control, Foc TR4, Fusarium wilt, Panama disease, plant growth promoter, *Pseudomonas aeruginosa*, siderophore

INTRODUCTION

Bananas are cultivated in more than 135 countries and are a staple food for many developing nations in Africa and Asia. Banana production was valued at 38 billion USD in 2018 and had an export value of 14.7 billion USD in 2020 (Workman, 2021). However, the production of bananas is severely threatened by Panama disease, also known as Fusarium wilt, caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) (ProMusa, n.d.). Foc is classified into distinct races according to the banana varieties they infect, namely races 1, 2, 3, and 4. Foc Race 1 affected the Gros Michel banana and decimated plantations across Central America during the 1950s, prompting farmers to switch to the Cavendish because it was resistant to Foc Race 1 (Ploetz, 2005). However, a new and more virulent strain of Foc emerged in the 1970s, infecting the Cavendish bananas in Taiwan (Su et al., 1986). This new strain of Foc termed Foc Tropical Race 4 (TR4) can infect *Musa* spp. that is susceptible to Foc Race 1 and Race 2 in addition to the Cavendish. Furthermore, vegetative propagation of commercial banana plants results in daughter plants that are clones of the parent plant and are susceptible to the same disease. Increasing globalisation and the worldwide trading of bananas have also caused the Fusarium wilt to spread rapidly. The losses continue to rise as current prevention and management measures are ineffective and insufficient to control the disease (Scheerer et al., 2018). As such, Foc TR4 poses a serious threat to global banana production and food security (Food and Agriculture Organization of the United Nations [FAO], 2019).

Fusarium oxysporum f. sp. *cubense* TR4 is an ascomycete that reproduces asexually via microconidia, macroconidia, and chlamydospores (Ghag et al., 2015). According to VanderMolen et al. (1987), infection by its spores causes the host plant to form tyloses that gel up the xylems. The occlusion of the xylem prevents the host from transpiration and water transport, eventually killing the plant. Fusarium wilt is extremely difficult to control because the spores are durable and can persist in the soil for up to 30 years (Agrios, 2005; Hennessy et al., 2005). Therefore, finding reliable and efficient prevention methods is crucial for the survival of the banana industry and the livelihoods of communities that rely on banana production. While the development of disease-resistant cultivars is highly desirable, it is costly and time-consuming (Dita et al., 2018; Zuo et al., 2018). On the other hand, fungicides and sterilants have limited efficacy and are considered unsustainable due to the possible adverse

effects on human health and the ecosystem (ProMusa, n.d.). Meanwhile, biological control or biocontrol agents reliant on an organism's natural functions to control a pest or a disease (Vincent et al., 2007) are progressively being investigated for the potential as a sustainable option to curb the spread of Foc TR4 (Sharma et al., 2017; Vincent et al., 2007).

Potential biocontrol candidates come from the genus *Pseudomonas* spp., ubiquitous Gram-negative, motile, flagellated, rod-shaped, and aerobic gammaproteobacteria (Jenny & Kingsbury, 2018; Liao, 2006; Schroth et al., 2018; Weller, 2007). Members of *Pseudomonas* spp. have exhibited suppressive ability towards plant diseases such as the tomato disease caused by *Pythium aphanidermatum* (Jayaraj et al., 2007), pepper blight caused by *Phytophthora capsici* (Sang & Kim, 2014), and pink snow mould of wheat caused by *Microdochium nivale* (Andersson et al., 2012). In addition, some strains of *Pseudomonas* spp. produce fluorescent pigments, such as pyoverdine or pyochelin during iron-deficit conditions, are observable under ultraviolet (UV) light (Budzikiewicz, 1996). These fluorescent pigments are iron chelators known as siderophores that allow the bacteria to scavenge ferric ions (Fe^{3+}) from the surroundings for DNA synthesis, oxygen transport, incorporation into proteins, and nitrogen fixation (Andrews et al., 2003; Cornelis & Matthijs, 2007). Simeoni (1987) found that the germination of *F. oxysporum* f. sp. *cucumerinum* chlamydospores was inhibited when Fe^{3+} concentration in the soil reached 10^{-19} M and Fe^{3+} concentrations of 10^{-22} to 10^{-27} M produced optimal fungal inhibition. Meanwhile, Sayyed and Patel (2011) discovered that siderophore rich broth and supernatants exhibited strong antifungal activity against several types of fungi, including *Aspergillus niger* and *F. oxysporum*. These findings indicated that siderophore production and iron availability is strongly correlated to the antagonistic ability of *Pseudomonas* spp. against fungi.

Therefore, this study aimed to isolate potential biocontrol bacterial agents to suppress Foc TR4 by screening for fluorescent siderophore-producing bacteria and evaluating the antagonistic activities of the isolates against Foc TR4. Isolates exhibiting antagonistic activity were subsequently identified using 16s rRNA sequencing. In addition, the isolate that demonstrated more than 50% *in vitro* inhibition rate against Foc TR4 was further characterised using biochemical assays.

MATERIALS AND METHODS

Isolation and Screening of Fluorescent Isolates

Soil samples were collected from four points (north, south, east, west) at a depth of 20 cm around banana plants from Pahang and Selangor, Malaysia (Table 1). Healthy (N = 44) and affected (N = 11) banana plants were selected randomly to increase the possibility of getting various bacterial samples.

Table 1
List of sampling sites and their corresponding coordinates

Location	Coordinates	State
Felda Lembah Klau, Raub	N3˚41'29.454''; E102˚0'20.214''	Pahang
Sungai Pelek, Sepang	N2˚40'15.4''; E101˚41'22.4''	Selangor
Ladang Kongsi, UPM	N2°58'51.2"; E101°42'45.4"	Selangor
Kolej Kedua, UPM	N2°59'40.6"; E101°42'20.6"	Selangor
Kolej Tun Perak, UPM	N2°59'29.9"; E101°42'26.8"	Selangor
Jabatan Biology, UPM	N3˚0'4.8204''; E101˚42'17.0604''	Selangor

The samples were stored at 4 ˚C and processed two weeks after collection. A modified protocol of Sudarma and Suprapta (2011) and Nawangsih and Purba (2013) was used for screening. First, samples collected from the four points of each plant were mixed, then one gram of the mixed soil sample was taken and diluted in 99 mL of sterilised water to make up to 100 mL. After five minutes of agitation, the suspension of each mixed sample was used to prepare three spread plates on King's B agar (Pronadisa, Condalab, Spain) to isolate fluorescent bacteria, each with 1 mL of suspension. The plates were incubated at 25 ˚C ± 2 for 48 h, then viewed under UV light in an Enduro™ GDS-1302 gel documentation system (Labnet International, USA). Fluorescent colonies were picked and streaked on King's B agar, then incubated at 25 ˚C ± 2 for 24 h.

Determination of Siderophore Production using Chrome Azurol S Overlay

Blue Chrome Azurol S (CAS) dye was prepared according to Pérez-Miranda et al. (2007). A 7:3 mixture of blue CAS agar was prepared by slowly adding the dye to a 1% (w/v) agarose solution, then poured onto the Petri dish containing 24 h colonies of a single isolate, and then incubated at 25 ˚C for 2 h. The development of an orange-yellow zone indicated positive siderophore production. Therefore, colonies with positive siderophore production were chosen for the *in vitro* fungal antagonistic test. An uninoculated filter paper disc (4 mm) in place of bacterial culture was used as a negative control. All experiments were repeated at least twice with three replicates for each isolate.

In vitro Antagonistic Assay

The Foc TR4 (culture collection number: 9888, isolated initially from Fusarium wilt infected banana, Kuala Terengganu, Terengganu) for the antagonistic test was provided by the Fusarium Collection Centre, Plant Pathology Laboratory, Universiti Sains Malaysia (USM). A dual culture *in vitro* antagonistic test was performed. First, a filter paper disc (4 mm) was soaked in 24 h cultures of the isolate, then placed at 5 cm from a mycelial plug (4 mm) of Foc TR4 on

potato dextrose agar (PDA) (Merck, USA). After that, the plate was incubated at 25 ˚C ± 2 for 10 days. This assay was carried out in triplicate. An uninoculated filter paper disc was used as a negative control. The percentage of inhibition was calculated according to the formula provided in Chaiharn et al. (2009).

$$\%\ of\ inhibition = \left(\frac{D_c - D_t}{D_c}\right) x\ 100$$

Where Dc = distance of fungal growth in the control plate when it was cultured opposite a blank disc, and Dt = distance of fungal growth when it was cultured opposite a filter paper disc inoculated with the isolate. A one-way analysis of variance (ANOVA) was performed, followed by Tukey's honest significant difference (HSD) post-hoc test to determine if the inhibition rates were significantly different (GraphPad Prism version 9.2.0 for Windows, GraphPad Software, USA, www.graphpad.com). The isolates were then identified via molecular means.

Molecular Identification of Bacterial Isolates

The DNA of each isolate was extracted using the methods described in Nasiri et al. (2005) with slight modification by substituting the mentioned laundry powder with 25 mg/mL of enzymatic Daia washing powder (PT. Sayap Mas Utama, Indonesia). Amplification of the 16s rRNA region was performed on PCRmax Alpha Cycler (PCR Max, USA) using primers 16SF (5'-CGGTTACCTTGTTACGACTT-3') and 1387R (5'-GCCCGGGAACGTATTCACCG-3') obtained from Nawangsih and Purba (2013), and GoTaq® Flexi DNA Polymerase reagents (Promega, USA). In each 20 µL reaction, it contained 2 µL GoTaq® buffer (5x), 1 µL magnesium chloride (25 mM), 2 µL dNTPs (2 µM), 2 µL of each forward primer (2 µM) and reverse primer (2 µM), 1 µL GoTaq® polymerase (0.5U/µL), 2 µL DNA (10-20 ng), and 8 µL of distilled water. The cycling profile started with an initial denaturation of template DNA at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, annealing at 64 °C for 30 s, elongation at 72 °C for 30 s, and a single cycle of final elongation at 72 °C for 5 min. The PCR products were sequenced using the service provided by Apical Scientific Sdn. Bhd. (Seri Kembangan, Malaysia). The 16s rRNA sequences were searched against the National Centre of Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990), and the Ribosomal Database Project (RDP) using the sequence aligner (Q. Wang et al., 2007). The 16s rRNA sequences of all isolates were deposited into the NCBI database with the accession numbers of MN203661 (JBAA132), MN203662 (K2B121), MN203663 (K2B131), MN203664 (K2B421), MN203665 (K2B431), MN203666 (KTP211), MN203667 (KTP231), MN203668 (SNH212), MN203669 (SNH222), MN203670 (SNH231), MN203671 (SNH232), MN203672 (TGB111), MN203673 (TGB112), MN203674 (TGB131).

Phylogenetic Analysis

The 16s rRNA sequences of the most significant match for each isolate based on Nucleotide BLAST (BLASTn) were retrieved from NCBI and used for phylogenetic analysis in Molecular Evolutionary Genetics Analysis (MEGA)-X (version 10.2.6) (Kumar et al., 2018). An unweighted pair group method with arithmetic mean (UPGMA) tree (Sneath & Sokal, 1963) was constructed with 1,500 bootstrap replicates using the Tamura-Nei model (Tamura & Kumar, 2002) and rooted using *Aquaspirillum polymorphum* NRBC 13961 (NR_104710.1) as an outgroup. Branches corresponding to partitions reproduced in less than 70% of bootstrap replicates were collapsed.

Biochemical Characterisation

The isolate that displayed more than 50% of *in vitro* antagonistic activity and defined inhibition pattern was chosen for further characterisation. Next, the nitrogen-fixing test was performed using the methods explained in Baldani et al. (2014). Meanwhile, the phosphate solubilisation test was conducted based on the protocols provided in Chatli et al. (2008). Lastly, the catalase test, cetrimide test, citrate test, decarboxylase test (arginine, lysine), gelatine liquefaction test, indole production, lipase solubilisation test, Methyl Red-Voges Prokaur (MR-VP), nitrate reduction, oxidase test, oxidative-fermentative test (dextrose, inulin, lactose, mannitol, sucrose), and sulphate-indole-motility test were all performed according to the protocols described in Cappuccino and Sherman (2008).

RESULTS AND DISCUSSION

Isolation, Screening, and Siderophore Production Assay

A total of 55 soil samples were collected from the immediate vicinity of banana plants. From these samples, 32 fluorescent isolates were obtained. However, only 14 of the fluorescent isolates—KTP231, JBAA132, SNH212, K2B421, TGB112, SNH222, SNH232, TGB131, K2B431, KTP211, TGB111, K2B131, SNH231, and K2B121—were positive for siderophore production. Figure 1 shows the differences observed in control a non-siderophore producer and a siderophore producer. The orange halos surrounding the colonies indicate the presence of siderophores.

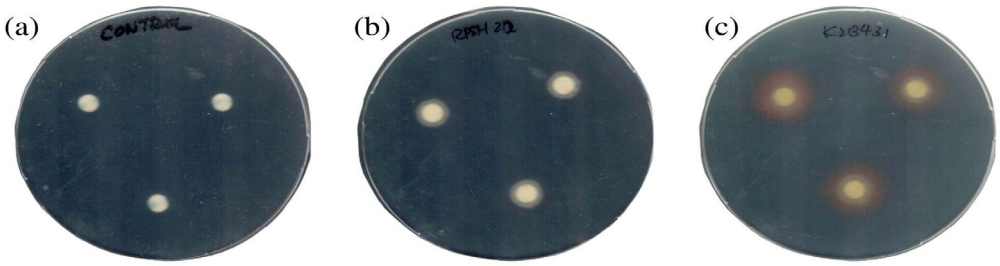

Figure 1. Siderophore production assays. (a) Control plate with uninoculated filter paper discs; (b) plate showing a lack of orange halo around the colonies, thus indicating a non-siderophore producer; (c) plate showing orange halo around colonies, thus indicating a siderophore producer

In vitro Antagonistic Assay

The antagonistic ability of the 14 siderophore producers against Foc TR4 was tested on PDA. All 14 isolates demonstrated various inhibition rates after ten days of incubation. Compared to the control plate, inhibitions were observed in all the plates indicated by the halt of mycelial growth towards the bacterial isolates. Figure 2 shows the Foc TR4 mycelial growth in the control plate (a) and cultured with inhibitive isolates (b-o) after ten days of incubation. The inhibition of the mycelial growth can be clearly seen in plates (b) JBAA132, (c) K2B121, (d) K2B131, (e) K2B421, and (h) KTP231. A less defined inhibition zone was observed in all other plates, with plates (m) TGB111 and (o) TGB131 displaying the weakest inhibition abilities. Among the isolates screened, seven isolates showed more than 40% *in vitro* inhibition rates. The percentages of inhibition for all 14 isolates were plotted and are shown in Figure 3. One-way ANOVA revealed a significant difference in the inhibition rate ($p < 0.0001$). The Tukey's HSD post-hoc test found that the inhibition rates of JBAA132, K2B131, KTP231, SNH212, K2B121, KTP211, SNH232, TGB111, SNH222, TGB112, K2B43, and K2B4211 were significantly different to the control ($p < 0.0001$). However, there was no significant difference among the isolates except for JBAA132 vs. SNH231 and JBAA132 vs. TGB131.

Antagonistic bacteria play an important role in suppressing soil-borne plant diseases and are potential biocontrol agents. The fungal inhibition efficiency of various bacterial species against Foc has been studied over the years. Li et al. (2012) studied 45 isolates of *Pseudomonas* spp. and discovered that these isolates demonstrated an inhibition rate that ranged from 38.30% to 67.14% against Foc. Meanwhile, Yuan et al. (2012) showed that volatile compounds from *Bacillus amyloliquefaciens* achieved 30-40% inhibition in dual culture plates against Foc. Zacky and Ting (2013) discovered that cell and cell-free extracts of *Streptomyces griseus* produced a 54% and 33% rate of inhibition on Foc-TR4. Meanwhile, the usage of *Burkholderia cenocepacia* in Ho et al. (2014) demonstrated an inhibition rate of 44.4% against Foc TR4. Similarly, a study by Islam et al. (2018) demonstrated that *P. aeruginosa* BA5 could inhibit Foc growth at a rate of up to 58.33%. It is plausible that isolate JBAA132 shows a comparable inhibition rate at 50.38% against Foc TR4.

Molecular Identification of Isolates

Molecular identification of the 14 antagonistic isolates using 16S rRNA revealed that all isolates were from the genus *Pseudomonas*, except for isolates KTP211 and SNH231. Similarity searches against both NCBI and RDP databases returned similar significant matches for each isolate. For example, a BLAST search of the 16s rRNA sequence of isolate JBAA132 showed a 99.48% similarity to *Pseudomonas aeruginosa*, followed by *Pseudomonas otiditis* (98.07%) and *Pseudomonas guezennei* (97.96%). Similarity search against RDP also returned *P. aeruginosa* as the top match but only

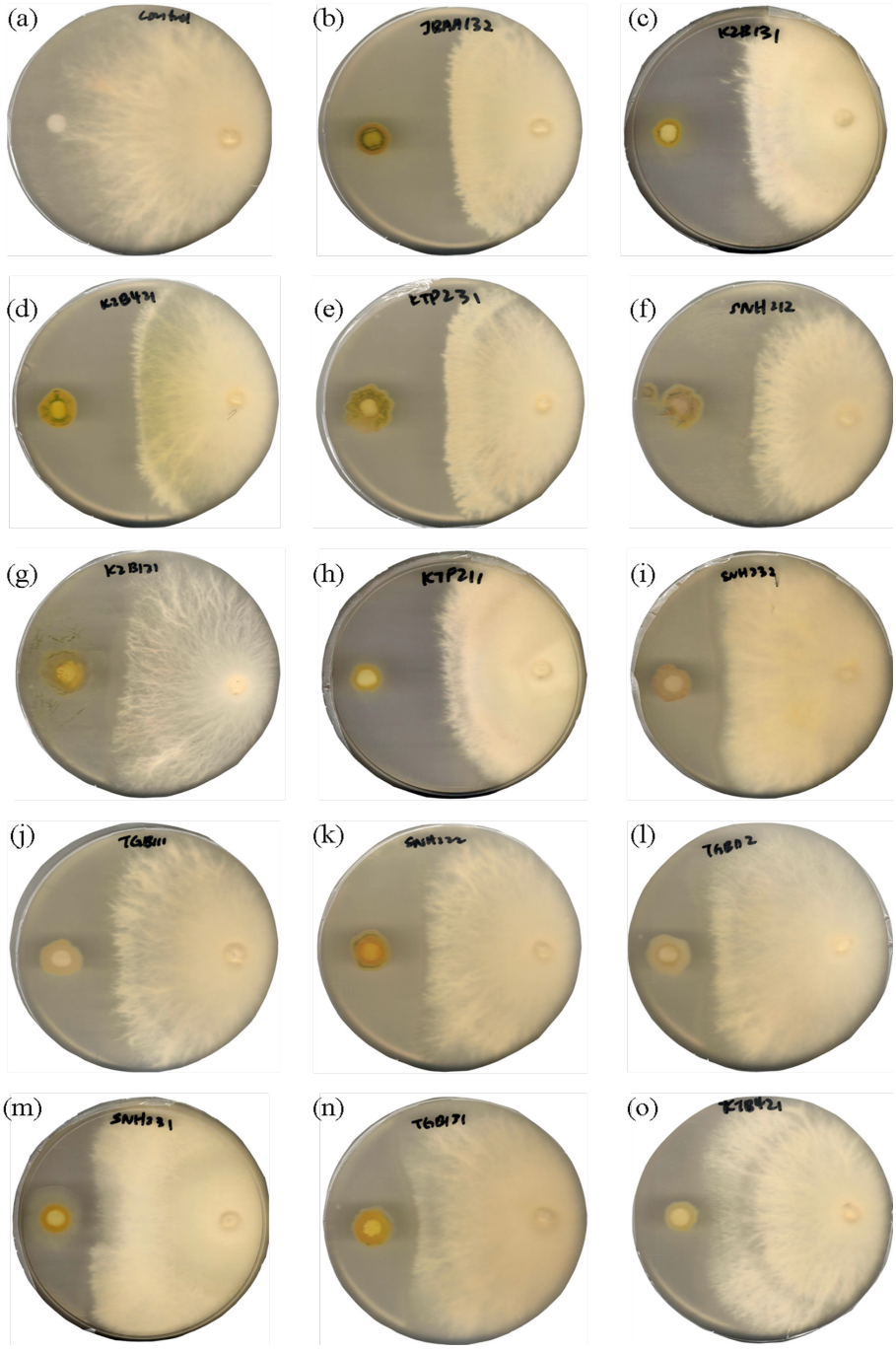

Figure 2. *In vitro* antagonistic assays. (a) Control with filter paper placed opposite a mycelial plug of Foc TR4; (b) JBAA132; (c) K2B121; (d) K2B131; (e) K2B421; (f) K2B431; (g) KTP211; (h) KTP231; (i) SNH212; (j) SNH222; (k) SNH231; (l) SNH232; (m) TGB111; (n) TGB112; (o) TGB131

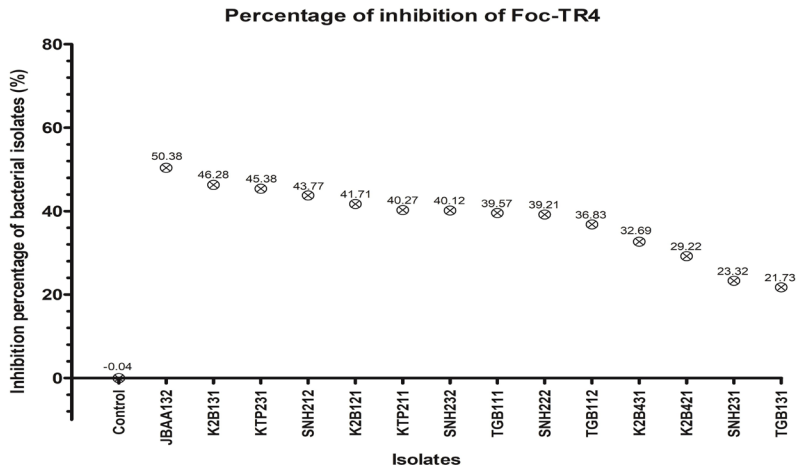

Figure 3. Percentages of inhibition of Foc TR4 by the 14 bacterial isolates

87.3% sequence similarity. The results of molecular identification via 16s rRNA based on the two databases are displayed in Table 2 and Table 3.

Ten out of 14 isolates were identified as *P. aeruginosa*, and all matched significantly to type strain DSM50071. While two isolates were identified as *Pseudomonas plecoglossicida* and *Pseudomonas taiwanensis*. The abundance of *Pseudomonas* spp. found in this study collaborated well the findings of Zhou et al. (2019), who reported that *Pseudomonas* spp. were the third most abundant genus in samples taken from banana rhizospheres. Zhou et al. (2019) also indicated that the top two dominant genera were *Bacillus* and *Lactococcus*. However, members of these genera would have likely been screened out by the siderophore production assay in our study. In another study, Kaushal et al. (2020) reported that *Pseudomonas* spp. was the second most abundant genus in Kilimanjaro revealed by an analysis of soil microbial species around infected and non-infected banana plants. Members from the genera *Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Variovorax* are often associated with the plant-growth-promoting property. Thus, they are often found in close proximity with numerous plant species (Habibi et al., 2019; Saxena et al., 2020).

In addition to *Pseudomonas* spp., two non-*Pseudomonas* siderophore-producing bacteria were also present among the isolates, namely *Pantoea septica* (isolate KTP211) and *Serratia nematodiphila* (isolate SNH231). While there have been no reports of *Pantoea septica* being isolated from the soil surrounding banana plants prior to this study, Walterson and Stavrinides (2015) asserted that some members of the genus *Pantoea* isolated from soil environments have plant-growth-promoting properties. In another study, Chakdar et al. (2018) discovered a phosphate-solubilising

soil isolate similar to *Pantoea septica* LMG 5345 from termitorial soil in India, indicating its plant growth-promoting capabilities. Finally, Marcano et al. (2016) studied the bacteria community from the soil surrounding banana plants. Most of the isolates in the study came from the genus *Pseudomonas* and *Actinobacter*. However, they also identified a bacterial isolate that showed a high similarity to *Serratia nematodiphila* based on 16s rRNA sequencing.

Table 2

Percentages of similarity and coverage of antagonistic isolates capable of inhibition of Foc TR4 based on BLAST search against NCBI database

Isolate	BLAST NCBI	Similarity (%)	Coverage (%)
JBAA132	*Pseudomonas aeruginosa* DSM50071	99.48	99
K2B121	*Pseudomonas aeruginosa* DSM50071	96.68	93
K2B131	*Pseudomonas aeruginosa* DSM50071	96.51	93
K2B421	*Pseudomonas aeruginosa* DSM50071	100	100
K2B431	*Pseudomonas aeruginosa* DSM50071	97.24	91
KTP211	*Pantoea septica* LMG 5345	99.55	100
KTP231	*Pseudomonas plecoglossicida* NBRC 103162	100	100
SNH212	*Pseudomonas aeruginosa* DSM50071	99.64	100
SNH222	*Pseudomonas aeruginosa* DSM50071	99.82	100
SNH231	*Serratia nematodiphila* DZ0503SBS1	99.56	100
SNH232	*Pseudomonas aeruginosa* DSM50071	99.91	100
TGB111	*Pseudomonas taiwanensis* BCRC 17751	98.35	93
TGB112	*Pseudomonas aeruginosa* DSM50071	96.84	89
TGB131	*Pseudomonas aeruginosa* DSM50071	99.91	100

Table 3

Percentage of similarity of antagonistic isolates capable of inhibition of Foc TR4 based on similarity search against the database of Ribosomal Database Project (RDP)

Isolate	RDP	Similarity (%)
JBAA132	*Pseudomonas aeruginosa* DSM50071	87.3
K2B121	*Pseudomonas aeruginosa* DSM50071	96.7
K2B131	*Pseudomonas aeruginosa* DSM50071	80.5

Table 3 (*Continue*)

Isolate	RDP	Similarity (%)
K2B421	*Pseudomonas aeruginosa* DSM50071	99.6
K2B431	*Pseudomonas aeruginosa* DSM50071	87.1
KTP211	*Pantoea septica* LMG 5345	97
KTP231	*Pseudomonas plecoglossicida* FPC951	99.8
SNH212	*Pseudomonas aeruginosa* DSM50071	97.7
SNH222	*Pseudomonas aeruginosa* DSM50071	98.3
SNH231	*Serratia nematodiphila* DZ0503SBS1	98
SNH232	*Pseudomonas aeruginosa* DSM50071	99.3
TGB111	*Pseudomonas taiwanensis* BCRC 17751	85.2
TGB112	*Pseudomonas aeruginosa* DSM50071	96.7
TGB131	*Pseudomonas aeruginosa* DSM50071	99

Phylogenetic Analysis

The phylogenetic tree inferred from the 16S rRNA sequences showed clustering of isolates and species (Figure 4) congruent with the similarity search results. Two main clades were formed, where one of the clades (I) consisted of only *Pseudomonas* spp. In contrast, the other clade comprised only non-*Pseudomonas* species (II). The ten isolates (i.e., JBAA132, K2B121, K2B131, K2B421, K2B431, SNH212, SNH222, SNH232, TGB112, and TGB131) identified as *P. aeruginosa* based on BLAST similarity searches were grouped with various *Pseudomonas* spp. into a clade with a strong bootstrap value indicating genetic closeness. While isolate KTP231 identified as *P. plecoglossicida* and TGB111 identified as *P. taiwanensis* based on similarity searches, were clustered in the same subclade with several other *Pseudomonas* spp., which also included *P. plecoglossicida* and *P. taiwanensis*, with a strong bootstrap value of 98%. Meanwhile, isolate KTP211 was placed in the same subclade with *Pantoea septica*. In contrast, isolate SNH231, identified as *Serratia nematodiphila*, was clustered together with *Serratia marcescens* and *Serratia nematodiphila* with a strong bootstrap value of 100%.

Among the 14 fluorescent siderophore-producing isolates capable of inhibiting Foc TR4, 12 were identified as *Pseudomonas* spp. i.e., *P. aeruginosa*, *P. plecoglossicida*, and *P. taiwanensis*. Isolate JBAA132, the only isolate that displayed an *in vitro* inhibitive activity of more than 50% against Foc TR4 in this study, was identified as *P. aeruginosa*. *Pseudomonas* spp. are ubiquitous (Wu et al., 2015) and have been isolated from many environmental samples such as soil (AL-Saleh & Akbar, 2015) and water (Nasreen et al., 2015). *Pseudomonas aeruginosa* is a common soil bacterium

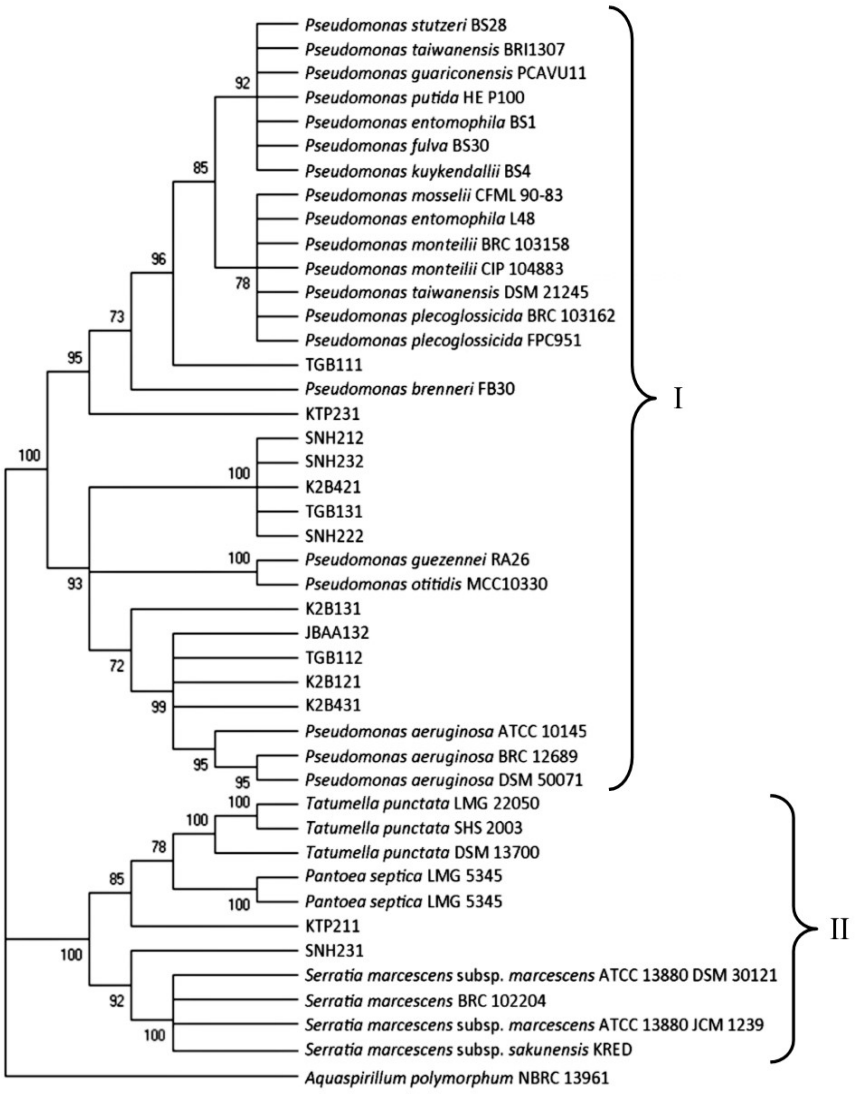

Figure 4. UPGMA tree constructed with MEGA-X using the Tamura-Nei model with 1,500 bootstraps and rooted using *Aquaspirillum polymorphum* NRBC 13961

(Gupta et al., 1999) that has been studied extensively and was reported to have biocontrol properties. Sekhar and Thomas (2015) discovered that *P. aeruginosa* was one of the bacteria capable of antagonistic ability against Foc. In addition, Lahkar et al. (2015) revealed the ability of the strain JS29 to inhibit the growth of *Alternaria solani*, the causal agent of tomato blight, by 73%. Meanwhile, *P. aeruginosa* ID 4365 and SBC 5 were demonstrated to have good antifungal ability against *Sclerotium rolfsii*,

the causal agent of Southern blight on vegetables and melons (Rane et al., 2008).

Other species within the same genus, isolate KTP231 (identified as *P. plecoglossicida*) and isolate TGB111 (identified as *P. taiwanensis*), showed moderate inhibitive activity (39.57%–45.38%) against the growth of Foc TR4 in this study. *Pseudomonas plecoglossicida* was first isolated from cultured ayu or sweetfish (*Plecoglossus altivelis*) by Nishimori et al. (2000). It was identified as a potential bioremediation agent for hazardous compounds (Boricha & Fulekar, 2009) but has not been assessed for biocontrol ability prior to this. *Pseudomonas taiwanensis* is a *Pseudomonas* species recently isolated from soil (L.-T. Wang et al., 2010). Previous studies revealed that *P. taiwanensis* is a potential biocontrol agent against plant pathogens such as *Xanthomonas axonopodis* pv. *dieffenbachiae* (Dhanya et al., 2020), and *Xanthomonas oryzae* pv. *oryzae* (Chen et al., 2016). However, no study has investigated the antagonistic activity of *P. taiwanensis* against Foc TR4.

Among the inhibitive isolates, *P. septica* and *S. nematodiphila* were the only two non-*Pseudomonas* spp. identified. Thus far, *P. septica* has never been investigated for or associated with biocontrol properties. Interestingly, isolate KTP211 (identified as *P. septica*) exhibited antagonistic activity against Foc TR4 with a moderate *in vitro* inhibition rate of 40.27% in this study. On the contrary, based on the low inhibition activity (23.32%) observed in isolate SNH231 (*S. nematodiphila*), it is unlikely a potential biocontrol agent against Foc TR4. Nonetheless, Khoa et al. (2016) had demonstrated the biocontrol properties of this species against other plant diseases such as the bacterial leaf blight in rice.

Biochemical Characterisation

Biochemical assays showed that isolate JBAA132 was catalase-positive and oxidase-positive. It demonstrated the ability to utilise citrate and mannitol and was also positive for gelatinase, tryptophanase, and cysteine desulfurase. The isolate also produced a bright green colour typical of the genus *Pseudomonas* when cultured on cetrimide agar. Isolate JBAA132 could fix nitrogen and solubilise phosphorus but could not decarboxylate arginine, asparagine, and glutamine. The results are summarised in Table 4.

There are evident differences in the biochemical characteristics between isolate JBAA132 and the type strain *P. aeruginosa* DSM50071 (Reimer et al., 2019). Type strain DSM50071 is purported to produce arginine, asparagine, and glutamine decarboxylases (Reimer et al., 2019). However, isolate JBAA132 did not produce these three types of decarboxylases. In the oxidative-fermentation test, isolate JBAA132 showed different characteristics from type strain DSM50071. Isolate JBAA132 produced a colour change in the media from green to blue when cultured in lactose and sucrose. The colour change from green to blue in the basal media indicated that even though the bacterial strain could not utilise the substrates, it could break

down the peptones contained in the basal media into alkaline products that turned the media blue (Hanson, 2008). Furthermore, isolate JBAA132 utilised dextrose under fermentative and mannitol under oxidative conditions, which were not associated with the type strain DSM50071 (Reimer et al., 2019). In addition, isolate JBAA132 was also positive for hydrogen sulphide production, which has not been reported in the type strain. It indicates a likelihood of cysteine desulfurase biosynthesis in isolating JBAA132 (J. Wang et al., 2019). Differences in the biochemical capabilities signify that isolating JBAA132 may be differ from the type strain DSM50071. This finding is congruent with the similarity results obtained from the search against the RDP, which showed only 87.3% similarity between isolate JBAA132 and the type strain. In addition, isolate JBAA132 is also capable of phosphorus solubilisation and nitrogen fixation, mechanisms found in most plant-growth-promoting bacteria (Gamalero & Glick, 2011).

Table 4

Biochemical characterisation of isolate JBAA132 and comparison with available data for Pseudomonas aeruginosa *DSM50071*

Biochemical Test		JBAA132	DSM50071
Catalase production		+	+
Cetrimide		+	+
Citrate agar		+	+
Decarboxylase activity	Arginine	-	+
	Asparagine	-	+
	Glutamine	-	+
	Lysine	-	-
	Tyrosine	-	n.a.
Gelatine liquefaction		$++_a$	+
Indole production		-	-
Lipase		-	n.a.
Methyl Red-Voges Prokaur	MR	-	-
	VP	-	-
Nitrate reduction		+	+
Nitrogen fixation		+	n.a.
Oxidase production		+	+

Table 4 (*Continue*)

Biochemical Test	JBAA132	DSM50071
Dextrose Oxidation	±$_b$	+
Dextrose Fermentation	+	-
Inulin Oxidation	±$_b$	n.a.
Inulin Fermentation	-	n.a.
Lactose Oxidation	±$_b$	-
Lactose Fermentation	-	n.a.
Sucrose Oxidation	±$_b$	-
Sucrose Fermentation	-	n.a.
Mannitol Oxidation	+	-
Mannitol Fermentation	-	n.a.
Phosphate solubilisation	+	n.a.
Motility	+	+
Hydrogen Sulphide Production	+	-

Note. $_a$ indicates very rapid liquefaction of gelatine, whereas $_b$ indicates the production of alkaline products. n.a. indicates that no information regarding this test is available at present

CONCLUSION

Antagonistic bacteria hold great potential as biocontrol agents in suppressing Foc TR4. In this study, siderophore-producing fluorescent soil isolates of genera *Pseudomonas*, *Pantoea*, and *Serratia* exhibited inhibitive activities against Foc TR4 during *in vitro* antagonistic assays were isolated. Isolate JBAA132 displayed high inhibition activity against Foc TR4 at a rate of 50.38%. Furthermore, the isolate showed high sequence similarity with *P. aeruginosa* DSM50071 based on 16s rRNA sequence searched against the NCBI database. However, it only showed 87.3% of sequence similarity to *P. aeruginosa* DSM50071 when searched against RDP. Furthermore, the isolate also displayed several biochemical characteristics different from type strain DSM50071. These findings indicate that isolating JBAA132 may be a different strain of *P. aeruginosa*. In addition, isolate JBAA132 showed biochemical activities that suggest its potential as a plant growth promoter. Therefore, it is proposed that isolates with more than 40.0% *in vitro* inhibition against Foc TR4 may be further explored for their potential *in vivo* inhibitive abilities. Nonetheless, the pathogenicity of the isolates on the animal must be investigated and determined prior to further *in vivo* studies.

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REFERENCES

Agrios, G. N. (2005). Plant diseases caused by fungi. In *Plant pathology* (5th ed., pp. 385–614). Elsevier Academic Press. https://doi.org/10.1016/b978-0-08-047378-9.50017-8

AL-Saleh, E., & Akbar, A. (2015). Occurrence of *Pseudomonas aeruginosa* in Kuwait soil. *Chemosphere*, *120*, 100–107. https://doi.org/10.1016/j.chemosphere.2014.06.031

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Andersson, P. F., Levenfors, J., & Broberg, A. (2012). Metabolites from *Pseudomonas brassicacearum* with activity against the pink snow mould causing pathogen *Microdochium nivale*. *BioControl*, *57*(3), 463–469. https://doi.org/10.1007/s10526-011-9411-5

Andrews, S. C., Robinson, A. K., & Rodríguez-Quiñones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiology Reviews*, *27*(2–3), 215–237. https://doi.org/10.1016/s0168-6445(03)00055-x

Baldani, J. I., Reis, V. M., Videira, S. S., Boddey, L. H., & Baldani, V. L. D. (2014). The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: A practical guide for microbiologists. *Plant and Soil*, *384*(1–2), 413–431. https://doi.org/10.1007/s11104-014-2186-6

Boricha, H., & Fulekar, M. (2009). *Pseudomonas plecoglossicida* as a novel organism for the bioremediation of cypermethrin. *Biology and Medicine*, *1*(4), 10.

Budzikiewicz, H. (1996). Siderophores from fluorescent *Pseudomonas*. In Atta-ur-Rahman (Ed.), *Studies in natural products chemistry* (Vol. 19, pp. 793–835). Elsevier. https://doi.org/10.1016/s1572-5995(96)80019-6

Cappuccino, J. G., & Sherman, N. (2008). *Microbiology: A laboratory manual* (8th ed.). Pearson Education.

Chaiharn, M., Chunhaleuchanon, S., & Lumyong, S. (2009). Screening siderophore producing bacteria as potential biological control agent for fungal rice pathogens in Thailand. *World Journal of Microbiology and Biotechnology*, *25*(11), 1919–1928. https://doi.org/10.1007/s11274-009-0090-7

Chakdar, H., Dastager, S. G., Khire, J. M., Rane, D., & Dharne, M. S. (2018). Characterization of mineral phosphate solubilizing and plant growth promoting bacteria from termite soil of arid region. *3 Biotech*, *8*(11), 463. https://doi.org/10.1007/s13205-018-1488-4

Chatli, A. S., Beri, V., & Sidhu, B. S. (2008). Isolation and characterisation of phosphate solubilising microorganisms from the cold desert habitat of *Salix alba* Linn. in trans Himalayan region of Himachal Pradesh. *Indian Journal of Microbiology*, *48*(2), 267–273. https://doi.org/10.1007/s12088-008-0037-y

Chen, W.-J., Kuo, T.-Y., Hsieh, F.-C., Chen, P.-Y., Wang, C.-S., Shih, Y.-L., Lai, Y.-M., Liu, J.-R., Yang, Y.-L., & Shih, M.-C. (2016). Involvement of type VI secretion system in secretion of iron chelator pyoverdine in *Pseudomonas taiwanensis*. *Scientific Reports*, *6*(1), 32950. https://doi.org/10.1038/srep32950

Cornelis, P., & Matthijs, S. (2007). *Pseudomonas* siderophores and their biological significance. In A. Varma & S. B. Chincholkar (Eds.), *Soil biology* (Vol. 12, pp. 193–203). Springer-Verlag. https://doi.org/10.1007/978-3-540-71160-5_9

Dhanya, S., Sherin, V., Divya, K., Sreekumar, J., & Jisha, M. S. (2020). *Pseudomonas taiwanensis* (MTCC11631) mediated induction of systemic resistance in *Anthurium andreanum* L. against blight disease and visualisation of defence related secondary metabolites using confocal laser scanning microscopy. *Biocatalysis and Agricultural Biotechnology*, *24*, 101561. https://doi.org/10.1016/j.bcab.2020.101561

Dita, M., Barquero, M., Heck, D., Mizubuti, E. S. G., & Staver, C. P. (2018). Fusarium wilt of banana: Current knowledge on epidemiology and research needs toward sustainable disease management. *Frontiers in Plant Science*, *9*, 1468. https://doi.org/10.3389/fpls.2018.01468

Food and Agriculture Organization of the United Nations. (2019). *Food outlook — Biannual report on global food markets*. FAO. https://doi.org/10.1787/agr_outlook-2019-en

Gamalero, E., & Glick, B. R. (2011). Mechanisms used by plant growth-promoting bacteria. In D. K. Maheshwari (Ed.), *Bacteria in agrobiology: Plant nutrient management* (pp. 17–46). Springer. http://dx.doi.org/10.1007/978-3-642-21061-7_2

Ghag, S. B., Shekhawat, U. K. S., & Ganapathi, T. R. (2015). Fusarium wilt of banana: Biology, epidemiology and management. *International Journal of Pest Management*, *61*(3), 250–263. https://doi.org/10.1080/09670874.2015.1043972

Gupta, C. P., Sharma, A., Dubey, R. C., & Maheshwari, D. K. (1999). *Pseudomonas aeruginosa* (GRC1) as a strong antagonist of *Macrophomina phaseolina* and *Fusarium oxysporum*. *Cytobios*, *99*(392), 183–189.

Habibi, S., Djedidi, S., Ohkama-Ohtsu, N., Sarhadi, W. A., Kojima, K., Rallos, R. V., Ramirez, M. D. A., Yamaya, H., Sekimoto, H., & Yokoyama, T. (2019). Isolation and screening of indigenous plant growth-promoting rhizobacteria from different rice cultivars in Afghanistan soils. *Microbes and Environments*, *34*(4), 347–355. https://doi.org/10.1264/jsme2.ME18168

Hanson, A. (2008). *Oxidative-fermentative test protocol*. https://web.archive.org/web/20111014202803/http://www.microbelibrary.org/component/resource/laboratory-test/3151-oxidative-fermentative-test-protocol

Hennessy, C., Walduck, G., Daly, A., & Padovan, A. (2005). Weed hosts of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 in northern Australia. *Australasian Plant Pathology*, *34*(1), 115-117. https://doi.org/10.1071/ap04091

Ho, Y.-N., Chiang, H.-M., Chao, C.-P., Su, C.-C., Hsu, H.-F., Guo, C., Hsieh, J.-L., & Huang, C.-C. (2014). *In planta* biocontrol of soilborne Fusarium wilt of banana through a plant endophytic bacterium, *Burkholderia cenocepacia* 869T2. *Plant and Soil*, *387*(1–2), 295–306. https://doi.org/10.1007/s11104-014-2297-0

Islam, Md. A., Nain, Z., Alam, Md. K., Banu, N. A., & Islam, Md. R. (2018). *In vitro* study of biocontrol potential of rhizospheric *Pseudomonas aeruginosa* against *Fusarium oxysporum* f. sp. *cucumerinum*. *Egyptian Journal of Biological Pest Control*, *28*(1), 90. https://doi.org/10.1186/s41938-018-0097-1

Jayaraj, J., Parthasarathi, T., & Radhakrishnan, N. V. (2007). Characterization of a *Pseudomonas fluorescens* strain from tomato rhizosphere and its use for integrated management of tomato damping-off. *BioControl*, *52*(5), 683–702. https://doi.org/10.1007/s10526-006-9046-0

Jenny, M., & Kingsbury, J. (2018). Properties and prevention: A review of *Pseudomonas aeruginosa*. *Journal of Biology and Medical Research*, *2*(3), 18.

Kaushal, M., Swennen, R., & Mahuku, G. (2020). Unlocking the microbiome communities of banana (*Musa* spp.) under disease stressed

(Fusarium wilt) and non-stressed conditions. *Microorganisms*, *8*(3), 443. https://doi.org/10.3390/microorganisms8030443

Khoa, N. Đ., Giàu, N. Đ. N., & Tuấn, T. Q. (2016). Effects of *Serratia nematodiphila* CT-78 on rice bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*. *Biological Control*, *103*, 1–10. https://doi.org/10.1016/j.biocontrol.2016.07.010

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Lahkar, J., Borah, S. N., Deka, S., & Ahmed, G. (2015). Biosurfactant of *Pseudomonas aeruginosa* JS29 against *Alternaria solani*: The causal organism of early blight of tomato. *BioControl*, *60*(3), 401–411. https://doi.org/10.1007/s10526-015-9650-y

Li, P., Ma, L., Feng, Y. L., Mo, M. H., Yang, F. X., Dai, H. F., & Zhao, Y. X. (2012). Diversity and chemotaxis of soil bacteria with antifungal activity against Fusarium wilt of banana. *Journal of Industrial Microbiology and Biotechnology*, *39*(10), 1495–1505. https://doi.org/10.1007/s10295-012-1163-4

Liao, C. H. (2006). *Pseudomonas* and related genera. In C. de W. Blackburn (Ed.), *Food spoilage microorganisms* (pp. 507–540). Woodhead Publishing Limited. https://doi.org/10.1533/9781845691417.5.507

Marcano, I.-E., Díaz-Alcántara, C.-A., Urbano, B., & González-Andrés, F. (2016). Assessment of bacterial populations associated with banana tree roots and development of successful plant probiotics for banana crop. *Soil Biology and Biochemistry*, *99*, 1–20. https://doi.org/10.1016/j.soilbio.2016.04.013

Nasiri, H., Forouzandeh, M., Rasaee, M. J., & Rahbarizadeh, F. (2005). Modified salting-out method: High-yield, high-quality genomic DNA extraction from whole blood using laundry detergent. *Journal of Clinical Laboratory Analysis*, *19*(6), 229–232. https://doi.org/10.1002/jcla.20083

Nasreen, M., Sarker, A., Malek, M. A., Ansaruzzaman, Md., & Rahman, M. (2015). Prevalence and resistance pattern of *Pseudomonas aeruginosa* isolated from surface water. *Advances in Microbiology*, *5*(1), 74–81. https://doi.org/10.4236/aim.2015.51008

Nawangsih, A. A., & Purba, F. (2013). Isolation of fluorescent pseudomonads, heat tolerant and chitinolytic bacteria in banana rhizosphere with antagonistic activities against *Fusarium oxysporum* f. sp. *cubense in vitro* and molecular identification of selected isolates. *International Society for Southeast Asian Agricultural Sciences*, *19*(2), 30–40.

Nishimori, E., Kita-Tsukamoto, K., & Wakabayashi, H. (2000). *Pseudomonas plecoglossicida* sp. nov., the causative agent of bacterial haemorrhagic ascites of ayu, *Plecoglossus altivelis*. *International Journal of Systematic and Evolutionary Microbiology*, *50*(1), 83–89. https://doi.org/10.1099/00207713-50-1-83

Pérez-Miranda, S., Cabirol, N., George-Téllez, R., Zamudio-Rivera, L. S., & Fernández, F. J. (2007). O-CAS, a fast and universal method for siderophore detection. *Journal of Microbiological Methods*, *70*(1), 127–131. https://doi.org/10.1016/j.mimet.2007.03.023

Ploetz, R. C. (2005). Panama disease: An old nemesis rears its ugly head: Part 1. The beginnings of the banana export trades. *Plant Health Progress*, *6*(1), 18. https://doi.org/10.1094/PHP-2005-1221-01-RV

ProMusa. (n.d.). *Fusarium wilt of banana*. http://www.promusa.org/Fusarium+wilt

Rane, M. R., Sarode, P. D., Chaudhari, B. L., & Chincholkar, S. B. (2008). Exploring antagonistic metabolites of established biocontrol agent of marine origin. *Applied Biochemistry and*

Biotechnology, *151*(2–3), 665–675. https://doi.org/10.1007/s12010-008-8288-y

Reimer, L.C., Vetcininova, A., Carbasse J. S., Sohngen, C., Gleim, D., Ebeling, C. & Overmann, J. (2019). Bac*Dive* in 2019: Bacterial phenotypic data for High-throughput biodiversity analysis. *Nucleic Acids Research*, 47(D1), D631-D636. https://doi.org/10.1093/nar/gky879

Sang, M. K., & Kim, K. D. (2014). Biocontrol activity and root colonization by *Pseudomonas corrugata* strains CCR04 and CCR80 against Phytophthora blight of pepper. *BioControl*, *59*(4), 437–448. https://doi.org/10.1007/s10526-014-9584-9

Saxena, A. K., Kumar, M., Chakdar, H., Anuroopa, N., & Bagyaraj, D. J. (2020). *Bacillus* species in soil as a natural resource for plant health and nutrition. *Journal of Applied Microbiology*, *128*(6), 1583–1594. https://doi.org/10.1111/jam.14506

Sayyed, R. Z., & Patel, P. R. (2011). Biocontrol potential of siderophore producing heavy metal resistant *Alcaligenes* sp. and *Pseudomonas aeruginosa* RZS3 vis-à-vis organophosphorus fungicide. *Indian Journal of Microbiology*, *51*(3), 266–272. https://doi.org/10.1007/s12088-011-0170-x

Scheerer, L., Pemsl, D., Dita, M., Vicente, L. P., & Staver, C. (2018). A quantified approach to projecting losses caused by Fusarium wilt Tropical Race 4. *Acta Horticulturae*, *1196*, 211–218. https://doi.org/10.17660/ActaHortic.2018.1196.26

Schroth, M. N., Cho, J. J., Green, S. K., & Kominos, S. D. (2018). Epidemiology of *Pseudomonas aeruginosa* in agricultural areas. *Journal of Medical Microbiology*, *67*(8), 1191–1201. https://doi.org/10.1099/jmm.0.000758

Sekhar, A. C., & Thomas, P. (2015). Isolation and identification of shoot-tip associated endophytic bacteria from banana cv. Grand Naine and testing for antagonistic activity against *Fusarium oxysporum* f. sp. c*ubense*. *American Journal of Plant Sciences*, *06*(07), 943–954. https://doi.org/10.4236/ajps.2015.67101

Sharma, M., Tarafdar, A., Ghosh, R., & Gopalakrishanan, S. (2017). Biological control as a tool for eco-friendly management of plant pathogens. In T. K. Adhya, B. B. Mishra, K. Annapurna, D. K. Verma, & U. Kumar (Eds.), *Advances in soil microbiology: Recent trends and future prospects* (Vol. 2, pp. 153–188). Springer Singapore. https://doi.org/10.1007/978-981-10-7380-9_8

Simeoni, L. A. (1987). Critical iron level associated with biological control of Fusarium wilt. *Phytopathology*, *77*(6), 1057-1061. https://doi.org/10.1094/phyto-77-1057

Sneath, P. H. A., & Sokal, R. R. (1963). *Numerical taxonomy: The principles and practice of numerical classification*. W. H. Freeman and Company.

Su, H., Hwang, S., & Ko, W. (1986). Fusarial wilt of Cavendish bananas in Taiwan. *Plant Disease*, *70*(9), 814-818. https://doi.org/10.1094/pd-70-814

Sudarma, I. M., & Suprapta, D. N. (2011). Diversity of soil microorganisms in banana habitats with and without Fusarium wilt symptom. *Journal of International Society for Southeast Asian Agricultural Sciences*, *17*(1), 147–159.

Tamura, K., & Kumar, S. (2002). Evolutionary distance estimation under heterogeneous substitution pattern among lineages. *Molecular Biology and Evolution*, *19*(10), 1727–1736. https://doi.org/10.1093/oxfordjournals.molbev.a003995

VanderMolen, G. E., Beckman, C. H., & Rodehorst, E. (1987). The ultrastructure of tylose formation in resistant banana following inoculation with *Fusarium oxysporum* f. sp. *cubense*. *Physiological and Molecular Plant Pathology*, *31*(2), 185–200. https://doi.org/10.1016/0885-5765(87)90063-4

Vincent, C., Goettel, M. S., & Lazarovits, G. (Eds.). (2007). *Biological control: A global perspective*. Centre for Agriculture and Biosciences International.

Walterson, A. M., & Stavrinides, J. (2015). *Pantoea:* Insights into a highly versatile and diverse genus within the Enterobacteriaceae. *FEMS Microbiology Reviews*, *39*(6), 968–984. https://doi.org/10.1093/femsre/fuv027

Wang, J., Guo, X., Li, H., Qi, H., Qian, J., Yan, S., Shi, J., & Niu, W. (2019). Hydrogen sulfide from cysteine desulfurase, not 3-mercaptopyruvate sulfurtransferase, contributes to sustaining cell growth and bioenergetics in *E. coli* under anaerobic conditions. *Frontiers in Microbiology*, *10*, 2357. https://doi.org/10.3389/fmicb.2019.02357

Wang, L.-T., Tai, C.-J., Wu, Y.-C., Chen, Y.-B., Lee, F.-L., & Wang, S.-L. (2010). *Pseudomonas taiwanensis* sp. nov., isolated from soil. *International Journal of Systematic and Evolutionary Microbiology*, *60*(9), 2094–2098. https://doi.org/10.1099/ijs.0.014779-0

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261–5267. https://doi.org/10.1128/AEM.00062-07

Wu, W., Jin, Y., Bai, F., & Jin, S. (2015). *Pseudomonas aeruginosa*. In Y.-W. Tang, M. Sussman, D. Liu, I. Poxton, & J. Schwartzman (Eds.), *Molecular medical microbiology* (pp. 753–767). Elsevier. http://doi.org/10.1016/b978-0-12-397169-2.00041-x

Weller, D. M. (2007). *Pseudomonas* biocontrol agents of soilborne pathogens: Looking back over 30 years. *Phytopathology*, *97*(2), 250–256. https://doi.org/10.1094/phyto-97-2-0250

Workman, D. (2021). *Bananas exports by country 2020*. https://www.worldstopexports.com/bananas-exports-country/

Yuan, J., Raza, W., Shen, Q., & Huang, Q. (2012). Antifungal activity of *Bacillus amyloliquefaciens* NJN-6 volatile compounds against *Fusarium oxysporum* f. sp. *cubense*. *Applied and Environmental Microbiology*, *78*(16), 5942–5944. https://doi.org/10.1128/AEM.01357-12

Zacky, F. A., & Ting, A. S. Y. (2013). Investigating the bioactivity of cells and cell-free extracts of *Streptomyces griseus* towards *Fusarium oxysporum* f. sp. *cubense* race 4. *Biological Control*, *66*(3), 204–208. https://doi.org/10.1016/j.biocontrol.2013.06.001

Zhou, D., Jing, T., Chen, Y., Wang, F., Qi, D., Feng, R., Xie, J., & Li, H. (2019). Deciphering microbial diversity associated with Fusarium wilt-diseased and disease-free banana rhizosphere soil. *BMC Microbiology*, *19*(1), 161. https://doi.org/10.1186/s12866-019-1531-6

Zuo, C., Deng, G., Li, B., Huo, H., Li, C., Hu, C., Kuang, R., Yang, Q., Dong, T., Sheng, O., & Yi, G. (2018). Germplasm screening of *Musa* spp. for resistance to *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4). *European Journal of Plant Pathology*, *151*(3), 723–734. https://doi.org/10.1007/s10658-017-1406-3

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Investigating the Potential of Endophytic Lactic Acid Bacteria Isolated from Papaya Seeds as Plant Growth Promoter and Antifungal Agent

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ABSTRACT

Endophytic lactic acid bacteria (LAB) isolated from papaya seeds, including a consortium of two LAB isolates, *Weissella cibaria* PPKSD19 and *Lactococcus lactis* subsp. *lactis* PPSSD39 could previously inhibit papaya dieback disease causative agent, *Erwinia mallotivora* BT-MARDI *in vitro*, indicating their potential as biofertilizer. However, further characterizations on other plant growth-promoting (PGP) properties of the LABs are pre-requisite to use in agricultural settings as bio-inoculum. Hence, this study aimed to evaluate PGP potentials further and *in vitro* antifungal activity of the LABs against various plant pathogens. The LAB isolates were tested positive in indole-3-acetic acid (IAA), siderophore, and ammonia production and could solubilize phosphate. *Weissella cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 exhibited the strongest *in vitro* antifungal activity against *Fusarium oxysporum* TKA and *Curvularia lunata*. Inoculum concentration of $1x10^8$ cfu/ml of

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W. cibaria PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 mixture showed the highest increment in shoot and root dry weight. In conclusion, *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 consortium displayed promising plant probiotic potential. These results highlighted the possibility of the bacterial consortium to be exploited as bioinoculant to promote plant growth and inhibit phytopathogens causing plant diseases.

Keywords: Antimicrobial activity, bioinoculant, endophytes, lactic acid bacteria, plant growth promotion

INTRODUCTION

Plant growth-promoting bacteria (PGPB) are the bacterial group that stimulates the growth of their plant host, resulting in yield improvement of crops. Their functions are highly diverse, such as nitrogen fixer (Pham et al., 2017), secondary metabolites inducer (Doumbou et al., 2001), nutrient solubilizer (Giassi et al., 2016), and also as biological control agents (Morales-cedeno et al., 2020). PGPB can supply indole-3-acetic acid (IAA) that improves the fitness of the plant root growth and plant nourishment uptake (Nimnoi & Pongslip, 2009; Passari et al., 2015). The PGPB produces several numbers of antibiotics that are good phytopathogen combatants. Certain PGPB could produce an enzyme that is essential in helping the bacteria to allocate itself into the plant tissue. Despite wealth resources on the use of rhizobacteria as a plant growth promoter, the information on lactic acid bacteria (LAB), which are commonly found in the plant endosphere, is rather limited.

LAB is a Gram-positive bacterium that yields lactic acid as one of the end products of the fermentation process. Among the plant, growth-promoting mechanisms of LAB is the production of organic acids, which result in a decrease in pH, accumulation of hydrogen peroxide, and antimicrobial secondary metabolites (Caplice & Fitzgerald, 1999). Due to their antimicrobial properties and Generally Regarded as Safe (GRAS) status, LABs are used in the food processing industry for various purposes. It suggests that the LAB is also potentially utilized in the agriculture industry as it poses no or little safety risk towards edible crops (Lutz et al., 2012), livestock, and human (Stiles & Holzapfel, 1997). LAB, such as *Lactobacillus,* has been proven to promote the growth of various plants (Shrestha et al., 2014). Plants treated with the bacteria showed higher yield, raised plant biomass and chlorophyll content, and enhanced the seedlings' growth rate (Mohite, 2013; Rzheyskaya et al., 2013; Shrestha et al., 2014; S. M. Kang et al., 2015).

Microbial inoculant is gaining more attention among farmers nowadays due to their agricultural sustainability and safety compared to chemical pesticides or fertilizers. To develop a good multi inoculant according to Bashan et al. (2014), 1) the efficiency of their resulting plant growth-promoting effects, 2) compatibility between the strains, 3) the symbiotic effect between the strains, and also 4) the potential

of biofilm formation must be explored. A microbial inoculant that can attack several pathogens simultaneously, besides promoting plant growth, might be more economical and attractive to consumers.

This study explored several LABs isolated from papaya seeds, then assessed the selected two antibacterial LABs, *L. lactis*, and *Weissella* sp., on plant growth promotion and antifungal properties. *Lactococcus lactis* is widely used in food fermentation, especially cheese and yogurt production. It has been isolated from various kinds of plants, including *Eucalyptus* (Procópio et al., 2009), sugar cane (Beneduzi et al., 2013), and pepper-rosmarin (da Silva et al., 2013). It is, however, still under-utilized in agriculture until recently. According to several previous studies, *L. lactis* displayed significant plant growth-promoting activity in greenhouse trials with cabbage and later on crops (Grönemeyer et al., 2012; Somers et al., 2007). In addition to its PGP trait, *L. lactis* showed remarkable antibacterial activity against *Listeria monocytogenes* and *Staphylococcus aureus* (Enan et al., 2013). Likewise, *W. cibaria* was one of the LABs that could show antimicrobial ability (Kamboj et al., 2015). However, information regarding its capabilities in agriculture was still limited. In our previous study, *L. lactis* and *W. cibaria* have been shown to inhibit *E. mallotivora*, the causal agent of papaya dieback disease (PDD), *in vitro* (Taha et al., 2019).

As a continuation of the study by Taha et al. (2019), this study aimed to 1) determine *in vitro* plant growth-promoting property of the endophytic LAB isolates previously isolated from papaya seeds (Taha et al., 2019), 2) assess their inhibitory activity against several fungal plant pathogens *in vitro*, and 3) examining the impact of selected life bacterial inoculum (consortium of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39) of different concentrations on promoting papaya plant growth. Twenty-three antagonistic *L. lactis* and *Weissella* sp. isolates were screened *in vitro* for their plant growth-promoting potential, i.e., phosphate solubilization, IAA formation, ammonia, siderophore, and hydrogen cyanide (HCN) production and pectinase assay. A bacterial consortium consists of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 that previously showed the best disease suppression against PDD *in planta* (Taha et al., 2019) were selected and further subjected to dual culture assay and poisoned agar test *in vitro* against common plant pathogens; i.e., *C. lunata*, *F. oxysporum* TKA, *Fusarium proliferatum* B68C, *Fusarium verticillioides* B106C, and *Fusarium verticillioides* J44C. Biofilm formation capability was also evaluated. The isolates were then tested for plant growth promotion under greenhouse conditions using three-month-old papaya seedlings as a model plant. Different concentrations of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 were applied to the papaya plantlets, and shoot length and plant biomass were measured. This study highlighted the potential application of the consortium of LAB as a plant growth promoter *in planta*.

MATERIALS AND METHODS

Bacterial Sources and Culture Maintenance

Twenty-three LAB isolates that were previously shown to inhibit *E. mallotivora* (Taha et al., 2019) were utilized in this study. The strains were isolated from papaya seed, and the species identity was determined using 16S rRNA gene sequencing by Taha et al. (2019) (Table S1). The isolates were maintained in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany) supplemented with 20% glycerol (Merck, Germany) and stored at -20 ºC freezer (Bosch, Germany).

Plant Growth Promoting Assay *In vitro*

Phosphate Solubilisation. The antagonistic LAB isolates were grown on Pikovskaya's (PKV) agar media supplemented with 0.5% tricalcium phosphate ($Ca_3(PO_4)_2$) (Merck, Germany). Bacterial suspensions grown for 16–24 h in MRS broth were pipetted onto the PKV agar media and incubated at 30 °C for two days. Clear halo zone formation of more than 0.01 cm surrounding the colonies indicates positive phosphorus (P)-solubilization ability (Passari et al., 2015). Positive control (*Klebsiella aerogenes*) was used as a comparison.

Indole-3-acetic Acid (IAA) Evaluation. The ability of LAB to manufacture IAA was calculated according to Passari et al. (2015) and Ehmann (1977). The isolates were grown in MRS broth (Merck, Germany) at 30 °C for three days. After incubation, 1 ml of each culture was pipetted into Eppendorf tubes (Eppendorf, United Kingdom). The cultures were then centrifuged at a maximum speed of 4,293 x *g* for 5 min. A volume of 1 ml of the resulting supernatant was transferred into test tubes containing 2 ml of Salkowski's reagent before being incubated for another 25 min at 30 °C in dark conditions. The positive result of IAA production is indicated by pink color development. Positive control (*Escherichia coli*) was used as a comparison. The optical densities (OD) of isolates were measured at 530 nm wavelength using a spectrophotometer (SPECTRO 23, USA). The result was correlated with the standard curve of IAA to obtain the amount of IAA produced.

Ammonia Production. The LAB isolates were tested for ammonia production according to Cappuccino and Sherman (1996). A volume of 20 µl of LAB culture was inoculated into 10 ml of peptone water (Himedia, India). The culture was then incubated at 30 °C in an incubator shaker (Yihder Technology Co. Ltd., Taiwan) at 150 rpm for 24 h. Post-incubation, 0.5 ml of Nessler's reagent (Sigma, Germany) was pipetted into the culture. Ammonia production was observed through color changes from brown to yellow. Then, OD was taken at wavelength 570 nm, and the ammonia concentration was measured by constructing the standard curve of ammonium sulfate of known concentrations. *Klebsiella pneumonia* was used as the positive control.

Production of Siderophore. LAB siderophore development was tested using Chrome Azurol S (CAS) agar (Schwyn & Neilands, 1987). Wells of 10 mm were made on the CAS agar using a sterile cork borer. The LAB antagonists were cultured in MRS broth at 30 °C overnight. Afterward, 0.5 µl of an overnight culture of LAB isolates was pipetted into the CAS agar well. The diameter of the orange-halo zone and the size of the agar well were measured using a vernier caliper (Mitutoyo, Japan). The strength of siderophore production was determined according to the following: + represents < 5 mm wide halo zone (weak), ++ represents 5 to 10 mm wide halo zone (moderate), +++ represents > 10 mm wide halo zone (strong) (Gull & Hafeez, 2012). *Escherichia coli* was used as the positive control.

Hydrogen Cyanide (HCN) Production. The formation of HCN was assessed according to a protocol from Lorck (1948). Bennett agar was amended with 4.4 g l^{-1} glycine and inoculated with LAB isolates. A Whatman filter paper was dipped into 0.5% picric acid in 2% sodium carbonate for 1 min, and the filter paper was placed underneath the Petri dish lids. After overnight incubation at 30 °C, color changes to red on the filter paper indicate positive HCN production. *Klebsiella aerogenes* was used as the positive control for comparison.

Pectinase Assay. Screening for pectinase-producing LAB was performed using a pectin agar medium (Nawawi et al., 2017; Singh et al., 2015). Basal agar medium was used to screen pectinolytic-producing bacteria supplemented with 1% pectin from the citrus peel to prepare the pectin agar. Sodium carbonate was used to modify the pH of the media, and the media were sterilized by autoclaving. The wells on the pectin agar were made using a sterile cork borer. Then, 100 µl of LAB suspension grown in MRS broth were loaded into the wells of pectin agar medium. Plates were incubated at 30 °C for 24 h. Plates were overflowed with Gram's iodine (Kasana et al., 2008) to observe the halo zone, indicating positive pectinase production. Positive control (*Bacillus subtilis*) was used as a comparison in determining the clear zone (Ho, 2015; Singh et al., 2015).

Antifungal Activity

Dual Culture Assay. Dual culture assay was carried out following the methods by Tiru et al. (2013) with some modifications. Plate culture of fungal pathogens, *F. oxysporum* TKA, *F. proliferatum* B68C, *F. verticillioides* B106C, and *F. verticillioides* J44C were obtained from the Faculty of Science, Universiti Putra Malaysia. The pure culture of *C. lunata* was collected from the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. An agar disk of 5 mm diameter was cut out from a young culture of fungal pathogen and placed in the middle of potato dextrose agar (PDA) (BD Difco™, USA) agar plates. A loopful of fresh LAB isolate from the plate culture was streaked 2 cm away from the fungal disc on both sides. The paired culture was incubated

at 30 °C for 5–10 days. All pairings were carried out in triplicates. The monoculture of each fungal pathogen served as control. The diameter of fungal growth in the treatment plate was measured immediately when the mycelia of the pathogen in the control plate reached the edge of the plate. The percentage inhibition of radial growth (PIRG) was calculated by using the formula:

$$\text{PIRG (\%)} = \frac{(\text{Dc}-\text{Ds})}{\text{Dc}} \times 100\% \quad [1]$$

where Dc represents the diameter (measured in cm) of fungal growth on the control plate and Ds is the diameter (measured in cm) of fungal growth on the plate containing bacteria.

Poisoned Agar Test. A poisoned agar test was carried out following the method by Rahman et al. (2009) with some modifications. This method involved several fundamental steps: preparation of culture filtrates, preparation of poisoned agar plates, and poisoned agar test.

Preparation of Culture Filtrates. For each LAB isolate, 300 ml of potato dextrose broth (PDB) (BD Difco™, USA) was prepared in conical flasks and autoclaved for 15 min at 121 °C/1.05 kg/cm^2 pressure. A loopful of fresh LAB isolate from the plate culture was inoculated into the broth. The flasks were incubated in an incubator shaker (Yihder Technology Co. Ltd., Taiwan) for 16 to 24 h at 150 rpm at 30 °C. After the incubation period, the overnight cultures of the LAB isolates were filtered using a 0.22 µm membrane filter.

Preparation of Poisoned Agar Plates. Initially, 90%, 80%, 70%, 60%, 50%, 40%, and 20% of PDA (BD Difco™, USA) were prepared in conical flasks added with the culture filtrates. The agar was autoclaved for 15 min at 121 °C. Seven concentrations, 10%, 20%, 30%, 40%, 50%, 60%, and 80% (v/v) of the sterilized culture filtrates of LAB isolates were prepared and added into 90%, 80%, 70%, 60%, 50%, 40%, and 20% of prepared PDA (BD Difco™, USA) respectively. The molten PDA (BD Difco™, USA) containing different culture filtrates concentrations was poured into Petri plates and allowed to solidify.

Poisoned Agar Test. The plates were labelled as 10%, 20%, 30%, 40%, 50%, 60%, and 80% concentration of LAB culture filtrates. An agar disc of 5 mm diameter was cut out from the young culture of fungal pathogen and placed at the middle of the prepared poisoned agar plates. Inhibitory activity test for each concentration of the LAB culture filtrate against each fungal pathogen was conducted in triplicates. The Petri dishes were incubated for 5-10 days at 30 °C. The monoculture of each fungal pathogen served as control. The diameter of fungal growth in the treatment plate was measured immediately when the mycelia of the pathogen in the control plate reached the edge of the plate. The PIRG was calculated by using the formula:

$$\text{PIRG (\%)} = \frac{(\text{Dc}-\text{Ds})}{\text{Dc}} \times 100\% \quad [2]$$

where Dc represents the diameter of fungal growth on the control plate, and Ds

represents the diameter of fungal growth on the plate containing bacterial metabolites.

Plant Growth-Promoting Assay *In planta*

Weissella cibaria PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 were grown in MRS broth, shaking at 150 rpm at 30 °C for 16-24 h. The cells were centrifuged at 4,629 x *g* for 15 min. The cell pellets were adjusted to OD_{600}:1.0 with distilled water before final concentration adjustment to 10^2, 10^4, 10^6, and 10^8 cfu ml^{-1}. Both LAB suspensions were then mixed at a 1:1 (v/v) ratio. The diluted cultures were maintained at 4 °C. Finally, the LAB mixture was applied to the papaya plantlets under greenhouse conditions.

The *in planta* experiment was performed in a plant nursery located at Universiti Putra Malaysia (UPM) Serdang, Selangor (GPS coordinate: 3.0083354, 101.7047198). The average daily temperature was 28 °C with a relative humidity of 80% and 12 h of days, and 12 h of night every day (Time and Date, n.d.). All plantlets with height ranging from 30–70 cm were planted in small polyethylene bags filled with peat moss soil. All plants were labeled appropriately and organized in a randomized complete block design. Six treatments included TK: Treatment with chitosan; TC: Un-inoculated as control; T2: Treatment with 1:1 mixture strain at $1x10^2$ cfu ml^{-1} final concentration of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39; T4: Treatment of 1:1 mixture strain at $1x10^4$ cfu ml^{-1} final concentration of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39; T6: Treatment of 1:1 mixture strain at $1x10^6$ cfu ml^{-1} final concentration of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39; T8: Treatment of 1:1 mixture strain at $1x10^8$ cfu ml^{-1} final concentration of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39. The inoculation treatment was performed using the soil drench method of Abdel-Kader et al. (2012) and foliar spraying by Jaber and Enkerli (2017). Then, 5 ml bacterial mixture was applied using the soil drench method and another 5 ml using foliar spraying. Each treatment was carried out in 14 replicates. Plant height was recorded at 0 d, 30 d, and 45 d, while the fresh and dry weight of both shoot and root were recorded at the end of the experiment, which was at 45 d.

Quantitative Biofilm Production

This method was performed according to Christensen et al. (1995). *Weissella cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 isolates were grown in 10 ml of trypticase soy broth (TSB) (Merck, Germany) supplemented with 1% glucose (Merck, Germany) and incubated at 30 °C overnight. The overnight culture was diluted at a ratio of 1:100 (v/v), and 200 µl of the culture was dispensed into the wells of 96-well plate. The cultures were grown at 30 °C for 24 h. Post-incubation period, the culture of all the isolates in the 96-well were discarded. Then, the wells were washed up with 0.2 ml of phosphate-buffered saline (PBS) (pH 7.2) four times. Then, 2% sodium acetate (Merck, Germany) was added to fix the biofilm formed adhered to the wells

and stained by 0.1% crystal violet (Merck, Germany). Excess stain was discarded, and the wells were washed using deionized water. The 96-well plates were dried at room temperature, and the optical density of the stained bacteria biofilm was measured using a microplate auto reader (Tecan infinite F-50, Switzerland) at 570 nm. The experiment was repeated three times.

Statistical Analysis

All univariate statistical analyses were conducted using SPSS v. 22.0 (IBM SPSS Inc., USA). The normality of the data was analyzed using the Shapiro-Wilk test. In addition, data from the antifungal activity, plant growth-promoting assay *in planta*, and biofilm formation were subjected to analysis of variance (ANOVA), and pair-wise comparisons between the means of the treatments were analyzed by Tukey's test ($p < 0.05$).

RESULTS

Plant Growth Promoting Assay *In vitro*

Phosphate Solubilization and IAA Production. Table 1 shows the plant growth-promoting capabilities of the 23 isolated antagonists LAB. Among the 23 isolates, all LAB colonies displayed clear halo zones on PKV media, indicating their capabilities to solubilize phosphate. The diameter of the clear zones ranges from 2.44 ± 0.16 cm to 3.17 ± 0.08 cm. The lowest index was shown by *L. lactis* subsp. *lactis* PPKST 11 and *L. lactis* subsp. *lactis* PPKST4S and the highest was exhibited by *L. lactis* subsp. *lactis* PPSSD38.

All the isolates were also positive for IAA production, ranging between 2.029 ± 0.352 μg ml^{-1} to 15.223 ± 0.329 μg ml^{-1}. *Weissella cibaria* PPKSD9 and *L. lactis* subsp. *lactis* PPKST3 produced the highest IAA production with 15.223 μg ml^{-1} and 15.086 μg ml^{-1}, respectively.

All lactic acid bacteria isolates produced ammonia at levels ranging from 4.09 ± 0.27 mg ml^{-1} to 14.31 ± 0.72 mg ml^{-1}. *Lactococcus lactis* subsp. *lactis* PPKST37 and *L. lactis* subsp. *lactis* PPKST2 produced the highest ammonia value, while *W. confusa* PPKSD39 had the lowest value. All isolates were tested negative for HCN production as there were no color changes on filter papers.

Siderophore Production. All bacterial isolates also produced siderophore as indicated by clear orange halo zone formation around the colonies on the CAS agar media. The siderophore production index ranged from 0.296 ± 0.084 to 0.704 ± 0.084. The weakest producer was *W. cibaria* PPKSD29 (11.7 mm in diameter) and *L. lactis* subsp. *lactis* PPSST25, while the strongest producer was *L. lactis* subsp. *lactis* PPKST11 (15.3 mm in diameter).

Pectinase Enzyme Production Activity. Of the 23 isolates, 21 showed negative results for pectinase enzyme activity, whereas only two showed a halo zone after iodine application. The two isolates were *W. confusa* PPKSD39 and *L. lactis* subsp. *lactis* PPKSD8 with clear zone size 0.20 ± 0.02 cm and 0.15 ± 0.01 cm, respectively.

Table 1

Lactic acid bacteria isolates and their plant growth-promoting traits

Isolates	IAA production (μg ml^{-1})	Evaluation for IAA[*]	Siderophore production[£]	Phosphate solubilization[€]	HCN production[¶]	Ammonia production (mg ml^{-1})	Ammonia production[¥]	Pectinase assay[©]
Negative	0.385 ± 0.132[a]	NA	NA	NA	NA	0[a]	NA	NA
Positive	8.594 ± 0.255[b]	++	+++	+	+	13.45 ± 0.42[b]	+	+
PPKSD19[∂]	14.88 ± 0.387[b]	+++	+++	+	-	9.94 ± 0.20[b]	+	-
PPKSD29[∂]	8.986 ± 0.573[b]	++	+++	+	-	8.23 ± 0.13[b]	+	-
PPKSD34[∂]	6.812 ± 0.641[b]	++	+++	+	-	7.97 ± 0.34[b]	+	-
PPKSD37[∂]	11.667 ± 0.908[b]	+++	+++	+	-	7.63 ± 0.08[b]	+	-
PPKSD39[Ω]	14.742 ± 0.239[b]	+++	+++	+	-	4.09 ± 0.27[b]	+	+
PPKSD8[Δ]	14.261 ± 0.242[b]	+++	+++	+	-	9.06 ± 0.22[b]	+	+
PPKSD9[∂]	15.223 ± 0.329[b]	+++	+++	+	-	10.00 ± 0.44[b]	+	-
PPKST1[Δ]	7.595 ± 0.391[b]	++	+++	+	-	10.97 ± 1.15[b]	+	-
PPKST11[Δ]	2.174 ± 0.253[a]	+	+++	+	-	12.33 ± 0.14[b]	+	-
PPKST14[Δ]	8.406 ± 0.919[b]	++	+++	+	-	12.85 ± 0.25[b]	+	-
PPKST2[Δ]	10.378 ± 0.343[b]	+++	+++	+	-	14.05 ± 0.09[b]	+	-
PPKST3[Δ]	15.086 ± 0.801[b]	+++	+++	+	-	9.29 ± 0.32[b]	+	-
PPKST37[Δ]	13.574 ± 0.132[b]	+++	+++	+	-	14.31 ± 0.72[b]	+	-
PPKST4[Δ]	11.856 ± 0.151[b]	+++	+++	+	-	12.58 ± 0.03[b]	+	-
PPKST4B[Δ]	13.952 ± 0.132[b]	+++	+++	+	-	8.61 ± 0.28[b]	+	-

Table 1 (*Continue*)

Isolates	IAA production (µg ml^{-1})	Evaluation for IAA[*]	Siderophore production[£]	Phosphate solubilization[€]	HCN production[¶]	Ammonia production (mg ml^{-1})	Ammonia production[¥]	Pectinase assay[©]
PPKST4S[Δ]	13.093 ± 0.203[b]	+++	+++	+	-	13.20 ± 0.22[b]	+	-
PPKST5[Δ]	14.124 ± 0.373[b]	+++	+++	+	-	8.84 ± 0.28[b]	+	-
PPSSD1[∂]	4.130 ± 0.488[a]	+	+++	+	-	7.70 ±0.16[b]	+	-
PPSSD38[Δ]	6.377 ± 0.236[b]	++	+++	+	-	8.68 ± 0.47[b]	+	-
PPSSD39[Δ]	10.000 ± 0.388[b]	+++	+++	+	-	7.54 ± 0.26[b]	+	-
PPSSD7[Δ]	1.522 ± 0.434[a]	+	+++	+	-	11.12 ± 0.53[b]	+	-
PPSST25[Δ]	13.471 ± 0.376[b]	+++	+++	+	-	6.90 ± 0.41[b]	+	-
PPSST38[Δ]	2.029 ± 0.352[a]	+	+++	+	-	11.74 ± 0.99[b]	+	-

Note.

[*]For IAA production: (-): No activity (below 1 µg ml^{-1}); (+): Low activity (1.00 - 5.00 µg ml^{-1}); (++): Moderate activity (5.01-10.00 µg ml^{-1}); (+++): High activity (10.01 µg ml^{-1}and above)

[£]For siderophore production: (-): No clear zone; (+): Clear zone observed

[€]For Phosphate solubilizing index: (-): No clear zone; (+) Clear zone observed

[¶]For HCN Production: (-) No color changes; (+) Yellow color changes to orange

[¥]Ammonia production: (-) No color changes; (+) Brown color changes to yellow

[©]For siderophore production: (-): No clear zone; (+): Clear zone detected. + represents < 5 mm halo zone (weak); ++ represents 5 to 10 mm halo zone (moderate); +++ represents > 10 mm halo zone (strong). All isolates except negative control showed a diameter of orange-halo zone ranging from 11.7 to 15.3 mm

NA, no activity

[Δ]*Lactococcus lactis* subsp. *lactis*, [∂]*Weissella cibaria*, [Ω]*Weissella confusa* as determined by API® 50 CH or 16S rRNA gene sequencing (Taha et al., 2019)

For the IAA production assay, *Staphylococcus aureus* and *Escherichia coli* were used as negative and positive controls, respectively

For siderophore production assay, included *Bacillus subtilis* and *Escherichia coli* were used as negative control and positive control, respectively

For phosphate solubilization assay, *Klebsiella aerogenes* and *Proteus vulgaris* were used as positive and negative controls, respectively

Means with different letters are significantly different in comparison with negative control ($p < 0.05$) using ANOVA based on Tukey's Test

Antifungal Activity

Five plant fungal pathogens isolated from different plant hosts, viz. *C. lunata* (rice), *F. oxysporum* TKA (banana), *F. proliferatum* B68C (maize), *F. verticillioides* B106C (maize), and *F. verticillioides* J44C (maize) (Zainudin et al., 2017) were used to challenge the LAB isolates. All these fungal pathogens were the causal agents of many plant diseases worldwide. The mean radial growth of pathogens formed in the dual culture assay showed weak to moderate inhibition ability against fungal pathogens, ranging from 3.03% to 42.16% (Figure 1). The PIRG values of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39, relative to control, were recorded at 30.34% and 31.20%, against *C. lunata*, 42.16% and 38.73% against *F. oxysporum* TKA and 32.00% and 33.33% respectively against *F. vertcillioides* B106C, 3.03%, and 4.55% against *F. proliferatum* B68C, and 15.28% and 23.61% against *F. verticillioides* J44C (Figure 1). *Weissella cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 exhibited the highest antagonistic activity against *F. oxysporum* TKA at 42.16% and 38.73%. Both isolates, however, showed the least inhibitory activity against *F. proliferatum* B68C.

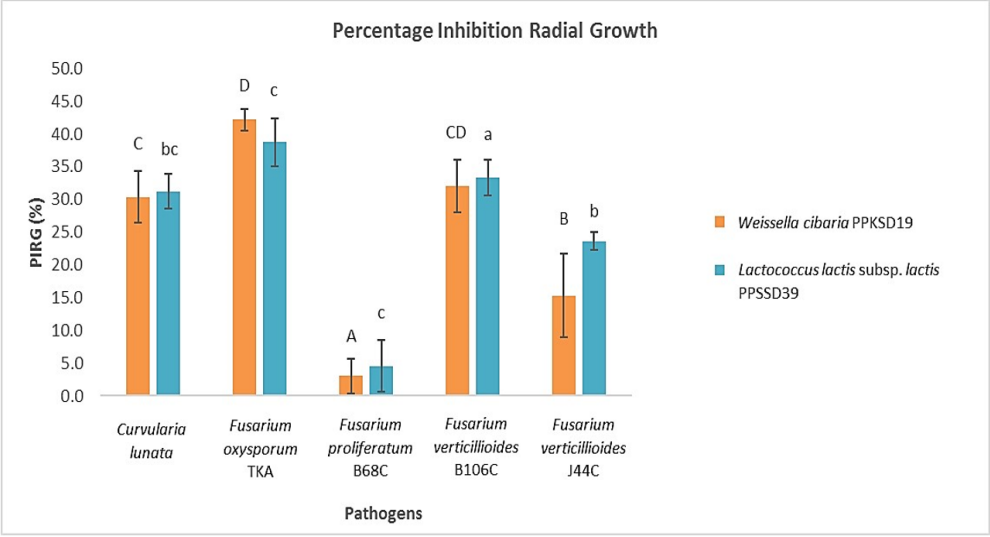
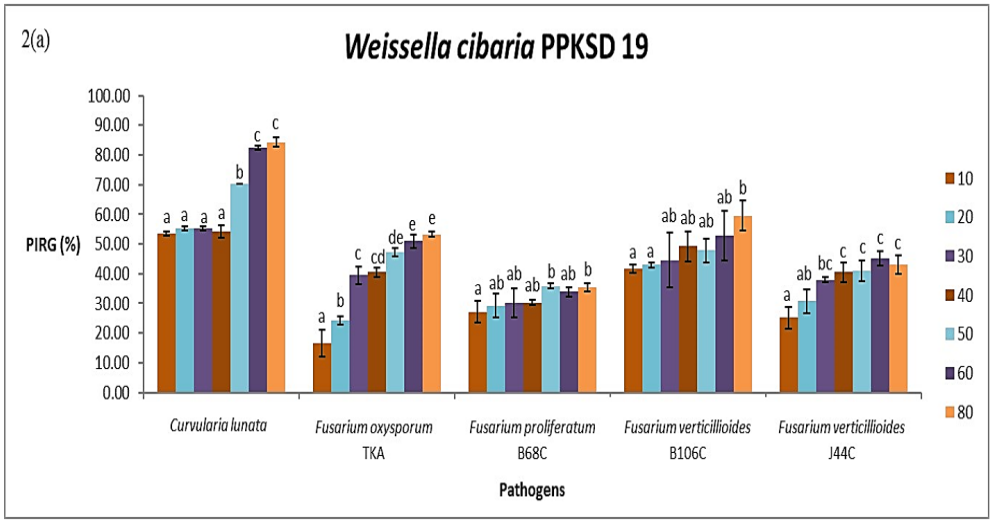


Figure 1. Percentage of inhibition of radial growth (PIRG, %) of isolates *Weissella cibaria* PPKSD19 and *Lactococcus lactis* subsp. *lactis* PPSSD39 in dual culture assay. Values are presented as means ± standard deviation (SD) of three replications. Means with different capital and small letters are significantly different ($p < 0.05$) using ANOVA based on Tukey's Test for *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39, respectively against five fungal pathogens

The antifungal activity of the LAB isolates against the representative pathogens was further confirmed with the poisoned agar test. The poisoned agar test was used to evaluate the action of secondary metabolites to control pathogen growth. The inhibition spectrum was in a dose-dependent manner as the percentage of culture filtrate containing secondary metabolites increased. The results showed weak to high inhibition against the fungal pathogens at different culture filtrate concentrations ranging from 16.67% to 84.36% (Figure 2a and Table S2). The PIRG value of *W. cibaria* PPKSD19 against *C. lunata* was comparatively higher than the other pathogens, with the lowest PIRG achieved was 53.50% at 10% of culture filtrate, and the highest PIRG was 84.36% at 80% culture filtrate (Table S2 in supplementary data). From Figure 2a, *W. cibaria* PPKSD19 had strong inhibition against *C. lunata* despite a very low percentage of culture filtrate as compared to other pathogens, which only inhibited 16.67% to 41.67% of mycelial growth at 10% of culture filtrate and 35.38% to 59.58% at 80% culture filtrate. Meanwhile, the *W. cibaria* PPKSD19 reduced the growth of *C. lunata* up to 84.36% at 80% of culture filtrate. *L. lactis* subsp. *lactis* PPSSD39 showed the highest inhibition against *F. oxysporum* TKA at 10% to 80% of culture filtrate (Figure 2b). The range of inhibition *L. lactis* subsp. *lactis* PPSSD39 against *F. oxysporum* TKA was from 43.81% to 59.05% (Table S2b and Figure S1 in supplementary data). The other pathogens, however, showed only at 22.56% to 47.33% inhibition at 10% of culture filtrate and 40.00% to 55.56% inhibition at 80% of *L. lactis* subsp. *lactis* PPSSD39 culture filtrate. It showed that *L. lactis* subsp. *lactis* PPSSD39 has high inhibitory activity against *F. oxysporum* TKA.



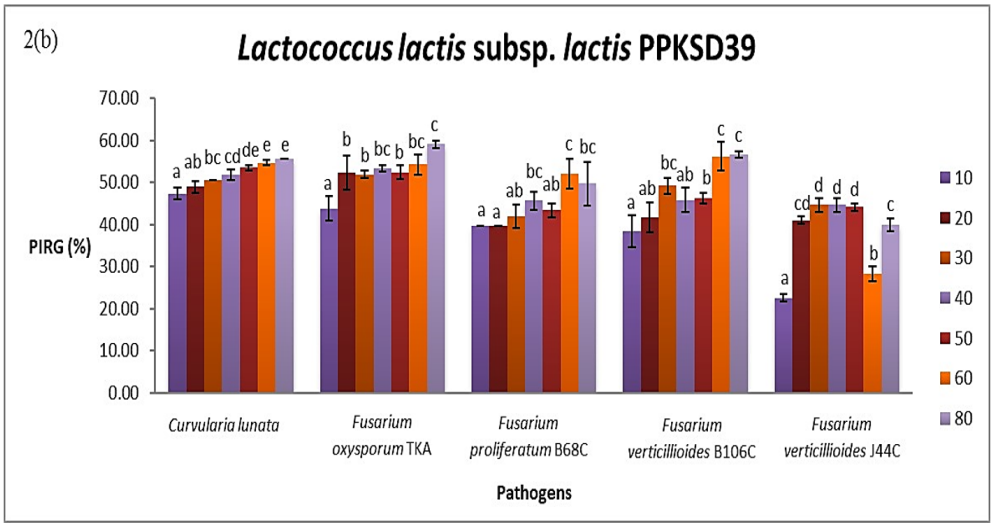


Figure 2. Percentage of inhibition of radial growth (PIRG, %) of isolates (a) *Weissella cibaria* PPKSD19 and (b) *Lactococcus lactis* subsp. *lactis* PPSSD39 in poisoned agar test. The error bars indicate standard deviations. Means with different letters are significantly different ($p < 0.05$) using ANOVA based on Tukey's test

Note. The 10, 20, 30, 40, 50, 60, and 80 indicate the percentage (%) of culture filtrate concentration (v/v)

Effect of Different Concentrations of *Weissella cibaria* PPKSD19 and *Lactococcus lactis* subsp. *lactis* PPSSD39 Mixture on the Growth Parameters of Papaya Plants

The use of chitosan as a control treatment in this study is widely used as a biofertilizer, where it could induce plant immune response towards the pathogen. Furthermore, chitosan supplementation may increase the availability of phosphate in the soil (Berger et al., 2013).

Assessment of different concentrations of the mixture of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 to promote the growth of papaya plant revealed that the growth of plantlets treated with the highest concentration of the mixture, 10^8 cfu ml^{-1}, showed a significant increment in fresh and dry weight of shoot ($p < 0.05$) as compared to the negative control (Figure 3a and Figure 3b). The dry weight of the root also increased significantly compared to negative control following treatment with 10^4, 10^6, and 10^8 cfu ml^{-1} of the LAB inoculant (Figure 3d). The fresh root also somewhat increased; however, the increment was not statistically significant ($p > 0.05$) (Figure 3c).

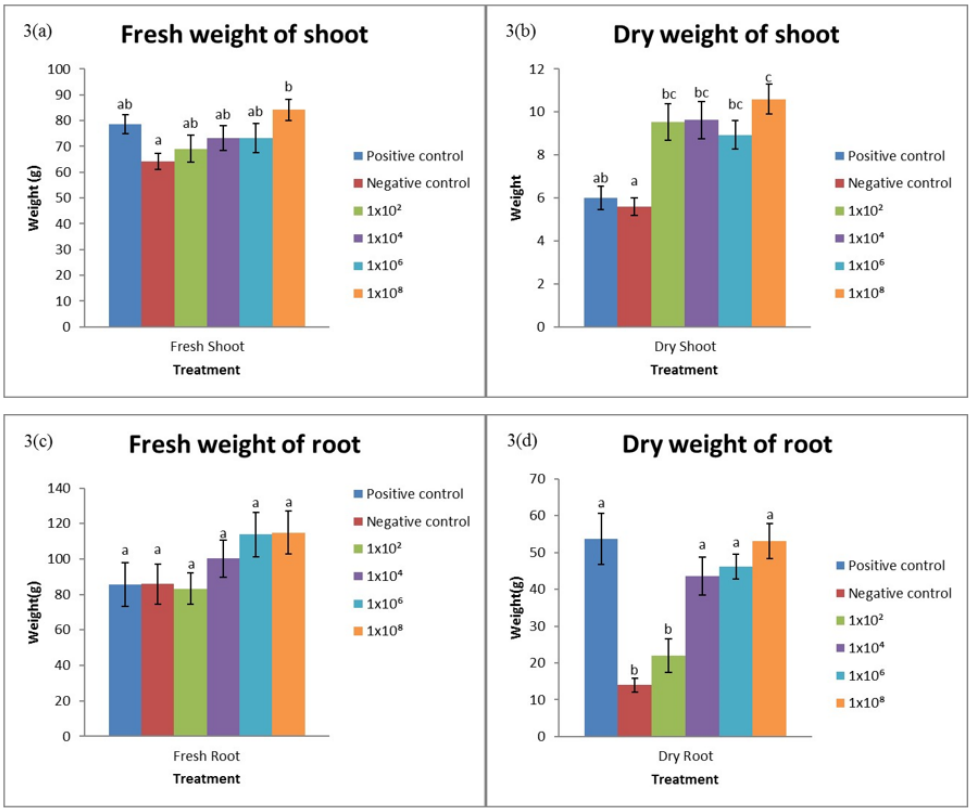


Figure 3. The effect of different concentrations of a mixture of *Weissella cibaria* PPKSD 19 and *Lactococcus lactis* subsp. *lactis* PPSSD 39 as main factors on the growth parameters of papaya plants at Day 45). (a) Fresh weight of shoot, (b) Dry weight of shoot, (c) Fresh weight of root, and (d) Dry weight of root against the different concentrations of a mixture of *W. cibaria* PPKSD 19 and *L. lactis* subsp. *lactis* PPSSD 39 treatment

Note. Each bar represents the means ± SD of 14 plant replicates. Means with different letters are significantly different ($p < 0.05$) using Kruskal Wallis (a, b, d) and ANOVA (c) based on Tukey's Test.

Biofilm Production

Quantitative biofilm production of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 isolates were determined using the microplate auto reader. There were light blue color changes in the microplate for both isolates. The low average OD_{570} nm measurement of both *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 compared to the positive control, indicating weak biofilm producers (Table 2).

Table 2

Evaluation of biofilm production by the LAB isolates

Isolates	Biofilm production (OD)*	Evaluation
Weissella cibaria PPKSD19	0.0783 ± 0.02^{a}	Weak
Lactococcus lactis subsp. *lactis* PPSSD39	0.0687 ± 0.03^{a}	Weak
¶*Listeria monocytogene*	0.2858 ± 0.05^{b}	Strong
¥Bacillus amyloliquefaciens	0.0602 ± 0.02^{a}	Weak

Note. Interpretation of biofilm production. Measurement was taken at OD_{570} nm for biofilm production
Optical density cut-off value (OD c) = Average OD of negative control + 3× standard deviation (SD) of negative control
≤ OD c / OD c < ~ ≤ 2x OD c = None/weak
2x OD c < ~ ≤ 4x OD c = Moderate
> 4x OD c = Strong
*Values are presented as means ± SD of three replications. Means with different superscript letters are significantly different ($p < 0.05$) using ANOVA based on Tukey's Test
¶Positive control strain (Strong)
¥Negative control (None/Weak)

DISCUSSION

The purpose of this study was to evaluate the plant growth-promoting ability *in vitro* and *in planta* of the 23 LAB isolates previously isolated from papaya seeds. In addition, IAA, HCN, siderophore and ammonia production, phosphate solubilization, and pectinase assay were carried out for the 23 bacterial isolates.

All of the endophytic LAB isolates could produce IAA, in which *W. cibaria* PPKSD9 and *L. lactis* subsp. *lactis* PPKSD3 showed the highest IAA production. Strafella et al. (2021) reported several *L. lactis* strains could produce a higher amount of IAA compared to the other LAB tested. Nimnoi and Pongslip (2009) reported that the IAA hormone affected plant cell elongation, and IAA-producing bacteria improved the root and shoot growth of *Raphanus sativus* and *Brassica oleracea*. Similarly, the IAA-producing *W. cibaria* and *L. lactis* used in this study have also enhanced the growth of papaya seedlings.

The highest phosphate solubilizing index was produced by *L. lactis* subsp. *lactis* PPSSD38. In a similar experiment conducted by Strafella et al. (2021), all tested *L. lactis* isolates could solubilize phosphate. Manufacturing of low molecular weight organic (Collavino et al., 2010), phytase, and alkaline phosphatase (de Lacerda et al., 2016) were proposed to mediate this property. Viruel et al. (2014) reported positive enhancement of maize plants after being treated with phosphate solubilizing bacteria. Phosphorous is one of the nutrients needed for plant growth (Khan et al., 2014). The availability of phosphorous, however, can be the limiting factor in a certain condition, thus affecting the growth of the plant significantly (Wang et al., 2010).

Hence, the phosphate-solubilizing LAB may help provide the plant with nutrients needed and increase growth.

All the tested LAB isolates were positive for ammonia production, with the highest production being *Lactococcus* sp. PPKST37 and *Lactococcus* sp. PPKST2. Ammonia production is valued as root and shoot elongation assistance, resulting in higher plant biomass. According to Marques et al. (2010), opportunistic phytopathogen can also be inhibited by the overproduction of ammonia. Earlier studies mentioned that HCN is commonly involved in disease suppression in plants (Wei et al., 1991). However, a study revealed that HCN was not involved in biocontrol against pathogens but rather regulated phosphate availability (Rijavec & Lapanje, 2016). According to Rijavec and Lapanje (2016), cyanide in the HCN can improve the mineral transportability and discharge of phosphate, resulting in phosphate availability. The LAB isolates used in this study, however, did not produce HCN.

Another plant growth-promoting feature tested was siderophore production. Through the iron-binding capabilities, bacteria that produce siderophores will have the upper hand in competing with the phytopathogens, making iron unavailable for them (Bashan et al., 1980; Siddiqui, 2005). All the endophytic LAB isolates were positive for siderophore production, with the largest halo zone created by *L. lactis* subsp. *lactis* PPKST11. Marag and Suman (2018) also found that one of their endophytic *Zea mays* L. isolates could produce siderophore and later identified as *L. lactis*. In the study of unique genomic cluster matching of two *L. lactis* strain, there were clusters of unique genes of *L. lactis,* similar to the siderophore production pathway of *B. subtilis* (Siezen et al., 2008), supporting the capabilities of the *L. lactis* to produce siderophore.

Pectinase enzyme production assay recorded only two positive results, *W. confusa* PPKSD39 and *L. lactis* subsp. *lactis* 1 PPKSD8. Assamoi et al. (2016) reported that *Weissella* isolates of Wc 115 displayed pectinase activity needed for cassava dough softening. The presence of pectinase helps the bacteria to break into the plant endosphere. As hydrolase, pectinase will break down the pectin of the plant structure to help the endophytic bacteria to get inside of the plant ecosystem (Hayat et al., 2010). Since *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 lack of pectinase production, slight wound, or pre-emptive seed colonization was made to allow the *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 to infiltrate into the plant endosphere. However, this does not rule out the possibility that *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 may utilize other ways to assist them in entering the plant endosphere.

All pathogens used in the antifungal assay were the severe causal agents of plant diseases of many important plantations worldwide. *Curvularia* sp. and *Fusarium* sp. previously showed capabilities in infecting papaya plants, causing various diseases. *Fusarium semifectum* and *F. oxysporum* in the papaya plant caused fruit

rot (Zakaria et al., 2012). The wilt-inducing strains of *F. oxysporum* were responsible for severe damage on many economically important plant species (Agbaglo et al., 2020; Fravel & Larkin, 2002), commonly banana plants. Meanwhile, *F. proliferatum* is always associated with *F. verticillioides* as they cause similar plant diseases. Bullerman and Tsai (1994) reported that soil-borne *F. proliferatum*, a closely related fungus to *Fusarium moniliforme* (syn. *F. verticillioides*), is found in all corn-growing regions. On the other hand, *C. lunata* was firstly discovered from an infected papaya plant causing post-harvest disease in the fruit (Helal et al., 2018). The pathogens can also infect many other plant species, such as rice, maize, and banana (Liu et al., 2011; Tann & Soytong, 2017). This fungus secreted mycotoxin, which is responsible for plant disease. The ability of our isolates to inhibit these globally recognized pathogens highlights their potential as universal biocontrol agents for various plant diseases.

By supporting our findings, *W. cibaria* and *L. lactis* from various sources were also shown to have inhibitory activity against various fungal pathogens in food, plants, and human. *Weissella cibaria* isolated from a semolina ecosystem completely inhibited all the tested fungal species that generally contaminated the bakery product, viz. *Aspergillus niger*, *Penicillium roqueforti*, and *Endomyces fibuliger* (Valerio et al., 2009) also agree with Ndagano et al. (2011), who found that *W. cibaria* FMF4B16 isolated from mill flour and fermented cassava showed antifungal activity against food molds. A study by Trias et al. (2008) reported that *W. cibaria* strain TM128 reduced the fungal rot diameter of the Golden Delicious apple by 50%. Kim (2005) reported that *L. lactis* subsp. *lactis* isolated from Kimchi had strong antagonistic activity against *Aspergillus fumigatus*, an opportunistic pathogen causing several human diseases. It has also been reported that *W. cibaria* can inhibit *F. oxysporum* (Valencia-Hernández et al., 2016), responsible for many plant diseases. Mauch et al. (2010) also reported that *W. cibaria* has fungistatic activity against *Fusarium* species. Interestingly, there were no past reports regarding antifungal activity of *W. cibaria* and *L. lactis* against *C. lunata*, *F. verticillioides*, and *F. proliferatum*. Overall, the antagonistic activities of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 against these pathogens suggests their biocontrol potential in suppressing a wider range of plant diseases. Further experiments need to be conducted to confirm this.

The potential of the *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 consortium that previously showed outstanding ability in suppressing papaya dieback disease *in planta* (Taha et al., 2019), while displaying positive *in vitro* PGP traits in this study were further tested for their ability in promoting papaya plant growth in the absence of the pathogen. Different doses of the consortium were applied to see at which concentration it can sufficiently enhance the papaya plant growth. Interestingly, the effects of the

LAB isolates on all growth parameters were also concentration-dependent (Figure 3), suggesting a positive growth enhancement effect on the papaya plantlets. It means that the higher the concentration of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39, the higher the increment of the papaya plant shoot growth, by which a significant improvement was seen between untreated and 10^8 cfu ml^{-1} of the consortium ($p < 0.05$). The effect was comparable to that of the positive control, chitosan, suggesting they are equally competent to promote plant growth. Evaluation for the plant height assessment revealed no significant difference between the treatments.

Meanwhile, the significantly higher dry weight for aerial and underground parts of papaya plants showed the effectiveness of the bacterial mixture treatment compared to the untreated plants (Figure 3b and Figure 3d). Many researchers recorded a similar increasing pattern in plant weight inoculated with PGP bacteria (Gholami et al., 2009; Passari et al., 2015; Shrestha et al., 2014). The positive effects of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 consortium to the papaya plantlets may be explained by their ability to produce IAA, ammonia, siderophore, and also solubilize phosphate.

Biofilm production assay showed no biofilm produced by *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39. The inability of our *L. lactis* subsp. *lactis* PPSSD39 to form biofilm was contradictory with a previous report, which stated that *Lactococcus* could produce biofilm (Zaidi et al., 2011). In a different study related to *W. cibaria*, the bacterium was discovered to inhibit the biofilm of *Streptococcus mutans* due to the production of water-soluble polymers from sucrose (M. S. Kang et al., 2006). Since *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 were mixed, *W. cibaria* PPKSD19 was likely to inhibit the biofilm production of *L. lactis* subsp. *lactis* PPSSD39, resulting in lower cell distribution. It remains to be elucidated in future work.

This study showed that all isolated LAB has great potential as a plant growth promoter, as all the isolates exhibited positive results in the IAA production, inorganic phosphorous solubilization, siderophores, and ammonia production tests, and two of them in the pectinase assay. *Weissella cibaria* PPKSD19 *and L. lactis* subsp. *lactis* PPSSD39 could inhibit fungal pathogens, viz. *C. lunata*, *F. oxysporum* TKA, *F. proliferatum* B68C, *F. verticillioides* B106C, and *F. verticillioides* J44C isolated from various plant hosts, suggesting their potential in controlling other plant diseases. *Weissella cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 inhibited *F. oxysporum* TKA and *C. lunata* the most. The greenhouse experiment showed that $1x10^8$ cfu ml^{-1} was the optimum concentration to enhance papaya plant growth significantly. These results highlighted the potential of both isolates as plant probiotics. However, further investigations must be performed to explore their potential to control various plant diseases while simultaneously increasing plant growth.

CONCLUSION

Weissella cibaria PPKSD19 and *Lactococcus lactis* subsp. *lactis* PPSSD39 consortium displayed promising plant probiotic potential by showing positive inhibition against multiple plant pathogens *in vitro*, as well as increasing fresh weight of shoot and dry weight of shoot and root of papaya plantlets. These results highlighted the possibility of the bacterial consortium being exploited as a versatile bioinoculant to promote plant growth and simultaneously suppress phytopathogens causing plant diseases. For future work, the antifungal activity of the consortium should be verified *in planta*. Furthermore, the storage stability of the consortium as bioinoculant should be optimized for longer shelf-life before commercialization and large-scale field application could be realized towards sustainable agriculture.

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REFERENCES

Abdel-Kader, M. M., El-Mougy, N. S., Aly, M. D. E., Lashin, S. M., & El-Mohamady, R. S. (2012). Soil drench with fungicides alternatives against root rot incidence of some vegetables under greenhouse conditions. *International Journal of Agriculture and Forestry*, *2*(2), 61–69. https://doi.org/10.5923/j.ijaf.20120202.10

Agbaglo, S. Y., Nyaku, S. T., Vigbedor, H. D., & Cornelius, E. W. (2020). Pathogenicity of *Meloidogyne incognita* and *Fusarium oxysporum* f. sp. *vasinfectum* on growth and yield of two okra varieties cultivated in Ghana. *International Journal of Agronomy*, *2020*, 8824165. https://doi.org/10.1155/2020/8824165

Assamoi, A. A., Krabi, E. R., Ehon, A. F., N`guessan, G. A., Niamké, L. S., & Thonart, P. (2016). Isolation and screening of *Weissella* strains for their potential use as starter during attiéké production. *Biotechnology, Agronomy, Society and Environment*, *20*(3), 355–362. https://doi.org/10.25518/1780-4507.13117

Bashan, Y., de-Bashan, L. E., Prabhu, S. R., & Hernandez, J. P. (2014). Advances in plant growth-promoting bacterial inoculant technology: Formulations and practical perspectives (1998-2013). *Plant Soil*, *378*(1–2), 1–33. https://doi.org/10.1007/s11104-013-1956-x

Bashan, Y., Okon, Y., & Henis, Y. (1980). Ammonia causes necrosis in tomato leaves infected with *Pseudomonas* tomato (Okabe) Alstatt. *Physiological Plant Pathology*, *17*(1), 111–114. https://doi.org/10.1016/0048-4059(80)90012-0

Beneduzi, A., Moreira, F., Costa, P. B., Vargas, L. K., Lisboa, B. B., Favreto, R., Baldani, J. I. & Passaglia, L. M. P. (2013). Diversity and plant growth promoting evaluation abilities of bacteria isolated from sugarcane cultivated in the South of Brazil. *Applied Soil Ecology*, *63*, 94–104. https://doi.org/10.1016/j.apsoil.2012.08.010

Berger, L. R., Stamford, N. P., Santos, C. E. R. S., Freitas, A. D. S., Franco, L. O., & Stamford, T. C. M. (2013). Plant and soil characteristics affected by biofertilizers from rocks and organic matter inoculated with diazotrophic bacteria and fungi that produce chitosan. *Journal of Soil Science and Plant Nutrition*, *13*(3), 592–603. https://doi.org/10.4067/S0718-95162013005000047

Bullerman, L. B., & Tsai, W. J. (1994). Incidence and levels of *Fusarium moniliforme*, *Fusarium proliferatum* and fumonisins in corn and corn-based foods and feeds. *Journal of Food Protection*, *57*(6), 541–546. https://doi.org/10.4315/0362-028X-57.6.541

Caplice, E., & Fitzgerald, G. F. (1999). Food fermentations: Role of microorganisms in food production and preservation. *International Journal of Food Microbiology*, *50*(1-2), 131–149. https://doi.org/10.1016/S0168-1605(99)00082-3

Cappuccino, J. G., & Sherman, N. (1996). *Instructor's guide for microbiology: A laboratory manual*. Benjamin-Cummings Publishing Company.

Christensen, G. D., Baldassarri, L., & Simpson, W. A. (1995). [38] Methods for studying microbial colonization of plastics. *Methods in Enzymology*, *253*, 477–500. https://doi.org/10.1016/s0076-6879(95)53040-1

Collavino, M. M., Sansberro, P. A., Mroginski, L. A., & Aguilar, O. M. (2010). Comparison of *in vitro* solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth. *Biology and Fertility of Soils*, *46*, 727–738. https://doi.org/10.1007/s00374-010-0480-x

da Silva, T. F., Vollú, R. E., Jurelevicius, D., Alviano, D. S., Alviano, C. S., Blank, A. F., & Seldin, L. (2013). Does the essential oil of *Lippia sidoides* Cham. (pepper-rosmarin) affect its endophytic microbial community?. *BMC Microbiology*, *13*, 29. https://doi.org/10.1186/1471-2180-13-29

de Lacerda, J. R. M., da Silva, T. F., Vollú, R. E., Marques, J. M., & Seldin, L. (2016). Generally recognized as safe (GRAS) *Lactococcus lactis* strains associated with *Lippia sidoides* Cham. are able to solubilize/mineralize phosphate. *SpringerPlus*, *5*, 828. https://doi.org/10.1186/s40064-016-2596-4

Doumbou, C. L., Salove, M. K. H., Crawford, D. L., & Beaulieu, C. (2001). Actinomycetes, promising tools to control plant diseases and to promote plant growth. *Phytoprotection*, *82*(3), 85–102. https://doi.org/10.7202/706219ar

Ehmann, A. (1977). The van Urk-Salkowski reagent-a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. *Journal of Chromatography*, *132*(2), 267–276. https://doi.org/10.1016/S0021-9673(00)89300-0

Enan, G., Abdel-shafi, S., Ouda, S., & Negm, S. (2013). Novel antibacterial activity of *Lactococcus lactis* subspecies *lactis* Z11 isolated from Zabady. *International Journal of Biomedical Science*, *9*(3), 174–180.

Fravel, D. R., & Larkin, R. P. (2002). Reduction of fusarium wilt of hydroponically grown basil by *Fusarium oxysporum* strain CS-20. *Crop Protection*, *21*(7), 539–543. https://doi.org/10.1016/S0261-2194(01)00143-0

Gholami, A., Shahsavani, S., & Nezarat, S. (2009). The effect of plant growth promoting rhizobacteria (PGPR) on germination, seedling growth and yield of maize. *World Academy of Science, Engineering and Technology*, *49*, 19–24. https://doi.org/10.5281/zenodo.1083385

Giassi, V., Kiritani, C., & Kupper, K. C. (2016). Bacteria as growth-promoting agents for citrus rootstocks. *Microbiological Research*, *190*, 46–54. https://doi.org/10.1016/j.micres.2015.12.006

Gull, M., & Hafeez, F. Y. (2012). Characterization of siderophore producing bacterial strain *Pseudomonas fluorescens* Mst 8.2 as plant growth promoting and biocontrol agent in wheat. *African Journal of Microbiology Research*, *6*(33), 6308-6318. https://doi.org/10.5897/AJMR12.1285

Grönemeyer, J. L., Burbano, C. S., Hurek, T., & Reinhold-Hurek, B. (2012). Isolation and characterization of root-associated bacteria from agricultural crops in the Kavango region of Namibia. *Plant Soil*, *356*(1), 67–82. https://doi.org/10.1007/s11104-011-0798-7

Hayat, R., Ali, S., Amara, U., Khalid, R., & Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: A review. *Annals of Microbiology*, *60*(4), 579–598. https://doi.org/10.1007/s13213-010-0117-1

Helal, R. B., Hosen, S., & Shamsi, S. (2018). Mycoflora associated with post-harvest disease of papaya (*Carica papaya* L.) and their pathogenic potentiality. *Bangladesh Journal of Botany*, *47*(3), 389–395. https://doi.org/10.3329/bjb.v47i3.38656

Ho, H. L. (2015). Xylanase production by *Bacillus subtilis* using carbon source of inexpensive agricultural wastes in two different approaches of submerged fermentation (SmF) and solid state fermentation (SsF). *Journal of Food Processing and Technology*, *6*(4), 1000437. https://doi.org/10.4172/2157-7110.1000437

Jaber, L. R., & Enkerli, J. (2017). Fungal entomopathogens as endophytes: Can they promote plant growth?. *Biocontrol Science and Technology*, *27*(1), 28–41. https://doi.org/10.1080/09583157.2016.1243227

Kamboj, K., Vasquez, A., & Balada-Llasat, J. M. (2015). Identification and significance of *Weissella* species infections. *Frontiers in Microbiology*, *6*, 1204. https://doi.org/10.3389/fmicb.2015.01204

Kang, M. S., Chung, J., Kim, S. M., Yang, K. H., & Oh, J. S. (2006). Effect of *Weissella cibaria* isolates on the formation of *Streptococcus* mutans biofilm. *Caries Research*, *40*(5), 418-425. https://doi.org/10.1159/000094288

Kang, S. M., Radhakrishnan, R., You, Y. H., Khan, A. L., Park, J. M., Lee, S. M., & Lee, I. J. (2015). Cucumber performance is improved by inoculation with plant growth-promoting microorganisms. *Acta Agriculturae Scandinavica, Section B — Soil and Plant Science*, *65*(1), 36–44. https://doi.org/10.1080/09064710.2014.960889

Kasana, R. C., Salwan, R., Dhar, H., Dutt, S., & Gulati, A. (2008). A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Current Microbiology*, *57*(5), 503–507. https://doi.org/10.1007/s00284-008-9276-8

Khan, M. S., Zaidi, A., & Ahmad, E. (2014). Mechanism of phosphate solubilization and physiological functions of phosphate-solubilizing microorganisms. In M. Khan, A. Zaidi, & J. Musarrat (Eds.), *Phosphate solubilizing microorganisms* (pp. 31–62). Springer. https://doi.org/10.1007/978-3-319-08216-5_2

Kim, J. D. (2005). Antifungal activity of lactic acid bacteria isolated from Kimchi against *Aspergillus fumigatus*. *Mycobiology*, *33*(4), 210–214. https://doi.org/10.4489/myco.2005.33.4.210

Liu, T., Xu, S., Liu, L., Zhou, F., Hou, J., & Chen, J. (2011). Cloning and characteristics of Brn1 gene in *Curvularia lunata* causing leaf spot in maize. *European Journal of Plant Pathology*, *131*, 211–219. https://doi.org/10.1007/s10658-011-9800-8

Lorck, H. (1948). Production of hydrocyanic acid by bacteria. *Physiologia Plantarum*, *1*(2), 142–146. https://doi.org/10.1111/j.1399-3054.1948.tb07118.x

Lutz, M. P., Michel, V., Martinez, C., & Camps, C. (2012). Lactic acid bacteria as biocontrol agents of soil-borne pathogens. *IOBC-WPRS Bulletin*, *78*, 285–288

Marag, P. S., & Suman, A. (2018). Growth stage and tissue specific colonization of endophytic bacteria having plant growth promoting traits in hybrid and composite maize (*Zea mays* L.). *Microbiological Research*, *214*, 101–113. https://doi.org/10.1016/j.micres.2018.05.016

Marques, A. P. G. C., Pires, C., Moreira, H., Rangel, A. O. S. S., & Castro, P. M. L. (2010). Assessment of the plant growth promotion abilities of six bacterial isolates using *Zea may*s

as indicator plant. *Soil Biology and Biochemistry*, *42*(8), 1229–1235. https://doi.org/10.1016/j.soilbio.2010.04.014

Mauch, A., Dal Bello, F., Coffey, A., & Arendt, E. K. (2010). The use of *Lactobacillus brevis* PS1 to *in vitro* inhibit the outgrowth of *Fusarium culmorum* and other common *Fusarium* species found on barley. *International Journal of Food Microbiology*, *141*(1–2), 116–121. https://doi.org/10.1016/j.ijfoodmicro.2010.05.002

Mohite, B. (2013). Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. *Journal of Soil Science and Plant Nutrition*, *13*(3), 638–649. https://doi.org/10.4067/S0718-95162013005000051

Morales-Cedeño, L. R., del Carmen Orozco-mosqueda, M., Loeza-lara, P. D., Parra-Cota, F. I., de los Santos-villalobos, S., & Santoyo, G. (2020). Plant growth-promoting bacterial endophytes as biocontrol agents of pre- and post-harvest diseases: Fundamentals, methods of application and future perspectives. *Microbiology Research*, *242*, 126612. https://doi.org/10.1016/j.micres.2020.126612

Nawawi, M. H., Mohamad, R., Tahir, P. M., & Saad, W. Z. (2017). Extracellular xylanopectinolytic enzymes by *Bacillus subtilis* ADI1 from EFB's compost. *International Scholarly Research Notices*, *2017*, 7831954. https://doi.org/10.1155/2017/7831954

Ndagano, D., Lamoureux, T., Dortu, C., Vandermoten, S., & Thonart, P. (2011). Antifungal activity of 2 lactic acid bacteria of the *Weissella* genus isolated from food. *Journal of Food Science*, *76*(6), M305–M311. https://doi.org/10.1111/j.1750-3841.2011.02257.x

Nimnoi, P., & Pongslip, N. (2009). Genetic diversity and plant-growth promoting ability of the indole-3-acetic acid (IAA) synthetic bacteria isolated from agricultural soil as well as rhizosphere, rhizoplane and root tissue of *Ficus religiosa* L., *Leucaena leucocephala* and *Piper sarmentosum* Roxb. *Research Journal of Agriculture and Biological Sciences*, *5*(1), 29–41.

Passari, A. K., Mishra, V. K., Gupta, V. K., Yadav, M. K., Saikia, R., & Singh, B. P. (2015). *In vitro* and *in vivo* plant growth promoting activities and DNA fingerprinting of antagonistic endophytic actinomycetes associates with medicinal plants. *PLOS One*, *10*(9), e0139468. https://doi.org/10.1371/journal.pone.0139468

Pham, V. T. K., Rediers, H., Ghequire, M. K. G., Nguyen, H. H., De Mot, R., Vanderleyden, J., & Spaepen, S. (2017). The plant growth-promoting effect of the nitrogen-fixing endophyte *Pseudomonas stutzeri* A15. *Archives of Microbiology*, *199*(3), 513–517. https://doi.org/10.1007/s00203-016-1332-3

Procópio, R. E. L., Araújo, W. L., Maccheroni Jr., W., & Azevedo, J. L. (2009). Characterization of an endophytic bacterial community associated with *Eucalyptus* spp. *Genetics and Molecular Research*, *8*(4), 1408–1422. https://doi.org/10.4238/vol8-4gmr691

Rahman, M. A., Begum, M. F., & Alam, M. F. (2009). Screening of *Trichoderma* isolates as a biological control agent against *Ceratocystis paradoxa* causing pineapple disease of sugarcane. *Mycobiology*, *37*(4), 277–285. https://doi.org/10.4489/MYCO.2009.37.4.277

Rijavec, T., & Lapanje, A. (2016). Hydrogen cyanide in the rhizosphere: Not suppressing plant pathogens, but rather regulating availability of phosphate. *Frontiers in Microbiology*, *7*, 1785. https://doi.org/10.3389/fmicb.2016.01785

Rzheyskaya, V. S., Teplitskaya, L. M., & Oturina, I. P. (2013). Colonization of rhizoplane of cucumber roots by microorganisms which are components of the microbial preparation "Embiko®". *Regulatory Mechanisms in Biosystems*, *4*(2), 63–70. https://doi.org/10.15421/021311

Schwyn, B., & Neilands, J. B. (1987). Universal chemical assay for the detection determination of siderophores. *Analytical Biochemistry*, *160*(1), 47–56. https://doi.org/10.1016/0003-2697(87)90612-9

Shrestha, A., Kim, B. S., & Park, D. H. (2014). Biological control of bacterial spot disease and plant growth-promoting effects of lactic acid bacteria on pepper. *Biocontrol Science and Technology*, *24*(7), 763–779. https://doi.org/10.1080/09583157.2014.894495

Siddiqui, Z. A. (2005). PGPR: Prospective biocontrol agents of plant pathogens. In Z. A. Siddiqui (Ed.), *PGPR: Biocontrol and biofertilization* (pp. 111–142). Springer. https://doi.org/10.1007/1-4020-4152-7_4

Siezen, R. J., Starrenburg, M. J. C., Boekhorst, J., Renckens, B., Molenaar, D., & van Hylckama Vlieg, J. E. (2008). Genome-scale genotype-phenotype matching of two *Lactococcus lactis* isolates from plants identifies mechanisms of adaptation to the plant niche. *Applied and Environmental Microbiology*, *74*(2), 424–436. https://doi.org/10.1128/AEM.01850-07

Singh, A., Kaur, A., Dua, A., & Mahajan, R. (2015). An efficient and improved methodology for the screening of industrially valuable xylano-pectino-cellulolytic microbes. *Enzyme Research*, *2015*, 725281. https://doi.org/10.1155/2015/725281

Somers, E., Amke, A., Croonenborghs, A., van Overbeek, L. S., & Vanderleyden, J. (2007, August 26-31). *Lactic acid bacteria in organic agricultural soils* [Poster presentation]. Rhizosphere 2 Conference 2007, Montpellier, France. https://research.wur.nl/en/publications/lactic-acid-bacteria-in-organic-agricultural-soils

Stiles, M. E., & Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology*, *36*(1), 1–29. https://doi.org/10.1016/S0168-1605(96)01233-0

Strafella, S., Simpson, D. J., Khanghahi, M. Y., De Angelis, M., Gänzle, M., Minervini, F., & Crecchio, C. (2021). Comparative genomics and *in vitro* plant growth promotion and biocontrol traits of lactic acid bacteria from the wheat rhizosphere. *Microorganisms*, *9*(1), 78. https://doi.org/10.3390/microorganisms9010078

Taha, M. D. M., Jaini, M. F. M., Saidi, N. B., Rahim, R. A., Shah, U. K. M., & Hashim, A. M. (2019). Biological control of *Erwinia mallotivora*, the causal agent of papaya dieback disease by indigenous seed-borne endophytic lactic acid bacteria consortium. *PLOS One*, *14*(12), e0224431. https://doi.org/10.1371/journal.pone.0224431

Tann, H., & Soytong, K. (2017). Biological control of brown leaf spot disease caused by *Curvularia lunata* and field application method on rice variety IR66 in Cambodia. *AGRIVITA Journal of Agricultural Science*, *39*(1), 111–117. https://doi.org/10.17503/agrivita.v39i1.768

Time and Date (n.d.). *Weather in Malaysia*. Retrieved from 5th April, 2021, from https://www.timeanddate.com/weather/malaysia/

Tiru, M., Muleta, D., Berecha, G., & Adugna, G. (2013). Antagonistic effects of rhizobacteria against coffee wilt disease caused by *Gibberella xylarioides*. *Asian Journal of Plant Pathology*, *7*(3), 109–122. https://doi.org/10.3923/ajppaj.2013.109.122

Trias, R., Bañeras, L., Montesinos, E., & Badosa, E. (2008). Lactic acid bacteria from fresh fruit and vegetables as biocontrol agents of phytopathogenic bacteria and fungi. *International Microbiology*, *11*(4), 231–236. https://doi.org/10.2436/20.1501.01.66

Valencia-Hernández, L. J., López-López, K., & Serna-Cock, L. (2016). *Weissella cibaria* fungistatic activity against *Fusarium* spp. affecting yellow pitahaya. *American Journal of Applied Sciences*, *13*(12), 1354–1364. https://doi.org/10.3844/ajassp.2016.1354.1364

Valerio, F., Favilla, M., De Bellis, P., Sisto, A., de Candia, S., & Lavermicocca, P. (2009). Antifungal activity of strains of lactic acid bacteria isolated from a semolina ecosystem against *Penicillium roqueforti*, *Aspergillus niger* and *Endomyces fibuliger* contaminating bakery products. *Sytematic and Applied Microbiology*, *32*(6), 438–448. https://doi.org/10.1016/j.syapm.2009.01.004

Viruel, E., Erazzú, L. E., Martínez Calsina, L., Ferrero, M. A., Lucca, M. E., & Siñeriz, F. (2014). Inoculation of maize with phosphate solubilizing bacteria: Effect on plant growth and yield. *Journal of Soil Science and Plant Nutrition*, *14*(4), 819–831. https://doi.org/10.4067/S0718-95162014005000065

Wang, X., Shen, J., & Liao, H. (2010). Acquisition or utilization, which is more critical for enhancing phosphorus efficiency in modern crops?. *Plant Science*, *179*(4), 302–306. https://doi.org/10.1016/j.plantsci.2010.06.007

Wei, G., Kloepper, J. W., & Tuzun, S. (1991). Induction of systemic resistance of cucumber to *Colletrichum orbiculare* by select strains of plant growth-promoting rhizobacteria. *Phytopathology*, *81*(11), 1508–1512. https://doi.org/10.1094/Phyto-81-1508

Zaidi, A. H., Bakkes, P. J., Krom, B. P., van der Mei, H. C., & Driessen, A. J. M. (2011). Cholate-stimulated biofilm formation by *Lactococcus lactis* cells. *Applied and Environmental Microbiology*, *77*(8), 2602–2610. https://doi.org/10.1128/AEM.01709-10

Zainudin, N. A. I. M., Hamzah, F. A., Kusai, N. A., Zambri, N. S., & Salleh, S. (2017). Characterization and pathogenicity of *Fusarium proliferatum* and *Fusarium verticillioides*, causal agents of *Fusarium* ear rot of corn. *Turkish Journal of Biology*, *41*(1), 220–230. https://doi.org/10.3906/biy-1606-25

Zakaria, L., Chik, M. W., Heng, K. W., & Salleh, B. (2012). *Fusarium* species associated with fruit rot of banana (*Musa* spp.), papaya (*Carica papaya*) and guava (*Psidium guajava*). *Malaysian Journal of Microbiology*, *8*(2), 127–130.

SUPPLEMENTARY DATA

Table S1

List of bacterial isolates used and its sources. Bacteria was isolated from papaya seed and sarcotesta (Taha et al., 2019)

Plant tissue source	Strain	Lactic acid bacteria
Seed	PPKSD8	*Lactococcus lactis* subsp. *lactis*
	PPKSD9	*Weissella cibaria*
	PPKSD19	*Weissella cibaria*
	PPKSD29	*Weissella cibaria*
	PPKSD34	*Weissella cibaria*
	PPKSD37	*Weissella cibaria*
	PPKSD39	*Weissella confusa*
	PPSSD1	*Weissella cibaria*
	PPSSD7	*Lactococcus lactis* subsp. *lactis*
	PPSSD38	*Lactococcus lactis* subsp. *lactis*
	PPSSD39	*Lactococcus lactis* subsp. *lactis*
Sarcotesta	PPKST1	*Lactococcus lactis* subsp. *lactis*
	PPKST2	*Lactococcus lactis* subsp. *lactis*
	PPKST3	*Lactococcus lactis* subsp. *lactis*
	PPKST4	*Lactococcus lactis* subsp. *lactis*
	PPKST4S	*Lactococcus lactis* subsp. *lactis*
	PPKST4B	*Lactococcus lactis* subsp. *lactis*
	PPKST5	*Lactococcus lactis* subsp. *lactis*
	PPKST11	*Lactococcus lactis* subsp. *lactis*
	PPKST14	*Lactococcus lactis* subsp. *lactis*
	PPSST25	*Lactococcus lactis* subsp. *lactis*
	PPSST38	*Lactococcus lactis* subsp. *lactis*

Table S2

Percentage of inhibition of radial growth (PIRG, %) of isolates (a) Weissella cibaria *PPKSD 19 and (b)* Lactococcus lactis *subsp.* lactis *PPSSD 39 in poisoned agar test*

(a) *Weissella cibaria* PPKSD19

PIRG (%)	*Curvularia lunata*	*Fusarium oxysporum* TKA	*Fusarium proliferatum* B68C	*Fusarium verticillioides* B106C	*Fusarium verticillioides* J44C
10	53.50 ± 0.71^{a}	16.67 ± 4.59^{a}	27.18 ± 3.55^{a}	41.67 ± 1.44^{a}	25.13 ± 3.55^{a}
20	55.14 ± 0.71^{a}	24.29 ± 1.43^{b}	29.23 ± 4.07^{ab}	42.92 ± 0.72^{a}	30.77 ± 4.07^{ab}
30	55.14 ± 0.71^{a}	39.52 ± 2.97^{c}	30.26 ± 4.95^{ab}	44.58 ± 9.21^{ab}	37.95 ± 0.89^{bc}
40	54.32 ± 2.14^{a}	40.48 ± 1.65^{cd}	30.26 ± 0.89^{ab}	49.17 ± 5.20^{ab}	40.51 ± 3.20^{c}
50	70.37 ± 0.00^{b}	47.14 ± 1.43^{de}	35.90 ± 0.89^{b}	47.92 ± 4.02^{ab}	41.03 ± 3.55^{c}
60	82.30 ± 0.71^{c}	50.95 ± 2.18^{e}	33.85 ± 1.54^{ab}	52.92 ± 8.32^{ab}	45.13 ± 2.35^{c}
80	84.36 ± 1.43^{c}	53.33 ± 0.82^{e}	35.38 ± 1.54^{b}	59.58 ± 5.05^{b}	43.08 ± 3.08^{c}

Means with different superscript letters within rows indicate significant difference at $p < 0.05$ using one-way ANOVA

(b) *Lactococcus lactis* subsp. *lactis* PPSSD39

PIRG (%)	*Curvularia lunata*	*Fusarium oxysporum* TKA	*Fusarium proliferatum* B68C	*Fusarium verticillioides* B106C	*Fusarium verticillioides* J44C
10	47.33 ± 1.43^{a}	43.81 ± 2.97^{a}	39.73 ± 0.00^{a}	38.33 ± 3.82^{a}	22.56 ± 0.89^{a}
20	48.97 ± 1.43^{ab}	52.38 ± 4.12^{b}	39.73 ± 0.00^{a}	41.67 ± 3.61^{a}	41.03 ± 0.89^{ab}
30	50.62 ± 0.00^{bc}	51.90 ± 0.82^{b}	42.01 ± 2.85^{ab}	49.17 ± 1.91^{ab}	44.62 ± 1.54^{bc}
40	51.85 ± 1.23^{cd}	53.33 ± 0.82^{bc}	45.66 ± 2.09^{bc}	45.83 ± 2.89^{ab}	44.62 ± 1.54^{c}
50	53.50 ± 0.71^{de}	52.38 ± 1.65^{b}	43.38 ± 1.58^{ab}	46.25 ± 1.25^{ab}	44.10 ± 0.89^{c}
60	54.73 ± 0.71^{e}	54.29 ± 2.47^{bc}	52.05 ± 3.62^{c}	56.25 ± 3.31^{ab}	28.21 ± 1.78^{c}
80	55.56 ± 0.00^{e}	59.05 ± 0.82^{c}	49.77 ± 5.19^{bc}	56.67 ± 0.72^{b}	40.00 ± 1.54^{c}

Means with different superscript letters within rows indicate significant difference at $p < 0.05$ using one-way ANOVA

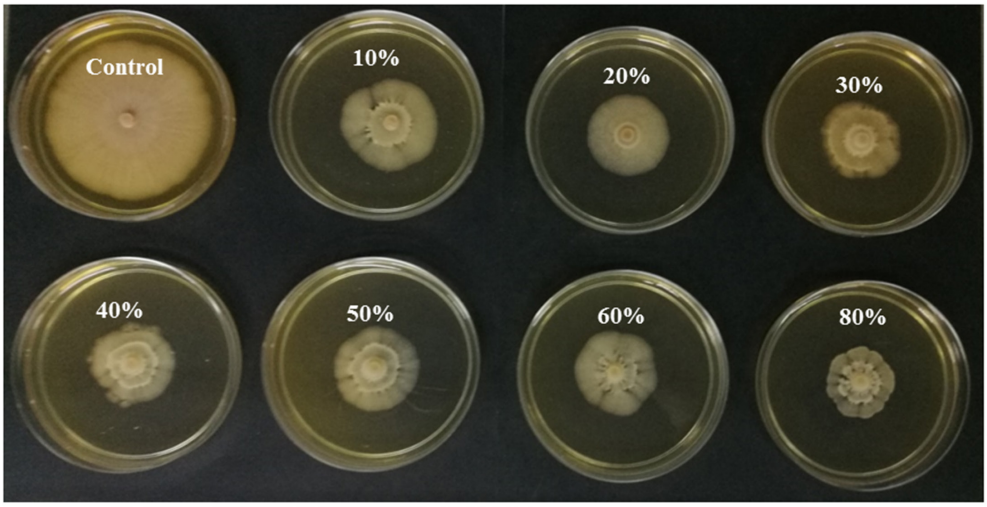

Figure S1. Antagonistic activity of different culture filtrate concentrations of *Lactococcus lactis* subsp. *lactis* PPSSD39 against *Fusarium oxysporum* TKA grown on PDA media. The PDA was incorporated with 10%, 20%, 30%, 40%, 50%, 60%, and 80% (v/v) culture filtrate concentration of 7-day old *L. lactis* subsp. *lactis* PPSSD39 cultures

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Short communication

Brief Communication: Caffeic Acid Derivatives and Polymethoxylated Flavonoids from Cat's Whiskers (*Orthosiphon stamineus*) Form Stable Complexes with SARS-CoV Molecular Targets: An *In silico* Analysis

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ABSTRACT

Cat's whiskers or the 'misai kucing' is an herbal plant native to the Southeast Asian region. The polyphenol enriched leaf extract contains numerous medicinal properties of major pharmaceutical interest. In this study, selected cat's whiskers polyphenols were screened computationally to predict the minimum binding affinities with severe acute respiratory syndrome coronavirus (SARS-CoV) molecular targets. Molecular docking analysis showed that the caffeic acid derivatives and polymethoxylated flavonoids from cat's whiskers bound stably to the binding pocket regions of SARS-CoV molecular targets at -4.2 to -7.1 kcal/mol. Furthermore, these cat's whiskers polyphenol-bound SARS-CoV complexes were held fairly strongly by hydrophobic interactions, hydrogen bonds, and electrostatic interactions at various extents.

Keywords: CoV-2, COVID-19, misai kucing, molecular docking, molecular target, polyphenol, SARS- therapeutics

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INTRODUCTION

The coronavirus disease (COVID-19) first emerged in Wuhan, China, in late 2019. It has been more than a year since the pandemic emerged, and the disease continues to impact the socio-economic lives of the world population. At this time of writing (9th November 2021), more than 250 million people have been infected with COVID-19, and up to 5 million deaths

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have been reported globally. Malaysia has reported 2.5 million COVID-19 cases and 29,291 deaths (www.worldometers.info).

COVID-19 is divided into three distinct phases, and each phase represents a unique clinical stage (Gu & Korteweg, 2007). The asymptomatic stage 1 (about 1-2 days upon infection) begins with the inhalation of airborne droplets of SARS-CoV-2. The virus binds to receptors localized at the epithelial cells in the nasal cavity. Stage 2 (2-5 days upon viral entry) marks the virus propagation at the upper airway and conducting airway, followed by migration heading downwards to the respiratory tract. During this stage, a robust innate immune response is triggered. About 80% of the COVID-19 infected patients experience mild symptoms when the virus manifestation becomes restricted to the upper and conducting airways (Wu & McGoogan, 2020). In stage 3, massive viral propagation develops into severe disease symptoms as a result of aggravated immune response (cytokine storm) (Tang et al., 2020).

Orthosiphon stamineus Benth. (*Clerodendrathus spicatus* Thunb.) is well-known as 'misai kucing' in Malaysia, 'java tea' in Indonesia and 'sen cha' in China (Abdullah et al., 2012; Li et al., 2021). It is one of the most widely grown and used medicinal plants in Asia, southern China, and Australia (Abdullah et al., 2012). The long-term consumption of *O. stamineus* extract (OE) has been shown to offer protection against various diseases (Pandey & Rizvi, 2009). For thousands of years, the leaves, in their dried and/or fresh form are consumed as a tea to treat multiple ailments such as urinary tract infection (Deipenbrock & Hensel, 2019), arthritis (Tabana et al., 2016), diabetes mellitus, hypertension, tonsillitis, rheumatism, and menstrual disorders (Cai et al., 2020; Zhong et al., 2012). Bioactive compounds pre-dominantly found in the leaves of *O. stamineus* have been reported to display anti-allergic, anti-angiogenic, anti-hypertensive, anti-inflammatory, antioxidant, and diuretic properties (Ashraf et al., 2018, 2020; Muhammad et al., 2011; Tabana et al. 2016). Despite being an important folk medicine, the potential activities of the *O. stamineus* bioactive compounds for COVID-19 management are least reported. Generally, the *O. stamineus* leaves constitute a broad range of bioactive compounds: pentacyclic triterpenes, phenolics, and small sterols. The *O. stamineus* leaves are especially enriched in polyphenols: up to 0.5 to 0.7% of the leaf extract contains flavonoids with methoxylated flavones and prenylated flavones, among the majority fraction (Deipenbrock & Hensel, 2019). In Malaysia, *O. stamineus* leaf extract has been reported to contain sinensetin, eupatorine, rosmarinic acid, caffeic acid, and gallic acid (Engku-Hasmah et al., 2013; Muhammad et al., 2011). In others, chloroform extracts of *O. staminieus* leaves showed high contents of sinensetin and eupatorine flavonoids, whilst the ethanolic leaf extract was characterized with high rosmarinic acid (Tabana et al. 2016).

In this study, the caffeic acid derivatives and polymethoxylated flavonoids from *O.*

stamineus were docked with SARS-CoV molecular targets at the binding pocket regions, and the minimum binding affinities of the resulting complexes were evaluated. Structural proteins governing key biological functions of the different stages of the SARS-CoV life cycle were identified from Protein Data Bank (PDB). The ligand-protein complexes with good minimum binding affinities were identified and further visualized. Our findings highlight the potentials of *O. stamineus* polyphenols for COVID-19 management strategies such as phytotherapy, preventive therapeutics, and immune health-enhancing and illness relief beverages/supplements.

METHODS

Data Collection and Target Pre-Processing

In this study, four different SARS-CoV structural proteins were selected and retrieved from the PDB (www.rcsb.org): membrane protein (PDB ID: 3I6G), main protease (PDB ID: 5RE4), and spike glycoprotein (PDB ID: 6VXX) (PDB ID: 6VYB). The binding pocket regions expressed as x, y, and z coordinates in a 3D space were identified using Biovia Discovery Studio 2021 version 4.5. In its native format (.PDB), each protein was manually pre-processed using the AutoDock tools described by Trott and Olson (2010); crystal water molecules were discarded, and hydrogen polar atoms and Kollman charges were added. The edited files were stored in the protein data bank, partial charge (Q), and atom type (T) (PDBQT) format.

The 3D structures of the following *O. stamineus* bioactive compounds (hereafter, designated as ligands) were searched and retrieved from PubChem (pubchem.ncbi.nlm.nih.gov): sinensetin (PubChem Compound ID number [CID]: 145659), gallic acid (PubChem CID: 370), caffeic acid (PubChem CID: 689043), rosmarinic acid (PubChem CID: 5281792), and eupatorine (PubChem CID: 97214). First, the structure data (SDF) format files were converted to PDB format using Open Babel Compiler version 2.3.2. Next, the ligand files were pre-processed using the AutoDock tools (Trott & Olson, 2010): set the number of torsions and detect the root atoms. Following the edits, all files were saved in the PDBQT format.

Ligand-Receptor Docking

A configuration file defining the binding pocket regions (grid box) of the receptors was prepared in txt. file. The selected ligands (sinensetin, gallic acid, caffeic acid, rosmarinic acid, and eupatorine) were docked against the binding pocket regions of the receptors using AutoDock Vina 1.1.2. (Trott & Olson, 2010). All docking runs were initiated at Command Prompt (Windows operating system). The output log files were evaluated, and the best-docked conformations were selected based on binding energy (kcal/mol), cluster RMSD value, number of stabilizing interactions such as hydrogen bonds, and other weak interactions. The 3D complex structure and interactions were visualized using the Biovia Discovery Studio version 4.5.

RESULTS AND DISCUSSION

The coronavirus disease-2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The SARS-CoV-2 pathogenesis mechanism is governed by structural proteins (surface spike protein, membrane protein, envelope protein, and main protease-replicase polyproteins) and a few non-structural proteins (nsp6, nsp8, and nsp10) (Walls et al., 2020). The global outbreak is one of the most challenging pandemics ever experienced, and effective drugs are yet to avail (November 2021). However, numerous research efforts, especially promising and high-throughput computational biology approaches for plant-based therapeutics, have emerged through molecular docking, modeling, and dynamic simulation studies (Skariyachan et al., 2020).

The *O. stamineus* is a traditional medicinal herbal plant with rich bioactive compounds. Extensively concentrated in the leaves, there are a large number of different types of bioactive compounds identified in *O. stamineus* (Li et al., 2021; Tezuka et al., 2000). As such, the total phenolic content of *O. stamineus* leaf 50% ethanol extract was 385 µg/ml, and the total flavonoid content, 57 µg/ml (Tabana et al., 2016). Rosmarinic acid is the main constituent of *O. stamineus* leaves. Previous studies have reported that *O. stamineus* leaves contain two different polyphenols in abundance: polymethoxylated flavonoids and caffeic acid derivatives (Engku-Hasmah et al., 2013; Muhammad et al., 2011; Tabana et al., 2016). This study selected the most abundantly occurring polyphenols in the *O. stamineus* leaves as ligands. Sinestine and eupatorine are polymethoxylated flavonoids, while rosmarinic acid, gallic acid, and caffeic acid belong to the caffeic acid derivatives group (Olah et al., 2003).

Four different structural SARS-CoV-related proteins were selected, namely the membrane protein (PDB ID: 3I6G), main proteases (PDB ID: 5RE4), and spike glycoproteins (PDB ID: 6VXX and 6VYB). The biological roles of the selected proteins are described as follows: 3I6G is a SARS-CoV M protein complexed with HLA-A*0201 obtained from X-ray diffraction method; 5RE4 is a crystal structure of SARS-CoV-2 main protease complexed with Z1129283193 obtained by X-ray diffraction method; 6VXX is a closed state model of SARS-CoV-2 spike glycoprotein obtained via electron microscopy, and 6VYB, an open state of SARS-CoV-2 spike ectodomain structure obtained through electron microscopy (www.rcsb.org).

The minimum binding affinities for all possible combinations of ligand (sinensetin, gallic acid, caffeic acid, rosmarinic acid, and eupatorine)-protein complexes were measured at root mean square deviation (RSMD) = 0. For complexes formed between the ligands and the 3I6G, the binding affinity ranged from -5.3 to -7.1 kcal/mol (Table 1). A various number of hydrogen bonds, hydrophobic, and electrostatic interactions were observed at the receptor residue (RR) of all 3I6G-bound complexes except the 3I6G-gallic acid complex (absence of

hydrophobic interaction) (Table 2). The rosmarinic acid-3I6G complex showed the highest binding affinity at -7.1 kcal/mol, followed by sinensetin-3I6G complex at -6.6 kcal/mol (Table 1). In contrast, the number of interactions was higher in the sinensetin-3I6G complex as compared to the rosmarinic acid-3I6G complex. There were three hydrogen bonds (RR: ARG97, TYR123, HIS114), three hydrophobic interactions (RR: VAL152, TRP147, ALA150), and one electrostatic interaction (ASP77) in the rosmarinic acid-3I6G complex. In comparison, the sinensetin-3I6G complex showed three hydrogen bonds (RR: TYR7, GLU63, LYS66), six hydrophobic interactions (RR: TYR7, TYR159, PHE9, HIS70, TRP167, LYS66), and a single electrostatic interaction (RR: LYS66) (Figure 1A) (Table 2). On the other hand, 3I6G-gallic acid showed the lowest binding affinity at 5.3 kcal/mol with a single hydrogen bond (RR: TRP14) and electrostatic interaction (RR: ASP77) (Tables 1 and 2).

The minimum binding affinities of ligands bound to 5RE4 ranged from -4.9 to -6.7 kcal/mol. The rosmarinic acid-5RE4 complex shows the least minimum binding affinity at -6.7 kcal/mol, followed by the eupatorine-5RE4 and sinensetin-5RE4 complexes at -5.7 kcal/mol and -5.6 kcal/mol, respectively (Table 1). Although the rosmarinic acid-5RE4 complex showed no presence of electrostatic interactions, a total of nine hydrogen bonds (RR: ARG76, LEU75, PHE66, ASP92, THR93, HIS64, LEU67, LEU75, VAL77) along two hydrophobic interactions (RR: ARG76, VAL73) were observed (Figure 1B). The eupatorine-5RE4 complex showed the presence of a hydrogen bond (RR: VAL77), hydrophobic interaction (RR: ARG76), and electrostatic (RR: ARG76) interaction each. Besides the rosmarinic acid-5RE4 complex, both the gallic acid-5RE4 and caffeic acid-5RE4 complexes showed an absence of electrostatic interaction (Table 2).

For the ligand-6VXX complexes, the minimum binding affinities range at -4.3 to -5.6 kcal/mol (Table 1), and electrostatic interactions were consistently absent in all complexes (Table 2). The eupatorine-6VXX complex showed the least minimum binding affinity at -5.6 kcal/mol with three hydrogen bonds (RR: PHE86, ASN87, ASN234)

Table 1
Molecular docking analysis of Orthosiphon stamineus *polyphenols and SARS-CoV molecular targets (proteins) performed using the Autodock Vina software*

	3I6G	5RE4	6VXX	6VYB
Sinensetin	-6.6	-5.6	-4.9	-4.8
Gallic acid	-5.3	-4.9	-4.7	-4.5
Caffeic acid	-5.4	-5.0	-4.3	-4.2
Rosmarinic acid	-7.1	-6.7	-5.5	-6.0
Eupatorine	-6.5	-5.7	-5.6	-5.6

Note. The first row denotes receptor ID (Protein Data Bank), and the first column represents polyphenols. All numerical values indicate the minimum binding affinities expressed in kcal/mol

Table 2
The interactions between the Orthosiphon stamineus *polyphenol and receptor residue*

Receptor-ligand complex	Hydrogen bond	Hydrophobic interaction	Electrostatic interaction
3I6G-sinensetin	TYR7, GLU63, LYS66	TYR7, TYR159, PHE9, HIS70, TRP167, LYS66	LYS66
3I6G-gallic acid	TRP14	-	ASP77
3I6G-caffeic acid	LYS66, ARG97, HIS114	TYR15	LYS66
3I6G-rosmarinic	ARG97, TYR123, HIS114	VAL152, TRP147, ALA150	ASP77
3I6G-eupatorine	TYR7, LYS66	TYR7, TYR159, LEU156, PHE9, LYS66	LYS66
5RE4-sinensetin	PHE66, LEU75	VAL73, LEU75, LEU67, ARG76	ARG76, ASP92
5RE4-gallic acid	GLN74, PHE66, VAL77	ARG76, VAL77	-
5RE4-caffeic acid	GLN74, PHE66, HIS64, VAL77	ARG76, VAL77	-
5RE4-rosmarinic	ARG76, LEU75, PHE66, ASP92, THR93, HIS64, LEU67, LEU75, VAL77	ARG76, VAL73	-
5RE4-eupatorine	VAL77,	ARG76	ARG76
6VXX-sinensetin	THR236, ILE233	ARG237, THR236	-
6VXX-gallic acid	ASN196, ILE237, ASN234, ILE235	-	-
6VXX-caffeic acid	-	-	-
6VXX-rosmarinic	ASP88, ASN196, THR236	-	-
6VXX-eupatorine	PHE86, ASN87, ASN234,	THR236, PHE86	-
6VYB-sinensetin	THR236, ASN234	THR236, ARG237	-
6VYB-gallic acid	THR108, LYS113, GLY232	ILE233	-
6VYB-caffeic acid	ILE235	-	-
6VYB-rosmarinic	THR236, ILE235	-	-
6VYB-eupatorine	-	-	-

and two hydrophobic interactions (RR: THR236, PHE86) (Figure 1C). The caffeic acid-6VXX complex showed the lowest binding affinity at -4.3 kcal/mol with no interactions (Table 1).

The minimum binding affinities for ligand-6VYB ranged from -4.2 to -6.0 kcal/mol. The rosmarinic acid-6VYB complex showed the least minimum binding affinity at -6.0 kcal/mol, followed by the eupatorine-6VYB complex at -5.6 kcal/mol. Caffeic acid-6VYB complex showed the least minimum binding affinity (-4.2 kcal/mol) among all the ligand-6VYB complexes (Table 1). All the ligand-6VYB complexes had no electrostatic interaction. Interestingly, the sinensetin-6VYB complex showed a relatively lower minimum binding affinity (-4.8 kcal/mol) than the rosmarinic acid-6VYB complex (-6.0 kcal/mol) and the number of interactions displayed a *vice versa* trend. The rosmarinic acid-6VYB complex showed two hydrogen bonds (RR: THR236, ILE235) only, while the

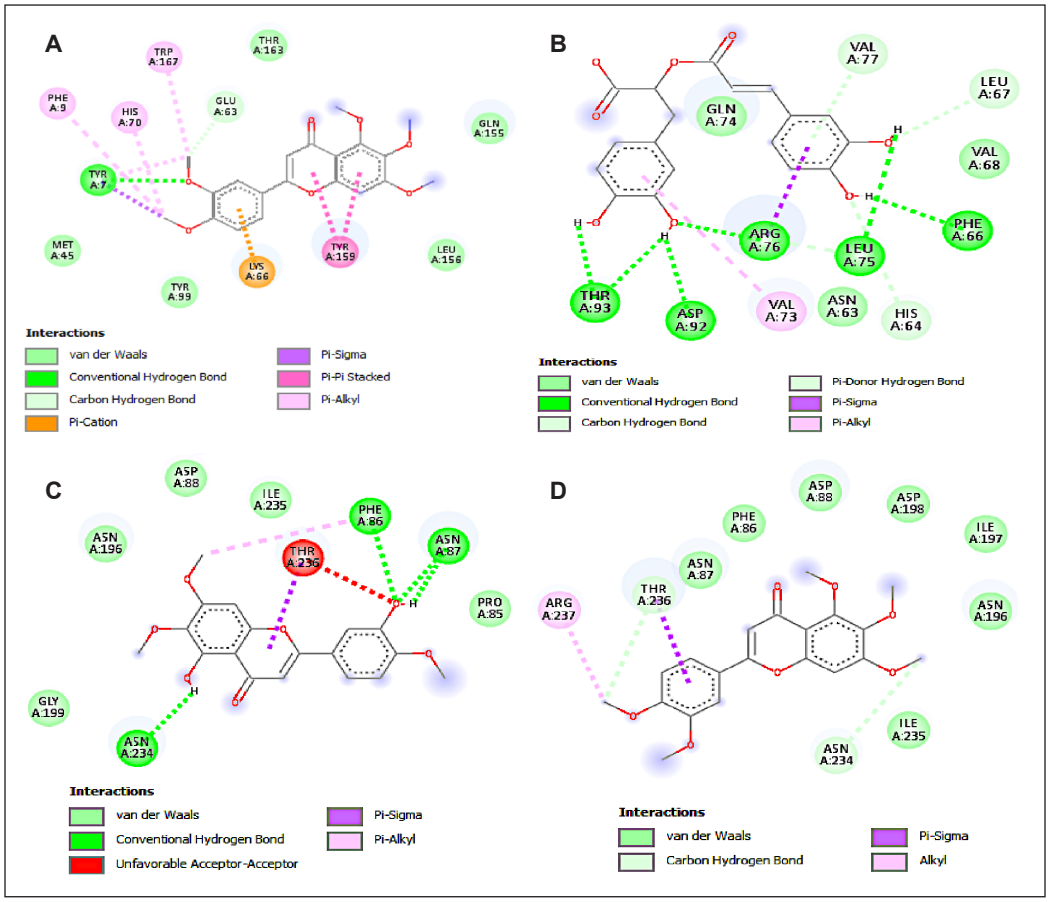

Figure 1. Interactions between the *Orthosiphon stamineus* polyphenols and SARS-CoV receptors. A) 3I6G-sinensetin complex; B) 5RE4-rosmarinic acid complex; C) 6VXX-eupatorine complex, and D) 6VYB-sinensetin complex. The best conformation complexes are selected based on the minimum binding affinity values and number of interactions at RMSD = 0. All 2- dimension *O. stamineus* polyphenol-bound SARS-CoV receptors complexes contain nodes, represent the receptor residue (RR), and dotted lines represent the interactions between ligand atom and RR

sinensetin-6VYB complex showed a greater number of interactions with two hydrogen bonds (RR: THR236, ASN234) and two hydrophobic interactions (RR: THR236, ARG237) (Figure 1D) (Table 2).

Plant-based natural products have been used to treat various diseases for a long time. These natural resources are mainly derived from beneficial traditional plant medicines. In time of the COVID-19 pandemic, the use of traditional medicinal plants as alternative medicine has been overwhelming, especially among Asian countries. Both China and the Indian government have recommended polyherbal decoctions for COVID-19 prevention and management: Qingfei Paidu, Huashi Baide, Xuanfei Badu, Toujie Quwen granules, Jinhua Qinggan granules (traditional Chinese medicine), Kabasura Kudineer

(traditional Siddha medicine). In Thailand, *Andrographis paniculata* (local name, 'hempedu bumi') is used for mild COVID-19 infection, whilst other herbal plants such as the lemon balm (*Melissa officinalis* L.), skullcap (*Scutellaria lateriflora*), Siberian ginseng (*Eleutherococcos senticosus*) are also widely employed throughout the world. As plant-based medicines pose minimal side effects compared to steroidal drugs, the ethnopharmacological relevance of traditional herbal plants for COVID-19 can be explored using extensive computational approaches, bioinformatics, and cheminformatics. In this study, five different polyphenols enriched in *O. stamineus* leaves (the sinensetin, caffeic acid, gallic acid, rosmarinic acid, and eupatorine) showed structural interactions with SARS-CoV proteins. In terms of enzymes encoding pathogenesis, the SARS-CoV is similar to SARS-CoV-2 (Lan et al., 2020). The structural insights of the potential interactions between the *O. staminineus* polyphenols and SARS-CoV proteins require further investigations to confirm and fully validate the inhibitory properties of *O. staminineus* polyphenols against SARS-CoV proteins. Other molecular dynamic simulation studies, drug-likeness analysis, and bioassay studies are necessary to identify the inhibitory and druggability roles of *O. staminineus* polyphenols against SARS-CoV-2 of COVID-19. The use of *O. stamineus* to treat multiple ailments could be stretched to viral diseases associated with SARS-CoV, such as the COVID-19.

CONCLUSION

The *in silico* analysis of *Orthosiphon stamineus* polyphenols against SARS-CoV molecular targets shows good interaction at a minimum binding affinity range of –4.2 to –7.1 kcal/mol coupled with numerous hydrogen bonds and hydrophobic interactions. The *O. stamineus* polyphenols are lucrative alternatives to synthetic drugs as they are comparably cheap and may pose fewer side effects in anti-viral treatments. With further research that complements the present preliminary findings, *O. staminineus* polyphenols could potentially be utilized for COVID-19 therapeutics development.

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REFERENCES

Abdullah, S., Shaari, A. R., & Azimi, A. (2012). Effect of drying methods on metabolites composition of misai kucing (*Orthosiphon stamineus*) leaves. *APCBEE Procedia*, *2*, 178–182. https://doi.org/10.1016/j.apcbee.2012.06.032

Ashraf, K., Halim, H., Lim, S. M., Ramasamy, K., & Sultan, S. (2020). *In vitro* antioxidant, antimicrobial and antiproliferative studies of four different extrscts of *Orthosiphon stamineus*, *Gynura procumbens* and *Ficus deltoidei*. *Saudi Journal of Biological Sciences*, *21*(1), 417-432. https://doi.org/10.1016/j.sjbs.2019.11.003

Ashraf, K., Sultan, S., & Adam, A. (2018). *Orthosiphon stamineus* Benth. is an outstanding food medicine: Review of phytochemical and

pharmacological activities. *Journal of Pharmacy and Bioallied Sciences*, *10*(3), 109-118. https://doi.org/10.4103/jpbs.JPBS_253_17

Cai, X., Zhang, L., Chen, X., Zhang, H., Xue, H., Lu, Y., Tang, J., & Lu, Y. (2020). *Orthosiphon stamineus* and rosmarinic acid reduce heat stress in laying hens. *Livestock Science*, *240*, 104124. https://doi.org/10.1016/j.livsci.2020.104124

Deipenbrock, M., & Hensel, A. (2019). Polymethoxylated flavones from *Orthosiphon stamineus* leaves as antiadhesive compounds against uropathogenic *E. coli*. *Fitoterapia*, *139*, 104387. https://doi.org/10.1016/j.fitote.2019.104387

Engku-Hasmah, E. A., Ahmad Tarmizi, S., Noor Ismawaty, N., & Abdul Ghani, O. (2013). Total phenolic, total flavonoid and rosmarinic acid contents in *Orthosiphon stamineus* (Misai Kucing) in relation to storage conditions. *Acta Horticulturae*, *1012*, 837–842. https://doi.org/10.17660/ActaHortic.2013.1012.113

Gu, J., & Korteweg, C. (2007). Pathology and pathogenesis of severe acute respiratory syndrome. *The American Journal of Pathology*, *170*(4), 1136–1147. https://doi.org/10.2353/ajpath.2007.061088

Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S. (2020). *Crystal structure of the 2019-nCoV spike receptor-binding domain bound with ACE2 receptor*. https://www.biorxiv.org/content/10.1101/2020.02.19.956235v1.full.pdf

Li, Z., Qu, B., Zhou, L., Chen, H., Wang, J., Zhang, W., & Chen, C. (2021). A new strategy to investigate the efficacy markers underlying the medicinal potentials of *Orthosiphon stamineus* Benth. *Frontiers in Pharmacology*, *12*, 748684. https://doi.org/10.3389/fphar.2021.748684

Muhammad, H., Gomes-Carneiro, M. R., Poa, K. S., De-Oliveira, A. C. A. X., Afzan, A., Sulaiman, S. A., Ismail, Z., & Paumgartten, F. J. R. (2011). Evaluation of the genotoxicity of *Orthosiphon stamineus* aqueous extract. *Journal of Ethnopharmacology*, *133*(2), 647–653.

Olah, N. K., Radu, L., Mogosan, C., Hanganu, D., & Gocan, S. (2003). Phytochemical and pharmacological studies on *Orthosiphon stamineus* Benth. (Lamiaceae) hydroalcoholic extracts. *Journal of Pharmaceutical and Biomedical Analysis*, *33*(1), 117-123. https://doi.org/10.1016/s0731-7085(03)00227-9

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*, *2*, 897484. https://doi.org/10.4161/oxim.2.5.9498

Skariyachan, S., Gopal, D., Chakrabarti, S., Kempanna, P., Uttarkar, A., Muddebihalkar, A. G., & Niranjan, V. (2020). Structural and molecular basis of the interaction mechanism of selected drugs towards multiple targets of SARS-CoV-2 by molecular docking and dynamic simulation studies-deciphering the scope of repurposed drugs. *Computational Biology Medicine*, *126*, 104054. https://doi.org/10.1016/j.compbiomed.2020.104054

Tabana, Y. M., Al-Suede, F. S., Ahamed, M. B., Dahham, S. S., Hassan, L. E., Khalilpour, S., Taleb-Agha, M., Sandai, D., Majid, A.S.A., & Majid, A. M. S. A. (2016). Cat's whiskers (*Orthosiphon stamineus*) tea modulates arthritis pathogenesis via the angiogenesis and inflammatory cascade. *BMC Complementary and Alternative Medicine*, *16*(1), 480. https://doi:10.1186/s12906- 016-1467-4

Tang, Y., Liu, J., Zhang, D., Xu, Z., Ji, J., & Wen, C. (2020). Cytokine storm in COVID-19: The current evidence and treatment strategies. *Frontiers in Immunology*, *11*, 1708. https://doi.org/10.3389/fimmu.2020.01708

Tezuka, Y., Stampoulis, P., Banskota, A. H., Awale, S., Tran, K. Q., Saiki, I., & Kadota, S. (2000). Constituents of the Vietnamese medicinal

plant *Orthosiphon stamineus*. *Chemical and Pharmaceutical Bulletin*, *48*(110),1711-1719.

Trott, O., & Olson, A. J. (2010). AutoDock Vina: Improving the speed and accuracy of docking with new scoring function, efficient optimization and multithreading. *Journal of Comptutational Chemistry*, *31*(2), 455-461. https://doi.org/10.1002/jcc.21334

Walls, C., Park., Y., Tortorici, M. A., Wall, A., McGuire, A. T., & Veesler, D. (2020). Structure, function and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell*, *181*(2), 281-292. https://doi.org/10.1016/j.cell.2020.02.058

Wu, Z., & McGoogan, J. M. (2020). Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: Summary of a report of 72314 cases from the Chinese Center for Disease Control and Prevention. *JAMA - Journal of the American Medical Association*, *323*(13), 1239–1242. https://doi.org/10.1001/jama.2020.2648

Zhong, Y. S., Yu, C. H., Ying, H. Z., Wang, Z. Y., & Cai, H. F. (2012). Prophylactic effects of *Orthisiphon stamineus* Benth. extracts on experimental induction of calcium oxalate nephrolithiasis in rats. *Journal of Ethnopharmacology*, *144*(3), 761-767.

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Effects of Planting Density on Growth and Yield Attributes of Rubber Trees (*Hevea brasiliensis*)

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ABSTRACT

This study aimed to identify rubber clones, suitable for rubber-timber production. An experiment was established in randomized complete block design to evaluate the effects of two different planting densities on girth, girth increment, bark thickness, latex yield per tapping per tree (g/t/t), incident of tapping panel dryness disease, and wood potential of nine rubber clones, including RRIV 2, RRIV 3, RRIV 4, RRIV 5, RRIV 107, PB 235, PB 260, PB 330, and RRIC 121. Data of girth, girth increment, bark thickness were collected in the 7th year, prior to opening for tapping, and 17th year of planting. In contrast, data of latex yield were collected in the 3rd and the 11th tapping year, and wood potential was compared based on the data collected in the 11th tapping year. As a result, there were no significant interactions between clones and planting density in girth growth, latex yield, tapping panel dryness and bark thickness, and the first tapping panel (BO-1). There were no significant interactions between clone and planting density on girth growth, latex yield, tapping panel dryness, and bark thickness when these clones were tapped on the first tapping panel (BO-1). Meanwhile, there were significant interactions between clones and planting density on girth growth, girth increment, and latex yield when the trees were tapped on the second tapping panel (BO-2). Statistical comparison of mean diameters at breast height and bole volume per tree of the same clones at two different planting densities showed that most of the studied clones gave significant differences. However, no significant differences resulted in the statistical comparison of the mean bole height of the same clones at two different planting densities. The total bole volume per hectare of all studied clones was larger at high planting density than at normal planting density. However, the bole volume per tree at high planting density was smaller

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than that at normal planting density. Clone RRIC 121 could be considered a suitable clone for latex and timber productions. The favorable planting density for commercial timber production is high.

Keywords: Growth, *Hevea brasiliensis*, latex yield, planting density, timber production

INTRODUCTION

The rubber tree (*Hevea brasiliensis* Müll. Arg.) is a deciduous perennial tree producing natural rubber belonging to the Euphorbiaceae family. *Hevea brasiliensis* is a native species of the Amazon basin and was imported into tropical countries in Asia and Africa during the late 19th century. In Vietnam, this species was brought into the country from Bogor (Indonesia) in 1897 by Alexandre Yersin (Lam et al., 2012). Since then, it has become one of the most important industrial crops and widely planted across the country. Currently, the total land planted with rubber trees in Vietnam is 932,400 hectares, and the natural rubber production is 1,226,100 tonnes with an average latex yield of 1,682 kg/ha/year. Most rubber plantations are in the Southeast region, followed by the Central Highlands, the Coastal region, and the newly developed areas in the Northern region.

Yield and timber production from the rubber tree is affected significantly by the size, the number, the distance, spatial arrangement of the adjacent trees, and planting density (Mäkinen, 1997). So far, the optimum planting density recommended for the rubber trees is 500–600 trees/ha is regardless of their genotypes or environmental conditions. At this density, the mature rubber trees would have enough space needed for their growth and development during their entire life cycles. Additionally, competition between the trees is in favor of the best production of dry rubber yield per hectare since planted in high densities, tree canopies could overlap, leading to the reduction of the size of the leaf canopy (Mäkinen, 1996), and the competition under the ground could also be high (Schroth, 1999). In rubber trees, stress induced by high tree densities was found to significantly reduce the girth and latex yield per tapping (grams/tree/tapping) (Obouayeba et al., 2005; Webster & Paardekooper, 1989). High planting densities contribute to delayed growth, taking a long time for the rubber trees to reach tappable girth and, therefore, the commencement of tapping.

Rubberwood is a by-product of rubber production as the rubber trees are mainly grown for latex and that this by-product is only available after 25–30 years when latex yield starts to decline significantly, and profit cannot compensate the cost of latex harvesting. At this time, replanting is required, and the old rubber trees need to be uprooted. At this stage, the by-product of the rubberwood could be sold as firewood for a brick manufacturing factory or even burnt on the spot for land clearing. However, in recent years, this by-product has gained increasing attention when technical problems in processing and utilization of rubberwood have been overcome, and rubberwood can be used to manufacture

a variety of products, especially furniture. That is why the combination of latex and wood production is becoming a popular trend in rubber-producing countries. Hence, one of the most important objectives in rubber breeding and selection is to produce rubber clones with high latex content, and high timber productivity, referred to as latex-timber clones. The present study aims to investigate the responses of growth, latex yield, and timber production of different rubber clones to two different planting densities to select the best rubber clones and their suitable planting density for latex-timber production.

MATERIALS AND METHODS

Experimental Design

The experiment was established in randomized complete block design with two replicates for two factors, including rubber clone (9 rubber clones: RRIV 2, RRIV 3, RRIV 4, RRIV 5, RRIV 107, PB 235, PB 260, PB 330, and RRIC 121) and planting density (571 and 1,111 trees/ha). Each experimental block was 1.0 ha. These trials were established in Lai Khe Experimental Station of the Rubber Research Institute of Vietnam located at Lai Hung commune, Bau Bang district, Binh Duong province, which is considered the traditional rubber growing region of Vietnam. For the planting density of 571 trees/ha, the inter-row spacing was 7.0 m, and the intra-row spacing was 2.5 m; meanwhile, the respective spacing for the planting density of 1,111 trees/ha was 3.0 m and 3.0 m.

Measurements

Growth measurements of rubber trees were taken annually in the immature stage, at which the girth of trees (cm) was measured at the height of 150 cm above the ground level using a graded tape measure. Girths of the tapped trees were measured annually at the height of 100 cm above the ground level, and girth increment under tapping (cm/year) was thereby calculated. The girth was measured at two different stages: the 7^{th} year of planting (the last year of immature phase and the first tapping year) and the 17^{th} year of planting (the 11^{th} tapping year). The girth increment under tapping was determined as the mean increase in girth per year between the 7^{th} and 17^{th} years of planting.

Latex is normally harvested after 6 - 7 years of planting when at least half of the total number of the trees in a plantation reaches the tappable girth of 50.0 cm or more at the height of 1.0 m above the ground. In order to harvest latex, the tappable rubber trees were opened for tapping at 1.3 m above the ground. The tapping system applied to harvest the annual latex yield of these two trials was the standardized one, S/2 d3 10 m/y, i.e., tapping the trunks in a half spiral (S/2) once every three days (d3) continuously for ten months of the year (10 m/y). On tapping days, latex was collected using the cup-coagulation method. Briefly, latex dripped into the plastic or ceramic cup equipped for each tapping tree, then, when latex flow stopped, 2–3% acetic acid solution was added into the cups and stirred well to coagulate the latex. Coagulated rubber from each cup was labeled carefully,

collected, and dried in the air for about one month before the dry rubber content of each rubber tree was weighed and calculated as gram per tree per tapping (g/t/t). Latex yield was recorded for two different stages of the tapping phase: the third tapping year (9-year-old trees were tapped on their first tapping panel) and the 11th tapping year (17-year-old trees were tapped on their second tapping panel, also referred to as 'BO-2 panel').

The thickness of virgin bark (mm) was measured prior to opening for tapping at 2–3 cm above the tapping panel using a bark gauge.

The bole height of rubber trees was measured using a laser hypsometer, namely Trimble LaserAce 1000 rangefinder (Trimble Navigation, USA). Bole volume per tree was calculated for each tree using stand volume models developed for rubber tree by Truong et al. (2003) as follows: $V = 10^{(-3,668)} \times D^{(1,629)} \times H^{(0,921)}$ where V is the bole volume (m^3/tree), D is the diameter measured at the breast height (1 m, cm), and H is the bole height (m), respectively. Latex yields were recorded at two different stages of the tapping phase: the third tapping year (9-year-old trees were tapped on their first tapping panel) and the 11th tapping year (17-year-old trees were tapped on their second tapping panel, also referred to as 'BO-2 panel').

The data of the criteria mentioned above were recorded on 100 rubber trees per clone per replicate, which were marked carefully by paint.

Tapping panel dryness was investigated and counted on the trees, which showed total bark dryness in each replicate of treatment and expressed as a percentage.

Statistical Analysis

The data collected from these trials were analyzed using the Statistical Analysis System (SAS) statistical package (SAS Institute Inc., 1999), independent sample *t*-test to compare means between the planting densities, and analysis of variance (PROC ANOVA) was implemented for the analysis of the balanced data; meanwhile, general linear model (PROC GLM) was applied to analyze the unbalanced data.

RESULTS AND DISCUSSION

Effects of Clones and Planting Density on Girth, Girth Increment, and Bark Thickness

Data on the girth of the rubber clones planted at two different densities were collected and compared at different growth and development phases. In this experiment, girth and bark thickness were compared when the rubber clones were 7-year-old, right before these clones were subjected to latex harvesting, and when these clones were 17-year-old, at this time, these clones were under the 11th tapping year. Girth increment per year calculated based on these two sets of data was referred to as girth increment under tapping in this study. As a result, a significant difference among clones was observed in girth at the 7th and the 17th year of planting, as well as in girth increment under the taping phase and bark thickness

in both planting densities (Table 1). Girth growth of the clones in the normal density was greater than that in the high density. Among the clones, RRIV 2 had the highest girth at the 7th and the 17th year of planting as well as the highest girth increment under tapping in both planting densities. Statistical analysis revealed that both clone and planting density significantly affected girth, bark thickness, and girth increment under tapping phase ($P < 0.001$) when these clones were at a different stage (Table 2). However, the interaction between clone and planting density was found on girth ($P < 0.001$) and

Table 1
Girth growth (cm) and girth increment under tapping (cm/year) of studied clones under two different planting densities

Clones	Planting density of 571 trees/ha			Planting density of 1,111 trees/ha		
	7th year of planting	17th year of planting	Girth increment	7th year of planting	17th year of planting	Girth increment
RRIV 2	54.7^{a}	74.5^{a}	2.00^{bcd}	44.5^{a}	64.5^{a}	2.68^{a}
RRIV 3	46.9^{cde}	65.6^{cd}	1.80^{cd}	38.1^{b}	52.3^{e}	1.14^{f}
RRIV 4	49.0^{bc}	65.3^{d}	1.64^{d}	40.0^{ab}	55.3^{d}	1.40^{ef}
RRIV 5	50.7^{b}	72.5^{ab}	2.19^{bc}	38.6^{b}	58.2^{bc}	1.73^{cde}
RRIV 107	48.1^{cd}	75.9^{a}	2.79^{a}	40.2^{ab}	58.8^{b}	2.24^{abc}
PB 235	48.4^{cd}	70.3^{bc}	2.23^{bc}	40.2^{ab}	64.0^{a}	2.04^{bcd}
PB 260	45.7^{e}	69.9^{bcd}	2.42^{ab}	38.5^{b}	56.0^{cd}	1.61^{def}
PB 330	47.7^{cde}	72.6^{ab}	2.48^{ab}	37.6^{b}	59.1^{b}	1.93^{cde}
RRIC 121	46.3^{de}	67.5^{cd}	2.07^{bcd}	39.3^{ab}	62.3^{a}	2.52^{ab}
CV (%)	1.77	2.81	9.11	5.9	1.67	11.91
F-value	20.15**	7.23**	6.44**	1.54^{ns}	34.79***	9.84**

Note. Means within columns with the same letter(s) are not significantly different at the 0.05 probability level. CV = Coefficient of variation; ^{ns}Non-significant; **Significant at 0.01 probability level; ***Significant at 0.001 probability level

Table 2
Effects of clone and planting density to growth and bark thickness

Source of variations	df	Mean square			
		Girth after 7th year of planting	Girth after 17th year of planting	Girth increment under tapping	Bark thickness
Replication	1	3.81551^{ns}	2.88434^{ns}	0.01174^{ns}	0.02054^{ns}
Clone	8	20.68630***	46.68405***	0.48846***	0.64070***
Density	1	717.16840***	1189.44514***	1.16280***	1.46410***
Clone x Density	8	2.72425^{ns}	15.49042***	0.29140**	0.13225**
Error	17	3.03136	2.33128	0.05666	0.01746
Mean	-	44.11199	64.66475	2.26979	6.09782
CV (%)	-	3.94683	2.36119	10.48218	2.16672

df = Degrees of freedom; CV = Coefficient of variation; ^{ns}Non-significant; **Significant at 0.01 probability level; ***Significant at 0.001 probability level

bark thickness ($P < 0.01$) girth increment under tapping ($P < 0.01$) when they were in the 17th year of planting, indicating that girth, girth increment, and bark thickness of the rubber clones seemed to be affected significantly by the planting densities during the tapping phase.

This study showed that the rubber trees planted in low density grew better than the trees planted in high density; therefore, they had larger circumference regardless of their ages. Better girth at the 7th year after planting indicated that trees planted at low density had a better growth rate than the trees planted closely. The previous study reported that during the tapping phase, the growth rate of the tree decreased significantly, and the growth during the immature stage played the key role in determining its future yields (Webster & Paardekooper, 1989) since low density during the immature phase supported the growth rate of those trees (Rodrigo et al., 1995). In addition, the finding of this study conformed to the finding reported by Naji and Sahri (2012) that trees, which were closely planted had slow growth rates.

Effects of Clone and Planting Density on Latex Yield and Tapping Panel Dryness

The mean latex yield of the studied clones at the 3rd and the 11th years of tapping in both planting densities were significantly different (Table 3). The results showed that the grams/tree/tapping yield (g/t/t) at the 3rd year of tapping of clone RRIV 4 was significantly greater than that of the other clones when this clone was planted in high planting densities. Meanwhile, RRIC 121 could be considered the best yielding clone

Table 3
Latex yield (grams/tree/tapping, g/t/t) and tapping panel dryness (TPD, %) of studied clones under two different planting densities

Clones	Planting density of 571 trees/ha			Planting density of 1,111 trees/ha		
	3rd year of tapping	11th year of tapping	TPD	3rd year of tapping	11th year of tapping	TPD
RRIV 2	52.88^{ab}	54.2^{bc}	13.39^{b}	43.89^{bc}	26.4^{cd}	10.09^{b}
RRIV 3	48.61^{abc}	53.6^{bc}	9.99^{b}	29.08^{e}	35.5^{bc}	16.45^{ab}
RRIV 4	59.17^{a}	52.1^{dc}	15.50^{b}	56.37^{a}	30.1^{bcd}	26.09^{ab}
RRIV 5	54.84^{ab}	55.7^{b}	9.93^{b}	47.64^{b}	34.7^{bc}	8.68^{b}
RRIV 107	56.11^{a}	50.1^{d}	12.01^{b}	34.67^{de}	23.8^{d}	21.57^{ab}
PB 235	54.38^{ab}	40.5^{e}	15.70^{b}	43.59^{bc}	30.1^{bcd}	28.01^{ab}
PB 260	47.78^{abc}	49.8^{d}	14.86^{b}	37.61^{cd}	36.1^{bc}	43.27^{a}
PB 330	43.18^{bc}	58.7^{a}	27.06^{a}	33.08^{de}	37.5^{b}	27.14^{ab}
RRIC 121	38.34^{c}	59.1^{a}	8.58^{b}	31.54^{de}	49.4^{a}	16.00^{ab}
CV (%)	9.68	2.68	31.51	7.80	12.29	56.84
F-value	3.74^{*}	33.32^{***}	3.07^{ns}	16.32^{***}	6.54^{**}	1.48^{ns}

Note. Means within columns with the same letter(s) are not significantly different at the 0.05 probability level. CV = Coefficient of variation; TPD = Tapping panel dryness; ^{ns}Non-significant; *Significant at 0.05 probability level; **Significant at 0.01 probability level; ***Significant at 0.001 probability level

in gram/tree/tapping at the 11th year of both planting densities. Regarding tapping panel dryness (TPD), there was no significant difference in TPD incidence among the clones in both planting densities.

It was revealed that there was no interaction between clones and planting densities on grams/tree/tapping yield at the 3rd year of tapping ($P > 0.05$) (Table 4), which suggested that planting density seemed to have similar effects on all studied clones during the tapping years on the first tapping panel (BO-1). Similarly, no marked link between the density of planting and the occurrence of tapping panel dryness ($P > 0.05$) indicated that the effect of planting density on clones was seemly similar (Table 4). This finding agreed with the previous study, which also showed no clear interaction between clone and planting density in TPD incidence (Obouayeba et al., 2005). Conversely, there was an interaction between clones and planting densities in grams/tree/tapping yield at the 11th year of tapping ($P < 0.01$) (Table 4), indicating that clones seemed to be affected by the planting density during the years of tapping on the second tapping panel (BO-2).

Although the yield per tree decreases, higher yields of timber production per hectare can be obtained by employing higher planting densities (Obouayeba et al., 2005), and optimum tree densities have been identified in consideration of this fact. Nevertheless, before implementing this cultural practice, it is very important to consider the costs of planting materials, planting practices, and maintenance of the plantations during the immature and mature phases, as well as costs of latex harvest and manufacture because these costs could be higher when a greater number of trees are maintained under high densities.

Effects of Clones and Planting Densities on Timber Production

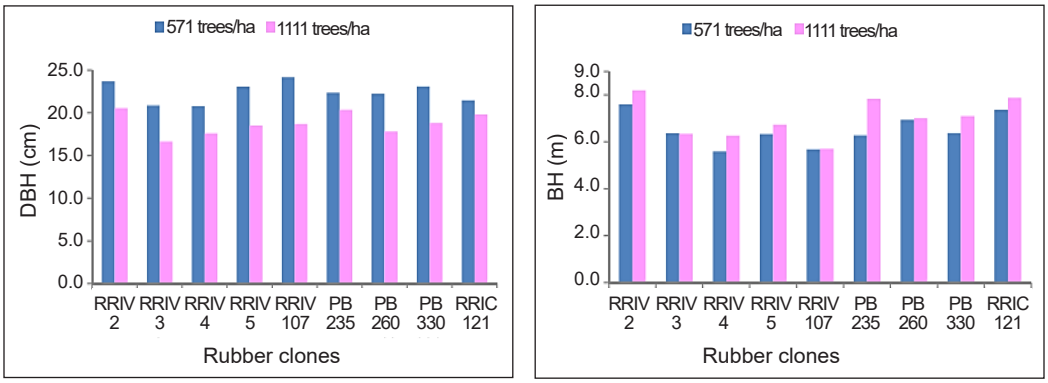
Diameter at Breast Height (DBH) and Bole Height (BH). In general, a negative correlation between DBH value and planting density was revealed, as shown in Figure

Table 4
Effects of clone and planting density to latex yield and tapping panel dryness

Source of variations	df	Mean square		
		Individual yield of 3rd year of tapping	Individual yield of 11th year of tapping	Tapping panel dryness (TPD)
Replication	1	0.93767^{ns}	45.11361^{ns}	979.48134^{ns}
Clone	8	210.50972***	136.35948***	199.77482^{ns}
Density	1	1063.73823***	3259.26810***	548.96490^{ns}
Clone x Density	8	35.83646^{ns}	42.71591**	90.32911^{ns}
Error	17	16.62380	10.741229	194.54552
Mean	-	45.15250	43.24500	18.01722
CV (%)	-	9.02991	7.578637	77.41458

CV = Coefficient of variation; ^{ns}Non-significant; **Significant at 0.01 probability level; ***Significant at 0.001 probability level

1a. Table 5 shows that the mean DBH calculated for each clone was smaller as the planting density increased from 571 trees/ha to 1,111 trees/ha. The results showed that, in the same planting density, the mean DBH was significantly different among the nine studied clones (Table 5). However, a statistical comparison of mean DBH of the same clones planted at two different planting densities revealed significant differences in five out of nine studied clones (Table 6). These results indicated that intra-row and inter-row spacing significantly affected the diameter of the rubber clones. The trees



(a) (b)

Figure 1. Effect of two different planting densities to diameter at breast height (a) and bole height (b) of the studied clones

Table 5
Diameter at breast height (DBH) and bole height (BH) of studied clones under two different planting densities

Clones	Planting density of 571 trees/ha				Planting density of 1,111 trees/ha			
	DBH (cm)		BH (m)		DBH (cm)		BH (m)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
RRIV 2	23.69^{a}	5.93	7.6^{a}	2.84	20.49^{a}	4.56	8.2^{a}	2.46
RRIV 3	20.87^{cd}	3.80	6.4^{c}	2.10	16.90^{e}	2.26	6.4^{bc}	1.58
RRIV 4	20.81^{d}	4.32	5.6^{d}	1.33	17.72^{d}	3.14	6.3^{bc}	1.39
RRIV 5	23.12^{ab}	5.56	6.4^{c}	1.96	18.54^{bc}	2.67	6.8^{abc}	1.49
RRIV 107	24.15^{a}	5.39	5.7^{d}	2.21	18.85^{b}	3.27	5.7^{c}	2.15
PB 235	22.39^{bc}	5.37	6.3^{c}	2.14	20.45^{a}	4.24	7.9^{ab}	1.75
PB 260	22.25^{bcd}	4.12	7.0^{b}	2.27	17.89^{cd}	2.55	7.0^{abc}	1.67
PB 330	23.11^{ab}	5.71	6.4^{c}	2.10	18.38^{b}	3.47	7.1^{abc}	1.60
RRIC 121	21.48^{cd}	3.69	7.4^{a}	1.96	20.20^{a}	4.05	7.9^{ab}	2.37
CV (%)	2.81	-	2.97	-	1.67	-	9.40	-
F-value	7.23**	-	24.91***	-	34.79***	-	3.29*	-

Note. Means within columns with the same letter(s) are not significantly different at the 0.05 probability level. CV = Coefficient of variation; DBH = Diameter at breast height; BH = Bole height; SD = Standard deviation; *Significant at 0.05 probability level; **Significant at 0.01 probability level; ***Significant at 0.001 probability level

Table 6
Independent t-test comparing the diameter at breast height, bole height and bole volume per tree of the same clones between two planting densities

Clones	Planting density	Diameter at breast height			Bole height			Bole volume per tree		
		t-value	Pr >|*t*|	SE	*t*-value	Pr >|*t*|	SE	*t*-value	Pr >|*t*|	SE
RRIV 2	D1-D2	0.65	0.5137	0.7506	-1.77	0.0790	0.3668	-0.56	0.5769	0.0222
RRIV 3	D1-D2	2.96	**0.0038**	0.5246	1.49	0.1401	0.2833	2.54	**0.0123**	0.0116
RRIV 4	D1-D2	-0.04	0.9659	0.5846	-1.04	0.3003	0.1653	-0.33	0.7400	0.0093
RRIV 5	D1-D2	1.92	**0.0496**	0.7776	0.01	0.9908	0.2638	1.90	**0.0498**	0.0151
RRIV 107	D1-D2	4.21	**<0.0001**	0.6594	-1.42	0.1584	0.2914	3.02	**0.0032**	0.0166
PB 235	D1-D2	0.80	0.4270	0.7030	-0.69	0.4943	0.2904	0.10	0.9182	0.0156
PB 260	D1-D2	1.95	**0.0476**	0.5967	0.09	0.9309	0.2870	1.91	**0.0482**	0.0141
PB 330	D1-D2	2.14	**0.0344**	0.7218	-0.87	0.3887	0.2668	1.98	**0.0457**	0.0161
RRIC 121	D1-D2	1.80	0.0744	0.4763	-1.06	0.2931	0.2610	0.41	0.6848	0.0117

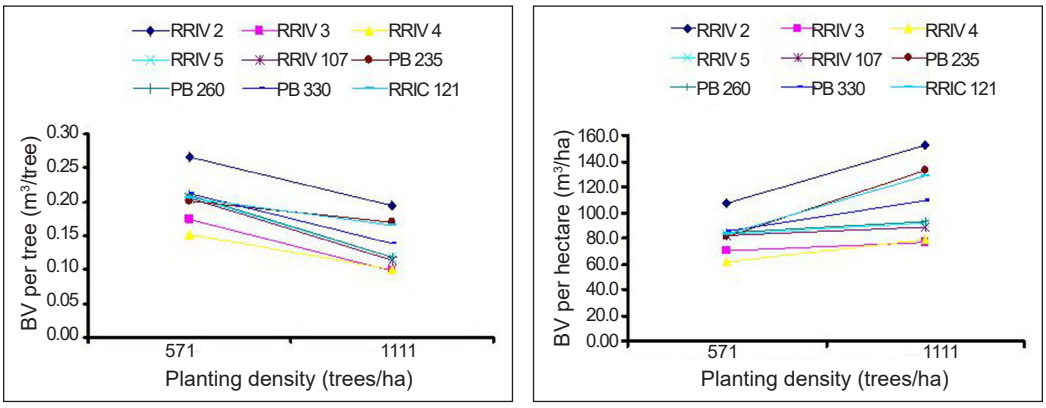
Note. Bold type indicates significant difference at the 0.05 probability level. SE = Standard error. D1 and D2 represent normal and high planting densities, respectively

with the largest diameter were found among trees planted at the density of 571 trees/ha, while the trees with the smallest diameter were seen in the high planting density of 1,111 trees/ha, as clearly shown in RRIV 107, RRIV 3, PB 330, PB 260, and RRIV 5. This result agreed with the previous studies, which reported that an increase in circumference of a tree depended on the growth ring and, therefore, depended on the increase of the diameter (Cockerham, 2004; Lei et al., 1997; Scott et al., 1998). Wider spacing supported plants to grow better, resulting in larger stem girth.

The mean BH of the studied rubber clones increased when these rubber clones were planted more densely (Figure 1b), suggesting that the BH of the rubber trees could have a was positive correlation with the tree density. The results showed that mean bole height was significantly different among nine rubber clones when these clones were planted in the same density (Table 5). Generally, intra-row and inter-row spacing significantly affected the bole height of the rubber trees. Those trees with the largest BH and smallest BH were found among the rubber trees planted in high density and low density. A similar result was reported and explained that in densely plantations, competition for light and less space for expansion promoted plants to grow in height (Nasir et al., 2006). However, statistical analysis revealed that the difference in the mean BH of the rubber trees of the same rubber clone was not statistically significant regardless of the planting densities (Table 6).

Bole Volume per Tree and per Hectare. Bole volume was calculated for each tree using the stand volume model developed for rubber trees by Truong et al. (2003). Total wood production/hectare was calculated for each rubber clone in each planting density using data of individual trees. The mean bole volume (BV) per tree was smaller in

planting density of 571 trees/ha than that in planting density of 1,111 trees/ha (Figure 2a), suggesting there was possibly a negative correlation between BV of individual trees and planting density. In contrast, the total bole volume (BV) per hectare was greater in trials of 1,111 trees/ha than that in trials of 571 trees/ha irrespective of the rubber clones (Figure 2b), indicating that there was likely a positive correlation between total BV per hectare and planting density. In addition, the results showed that the mean bole volume per tree and hectare was significantly different among the rubber clones regardless of the planting density (Table 7). The independent sample *t*-test proved that there



(a) (b)

Figure 2. Effect of two different planting densities to bole volume per tree (a) and total bole volume per hectare (b) of nine rubber clones

Table 7
Bole volume per tree and per hectare of studied clones at two different planting densities based on bole height and diameter at breast height

Clones	Planting density of 571 trees/ha		Planting density of 1,111 trees/ha	
	Bole volume per tree (m^3/tree)	Bole volume per hectare (m^3/ha)	Bole volume per tree (m^3/tree)	Bole volume per hectare (m^3/ha)
RRIV 2	0.270^{a}	106.70^{a}	0.195^{a}	152.35^{a}
PB 330	0.215^{b}	85.05^{b}	0.140^{bc}	108.95^{bcd}
PB 260	0.215^{b}	84.60^{b}	0.120^{bc}	92.65^{cd}
RRIV 5	0.210^{b}	83.70^{b}	0.120^{bc}	92.15^{dc}
RRIC 121	0.205^{bc}	82.85^{b}	0.165^{bc}	129.00^{abc}
RRIV 107	0.205^{bc}	82.20^{b}	0.115^{bc}	88.50^{d}
RRIV 3	0.175^{cd}	70.10^{c}	0.095^{c}	77.10^{d}
RRIV 4	0.155^{d}	61.05^{c}	0.100^{c}	79.15^{d}
PB 235	0.205^{bc}	80.90^{b}	0.170^{ab}	132.50^{ab}
CV (%)	6.18	5.44	16.17	15.11
F-value	12.06^{***}	15.28^{**}	4.90^{**}	5.50^{**}

Note. Means within columns with the same letter(s) are not significantly different at the 0.05 probability level. CV = Coefficient of variation; **Significant at 0.01 probability level; ***Significant at 0.001 probability level

was a statistically significant difference in the bole volume per tree recorded for those rubber clones of RRIV 107, RRIV 3, PB 330, PB 260, and RRIV 5 between two planting densities ($P < 0.05$) (Table 6). A greater total bole volume per hectare of each studied clone was found in high tree density compared to normal tree density (Table 7). These results were supported by findings reported in previous studies (Naji & Sahri, 2012; Wei et al., 2005), which revealed that better wood biomass could be obtained from intensive planting density for commercial production. In short, high planting density is a better choice for commercial rubberwood production. Although low planting density could help obtain bigger and heavier individual trees, it could not help to compensate for the difference in biomass brought by the total bole volume per hectare in densely planted populations. In addition to the findings mentioned above, this study also revealed that clones RRIV 2 were suitable for timber production when planted in both densities while RRIC 121 and PB 235 were suitable for timber production in high planting density as these clones had higher BV per hectare than the other clones.

CONCLUSION

Performances of the rubber clones in terms of girth, girth increment, bark thickness, latex yield, and rubberwood production were significantly affected by planting density and rubber clones, while these two factors did not affect the tapping incident panel dryness disease. There was no interaction between rubber clones and planting density on girth measured on the 7th year of planting (immature stage) and latex yield when tapped on the first tapping panel (BO-1, the third tapping year). Meanwhile, there were significant interactions between rubber clones and tree spacing on the girth increment as well as the latex yield when the trees were under tapping on the second tapping panel (BO-2, the 11th tapping year).

In high planting density, RRIV 2 and RRIC 121 were recorded as the suitable clones for timber production and latex yield, respectively. Regarding both latex and timber productions, RRIC 121 was recorded as the best clone that gave the high latex yield and the high timber production in the normal or high planting density.

Variations in the diameter measured at the breast height and the bole height due to planting density resulted in a significant reduction in the wood potential of the less dense rubber plantations. The diameter of *H. brasiliensis* measured at breast height was greater at normal planting density. Meanwhile, the bole height in high planting density was positively correlated with the number of rubber trees per hectare. As a result, planting the rubber trees more densely is a favorable solution for the commercial production of rubber timber.

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REFERENCES

Cockerham, S. T. (2004). Irrigation and planting density affect river red gum growth. *California Agriculture*, *58*(1), 40-43. https://doi.org/10.3733/ca.v058n01p40

Lam, L. V., Thanh, T., Trang, L. T. T., Truong, V. V., Lam, H. B., & Tuy, L. M. (2012). *Hevea* germplasm in Vietnam: Conservation, characterization, evaluation and utilization. In M. Caliskan (Ed.), *Genetic diversity in plants* (pp. 433-456). InTech Publisher. https://doi.org/10.5772/35086

Lei, H., Gartner, L. B., & Milota, M. R. (1997). Effect of growth rate on the anatomy, specific gravity, and bending properties of wood from 7-year-old red alder (*Alnus rubra)*. *Canadian Journal of Forest Research*, *27*(1), 80-85. https://doi.org/10.1139/x96-165

Mäkinen, H. (1996). Effect of inter tree competition on biomass production of *Pinus sylvestris* (L.) half-sib families. *Forest Ecology and Management*, *86*(1-3), 105-112. https://doi.org/10.1016/S0378-1127(96)03788-7

Mäkinen, H. (1997). Possibilities of competition indices to describe competitive differences between Scots pine families. *Silva Fennica*, *31*(1), 43-52. https://doi.org/10.14214/sf.a8509

Naji, H. R., & Sahri, M. H. (2012). Intra- and inter-clonal tree growth variations of *Hevea brasiliensis*. *Journal of Forestry Research*, *23*(3), 429-434. https://doi.org/10.1007/s11676-012-0280-2

Nasir, M. A., Aziz, A., Mohar, T. A., Rehman, M. A., & Ahmad, S. (2006). Effect of planting distance on tree growth and fruit quality of shamber grapefruit under agro climatic conditions of Sargodha. *Journal of Agricultural Research, 44*(4), 353-358.

Obouayeba, S., Dian, K., Boko, A. M. C., Gnagne, Y. M., & Ake, S. (2005). Effect of planting density on growth and yield productivity of *Hevea brasiliensis* Muell. Arg. clone PB 235. *Journal of Rubber Research*, *8*(4), 257-270.

Rodrigo, V. H. L., Nugawela, A., Pathiratna, L. S. S., Waidyanatha, U. P. S., Samaranayake, A. C. I., Kodikara, P. B., & Weeralal, J. L. K. (1995). Effect of plant density on growth, yield, and yield related factors and profitability of rubber (*Hevea brasiliensis* Muell. Arg.). *Journal of Rubber Research Institute of Sri Lanka*, *76*, 55-71.

SAS Institute Inc. (1999). *SAS/STAT user's guide* (version 8.01). SAS Institute.

Schroth, G. (1999). A review of below ground interactions in agroforestry, focusing on mechanisms and management options. *Agroforestry Systems*, *43*(1), 5-34. https://doi.org/10.1023/A:1026443018920

Scott, W., Meade, R., & Leon, R. (1998). Planting density and tree-size relations in coast Douglas-fir. *Canadian Journal of Forest Research*, *28*(1), 74-78. https://doi.org/10.1139/x97-190

Truong, V. V., Lam, L. V., Tuy, L. M., & Hoa, T. T. T. (2003, November 12-14). *An approach for estimation of wood volume of the main stem of rubber stands* [Paper presentation]. Proceedings of the Workshop on Rubber, Wood, Croping and Research, Bangkok, Thailand.

Webster, C. C., & Paardekooper, E. C. (1989). The botany of the rubber tree. In C. C. Webster & W. J. Baulkwill (Eds.), *Rubber* (pp. 57-84). Longman Scientific and Technical.

Wei, H. Y., Wang, Y., Wang, Z., & Yan, X. (2005). Effect of planting density on plant growth and camptothecin content of *Camptotheca acuminate* seedlings. *Journal of Forestry Research*, *16*(2), 137-139. https://doi.org/10.1007/BF02857907

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Nutritional Effects of Different Calcium Sources on Growth of Oil Palm Seedlings under Nursery Condition

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ABSTRACT

Calcium (Ca) is an essential secondary macronutrient which necessary plant mineral frequently added to fertilizers to promote plant development and resistance to abiotic and biotic stressors. Applying Ca to soils suffices to meet crops' Ca requirements. Regrettably, its function is obscure. Thus, it is critical to maintain enough nutrient availability through fertilizers or alter the soil environment for oil palm seedlings to grow and thrive. This study investigates the effects of different Ca sources on vegetative growth in oil palm seedlings. This experiment was carried out for nursery evaluation using 5-months old of oil palm seedlings with varying sources of Ca (C1–calcium chloride, $CaCl_2$; C2–calcium sulfate, $CaSO_4$; C3-calcium nitrate, $CaNO_3$; C4–calcium carbonate, $CaCO_3$; C5–calcium oxide, CaO, C6–calcium hydroxide, $Ca(OH)_2$; and C7–water leach purification and neutralization underflow, NUF-WLP) and grown in a polybag containing beach ridges interspersed with swales (BRIS) soil within six months in Malaysian Palm Oil Board (MPOB) nursery, Seksyen 15, Bandar Baru Bangi. Five concentration levels of Ca (T1–200 ppm, T2–250 ppm, T3–300 ppm, T4–1,000 ppm, and T5–1,500 ppm) were used in a completely randomized design

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(CRD) with ten replications for each. Based on the total biomass of oil palm seedlings at 24 weeks after treatment with various sources of Ca, the result confirmed that C2 oil palm seedlings were more significant in size and denser at the root than other Ca types. The result is an essential indicator that C2 effectively increased the total biomass of oil palm seedlings at 1,000 ppm of Ca (T4); hence it was the best Ca source to improve the growth and development of oil palm seedlings throughout the experimental period at $p \leq 0.05$.

Keywords: Calcium, non-soluble Ca, oil palms, optimum rate, water-soluble Ca

INTRODUCTION

Sustainable agriculture has been one of the most significant problems in agriculture in recent years. However, plant nutrient interactions and plant responses to stimuli are various. Nutrients are essential for plants' growth and development, as they provide vital and precise plant metabolism functions (Agrios, 2005). Therefore, it is crucial to maintain adequate nutrients in the soil through fertilizers or by manipulating soil conditions to maintain optimum nutrient availability in soil. Mineral nutrients would be an important metabolic regulator, widely used in fertilizers to promote plants' growth by controlling plant metabolism. Moreover, many macro and micro-nutrients are recognized as correlated with some plants' vegetative growth changes.

Ca is an essential macronutrient in plants, with shoot concentrations varying from 0.1% to over 5% per dry weight (Marschner, 1995; White & Broadley, 2003). It was found primarily on cell organelles such as the cell wall and the endoplasmic reticulum, and it was crucial in regulating plant growth and development (Thor, 2019). Therefore, it has a dual role, both as a structural part of cell walls and membranes as well as an intracellular second messenger. Consequently, the plant's uptake, delivery, and storage need to be closely controlled to fulfill both missions. In addition, Ca is also crucial for many plant functions, including cell division, cell wall growth, nitrate uptake, metabolism, enzyme activity, and starch metabolism. Thus, the Ca must be accessible to the plant in adequate quantities to fulfill the structural function.

The Ca is present in three primary forms in the soils, which are bound to exchangeable sites of clay minerals, organic matter, and minerals in soil solution (Ramírez-Builes et al., 2020). The soil solution contains only a minuscule proportion of the total Ca. Therefore, plant roots from the soil solution can absorb only calcium ions (Ca^{2+}). Several factors influence the availability of Ca in soil solution, including soil type, colloidal mineral fraction, pH, organic carbon concentration, humic acids, and exchangeable cation capacity (CEC). The Ca deficiency is most significant in acidic and sandy soils, as well as soils with high aluminum ion (Al^{3+}) saturation and a high Al^{3+} to Ca^{2+} ratio in soil solution (Bolan et al., 2007).

Ca is frequently administered to plant seedlings during the growing period to

boost their development potential (Chao et al., 2021). By adding Ca to oil palm, it can be an effective way to promote the growth and productivity of plants. The Ca used exists in the form of an exchangeable cation in the nutrient solution is the main component that can transfer to and absorb by plant roots (Rahman & Punja, 2007). Exchangeable Ca is generally predominant in a nutrient solution than potassium (K) and magnesium (Mg), the next two most abundant exchangeable cations (McLean, 1975). The Ca is withdrawn from the nutrient solution through plasma membrane channels expressed in roots (White et al., 2002).

The most vital sources of Ca fertilizer used in agriculture are calcium sulfate ($CaSO_4$) or gypsum; calcium carbonate ($CaCO_3$) promoted as ground limestone or screened chalk and ground chalk; calcium hydroxide ($Ca(OH)_2$) retailed as slaked lime or hydrated lime; and calcium oxide (CaO) promoted as burnt or quick lime (Minson, 1990). However, the ability of various sources of Ca as a fertilizer to improve growth and plant production in oil palm remains unknown. Based on a previous experiment, 1,000 ppm of Ca in nutrient solution established optimum growth for oil palm seedlings (Mayzaitul Azwa, 2021). However, further research is required to identify the potential effects of different Ca sources with an optimum concentration of Ca (1,000 ppm) on the vegetative growth of oil palm seedlings. This research was, therefore, conducted to determine the effects of different Ca sources on vegetative growth in oil palm seedlings.

MATERIALS AND METHODS

Study Site

The experiment was conducted under transgenic glasshouse conditions in Taman Pertanian Universiti, Universiti Putra Malaysia, Serdang, Selangor. Five-months-old oil palm seedlings were obtained from MPOB Kluang Research Station, Johor. All seedlings were planted in black polybags (35.56 cm height × 35.56 cm diameter) filled with 25 kg of sandy soil (> 95% sand) developed from marine sediments along the beach, commonly called the beach ridges interspersed with swales (BRIS) soil series collected from Forest Research Institute Malaysia (FRIM) Setiu Research Station, Terengganu. Sandy soil was selected as the growing media as it has zero nutrients and makes it possible to control the nutrient supply and uptake by oil palm seedlings. The experiment was conducted in a transgenic glasshouse, which had 24 hours controlled and monitored environment. It provided a sensor to detect temperature and relative humidity changes in a glasshouse. Thus, the temperature was maintained between 30°C and 36°C with relative humidity between 60% and 80%. The seedlings were maintained by watering twice daily and supplemented with monthly fertilizers throughout the experiment. For the water-soluble Ca experiment, the fertilizer is based on Hoagland solution (Table 1), while for non-soluble Ca experiment is based on basic fertilizer urea (nitrogen, N: 46%); monopotassium phosphate, MKP (phosphorus pentoxide, P_2O_5: 52%; potassium oxide, K_2O: 34%); muriate of

potash, MOP (potassium oxide, K_2O: 60%); kieserite (magnesium oxide, MgO: 25%); and borax (boron, B: 68%).

Table 1
Elements in Hoagland's solution based on Hoagland and Arnon (1950) for 120 L of stock solution

Stock solution	Type of fertilizer	Quantity
Stock A	Water	120 L
	EDTA-Fe	228 g
Stock B	Water	120 L
	KNO_3	8.4 kg
	KH_2PO_4	3.6 kg
	$MgSO_4$	4.8 kg
	EDTA-Mn	21.6 kg
	EDTA-Zn	18 g
	EDTA-Cu	4.8 g
	Ammonium molybdate	3.6 g
	Hibor	3.6 g

(Hoagland & Arnon, 1950)

Experimental Design and Treatments

A factorial experiment was conducted with ten replicates with three seedlings, each using a completely randomized design (CRD) experiment. Seven types of Ca sources were used in this experiment. The oil palm seedlings were treated with water-soluble and non-soluble Ca for six months throughout the experiment (Table 2). All the oil palm seedlings were sub-irrigated with modified Hoagland solution (different levels of Ca concentrations: T1–200 ppm, T2– 250 ppm, T3–300 ppm, T4–1,000 ppm, and T5–1,500 ppm). The pH of the nutrient solution was maintained at pH 6.0 by adjusting with 0.1 N hydrochloric acid (HCl) or 0.01 N sodium hydroxide (NaOH) solution and changed every week. A total of five treatment combinations were used in this study (Table 3). For the water-soluble Ca, the oil palm seedlings were supplemented with Ca fertilizer per 120 L of Hoagland solution per week throughout the six months of the experiment (Table 4). The rates of Ca concentration were T3 (300 ppm, 24 applications, total 7,200 ppm per seedlings), T4 (1,000 ppm, 24 applications, total 24,000 ppm per seedlings), and T5 (1,500 ppm, 24 applications, total 36,000 ppm). Whereas in the case of non-soluble Ca, the oil palms seedlings were added with Ca fertilizers per 25 kg of BRIS soil

Table 2
Treatments of oil palm seedlings with different Ca sources and solubility in water for plant growth analysis

	Calcium sources	Solubility in water
C1	Calcium chloride ($CaCl_2$)	Soluble
C2	Calcium sulphate ($CaSO_4$)	
C3	Calcium nitrate ($CaNO_3$)	
C4	Calcium carbonate ($CaCO_3$)	Non-soluble
C5	Calcium oxide (CaO)	
C6	Calcium hydroxide ($CaOH_2$)	
C7	Water leach purification and neutralization underflow (NUF-WLP)	

Table 3
Combination of treatments used

Treatment	Ca concentration (ppm)
T1	200
T2	250
T3	300
T4	1,000
T5	1,500

Note. T1, T2, and T3: Low concentration rate; T4: Optimum rate; T5: Excess concentration rate

Table 4
Application rate of Ca types of solubility in water

Ca sources	Application rate	Concentration of Ca per palm (ppm)				
		T1	T2	T3	T4	T5
C1	120 L of Hoagland solution	-	-	0.80 L	3.00 L	4.00 L
C2		-	-	0.88 L	2.70 L	4.40 L
C3		-	-	0.90 L	3.05 L	4.55 L
C4	25 kg of BRIS soil	7.00 g	16.80 g	14.00 g	-	-
C5		7.25 g	17.40 g	14.50 g	-	-
C6		12.51 g	30.02 g	25.0 g	-	-
C7		20.00 g	25.00 g	30.00 g	-	-

Note. A negative symbol is used to mention that no experiment for the treatment as mentioned above. C1 –$CaCl_2$, C2 – $CaSO_4$, C3 – $CaNO_3$, C4 – CaO, C5 – Ca $(OH)_2$, C6 – $CaCO_3$, and C7 – NUF-WLP (2 NUF:1 WLP). T1 – 200 ppm, T2 – 250 ppm, T3 – 300 ppm, T4 – 1,000 ppm, and T5 – 1,500 ppm

per month throughout the six months of the experiment (Table 4). The rates of Ca concentration were T1 (200 ppm, six applications, total 1,200 ppm per seedlings), T2 (250 ppm, six applications, total 1,500 ppm per seedlings), and T3 (1,000 ppm, six applications, total 6,000 ppm per seedlings).

No experiments were done for treatment T1 (200 ppm of Ca) and T2 (250 ppm of Ca) for water-soluble Ca. After being tested on their pH during pre-experiment, both treatments gave the range of soil pH below the optimal availability of plant nutrients (6.0 to 7.0). For non-soluble Ca, no experiments were done for treatment T4 (1,000 ppm) and T5 (1,500 ppm). Both treatments gave the range of soil pH above the optimal availability of plant nutrients (6.0 to 7.0).

Measurements of Vegetative Growth

The plant height (cm), girth diameter (mm), and the chlorophyll content (soil plant analysis development, SPAD) values were obtained three times throughout the experiment period, which was at two-, four-, and six-month intervals throughout the experiment period.

Data Analysis

The data were statistically analyzed using a two-way analysis of variance (ANOVA) from the Statistical Analysis Software (SAS) 9.2 package. Two-way ANOVA was used to understand whether there was an interaction between types of Ca sources on the growth of oil palm seedlings and different levels of Ca concentrations. Where types of Ca sources and different levels of Ca concentrations were independent variables, and the development of oil palm was the dependent variable. Means separation was conducted using the least significant difference (LSD) at a 5% significance level.

Pre-experiment (Water Field Capacity)

Water field capacity in BRIS soil was measured to calculate the amount of soil moisture or water content kept in the soil using the following formulae:

$$Moisture\ content\ (\%\ w/w) = \left[\left(\frac{weight\ of\ fresh\ soil\ -weight\ of\ dry\ soil}{weight\ of\ wet\ soil}\right) \times 100\right]$$

Pre-experiment (Soil pH of Oil Palm Seedlings)

All Ca sources used in the experiment were tested on their pH at a different concentration to determine which treatment gave the soil pH range the optimal availability of plant nutrients (6.0 to 7.0). The experiment was carried out for six months, and the soil pH was measured at two months intervals.

RESULTS AND DISCUSSION

Plants Height for Oil Palm Treated with Soluble and Non-soluble Ca Sources

Throughout the experiment period, there was no interaction between soluble Ca sources with Ca concentration rates at $p \geq 0.05$ (Table 5). Generally, the oil palm seedlings showed an increase in their height after two months treated with various types of water-soluble Ca at different concentration rates (low, optimum, and excess). At six months, the mean height of oil palm seedlings at 1,000 ppm of Ca (T4) treated with C3 was 7.23% higher than that in C2 and 10.50% higher than in C1. The mean height of oil palm seedlings at 1,000 ppm of Ca (T4) was almost the same as in C2 and C1, about 3.58%.

A similar trend was also observed in the height of oil palm seedlings treated with various types of non-soluble Ca (Table 6). As the seedlings grew old, the differences among the seedling's height became more pronounced. However, there was no interaction between non-soluble Ca sources with Ca concentration rates at $p \geq 0.05$. However, interactions existed between types of Ca used in the experiment with the time (month) at $p \leq 0.05$. All seedlings were observed to increase significantly in height after two to six months of treatment with various types of non-soluble Ca. After six months of treatment, the height of the seedlings at 300 ppm of Ca (T3) recorded in C7 was 3.19% significantly higher than in C6, 6.68% higher than in C4 15.48% higher than in C5. The seedlings' height treated with C6 was 3.61% higher than in C4 and 12.7% higher than in C5. The seedlings treated in C4 were 9.43% higher than in C5.

Girth for Oil Palm Treated with Soluble and Non-soluble Ca Sources

Throughout the experiment, an interaction between Ca water-soluble forms with different Ca concentrations was observed at $p \leq 0.05$ (Table 7). There was an interaction between soluble Ca sources with Ca concentration rates at $p \leq 0.05$ (Table 7). The girth of oil palm seedlings showed an improvement after two months of treatment with various forms of water-soluble Ca at different concentration rates (low, optimum, and excess). The mean value of girth at 1,000 ppm of Ca (T4) treated with C3 was 32.98% higher than that in C2 and 27.78% higher than in C1 over six months of experimental time. The mean value of the girth of oil palm seedlings at 1,000 ppm of

Table 5
Plant height for oil palm seedlings treated with soluble Ca

Sources of Ca	2 months						4 months						6 months					
	Ca concentration (ppm)																	
	T3		T4		T5		T3		T4		T5		T3		T4		T5	
C1	22.60c	± 0.48	23.10b	± 0.82	24.70a	± 0.99	45.20a	± 1.64	40.10b	± 0.70	44.15ab	± 1.86	66.30a	± 4.55	61.90a	± 4.54	66.40a	± 2.04
C2	28.40a	± 0.56	29.50a	± 1.06	22.10b	± 0.72	42.15a	± 4.74	46.18a	± 1.33	46.52a	± 2.48	65.50a	± 3.66	64.20a	± 1.99	64.40a	± 3.22
C3	25.60b	± 1.25	25.90b	± 0.87	23.50ab	± 0.98	47.15a	± 2.06	36.10b	± 2.00	41.30b	± 1.52	55.70a	± 7.71	69.20a	± 2.98	61.10a	± 4.29

Note. C1 - $CaCl_2$, C2 - $CaSO_4$, and C3 - $CaNO_3$. T3 - 300 ppm, T4 - 1,000 ppm, and T5 - 1,500 ppm. Data are means ± standard error using LSD ($p \leq 0.05$). Means with the same letter in the column are not significantly different

Table 6
Plant height for oil palm seedlings treated with non-soluble Ca

Sources of Ca	2 months						4 months						6 months					
	Ca concentration (ppm)																	
	T1		T2		T3		T1		T2		T3		T1		T2		T3	
C4	27.30a	± 1.10	42.30a	± 0.85	59.50ab	± 2.40	27.30a	± 2.12	39.70a	± 0.60	64.10a	± 1.56	28.10a	± 2.47	37.45a	± 1.91	55.70a	± 2.93
C5	23.00b	± 1.60	40.15a	± 1.12	53.00a	± 1.70	23.10b	± 4.55	37.35a	± 2.18	61.70a	± 1.28	25.80a	± 3.88	39.10a	± 3.19	63.80a	± 2.00
C6	23.40b	± 1.10	37.95a	± 1.50	60.80a	± 1.73	23.80b	± 2.10	37.50a	± 1.32	61.50a	± 0.83	27.60a	± 3.23	40.50a	± 3.86	61.50a	± 2.95
C7	24.40ab	± 1.41	40.30a	± 1.05	63.00a	± 1.19	24.50ab	± 1.37	39.05a	± 0.82	64.00a	± 1.57	24.20a	± 1.50	39.00a	± 2.21	65.90a	± 2.20

Note. C4 - $CaCO_3$, C5 - CaO, C6 - $Ca(OH)_2$, and C7 - NUF-WLP. T1 - 200 ppm, T2 - 250 ppm, T3 - 300 ppm, T4 - 1,000 ppm, and T5 - 1,500 ppm. Data are means ± standard error using LSD ($p \leq 0.05$). Means with the same letter in the column are not significantly different

Table 7
Girth for oil palm seedlings treated with soluble Ca

Sources of Ca	2 months						4 months						6 months					
	Ca concentration (ppm)																	
	T3		T4		T5		T3		T4		T5		T3		T4		T5	
C1	7.90b	± 0.45	12.25a	± 0.81	10.32a	± 0.59	18.39a	± 1.36	18.47a	± 1.14	18.32b	± 1.00	36.12ab	± 2.66	37.91b	± 2.50	38.60b	± 2.10
C2	10.58ab	± 0.56	10.27ab	± 0.72	11.59a	± 0.72	17.93a	± 2.30	22.46a	± 1.31	23.65a	± 1.30	32.08b	± 2.71	37.84b	± 3.37	37.24b	± 3.26
C3	12.17a	± 0.87	9.63b	± 0.84	11.91a	± 0.89	22.27a	± 1.35	22.10a	± 2.70	18.92b	± 1.68	43.37a	± 3.74	56.40a	± 1.95	57.54a	± 2.33

Note. C1 - $CaCl_2$, C2 - $CaSO_4$, and C3 - $CaNO_3$. T3 - 300 ppm, T4 -1,000 ppm, and T5 - 1,500 ppm. Data are means ± standard error using LSD ($p \leq 0.05$). Means with the same letter in the column are not significantly different

Table 8
Girth for oil palm seedlings treated with non-soluble Ca

Sources of Ca	2 months						4 months						6 months					
	Ca concentration (ppm)																	
	T1		T2		T3		T1		T2		T3		T1		T2		T3	
C4	10.03b	± 0.51	20.67a	± 0.49	30.94a	± 0.95	11.08a	± 1.04	20.44ab	± 0.77	32.71a	± 0.72	10.90a	± 1.09	20.45a	± 1.58	29.90a	± 1.72
C5	12.03ab	± 1.04	22.66a	± 0.84	33.50a	± 0.90	12.40a	± 1.14	23.08a	± 1.54	33.96a	± 1.05	12.86a	± 1.98	30.10a	± 1.15	34.60a	± 1.38
C6	12.80a	± 0.58	17.63a	± 0.70	30.05a	± 0.72	12.13a	± 1.74	18.41b	± 0.68	30.47q	± 0.18	12.20a	± 1.40	20.50a	± 1.40	30.47a	± 1.02
C7	11.20ab	± 0.78	22.81a	± 0.57	31.05a	± 0.54	11.41a	± 0.85	23.30a	± 1.36	35.52a	± 0.61	11.55a	± 1.82	30.00a	± 0.82	35.20a	± 1.09

Note. C4 - $CaCO_3$, C5 - CaO, C6 – $Ca(OH)_2$, and C7 - NUF-WLP. T1 - 200 ppm, T2 - 250 ppm, T3 - 300 ppm, T4 - 1,000 ppm, and T5 - 1,500 ppm. Data are means ± standard error using LSD ($p \leq 0.05$). Means with the same letter in the column are not significantly different

Ca (T4) in C1 and C2 was almost the same, around 0.16%.

A similar pattern in the girth of oil palm seedlings treated with different types of non-soluble Ca sources was observed (Table 8). As the seedlings grew old, the changes in the girth of seedlings became more pronounced. No interactions were found between non-soluble Ca sources with Ca concentration rates at $p \geq 0.05$ during the experiment period. However, interactions existed between types of Ca used in the experiment with the month at $p \leq 0.05$. After two to six months of treatment with different types of non-soluble Ca, all seedlings increased significantly in girth. After six months of treatment, the girth of seedlings at 300 ppm of Ca (T3) observed in C7 was significantly 1.70% higher than in C6, 13.44% higher than in C4, and 15.06% higher than in C5. The girth size of C6 treated seedlings was 11.94% higher than in C4 and 13.58% higher than in C5. A girth of C5 was 1.87% higher than in C4 seedlings treatment.

Chlorophyll Content for Oil Palm Treated with Soluble and Non-soluble Ca Sources

No interactions were observed between Ca water-soluble forms with Ca concentrations rates at $p \geq 0.05$ (Table 9). However, interactions were observed among the sources of Ca with the month over the entire experimental duration at $p \leq 0.05$. The results of this experiment generally showed an increase of the chlorophyll content (SPAD) in oil palm seedlings after two months of treatment with various forms of water-soluble Ca. The mean value of chlorophyll content (SPAD) at 1,000 ppm of Ca (T4) treated with C1 was 4.97% higher than that in C3 and 6.61% higher than in C2 over six months of experimental time. The mean value of the chlorophyll content (SPAD) in seedlings at 1,000 ppm of Ca (T4) in C3 and C2 was almost the same, around 2.36%.

A similar pattern in the chlorophyll content (SPAD) of oil palm seedlings treated with different sources of non-soluble Ca water was observed (Table 10). As the seedlings grew old, the changes in the chlorophyll content (SPAD) of the seedlings became more pronounced. There was no interaction between non-soluble Ca water sources with Ca concentration rates at $p \geq 0.05$ during the experiment duration. However, there were interactions between sources of Ca used in the experiment with the rate of Ca concentration at $p \leq 0.05$. All seedlings increased significantly in chlorophyll content (SPAD) after two to six months of treatment. After six months of treatment, the chlorophyll content (SPAD) of seedlings at 300 ppm of Ca (T3) observed in C6 was significantly 1.74% higher than in C4, 1.79% higher than in C7, and 13.47% higher than in C5. The chlorophyll content (SPAD) in C4 treated seedlings was 0.05% higher than in C7 and 11.93% higher than in C5. The C7 seedlings treatment showed chlorophyll content (SPAD) of 11.88 % more than in C5.

Total Biomass of Oil Palm Seedlings

There was no interaction between types of Ca used in the experiment, with the rate

Table 9
Chlorophyll content for oil palm seedlings treated with soluble Ca

Sources of Ca	2 months						4 months						6 months					
	Ca concentration (ppm)																	
	T3		T4		T5		T3		T4		T5		T3		T4		T5	
C1	24.02c	± 0.10	35.09a	± 0.85	25.16a	± 0.67	45.81a	± 4.05	46.75b	± 2.87	54.69a	± 2.59	59.44a	± 1.90	58.97a	± 2.47	52.80a	± 3.01
C2	27.01b	± 0.86	27.36b	± 1.06	30.21a	± 1.71	52.48a	± 4.41	53.62ab	± 0.99	58.12a	± 4.07	52.21b	± 2.64	55.07a	± 2.03	56.50a	± 2.47
C3	31.96a	± 1.39	24.72b	± 1.27	30.43a	± 3.23	45.85a	± 4.83	53.91a	± 3.00	52.52a	± 1.73	50.24b	± 1.53	56.40a	± 1.95	53.65a	± 2.52

Note. C1 - $CaCl_2$, C2 - $CaSO_4$, and C3 - $CaNO_3$. T3 - 300 ppm, T4 - 1,000 ppm, and T5 - 1,500 ppm. Data are means ± standard error using LSD ($p \leq 0.05$). Means with the same letter in the column are not significantly different

Table 10
Chlorophyll content for oil palm seedlings treated with non-soluble Ca

Sources of Ca	2 months						4 months						6 months					
	Ca concentration (ppm)																	
	T1		T2		T3		T1		T2		T3		T1		T2		T3	
C4	30.22a	± 1.15	55.29a	± 1.48	55.73a	± 1.31	28.35a	± 2.37	53.68a	± 2.16	49.18a	± 0.94	28.51ab	± 3.03	46.67a	± 1.76	47.75a	± 0.81
C5	27.15ab	± 0.56	51.03ab	± 1.67	51.25a	± 1.18	27.03a	± 2.78	52.80a	± 4.07	54.20a	± 2.75	28.35b	± 3.80	54.42a	± 2.46	55.18a	± 2.02
C6	25.33b	± 1.80	44.82b	± 1.08	43.22a	± 1.21	26.08a	± 2.6	50.20a	± 3.49	50.21a	± 3.21	26.46b	± 2.08	53.62a	± 2.03	54.22a	± 1.77
C7	25.39b	± 1.10	52.40ab	± 1.20	53.91a	± 1.37	28.39a	± 3.26	52.39a	± 1.84	53.60a	± 1.27	31.92a	± 2.34	52.46	± 2.07	54.19a	± 1.84

Note. C4 - $CaCO_3$, C5 - CaO, C6 – $Ca(OH)_2$, and C7 - NUF-WLP. T1 - 200 ppm, T2 - 250 ppm, T3 - 300 ppm, T4 - 1,000 ppm, and T5 - 1,500 ppm. Data are means ± standard error using LSD ($p \leq 0.05$). Means with the same letter in the column are not significantly different

of Ca concentration at $p \geq 0.05$. However, the total biomass of C2 seedlings was significantly higher than that of C1, C3, C4, C5, C6, and C7 seedlings after six months throughout the entire experimental period at $p \leq 0.05$ (Figure 1). The total biomass in C2 seedlings of oil palm was 23.01% larger than C4, followed by 23.37% larger than C7, 25.56% larger than C3, 26.44% larger than C5, 27.28% larger than C6, and 29.91% larger than C1. The C2 oil palm seedlings are bigger and denser at the root compared to C1, C3, C4, C5, C6, and C7 seedlings. These results suggested that the Ca source of C2 ($CaSO_4$) effectively increased the total biomass of oil palm seedlings.

The Soil pH

The effect of 300 ppm of various Ca sources used in the experiment on the BRIS soil pH is shown in Table 11. There was an interaction between types of Ca with the rate of Ca concentration at $p \leq 0.05$. The minimum and maximum values of BRIS soil pH were 6.71 and 8.10, respectively. These results suggested that the soil pH was ideal for plant growth ranging from 6.0 to 8.0, respectively.

Table 11
Soil pH for oil palm seedling treated with 300 ppm of Ca

Types of Ca	Average of soil pH	
C1	6.85ed	±0.17
C2	6.71e	±0.07
C3	6.92d	±0.08
C4	7.94ab	±0.16
C5	8.10a	±0.19
C6	7.78bc	±0.18
C7	7.75c	±0.15

Note. Data are means ± standard error using LSD ($p \leq 0.05$). Means with the same letter are not significantly different. C1 - $CaCl_2$, C2 - $CaSO_4$, C3 - $CaNO_3$, C4 - CaO, C5 – Ca $(OH)_2$, C6 - $CaCO_3$, and C7 - NUF-WLP

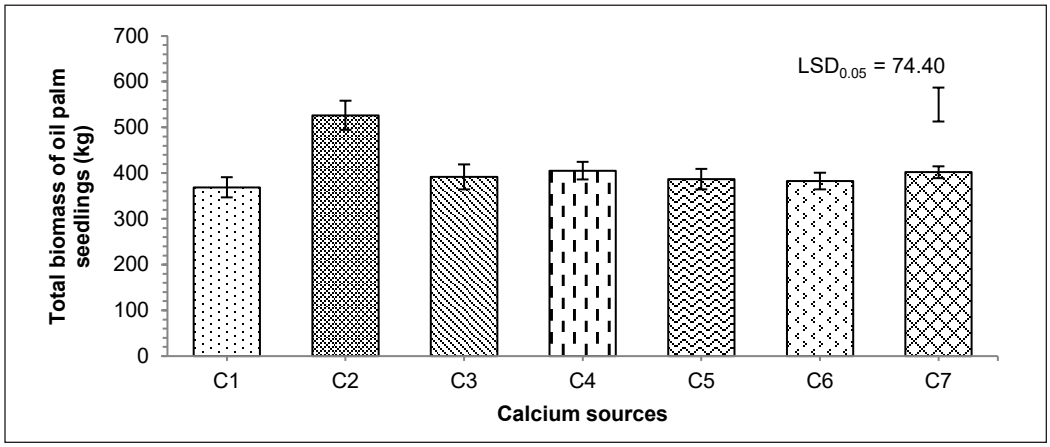

Figure 1. Total biomass of oil palm seedlings at 1,000 ppm of Ca (T4), 24 weeks after treatment with various sources of Ca
Note. C1 - $CaCl_2$, C2 - $CaSO_4$, C3 - $CaNO_3$, C4 - $CaCO_3$, C5 - CaO, C6 – Ca $(OH)_2$, and C7 - NUF-WLP. Data are means ± standard error using LSD ($p \leq 0.05$). Error bars represent LSD values between treatments. LSD bar below the LSD value shows the critical difference (CD) or least significant difference (LSD) in a graph to explain the differences between effects of different calcium sources on the total biomass of oil palm seedlings

The current study evaluated the effects of different types of Ca used in the experiment on oil palm seedlings' vegetative growth in the nursery. All oil palm seedlings increased in seedling height, girth diameter, and chlorophyll content after two months until six months treated with various types of Ca (water-soluble or non-soluble Ca) at different levels of Ca concentration. However, this experiment focused on treatment T4 (1,000 ppm of Ca), an optimum rate for oil palm seedlings in the nursery, following the previous research by Mayzaitul Azwa (2021). The results showed that C3 ($CaNO_3$) oil palm seedlings had the tallest plant height and most oversized girth diameter compared to other Ca sources. However, C1 ($CaCl_2$) showed the highest chlorophyll content than different types of Ca sources. From this experiment, the C2 ($CaSO_4$) treated oil palm seedlings at optimum Ca concentration rate (T4-1,000 ppm) were more prominent and denser at the root. This result was an essential indicator that C2 effectively increased the growth and development of oil palm seedlings, especially on total biomass. Thus, it was the best Ca source. It provides a natural source of Ca and S that can be directly absorbed by plants and is crucial for fertilization and healthy plant development. $CaSO_4$ application can enhance soil structure and physical (aeration, bulk density, and drainage) and chemical parameters (pH, CEC, EC, nutrients availability, and organic carbon) and biomass crop productivity (Alcívar et al., 2018; Kim et al., 2016; Lastiri-Hernández et al., 2019; Wang et al., 2017).

It is believed that there are three main theories involved in this research about how $CaSO_4$ changes the composition of the soil and its physical structure (Jason, 2013). First, it helps compacted soil reduce its compaction level, enhancing the quality of soil structure, which further improves the soil aeration and water permeability. The reduced compacted soil will allow the oil palm roots to penetrate deeper to acquire more usable nutrients. Second, Ca in $CaSO_4$ help to improve the soil by promoting the growth of soil organism, which then provides a more robust soil structure (Jason, 2013). The addition of $CaSO_4$ increased the soil organism's activity, which assisted in breaking down the organic material and dead plant matter. As a result, it helps bind soil particles together, stabilizing the soil structure. The transition from small particles to larger aggregates allows for greater water penetration and nutrients into the soil. Third, the application of $CaSO_4$ to the soil improves the size of soil pores (Jason, 2013). Thus, it helps to balance water drainage and holding capacity, on the other hand. The larger pores increase water and drainage flow, while the smaller pores hold the water longer and aid plant storage (Hopkins, 2013). These varying pore sizes are critical and provide the roots with the oxygen they need because of the improved aeration generated by better root penetration. Therefore, it makes the plants easier to receive more minerals and water to increase growth and development.

These findings are in accordance with the previous research findings by Winsor et al. (1963), who investigated the effect

of $CaSO_4$ on lettuce growth and reported that the application of soluble $CaSO_4$ had a comparative effect on lettuce growth mainly in terms of plant size and the fresh weight. Similar findings have been reported by Gharieb et al. (1998), explaining that supplementary $CaSO_4$ in tomato plants significantly increased all the growth parameters and physiological variables, such as plant growth, fruit yield, and membrane permeability as nutrients uptake from the nutrient solution. This outcome is further strengthened by Mahmood et al.'s (2009) findings, who reported that the application of 20 mg Ca as $CaSO_4$ along with N caused a significant improvement in plant height, straw, and grain yields wheat. These previous studies also reported that the application of Ca as $CaSO_4$ had significantly increased about 34 to 52% of plant growth and 25 to 43% of wheat yield production.

The Ca does not shift easily from elder leaves to the rising tips needed. Therefore, $CaSO_4$ is required as it is relatively soluble, which acts as a good medium-term source for releasing Ca and has fair mobility through the soil profile. However, the supplementation of $CaSO_4$ as a Ca fertilizer had little or no effect on the soil pH, thus increasing the plant growth. These outcomes followed the research findings demonstrated by Carvalho and van Raij (1997), whereby the addition of $CaSO_4$ in the soil had a very little significant effect in increasing the pH value, which only ranges from 0.05 units to 0.23 units.

The current study results showed that the application of $CaSO_4$ in the soil increased the chlorophyll content in oil palm seedlings. These results strongly correlated with the membrane permeability of treated $CaSO_4$ seedlings. This statement is in line with an investigation by Bolat et al. (2006) that the addition of 5 mM of $CaSO_4$ in root medium of plum (*Prunus domestica*), Marianna GF 8-1 (*Prunuscerasifera* × *munsoniana*), Myrobolan B (*Prunus cerasifera*), and Pixy (*Prunus insititia*) increased the total chlorophyll contents in their leaves. These results could be related to membrane permeability and electrolyte leakage. The membrane permeability improved as the concentration rate of $CaSO_4$ increased, reducing the percentage of electrolyte leakage and increasing the chlorophyll contents.

CONCLUSION

The results of this study revealed that Ca supplemented as $CaSO_4$ (C2) source was the best to be used for oil palm seedlings with an optimum rate of concentration (T4 - 1,000 ppm). Therefore, the addition of $CaSO_4$ could offer an economical and simple solution to enhance the growth and development of oil palm seedlings. There was a need to consider fertilizer nutrients using the $CaSO_4$ combined with an optimum concentration rate for further research as an alternative way to control plant disease, especially in oil palm caused by *Ganoderma boninense* fungi.

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REFERENCES

Agrios, N. G. (2005). *Plant pathology* (5th ed.). Elsevier Academic Press.

Alcívar, M., Zurita-Silva, A., Sandoval, M., Muñoz, C., & Schoebitz, M. (2018). Reclamation of saline–sodic soils with combined amendments: Impact on quinoa performance and biological soil quality. *Sustainability*, *10*(9), 3083. https://doi.org/10.3390/su10093083

Bolan, N. S., Loganathan, P., & Saggar, S. (2007). Calcium and magnesium in soils. In *Encyclopedia of soils in the environment* (pp 149-154). New York Academic Press. https://doi.10.1016/B0-12-348530-4/00223-X

Bolat, I., Kaya, C., Almaca, A., & Timucin, S. (2006). Calcium sulfate improves salinity tolerance in rootstocks of plum. *Journal of Plant Nutrition*, *29*(3), 553-564. https://doi.org/10.1080/01904160500526717

Carvalho, M., & van Raij, B. (1997). Calcium sulphate, phosphogypsum and calcium carbonate in the amelioration of acid subsoils for root growth. *Plant and Soil*, *192*, 37–48. https://doi.org/10.1023/A:1004285113189

Chao, Y. Y., Wang, W. J., & Liu, Y. T. (2021). Effect of calcium on the growth of Djulis (*Chenopodium formosanum* Koidz.) sprouts. *Agronomy*, *11*(1), 82. https://doi.org/10.3390/agronomy11010082

Gharieb, M. M., Sayer, J. A., & Gadd, G. M. (1998). Solubilization of natural gypsum ($CaSO_4.2H_2O$) and the formation of calcium oxalate by *Aspergillus niger* and *Serpula himantioides*. *Mycological Research*, *102*(7), 825–830. https://doi.org/10.1016/j.resconrec.2016.04.005

Hoagland, D. R., & Arnon, D. I. (1950). *The water-culture method for growing plants without soil*. https://ia601604.us.archive.org/22/items/waterculturemeth347hoag/waterculturemeth347hoag.pdf

Hopkins, M. (2013). *The role of gypsum in agriculture: 5 key benefits you should know*. https://www.croplife.com/crop-inputs/micronutrients/the-role-of-gypsum-in-agriculture-5-key-benefits-you-should-know/

Jason. (2013). *The benefits of calcium sulphate in agriculture. Calcium sulfate makes a difference in soil properties*. https://www.eco-gem.com/benefits-calcium-sulphate-agriculture/

Kim, H. S., Kim, K. R., Lee, S. H., Kunhikrishnan, A., Kim, W. I., & Kim, K. H. (2016). Effect of gypsum on exchangeable sodium percentage and electrical conductivity in the Daeho reclaimed tidal land soil in Korea - A field scale study. *Journal of Soils Sediments*, *18*, 336–341. https://doi.org/10.1007/s11368-016-1446-x

Lastiri-Hernández, M. A., Alvarez-Bernal, D., Bermúdez-Torres, K., Cruz Cárdenas, G., & Ceja-Torres, L. F. (2019). Phytodesalination of a moderately saline soil combined with two inorganic amendments. *Bragantia*, *78*(4), 579–586. https://doi.org/10.1590/1678-4499.20190031

Mahmood, I. A., Salim, M., Ali A., Arshadullah, M., Zaman, B., & Mir, A. (2009). Impact of calcium sulphate and calcium carbide on nitrogen use efficiency of wheat in normal and saline sodic soils. *Soil and Environment*, *28*(1), 29-37.

Marschner, H. (1995). Nutritional physiology. In *Mineral nutrition of higher plants* (pp. 417-426). Mineral Academic Press Limited.

Mayzaitul Azwa, J. N. (2021). *Calcium nutritional effects on suppression of* Ganoderma *disease in oil palm* [Unpublished Doctoral dissertation]. Universiti Putra Malaysia.

McLean, E. O. (1975). Calcium levels and availabilities in soils. *Communications in Soil Science and Plant Analysis*, *6*(3), 219-232. https://doi.org/10.1080/00103627509366563

Minson, D. J. (1990). Calcium. In *Forage in ruminant nutrition* (pp. 208-209). Academic Press.

Rahman, M., & Punja, Z. K. (2007). Calcium and plant disease. In L. E. Datnoff, W. H. Elmer, & D. M. Hubert (Eds.), *Mineral nutrition and plant disease* (pp. 79-93). APS Press.

Ramírez-Builes, V. H., Küsters, J., de Souza, T. R., & Simmes, C. (2020). Calcium nutrition in coffee and its influence on growth, stress tolerance, cations uptake, and productivity. *Frontiers in Agronomy*, *2*, 590892. http://doi.10.3389/fagro.2020.590892

Thor, K. (2019). Calcium - Nutrient and messenger. *Frontiers in Plant Science*, *10*, 440. https://doi.org/10.3389/fpls.2019.00440

Wang, S. J., Chen, Q., Li, Y., Zhuo, Y. Q., & Xu, L. Z. (2017). Research on saline-alkali soil amelioration with FGD gypsum. *Resource, Conservation and Recycling*, *121*, 82–92. https://doi.org/10.1016/j.resconrec.2016.04.005

White, P. J., & Broadley, M. R. (2003). Calcium in plants. *Annals of Botany*, *92*(4), 487- 511. https://doi.org/10.1093/aob/mcg164

White, P. J., Bowen, H. C., Demidchik, V., Nichols, C., & Davies, J. M. (2002). Genes for calcium-permeable channels in the plasma membrane of plant root cells. *Biochimica et Biophysica Acta - Biomembranes*, *1564*(2), 299–309. https://doi.org/10.1016/S0005-2736(02)00509-6

Winsor, G. W., Davies, J. N., & Massey, D. M. (1963). Soil salinity studies. I - Effect of calcium sulphate on the correlation between plant growth and electrical conductivity of soil extracts. *Journal of the Science Food and Agriculture*, *14*(1), 42-48. https://doi.org/10.1002/jsfa.2740140107

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Effects of Yeast on the Growth Performance of Sangkuriang Catfish Fingerlings (*Clarias gariepinus* var. Sangkuriang)

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ABSTRACT

The success of the intensive culture of Sangkuriang catfish (*Clarias gariepinus* var. Sangkuriang) highly depends on the availability of feed. The feed is the most significant share of cost production hamper (50-60%). Therefore, it is suggested that feed utilization is inefficient. One of the solutions is to enrich the commercial feed with yeast (*Saccharomyces cerevisiae*). The research aimed to identify the impacts of yeast (*S. cerevisiae*) enhanced feed on feed efficiency, growth, and survival rate of Sangkuriang catfish fingerlings. The treatments research was yeast (*S. cerevisiae*) enrichment in the commercial feed at the various dosages: 0 %/kg feed (A), 3 %/kg feed (B), 6 %/kg feed (C), 9 %/kg feed (D), and 12 %/kg feed (E). The yeast (*S. cerevisiae*) enrichment in the commercial feed increased feed efficiency and survival rate of Sangkuriang catfish fingerlings. The optimum dosage of *S. cerevisiae* for apparent digestibility coefficients for protein (ADCp), efficiency feed utilization (EFU), protein efficiency ratio (PER), and relative growth rate (RGR) ranged from 6.10% to 6.51%/kg feed.

Keywords: Aquaculture, catfish, cost, feed, growth performance, yeast

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INTRODUCTION

The success of the intensive culture of Sangkuriang catfish (*Clarias gariepinus* var. Sangkuriang) is highly related to feeding availability. Intensive aquaculture feed is the most significant share of cost production, around 50-60% (Rachmawati & Samidjan, 2018). Due to the inefficiency of feed to support growth, the enrichment of the commercial feed with yeast (*Saccharomyces cerevisiae*) is urgently needed. The yeast (*S. cerevisiae*) could boost digestive enzyme activities; therefore, it increased the breakdown of complex nutrients into simpler forms to be easily absorbed in the digestive tract (de Azevedo et al., 2016). Sitohang et al. (2012) stated that *S. cerevisiae* produced metabolic products, including amylase and peptidase proteolytic. Protease enzymes could hydrolyze protein into peptides and amino acids. In addition, *S. cerevisiae* produced cellulase enzyme that could break down cellulose into glucose; in turn, it decreased raw fiber and the decrease of raw fiber related to the increase of carbohydrate. Manurung and Mose (2018) disclosed that yeast (*S. cerevisiae*) could increase fish appetite by allowing catfish to feed intensively and increasing catfish growth.

Some studies on the effect yeast (*S. cerevisiae*) on freshwater and marine fish have been done, such as in *Barbonymus gonionotus* (Rachmawati et al., 2019a), *Cyprinus carpio* (Al-Refaiee et al., 2016), *Oreochromis niloticus* (de Azevedo et al., 2016), *Pangasius hypothalamus* (Rachmawati et al., 2019b), *Labeo rohita* (Tewary & Patra, 2011), and *Sarotherodon galileaus* (Abdel-Tawwab et al., 2010). However, there is a lack of information about the effect of yeast (*S. cerevisiae*) to enhance commercial feed by increasing feed efficiency and growth of Sangkuriang catfish fingerlings. The present study was to identify the impacts of yeast (*S. cerevisiae*) enhancing feed efficiency, growth, and survival rate of Sangkuriang catfish fingerlings (*Clarias gariepinus* var. Sangkuriang).

MATERIALS AND METHODS

Research Design

The study used a completely randomized design with five groups, and each treatment had three replications. The research was conducted in the Sido Makmur Catfish Farmers Association, which partnered with the researchers on May-July 2020. As many as 500 test fish used in the study were catfish fingerlings with a weight of 1.12±0.36 g/fish. The catfish was obtained from the Sido Makmur Catfish Farmers Association. Sangkuriang catfish fingerlings were adapted on feed and the environment in the fiber containers in $1.5 \times 1 \times 1.5$ m^3 of diameter for seven days. During adaptation, the fish was fed without *S. cerevisiae* in commercial feed. After the fish had adapted, one-day fasting was introduced to neutralize metabolic residual, not affecting the initial weight. Sangkuriang catfish fingerlings were selected based on several criteria: disease-free, malformation-free, healthy, energetic, and uniform size (Rachmawati et al., 2017).

Experimental Feed

Test study used experimental feed, which contains 30% protein, 0.5% chromium oxide (Cr_2O_3), and five dosages of yeast (*Saccharomyces cerevisiae*) treatment: A (0 %/kg feed), B (3 %/kg feed), C (6 %/kg feed), D (9 %/kg feed) and E (12 %/kg feed). *Saccharomyces cerevisiae* was purchased at the bread store. Commercial feed was first finely ground and mixed evenly with 0.5% Cr_2O_3. The mixture was formed as pellets with a 2 mm diameter, adjusted to the mouth size of the catfish fingerlings, and then dried at room temperature. Next, *S. cerevisiae* was diluted in 100 ml water for 1 kg feed (de Azevedo et al., 2016). *Saccharomyces cerevisiae* suspension was put in the sprayer bottle and then sprayed on an experimental feed containing Cr_2O_3. After mixing, the mixture was preserved at room temperature and packed in labeled plastic bags. Then it was stored in the refrigerator until further use (Vendrell et al., 2008). Experimental feed was 5%/ biomass weight/day for 63 days. Fish growth was identified by weighing the fish weekly. Fifteen plastic fiber containers were used in the research with $1 \times 1 \times 1$ m^3. Every treatment used three containers, equipped with a water circulation system and stocked with 50 Sangkuriang catfish fingerlings.

Analysis of Protein Digestion

The feeding at satiation was given twice a day, in the morning and afternoon. The feces were collected twice a day after being fed by siphoning the containers. First, the feces were collected in the plankton clothe net. The collected feces were then dried in the oven at 105 °C until constant weight. After the feces had dried, it was finely ground and stored at 4 °C until analyzed. Analyses for Cr_2O_3 content in the feed and feces were based on Association of Official Analytical Chemists (AOAC) (2005) using atomic absorption spectrometer.

Proximate Analysis

Proximate analysis of the feed and the fish body at the initial and final research was based AOAC (2005) method.

Moisture. Moisture was measured by 2 g of the samples in the oven at 105 ºC for 24 hours. Percentage of moisture was calculated using the following formula:

$$\text{Ka}\ (\%) = \frac{W_1 - W_2}{W_1} \times 100\% \qquad (1)$$

Ka = Percentage of moisture (wet weight)
W_1 = Weight of the sample (g)
W_2 = Weight of the dried sample (g)

Crude Protein. The crude protein content was determined using the micro-Kjeldahl method. Samples (2 g) were digested in the digestion unit for 45 minutes. The digester was then distilled in a distillation unit (Khjeltdahl System, VELP Scientifica Srl, Italy). It was titrated with 0.2 N hydrochloric acid (HCL), and crude protein was obtained by multiplying the total nitrogen by a conversion factor of 6.25.

$$\text{Crude protein (\%)} = \frac{\text{ml titration (blank} - \text{sample)} \times \text{N} \times 14{,}007 \times 6.25 \times 100\%}{\text{Weight of the sample (g)} \times 1000} \quad (2)$$

Crude Lipid. The content of crude lipid was analyzed using the Soxhlet extraction method. Samples with known constant weight were put into Soxhlet extracted using hexane or petroleum ether. After extraction, the sample was removed from the Soxhlet and dried.

$$\text{Crude lipid (\%)} = \frac{\text{Initial sample weight (g)} - \text{Final sample weight (g)}}{\text{Final sample weight (g)}} \times 100\% \quad (3)$$

Ash. The ash was obtained by putting samples into the furnace at 500 ºC for 10 hours. The cooled and stable ash results are then weighed so that the formula can calculate the total ash content:

$$\text{Ash (\%)} = \frac{W_1 - W_2}{W} \times 100\% \quad (4)$$

W = Sample weight before turn to ashes (g)
W_1 = Sample weight + porcelain dish after turn to ashes(g)
W_2 = Weight of an empty porcelain dish (g)

Observed Parameters

Parameters observed included feed conversion ratio (FCR), protein efficiency ratio (PER), relative growth rate (RGR), and efficiency feed utilization (EFU) based on National Research Council (NRC) (2011), apparent digestibility coefficients for protein (ADCp) based on Fenucci (1981), survival rate (SR) based on Tacon (2002). The equations to analyze the parameters were as follows:

ADC_P = 100(% Cr_2O_3 in the feed/% Cr_2O_3 in the feces) x (% protein in the feces/% protein in the feed)

EFU = 100 (final weight-initial weight/ the amount of feed consumed)

RGR = 100 ($W_t - W_o$)/(W_o x T); where W_o and W_t are the initial and final weight, respectively, and T is the number of days in the feeding period

FCR = 100 [feed intake (g) /weight gain (g)]

PER = 100 [weight gain (g) /protein intake (g)]

SR = 100 (final count/initial count)

Data Analysis

The impacts of yeast (*S. cerevisiae*) on experimental parameters were identified using analysis of variance (ANOVA). Suppose the treatments were significant (P<0.05) or highly significant (P<0.01), and then applied the Duncan's multiple range test. The optimum dosage of *S. cerevisiae* in the feed was analyzed using the polynomial orthogonal test in SAS (version 9) and Maple (version 12).

RESULTS

The proximate analysis (Table 1) indicated that the average percentage of raw protein was 30.27±0.28%, while the average percentage of raw fat was 8.25±0.24%. The values of ADCp, EFU, PER, RGR, and SR of Sangkuriang catfish fingerlings, which were given yeast (*S. cerevisiae*) enhanced feed, treatments B, C, D, and E were higher than that without yeast (*S. cerevisiae*) enhanced feed, treatment A. The yeast (*S. cerevisiae*) enhanced feed significantly affected ($P<0.05$) on ADCp, EFU, PER, and RGR; however, it did not affect significantly ($P>0.05$) on SR Sangkuriang catfish fingerlings (Table 2).

Table 1

The results of proximate analysis of test feed

Proximate analysis	Diets				
	A	B	C	D	E
Moisture (%)	6.98±0.12^{a}	7.18±0.11^{a}	6.78±0.21^{a}	7.03±0.15^{a}	6.88±0.10^{a}
Crude protein (%)	30.35±0.34^{a}	30.28±0.36^{a}	30.18±0.23^{a}	30.39±0.21^{a}	30.16±0.35^{a}
Crude lipid (%)	8.21±0.15^{a}	8.34±0.12^{a}	8.20±0.11^{a}	8.29±0.20^{a}	8.21±0.24^{a}
Ash (%)	10.23±0.25^{a}	10.31±0.20^{a}	10.73±0.12^{a}	10.28±0.22^{a}	10.13±0.24^{a}
Energy (kJ/g)	8.76±0.001^{a}	8.85±0.001^{a}	8.84±0.001^{a}	8.70±0.002^{a}	8.83±0.003^{a}

Note. Mean values ± SD with different superscript indicated a significant difference ($P<0.05$)
The proximate analysis performed at the Laboratory of Animal Feed, Faculty of Animal Sciences and Agriculture, Diponegoro University

Table 2

Growth performance of Sangkuriang catfish fingerlings fed yeast enriched feed

Parameters	Treatments				
	A	B	C	D	E
ADC_P (%)	64.14±0.22^{e}	78.89±0.29^{b}	85.12±0.25^{a}	73.34±0.21^{d}	68.23±0.20^{c}
EFU (%)	63.26±0.32^{e}	75.03±0.36^{b}	83.29±0.32^{a}	72.42±0.37^{d}	66.25±0.32^{c}
PER	2.05±0.24^{e}	3.58±0.22^{b}	4.63±0.23^{a}	3.23±0.24^{d}	2.96±0.22^{c}
RGR (%/day)	2.41±0.15^{c}	3.72±0.26^{c}	4.58±0.25^{a}	3.12±0.26^{b}	2.88±0.27^{b}
SR (%)	83.33±2.57^{a}	93.33±2.48^{a}	100±0.00^{a}	93.33±2.65^{a}	83.33±2.53^{a}

Note. Mean values ± SD in different superscript indicated a significant difference ($P<0.05$)

The results of the polynomial orthogonal test showed that the relation of yeast (*S. cerevisiae*) in the feed and ADCp has a quadratic pattern, Y = -0.4582x^2 + 5.5858x + 65.171, 0.857. The optimum dosage of 6.10 %/kg feed created a maximum value of ADCp as high as 85.73% (Figure 1). The relation of yeast (*S. cerevisiae*) in the feed and EFU was also in the quadratic form, Y = -0.4366x2 + 5.3514x + 63.517, R^2 = 0.882, with the optimum dosage of 6.12%/kg feed that generated a maximum value of EFU as much as 79.72% (Figure 2).

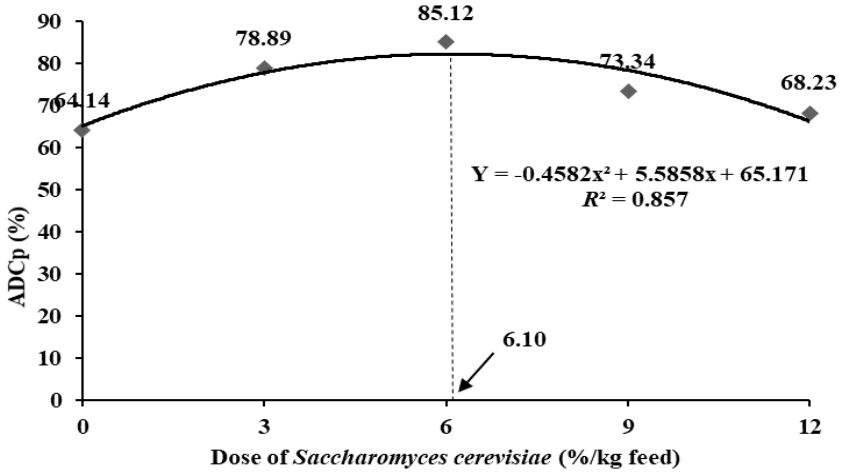

Figure 1. The relation of yeast (*Saccharomyces cerevisiae*) in the feed and ADCp

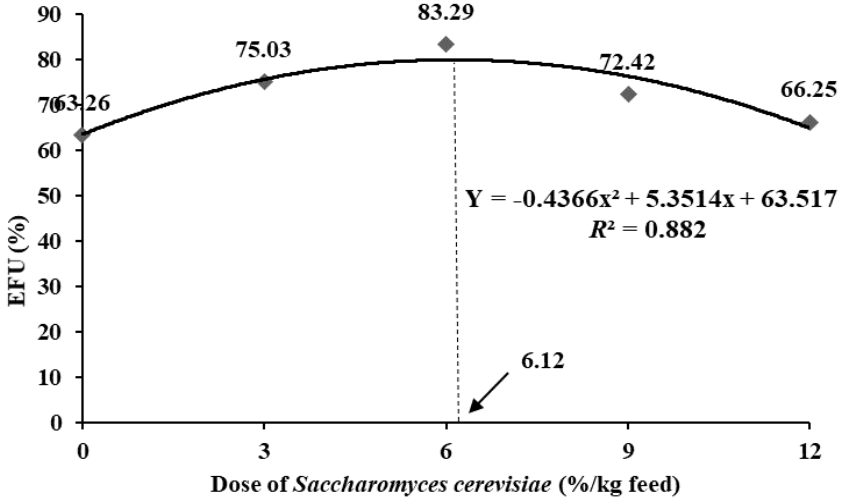

Figure 2. The relation of yeast (*Saccharomyces cerevisiae*) in the feed and EFU

The relation of yeast (*S. cerevisiae*) in the feed and PER were in the quadratic equation, $Y = -0.048x^2 + 0.6252x + 2.1317$, $R^2 = 0.801$. The maximum value of PER (4.17) was obtained from the optimum dosage of 6.51 %/kg feed (Figure 3). The relation of yeast (*S. cerevisiae*) in the feed and RGR has quadratic pattern, $Y = -0.043x^2 + 0.5275x + 2.4997$, $R^2 = 0.7512$. The maximum value of PER (4.12%/day) was obtained from the optimum dosage of 6.13%/kg feed (Figure 4). Water quality for Sangkuriang catfish cultivation during research was displayed in Table 3. The water quality was still in a viable condition for Sangkuriang catfish cultivation.

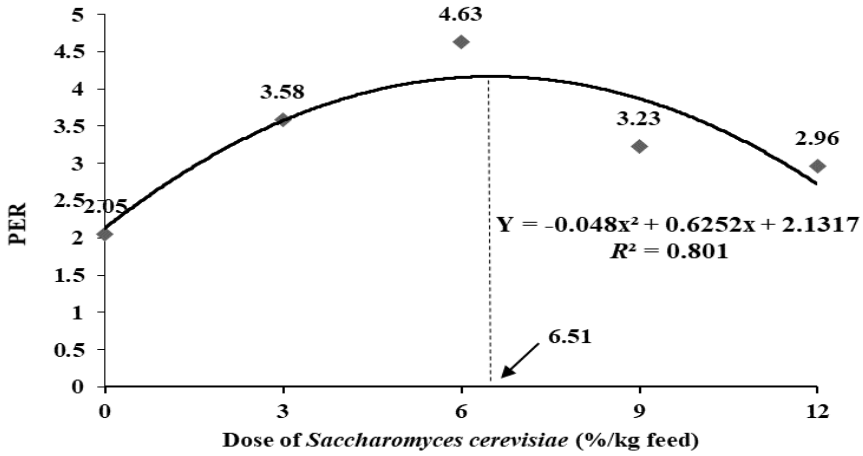

Figure 3. The relation of yeast (*Saccharomyces cerevisiae*) in the feed and PER

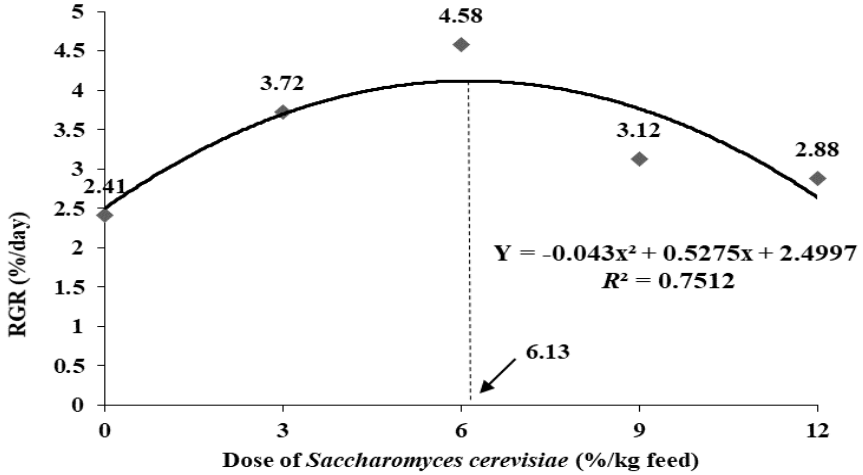

Figure 4. The relation of yeast (*Saccharomyces cerevisiae*) in the feed and RGR

Table 3
Water quality for Sangkuriang catfish cultivation during research

Treatment	Water Quality			
	Temperature (°C)	pH	DO (mg/l)	NH_3 (%)
A	26 - 30	7.26 - 7.48	5.01 – 5.78	0.002 - 0.002
B	26 - 30	7.31 - 7.52	5.23 – 5.64	0.002 - 0.002
C	26 - 30	7.29 - 7.53	5.12 – 5.69	0.002 - 0.002
D	26 - 30	7.32 - 7.47	5.17 – 5.76	0.002 - 0.002
Feasibility	14-38*	6.50 - 8.5*	>2*	<0.1*

Note. DO = Dissolved oxygen; NH_3 = Ammonia;* Data from the reference (Boyd, 2003)

DISCUSSION

Sangkuriang catfish fingerlings that were fed with the yeast *S. cerevisiae* enhanced feed (treatments B, C, D, and E) had a greater value of ADCp than that without the enrichment of yeast *S. cerevisiae* (A). It was indicated that the enrichment feed with *S. cerevisiae* could produce the digestive enzyme in the fish digestive tract (Welker et al., 2012). The highest value of ADCp (85.12%) was found in the catfish fed with *S. cerevisiae* enhanced feed with the dosage of 6 %/kg (C) and followed by the values of 78.89%, 73.34%, 68.23%, and 64.14% for B (3%), D (9%), E (12%), and A (0%). The highest value of ADCp in the catfish fed as in treatment C (6%) suggested that the dosage of yeast (*S. cerevisiae*) at 6% was the right amount of yeast addition to boosting digestive enzyme activities increased protein digestion optimally. In comparison, the dosage other than 6% resulted in not maximum enzyme activities. The same result was obtained in the *Barbonymus gonionotus* (Rachmawati et al., 2019a).

The yeast (*S. cerevisiae*) enhanced feed at the various dosages exhibited significant impacts (P<0.05) on EFU of Sangkuriang catfish fingerlings. It was suggested that the yeast (*S. cerevisiae*) supplementation in the feed could improve feed absorption; therefore, it increased feed efficiency utilization as the findings of the research by de Azevedo et al. (2016). Sangkuriang catfish fingerlings fed without the enhancement of *S. cerevisiae* (treatment A) has the lowest value of EFU 63.26% compared to the catfish fed with the enhancement of *S. cerevisiae* (treatments B, C, D, and E) with the values of 75.03%, 83.29%,72.42%, and 66.25% respectively. It was suspected that the absence of *S. cerevisiae* in the feed caused no enzyme activity in the digestive tract of the fish. Otherwise, the availability of *S. cerevisiae* in the feed could break down nutrients to easily be absorbed, so it could boost feed efficiency, as reported by Welker et al. (2012). It was suspected that yeast also raises enzymes activity in the digestive tract, such as peptidase, protease, and amylase.

According to the study by Tewary and Patra (2011), the yeast (*S. cerevisiae*) in the feed increased enzymes activity, such as peptidase, protease, and amylase in the digestive tract. Hence, it improved the decomposition of complex nutrients into simpler nutrients; in turn, it made absorption easier yeast can increase enzymes activity, such as peptidase, protease, and amylase in the digestive tract. As a result, the enzymes could decompose nutrients into a more straightforward form; therefore, the fish would easily absorb the nutrients (Tewary & Patra, 2011). According to Hurriyani (2017), the enrichment of yeast (*S. cerevisiae*) could improve feed digestion. *Saccharomyces cerevisiae* was known to produce vitamin B complex, especially biotin and vitamin B12 required by the fish digestion system. In addition, the content of peptides in the yeast plays an essential role in enzymatic digestion so that the fish can digest more efficiently. Similar results were reported by Rachmawati et al. (2019a) in the *Barbonymus gonionotus* and Abdel-Tawwab et al. (2010) in the *Sarotherodon galilaeus*.

The value of PER in the Sangkuriang catfish fingerlings fed with the yeast (*S. cerevisiae*), as in the treatments (B, C, D, and E), was higher than that without the yeast, as in treatment A. According to Tovar et al. (2002), the existence in the feed could boost protein digestion that supported an increased protein efficiency ratio. This finding was shown in Table 1 that explained the fish fed with the yeast (*S. cerevisiae*) enhanced feed has a higher value of ADCp compared to the fish fed without additional yeast in the feed. The fish fed with the additional *S. cerevisiae* dosage of 6% (C) generated the fish to efficiently consume the highest PER, which meant feed with additional yeast. In turn, it could hike protein retention to boost the protein efficiency ratio; however, the higher the yeast dosage caused the protein efficiency ratio to decrease. The findings were in line with Hurriyani's (2017) research that discovered that the additional yeast in the feed increased feed digestion and protein digestibility, resulting in more significant growth and feed efficiency. The level of protein efficiency ratio was also related to the size of the fish and feeding. The same results were found in the *Barbonymus gonionotus* (Rachmawati et al., 2019a) and *Oreochromis niloticus* (Abdel-Tawwab et al., 2008).

The yeast (*S. cerevisiae*) enhanced feed influenced significantly (P<0.05) on RGR Sangkuriang catfish fingerlings. It was suggested that *S. cerevisiae* contains nucleotides, significantly affecting the relative growth rate. The findings were supported by Manoppo and Kolopita's (2016) findings. They discovered that the additional yeast could hike growth because *S. cerevisiae* contains nucleotide in wet purine and pyrimidine as much as 0,9%. The increasing growth by adding yeast in the feed happened because the nucleotide in the yeast could increase fish appetite to feed; therefore, the feed absorption would increase. The highest value of RGR was 4.58%/day, which was obtained at the dosage of 6% *S. cerevisiae*, as in treatment

C. The result indicated that the dosage was just the right amount to optimize fish growth. According to Mohammadi et al. (2016), yeast stuck on the surface of the intestine and triggered amylase secretion and hiked digestive enzymes activity; therefore, it increased nutrient digestion. The additional yeast also improved the feeding pattern, boosting growth, and feed efficiency.

The yeast (*S. cerevisiae*) enrichment in the feed insignificantly influenced (P<0.05) on SR of Sangkuriang catfish fingerlings. The survival rate of Sangkuriang catfish fingerlings in the research ranged from 83.33% to 100%. A reasonable survival rate indicated that the additional yeast (*S. cerevisiae*) in the feed at the dosages of 0, 3, 6, 9, and 12 % did not cause mortality of the fish. The survival rate of Sangkuriang catfish is the percentage of surviving fish at the end study compared to the number of fish at the start of rearing. Therefore, the survival rate can be used to gauge the tolerance level and ability of the fish to survive. In addition, the survival rate was affected by abiotic factors, such as the ability to adjust to the environment, treatment, stocking density, competitors, diseases, age, and predators (Tacon et al., 2002).

CONCLUSION

Supplementing yeast (*Saccharomyces cerevisiae*) in the commercial feed could increase feed efficiency and growth in Sangkuriang catfish fingerlings. However, it did not affect the survival rate of Sangkuriang catfish fingerlings. The optimum dosages of the yeast (*S. cerevisiae*) in the commercial feed for ADCp, EFU, PER, and RGR ranged from 6.10% to 6.51%/kg feed.

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REFERENCES

Abdel-Tawwab, M., Mousa, M. A. A., & Mohammed, M. A. (2010). Use of live baker's yeast, *Saccharomyces cerevisiae*, in practical diet to enhance the growth performance of Galilee tilapia, *Sarotherodon galilaeus* (L.), and its resistance to environmental copper toxicity. *Journal of the World Aquaculture Society*, *41*(S2), 214-223. https://doi.org/10.1111/j.1749-7345.2010.00361.x

Abdel-Tawwab, M., Khattab, Y. A. E., Ahmad, M. H., & Shalaby, A. M. E. (2008). Compensatory growth, feed utilization, whole-body composition and haematological changes in starved juvenile Nile tilapia, *Oreochromis niloticus* (L.). *Journal of Applied Aquaculture*, *18*(3), 17-36. https://doi.org/10.1300/J028v18n03_02

Al-Refaiee, I. H., Abdulrahman, N. M., & Mutter, H. A. (2016). Replacement of commercial dry yeast (*Saccharomyces cerevisiae*) with animal protein concentrate and its effect in some blood parameters for fingerlings common carp

Cyprinus carpio L. *Basrah Journal of Veterinary Research*, *15*(3), 312-332.

Association of Official Analytical Chemists. (2005). *Official methods of analysis of AOAC International* (18th ed.). AOAC International.

Boyd, C. E. (2003). Guidelines for aquaculture effluent management at the farm level. *Aquaculture*, *226*(1-4), 101–112. https://doi.org/10.1016/S0044-8486(03)00471-X

de Azevedo, R.V., Filho, J. C. F., Pereira, S. L., Cardoso, L. D., de Andrade, D. R., & Júnior, M. V. V. (2016). Dietary mannan oligosaccharide and *Bacillus subtilis* in diets for Nile tilapia (O*reochromis niloticus*). *Acta Scietarium Animal Science*, *38*(4), 347-353. https://doi.org/10.4025/actascianimsci.v38i4.31360

Fenucci, J. L. (1981). *Studies on the nutrition of marine shrimp of the* Penaeus [Unpublished Doctoral dissertation]. University of Houston.

Hurriyani, Y. (2017, May 23-24). *Evaluasi penambahan ragi roti (*Saccharomyces cerevisiae*) dalam feed terhadap kinerja pertumbuhan benih ikan Jelawat (*Leptobarbus hoevenii*)* [Evaluation of addition of bread yeast (*Saccharomyces cerevisiae*) in feed on growth performance of Jelawat fish (*Leptobarbus hoevenii*)] [Paper presentation]. Prosiding Seminar Nasional Penerapan Ilmu Pengetahuan and Teknologi 2017, Pontianak, Indonesia. https://www.researchgate.net/publication/325013236_EVALUASI_PENAMBAHAN_RAGI_ROTI_Saccharomyces_cerevisiae_DALAM_PAKAN_TERHADAP_KINERJA_PERTUMBUHAN_BENIH_IKAN_JELAWAT_Leptobarbus_Hoevenii

Manoppo, H., & Kolopita, M. E. F. (2016). Penggunaan ragi roti (*Saccharomyces cerevisiae*) sebagai imunostimulan untuk meningkatkan resistensi ikan mas (*Cyprinus carpio* L.) terhadap infeksi bakteri *Aeromonas hydrophila* [The use of baker's yeast (*Saccharomyces cerevisiae*) as immunostimulant to enhance resistance of gold fish, (*Cyprinus carpio* L.) to *Aeromonas hydrophila* infection]. *Jurnal Budidaya Perairan*, *4*(3), 37-47. https://doi.org/10.35800/bdp.4.3.2016.14945

Manurung, U. N., & Mose, N. I. (2018). Peningkatan pertumbuhan and sintasan hidup ikan bawal (*Colossoma macropumum*) dengan penambahan ragi roti dalam feed [The improvement growth and survival rate of pomfret fish (*Colossoma macropumum*) with yeast addition in feed]. *Jurnal Saintek Lahan Kering*, *1*(2), 26-27. https://doi.org/10.32938/slk.v1i2.546

Mohammadi, F., Mousavi, S. M., Zakeri, M., & Ahmadmoradi, E. (2016). Effect of dietary probiotic, *Saccharomyces cerevisiae* on growth performance, survival rate and body biochemical composition of three spot cichlid (*Cichlasoma trimaculatum*). *AACL Bioflux*, *9*(3), 451-457.

National Research Council. (2011). Proteins and amino acids. In *Nutrient requirements of fish and shrimp* (pp. 57-101). National Academy Press.

Rachmawati, D., & Samidjan, I. (2018). The effects of papain enzyme supplement in feed on protein digestibility, growth and survival rate in Sangkuriang catfish (*Clarias* sp.). *Omni-Akuatika*, *14*(2), 91- 99. https://doi.org/10.20884/1.oa.2018.14.2.551

Rachmawati, D., Hutabarat, J., Samidjan, I., Herawati, V. E., & Windarto, S. (2019a). The effects of *Saccharomyces cerevisiae*-enhanced diet on feed usage efficiency, growth performance and survival rate in Java barb (*Barbonymus gonionotus*) fingerlings. *AACL Bioflux*, *12*(5), 1841-1849.

Rachmawati, D., Istiyanto, S., Ristiawan, A. N., & Susilowati T. (2019b). Effects of *Sacharomyces cereviceae* incorporated diet on growth performance, apparent digestibility coefficient of protein and survival rate of catfish (*Pangasius hypothalamus*). *Aquacultura Indonesiana*, *20*(1), 8-14. https://doi.org/10.21534/ai.v20i2.140

Rachmawati, D., Samidjan, I., & Mel, M. (2017). Effect of phytase on growth performance, feed utilization efficiency and nutrient digestibility in fingerlings of *Chanos chanos* (Forsskal 1775). *Philippine Journal of Science*, *146*(3), 237-245.

Sitohang, R., Herawati, V., T., & Lili, W. (2012). Pengaruh pemberian dedak padi hasil fermentasi ragi (*Saccharomyces cerevisiae*) terhadap pertumbuhan biomassa *Daphnia* sp. [Effect of yeast fermentation rice bran (*Saccharomyces cerevisiae*) on the growth of *Daphnia* sp. biomass]. *Jurnal Perikanan and Kelautan*, *3*(1), 65-72.

Tacon, A. G. J., Cody, J. J., Conquest, L. D., Divakaran, S., Forster, I. P., & Decamp, O. E. (2002). Effect of culture system on the nutrition and growth performance of Pacific white shrimp *Litopenaeus vannamei* (Boone) fed different feeds. *Aquaculture Nutrition*, *8*(2), 121-137. https://doi.org/10.1046/j.1365-2095.2002.00199.x

Tewary, A., & Patra, B. C. (2011). Oral administration of baker's yeast (*Saccharomyces cerevisiae*) acts as a growth promoter and immunomodulator in *Labeo rohita* (Ham.). *Journal of Aquaculture Research and Development*, *2*(1), 1000109. https://doi.org/10.4172/2155-9546.1000109

Tovar, D., Zambonino, J., Cahu, C., Gatesoupe, F. J., Va´zquez-Ju´arez, R., & Le´sel, R. (2002). Effect of live yeast incorporation in compound diet on digestive enzyme activity in sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture*, *204*(1-2), 113-123. https://doi.org/10.1016/S0044-8486(01)00650-0

Vendrell, D., Balcazar, J. L., de Blas, I., Ruiz-Zarzuela, I., Girones, O., & Muzquiz, J. L. (2008). Protection of rain-bow trout (*Oncorhynchus mykiss*) from lactococcosis by probiotic bacteria. *Comparative Immunology, Microbiology and Infectious Diseases*, *31*(4), 337–345. https://doi.org/10.1016/j.cimid.2007.04.002

Welker, T. L., Lim, C., Yildirim-Aksoy, M., & Klesius, P. H. (2012). Effect of short-term feeding duration of diets containing commercial whole-cell yeast or yeast subcomponents on immune function and disease resistance in channel catfish, *Ictalurus punctatus*. *Journal of Animal Physiology and Animal Nutrition*, *96*(2), 159-171. https://doi.org/10.1111/j.1439-0396.2011.01127.x

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The Potential of Rhamnolipid as Biofungicide against *Rigidoporus microporus* Isolated from Rubber Tree (*Hevea brasiliensis*)

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ABSTRACT

Rigidoporus microporus is the main causal of white root disease (WRD) in rubber trees (*Hevea brasiliensis*). The present study investigates the use of rhamnolipid, a biosurfactant produced by *Pseudomonas aeruginosa* USM-AR2 against *R. microporus*. *In vitro* dose-responses towards rhamnolipid were determined on different isolates of *R. microporus* using the poisoned food technique (PFT). Inhibition of mycelial growth was found to be dose-dependent, with the highest inhibition of 76.74% at 200 ppm (pH 6.29) on SEG isolate. On the contrary, the lowest concentration of rhamnolipid applied at 10 ppm (pH 5.97) had effectively inhibited the growth of RL 19 to 34.36%. AM isolate was assumed to be the most aggressive pathogen due to the lowest inhibition recorded on all rhamnolipid concentrations tested. At the same time, RL 19 was the least aggressive pathogen compared to the other *R. microporus* isolates. The rhamnolipid concentrations (ppm), which reduced mycelial growth at 50% (EC_{50}), were recorded at 17.82 ppm for AM isolate, 12.52 ppm for RL 26, and 11.80 ppm for RL 19 isolate. This result indicated that rhamnolipid concentrations to inhibit 50% of mycelial growth might vary based on the aggressiveness and the virulence levels of different *R. microporus* isolates. It was found that pH changes after incorporating rhamnolipid into the PDA were not the main factor affecting the inhibition of *R. microporus* isolates. It is obvious that rhamnolipid had an inhibitory effect on fungal growth *in vitro*. It is the first report on rhamnolipid that has been shown to control *R. microporus* potentially.

Keywords: EC_{50}, inhibition, isolate, *in-vitro*

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INTRODUCTION

White root disease, caused by the pathogenic fungus *Rigidoporus microporus*, is the most destructive root disease of *Hevea brasiliensis* Muell. Arg. worldwide (Oghenekaro et al., 2016). Rubber industries worldwide have been facing significant reductions in economic returns due to this disease since the infection kills rubber trees irrespective of age (Soytong & Kaewchai, 2014). *Hevea brasiliensis*, regardless of clone and age, are susceptible to white root disease (Farhana et al., 2017). This disease occurs in young rubber plantations, virgin jungles, and replanted areas. It has been determined to be more severe in young rubber plantations, especially after two years of planting (Prasetyo et al., 2009). In Malaysia, a survey of rubber diseases conducted by the Malaysian Rubber Board in 2012 revealed that the incidence of white root disease occurred in 10–15% of Peninsular Malaysia, 20–30% of Sabah, and 9–20% in Sarawak out of the total area of rubber plantations, which amounts to 1,065,630 hectares (Atan, 2015). Furthermore, white root disease has resulted in more tree losses than red or brown root disease, particularly between the first to fourth years after planting (Nicole & Benhamou, 1991; Wattanasilakorn et al., 2017). The application of sulfur as one of the standard preventive methods of white root disease during the early stage of rubber cultivation, has been adopted widely in rubber growing countries (Ismail & Azaldin, 1985; Rodesuchit et al., 2012; Satchuthananthavale & Halangoda, 1971).

In addition, drenching with triazole group of fungicide, propiconazole was believed to be an effective method to control *R. microporus* as the procedure was easy and fast (Hashim & Chew, 1997). Although effective, this fungicide has caused a variety of problems for natural environments, including a significant impact on soil microbial diversity and human health, as well as being quite expensive when it is required to be used consistently (Go et al., 2013; Jayasuriya & Thennakoon, 2007; Ogbebor et al., 2015; Satapute & Kaliwal, 2015). Moreover, the incidence of white root disease is still widespread, prompting immediate further investigation into safer sources of natural fungicides for the control of *R. microporus*. Monnier et al. (2020) mentioned that rhamnolipids (RLs) are natural glycolipids mainly produced by the bacteria *Pseudomonas aeruginosa*. The robust surface activity of dirhamnolipid could be related to antifungals produced by rhamnolipid that inhibit the growth modes of dimorphic fungi resulting in the disruption of the cell membrane spore (Sha & Meng, 2016). Aside from the recognized antifungal properties, rhamnolipids have never been studied for antifungal activities against *R. microporus*. RLs have a hydrophobic tail containing one or two fatty acids attached to the carboxyl end of one or two rhaminose molecules (Charles Oluwaseun et al., 2017). A few studies have shown a significant impact of rhamnolipid treatments in controlling plant diseases (Borah et al., 2016).

Pseudomonas aeruginosa USM-AR2, a hydrocarbon-utilizing bacterium, has been shown to secrete copious amounts of rhamnolipid when grown on water-immiscible substrates (Md Noh et al., 2014; Noh et al., 2012). Rhamnolipid has been explored as a potential antifungal agent for environmental-friendly agricultural practice. The concern lies in the repeated use of fungicides, which are harmful to the environment and increase the chemical resistance in the target organisms. Should rhamnolipid supplementation prove to be a viable alternative treatment for *R. microporus*, further study must be carried out to identify its mode of action and explore its potential in curbing the spread of white root disease. Hence, the current study has been intended to examine the ability of rhamnolipid to suppress *R. microporus in vitro* and its subsequent use to control white root disease of rubber *in vivo*.

MATERIALS AND METHODS

Source of Isolate

In this study, five isolates of *R. microporus* obtained from the Integrated and Disease Management Unit, Malaysian Rubber Board were RL 18, RL 19, RL 26, SEG, and AM. The locations of where the isolates were collected are presented in Table 1.

Table 1
List of Rigidoporus microporus *isolates used in this study*

Isolate	Locality of Collection
RL 18	Sungai Buloh, Selangor
RL 19	Seremban, Negeri Sembilan
RL 26	Kota Tinggi, Johor
SEG	Segamat, Johor
AM	Ayer Molek, Melaka

Production of Rhamnolipid

Rhamnolipid production was performed by cultivating *P. aeruginosa* USM-AR2 via a submerged batch fermentation. Cultivation was done in a 3.6 L stirred tank bioreactor (Labfors 4, INFORS HT, Switzerland) with a 1.5 L working volume at room temperature (27-30°C), agitated at 400 rpm aerated at an airflow rate of 0.5 vvm. The pH was left uncontrolled. A minimal salt medium (MSM) was used to cultivate *P. aeruginosa* USM-AR2 containing the ingredients as follows (per liter): sodium nitrate ($NaNO_3$) 8.25 g/L, magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) 0.75 g/L, potassium chloride (KCl) 1.5 g/L, dipotassium hydrogen phosphate (K_2HPO_4) 0.45 g/L, waste cooking oil 5% (v/v). Seed culture was prepared in nutrient broth before inoculation into the production medium. A 2% (v/v) cell suspension of a 24-h culture (OD_{540} = 2) was used as the inoculum.

Rhamnolipid Recovery

The fermentation broth was centrifuged (Hettich Zentrifugen, Universal-320R, Germany) at 8000 × *g* for 20 minutes to separate the supernatant containing rhamnolipid from the cells. Subsequently, the supernatant was transferred into a 500 ml shake flask and added ethyl acetate at a 1:1 volume ratio. The supernatant-solvent mixture was shaken at 200 rpm for 1 hour in an orbital shaker (Thermo Scientific, MaxQ-4000, USA). It was subsequently transferred into a separating funnel, where it was left to stand overnight to form separate layers. The upper aqueous layer containing rhamnolipid

was carefully transferred into a glass Petri dish and dried overnight in a fume hood chamber. Dried crude rhamnolipid was scraped from the surface of the glass Petri dish and stored at 4°C for further use.

Agar Preparation

Different rhamnolipid concentrations were screened against *R. microporus* isolates while commercial triazole group fungicide, propiconazole was tested against the most aggressive isolate of *R. microporus* using poisoned food technique (PFT) (Balamurugan, 2014; Durgeshlal et al., 2019) with slight modification. Firstly, 3,000 ppm of propiconazole and rhamnolipid were prepared as a stock of treatments and autoclaved at 121ºC, 1.05 kg/cm^2 for 21 minutes. Then, a specific amount of propiconazole and rhamnolipid stocks was incorporated into autoclaved potato dextrose agar (PDA) to achieve the desired concentration of 10, 25, 50, 100, and 200 ppm. The Petri dishes were shaken gently and laterally to allow propiconazole and rhamnolipid to distribute evenly in the PDA medium. PDA without rhamnolipid served as a negative control, while PDA amended with propiconazole serves as a positive control. The pH values of the prepared medium were recorded using Delta 320 pH meter (Mettler Toledo Instruments (Shanghai) Co. Ltd, China). The pH value of unamended PDA was recorded to be 6.0. Additionally, the pH values recorded for propiconazole and rhamnolipid ranged between 5.45 to 5.91 and 5.39 to 6.29, respectively, with the different concentrations applied into amended PDA. Subsequently, the agar solutions were decanted into Petri dishes and left to set before being incubated for two days to ensure no contamination.

Antifungal Activity Assay

A five mm diametric mycelial plug of 7 days old culture of *R. microporus* was placed at the center of the Petri dishes. The Petri dishes were then sealed with parafilm (Pechiney, USA) and incubated at room temperature. Data on the radial colony diameter were recorded seven days after incubation or when the growth of the control treatment completely covered the Petri dishes. In addition, a colony radius on PDA for each rhamnolipid concentration was measured from the bottom side of the Petri dishes. The mycelium was observed in-situ under an Olympus CX41 light compound microscope (Olympus Optical Co. Ltd. Tokyo, Japan) with a magnification of 10x and images recorded using an XCAM-a camera (The Imaging Source GmbH, Germany). Percentage inhibition of radial growth (PIRG) was calculated using the following formula developed by Skidmore and Dickinson (1976):

$$\text{PIRG (\%)} = \left(\frac{fungal\ growth - control\ growth}{control\ growth}\right) \times 100\%$$

Experimental Design and Data Analysis

The experiments were conducted in a completely randomized design (CRD) with three replications. An analysis of variance (ANOVA) was performed using SPSS®

24.0 for the Windows program. Factorial ANOVA analyses and Duncan's multiple range test (DMRT) were used to detect significant differences between treatments, and differences were considered significant when $p \leq 0.05$. All data were expressed as mean ± standard error. EC_{50} represents the concentration at which a rhamnolipid exerts half of its maximal response was analyzed using EC_{50} calculator software (AAT Bioquest Inc, n.d.).

RESULTS AND DISCUSSION

The ability of *P. aeruginosa* culture to produce rhamnolipid when grown on oil substrates has been widely reported (Ndlovu et al., 2017; Shi et al., 2021; Vanavil & Seshagiri, 2018). In this study, *P. aeruginosa* USM-AR2 was grown in a medium supplemented with waste cooking oil. Many studies have shown waste cooking oil as a renewable and low-cost substrate for rhamnolipid production (Chen et al., 2018; Radzuan et al., 2017). Waste cooking oil contains palm oil and other nutrient-rich compounds primarily to support microbial growth. Palm oil in waste cooking oil consists of saturated and unsaturated fats, made up of triglycerides, diglycerides, monoglycerides, and free fatty acids.

After five days of fermentation, *P. aeruginosa* USM-AR2 produced the rhamnolipid concentration of 3.0 g/L. Rhamnolipid is a secondary metabolite characterized by its production during the stationary phase. Towards the end of fermentation, an increase in rhamnolipid production was observed after microbial growth had ceased (Md Noh et al., 2014). Rhamnolipid is an extracellular metabolite; hence, it is secreted into the culture broth. It was harvested and recovered from the culture supernatant. Subsequently, the crude rhamnolipid suspension was used to screen antifungal activity against *R. microporus*.

It was found that rhamnolipid could inhibit the growth of *R. microporus* (RL 19 isolate) to 34.36% at the lowest concentration of rhamnolipid (10 ppm) used. In comparison, the highest concentration (200 ppm) of rhamnolipid had inhibited 76.74% growth of SEG isolate of *R. microporus* (Table 2). In addition, three

Table 2
Mean inhibition of Rigidoporus microporus isolates *at different rhamnolipid concentrations in amended PDA*

Concentration of rhamnolipid (ppm)	Percentage inhibition of radial growth (%)				
	RL 18	RL 19	RL 26	SEG	AM
0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
10	30.39 ± 1.71^{b}	34.37 ± 1.83^{b}	29.52 ± 3.35^{b}	25.64 ± 2.37^{b}	24.66 ±2.47^{b}
25	57.06 ± 0.34^{c}	56.51 ± 0.99^{c}	55.02 ± 1.12^{c}	57.26 ± 0.25^{c}	47.26 ± 3.89^{c}
50	65.29 ± 0.59^{d}	67.98 ± 0.58^{d}	60.24 ± 1.20^{d}	64.41 ± 1.45^{d}	59.81 ± 1.65^{d}
100	69.22 ± 1.09^{e}	71.53 ± 1.00^{e}	64.66 ± 0.20^{d}	69.98 ± 0.34^{e}	64.38 ± 0.68^{d}
200	75.10 ± 0.85^{f}	76.47 ± 0.90^{f}	73.89 ± 0.72^{e}	76.74 ± 0.38^{f}	74.66 ± 5.14^{e}

Note. Data are means of three replicates ± SE. Different letters within each column indicate significantly different values ($p \leq 0.05$) according to Duncan's multiple range test (DMRT)

isolates of *R. microporus* known as RL 19, RL 26, and AM collected from different locations in Peninsular Malaysia (Table 1) showed significant differences after exposure against rhamnolipid (Table 2 and Figure 1). Indeed, the effectiveness of rhamnolipid in promoting plant growth and controlling plant diseases has been discussed in many studies (Goswami et al., 2015; Jishma et al., 2021; Monnier et al., 2020).

The effectiveness of rhamnolipid in reducing the mycelial growth of the *R. microporus* depends highly on its concentration. It showed a significant difference among the total concentrations (ppm) applied compared to control (without rhamnolipid addition) (Figures 2 and 3). The results obtained were similar to Deepika et al. (2015), who claimed a significant reduction in tomato disease severity with the increased concentration of

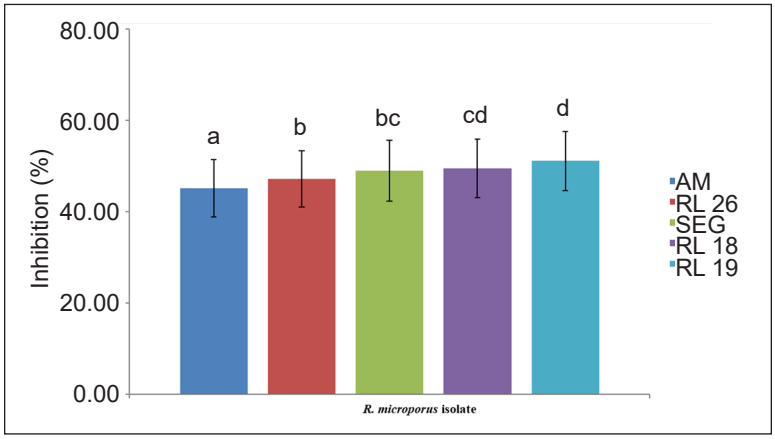


Figure 1. Inhibitory effect of rhamnolipid on mycelial growth of five (5) *Rigidoporus microporus* isolates on amended PDA with rhamnolipid. Error bars represent standard error (SE). Different letters indicate significantly different values at $p \leq 0.05$ according to Duncan's multiple range test (DMRT)

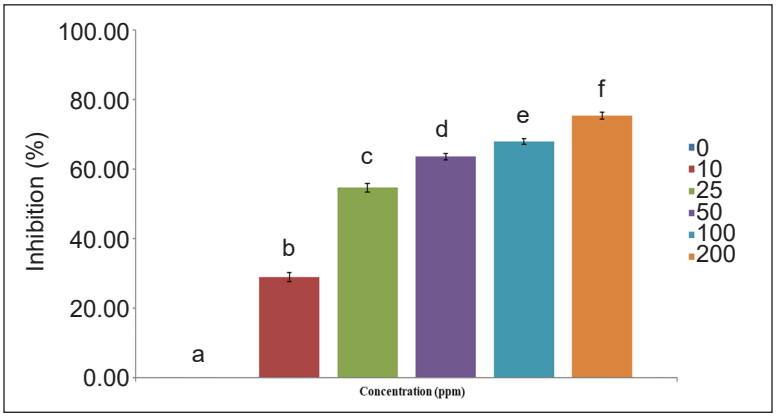


Figure 2. Inhibitory effect of different rhamnolipid concentrations on mycelial growth of *Rigidoporus microporus* isolates on PDA. Error bars represent standard error (SE). Different letters indicate significantly different values at $p \leq 0.05$ according to Duncan's multiple range test (DMRT)

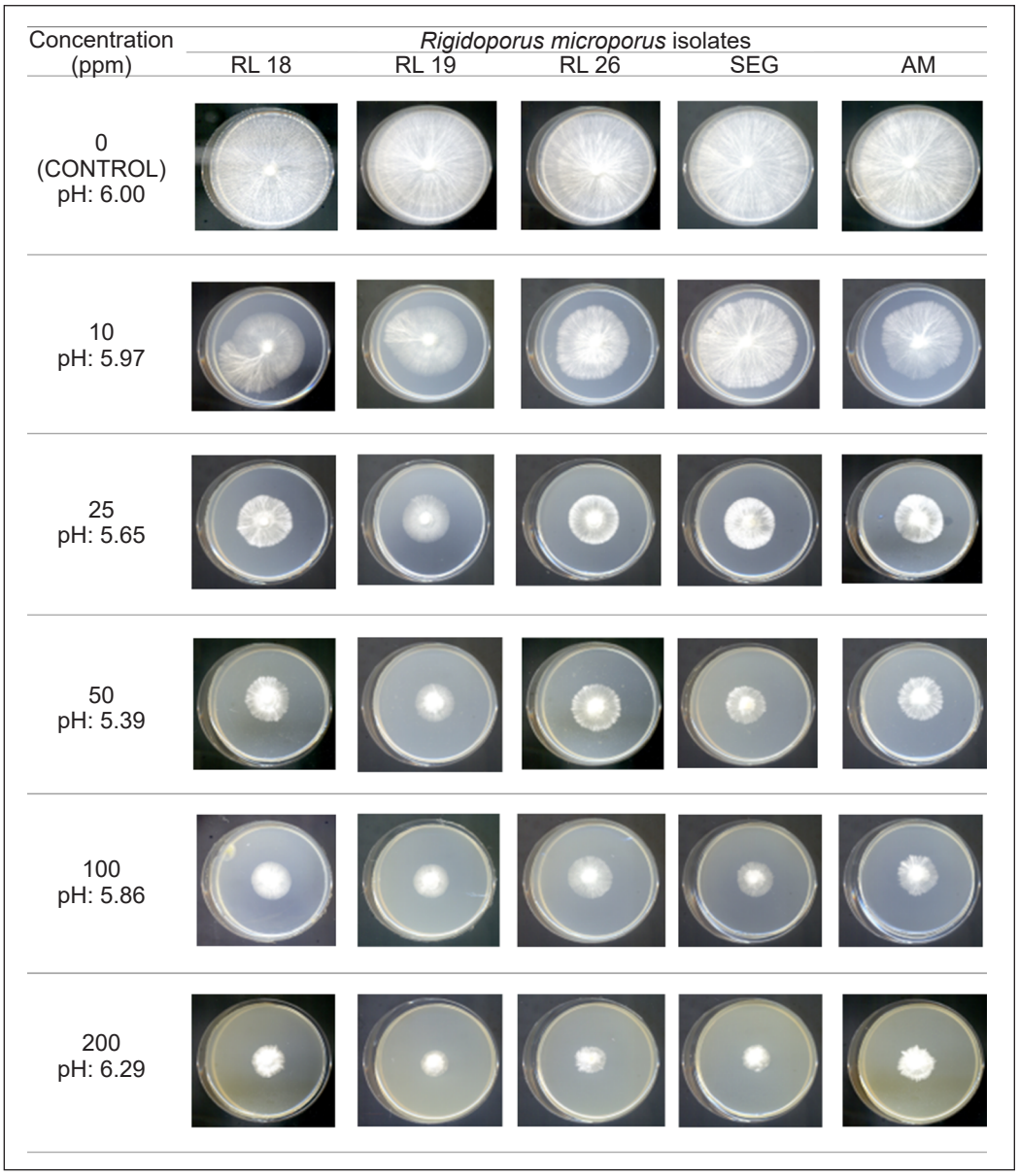


Figure 3. Cultures of *Rigidoporus microporus* in control and different concentrations of rhamnolipid incorporated into the PDA after 7 days of incubation

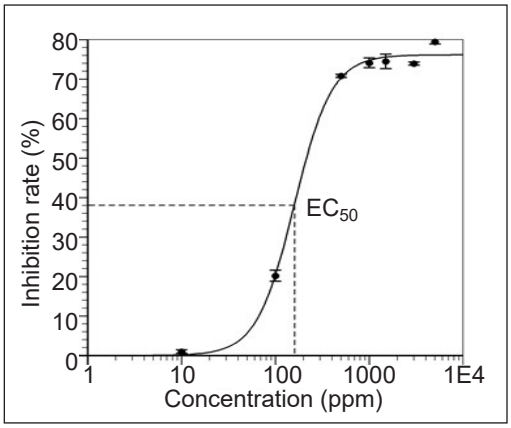
rhamnolipids used as treatment. Goswami et al. (2015) mentioned that more than 60 % reduction occurred on spore germination of *Colletotrichum falcatum* after exposure to the rhamnolipid at the concentration of 50 μgml^{-1}. Simlarly, Sha and Meng (2016) found that the application of 60 μgml^{-1} rhamnolipids had inhibited the colony growth of *Verticillium dahliae* ATCC 7611 up to 73%.

In addition, rhamnolipid biosurfactant was observed to be highly effective not

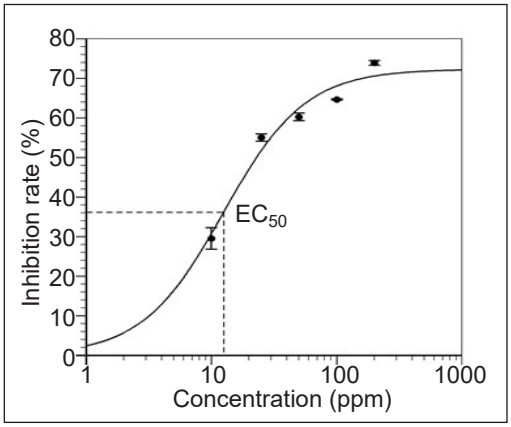
only in completely inhibiting the eggplant disease severity caused by *Fusariym oxysproum* f. sp. *melongenae* (*Fomg*) was also environmentally friendly (Nalini & Parthasarathi, 2018). On the contrary, Yan et al. (2014) revealed that rhamnolipid alone was not practical to control the growth of *Alternaria alternata* infection in cherry tomato fruit *in vivo*, but it was more efficient when combined with *Rhodotorula glutinis*. This finding was contended by Borah et al. (2016), who found the complete inhibition of stalk and ear rot disease in maize cv. PAC740 in a single application of rhamnolipid at 50 mg l^{-1}.

On average, AM isolate exhibited the lowest inhibition rate (%) compared to RL 19 isolate after being treated with different rhamnolipid concentrations (ppm). It is speculated that this is due to the different levels of virulence among isolates. Based on phylogenetic analysis of the β-tubulin gene region, it was discovered that *R. microporus* isolates collected from various areas in Malaysia using cultural and molecular characteristics had a distinct geographical origin among 27 local isolates (Andrew et al., 2021). Additionally, Siddiqui et al. (2017) claimed that AM isolate had been classified as an aggressive pathogen due to the expression of a higher number of proteins in AM-infected samples.

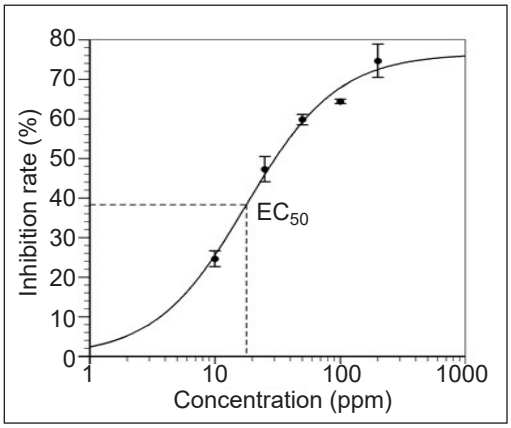
It was observed that the concentrations of rhamnolipid required to reduce mycelial growth at 50 % (EC_{50}) on RL 19, RL 26, and AM were recorded at 11.80 ppm, 12.52 ppm, and 17.81 ppm, respectively (Figure 4). The minimal concentration of



(a)



(b)



(c)

Figure 4. EC_{50} values of rhamnolipid concentration (ppm) on mycelium growth of three (3) isolates of *Rigidoporus microporus* on PDA. (a) EC_{50} of RL 19 = 11.80 ppm; (b) EC_{50} of RL 26 = 12.52 ppm; (c) EC_{50} of AM = 17.81 ppm

rhamnolipid needed to reduce 50% of the *R. microporus* growth implies the efficacy of rhamnolipid as an alternative method to control the incidence of white root disease in rubber. In comparison to propiconazole, the efficacy of rhamnolipid requires a bit higher concentration to reduce 50% of fungal growth. The results were similar to the study on the effect of rhamnolipid towards *A. alternata* on cherry tomato fruit, where higher rhamnolipid concentration was required to control the pathogen compared to synthetic fungicides (Yan et al. 2014).

On the contrary, the application of the triazole group of fungicide, propiconazole, showed the highest inhibition growth of the *R. microporus* even at the minimal concentration of 10 ppm (76.27%) (Table 3). Although effective in inhibiting the *R. microporus* growth, propiconazole could be detrimental to the environment and human health. Knebel et al. (2018) as well as Satapute and Kaliwal (2015) revealed that propiconazole was discovered to be cytotoxic to human cancer cell line, exhibits an anticancer property, and is toxic to the liver. Furthermore, the Risk Assessment Committee (RAC) of the European Chemicals Agency (ECHA) has proposed that propiconazole be classified as toxic for reproduction category 1B, in accordance with the provisions of Regulation (EC) No 1272/2008, due to the toxic effects on the endocrine organs and the contamination of groundwater (Arena et al., 2017).

Although there is a concern on cost-effectiveness to produce a higher yield of rhamnolipid, Nalini and Parthasarathi (2018) suggested the application of solid-state fermentation, which was more efficient and cheaper in the production of biosurfactant compared to the conventional method by using submerged fermentation. Furthermore, they mentioned that rhamnolipid is a better alternative to chemical surfactants because of its low toxicity, greater biodegradability, environmentally friendly, and ability to reduce agrochemicals. Besides, rhamnolipid could also be applied directly to the diseased plant to control the fungal growth *in vivo* (Monnier et al., 2020).

Presumably, rhamnolipid incorporated into the PDA had not caused any significant changes in the pH values compared to the control (Figure 3). According to de Freitas Ferreira et al. (2019), rhamnolipid increased the antimicrobial activity in acidic conditions, and it can be classified as a pH-dependent biosurfactant. This finding seems contradictory with the result in this

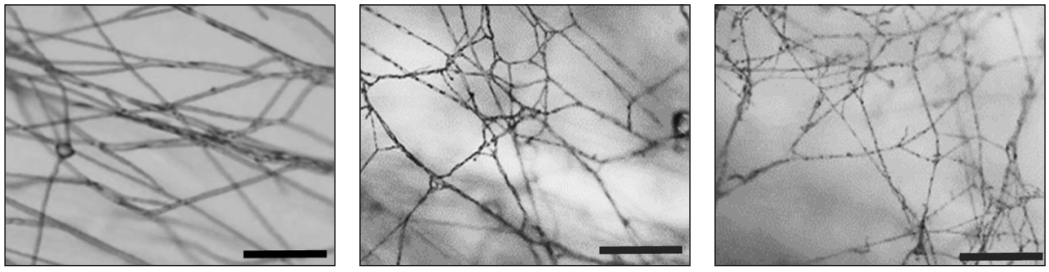
Table 3
Mean inhibition of Rigidoporus microporus *(AM isolate) at different propiconazole concentrations in amended PDA*

Percentage inhibition of radial growth (%)	Concentration of propiconazole (ppm)					
	0	10	25	50	100	200
AM	0.00 ± 0.00^{a}	76.27 ± 0.90^{b}	100.00 ± 0.00^{c}	100.00 ± 0.00^{c}	100.00 ± 0.00^{c}	100.00 ± 0.00^{c}

Note. Data are means of three replicates ± SE. Different letters within each column indicate significantly different values ($p \leq 0.05$) according to Duncan's multiple range test (DMRT)

study that showed the highest inhibition (>70%) of *R. microporus* isolates at the pH 6.29 (neutral condition) compared to a low inhibition (%) at a lower pH (more acidic condition). In the other study, Hadi et al. (2021) revealed that the higher pH of PDA after amended with soluble silicon did affect the growth of *R. microporus* (AM isolate), whereas no inhibition of *R. microporus* isolates was recorded in amended PDA without soluble silicon at higher pH. This finding was supported by the other study that mentioned the increase in pH values after incorporating soluble silicon into PDA did not solely cause the inhibition of mycelial growth of *Phytophthora cinnamomi* as the comparison test by increasing the pH values of PDA by using potassium hydroxide (KOH) had not given a significant reduction in the growth of *P. cinnamomi* mycelial (Kaiser et al., 2005). On the other hand, Prasetyo et al. (2009) mentioned that the growth of *R. microporus* in the rubber plantation was enhanced in the porous soil with neutral soil pH approximately between 6 to 7. Thus, the pH values can be ruled out as the main cause of the *R. microporus* growth inhibition.

The observation of *R. microporus* mycelial under a light compound microscope revealed the morphological changes of the mycelial structure comparing the untreated mycelial of *R. microporus* with the treated mycelial in amended PDA with 10 ppm and 200 ppm of rhamnolipid (Figure 5). The untreated mycelial were long, even, and the round hyphal with a smooth surface (Figure 5a). However, it was observed that the treated mycelial of *R. microporus* with rhamnolipid had shown sparse, asymmetric, curling, and twisting mycelium (Figure 5b); thinner and distorted newborn hyphae (Figure 5c). This result is similar to the scanning electron microscope observations of Borah et al. (2016), who found that the mycelial of *Fusarium verticillioides* FS7 was exhibited an irregular shape with an uneven surface, severely reduced thickness, and breakage after treated with rhamnolipid at the concentration of 200 mg l^{-1} in potato dextrose broth as compared to untreated mycelial. A fungus contains the vegetative structure known as mycelium that plays a crucial role in asexual reproduction and disease progression. As a result, any disruption to the mycelial integrity of a



(a) (b) (c)

Figure 5. Olympus CX41 light compound microscope images of (a) the untreated mycelial of *Rigidoporus microporus* (AM isolate); (b) mycelial treated with 10 ppm rhamnolipid; (c) mycelial treated with 200 ppm of rhamnolipid (scale bar: 200 µm; magnification at 10×)

fungus may have a detrimental effect on the pathogenicity of the fungus.

In this study, the light compound microscope was used to assess the effect of rhamnolipid on the mycelia of *R. microporus*. The findings demonstrated that rhamnolipid, a biosurfactant generated by *P. aeruginosa* USM-AR2, could severely modify the morphology of fungal mycelia. It has previously been observed that rhamnolipid influences the mycelial structure of fungi (Borah et al., 2016; Yan et al., 2015). The damage could be caused by the rhamnolipid's surfactant activity, which causes the breakdown of the phospholipid bilayer of the cell membrane, resulting in the leakage of electrolytes, proteins, and DNA (Bharali et al., 2013; Yan et al., 2015). According to Sotirova et al. (2012) and Monnier et al. (2019), the ability of rhamnolipid to inhibit fungal mycelial growth was attributed to the degradation of the cell membrane. Based on these results, early predictions can be made where rhamnolipid could have an inhibitory effect on fungal growth *in vitro*, mostly fungicidal.

CONCLUSION

The results of the present investigation reveal that the rhamnolipid biosurfactant produced by the bacterial strain *Pseudomonas aeruginosa* USM-AR2 has strong antifungal activity against *Rigidoporus microporus*, which may offer the possibility of its application as an alternative fungicide. In addition, the production of rhamnolipid from recycled material, such as waste cooking oil, is expected to be more economical and environmentally friendly than a current commercial fungicide. However, the concentration at which complete suppression of a particular fungus occurs varies and must be determined *in vitro* before *in vivo* investigations are initiated. Therefore, pot and field trials are suggested to be carried out in the near future to confirm the efficacy of rhamnolipid against *R. microporus in vivo*.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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REFERENCES

AAT Bioquest Inc. (n.d.). *Quest Graph™ EC50 calculator*. https://www.aatbio.com/tools/ec50-calculator

Andrew, B., Ahmad, K., Ismail, S. I., Ahmad, M. F., Ahmed, O. H., & Yun, W. M. (2021). Disease prevalence and molecular characterisation of *Rigidoporus microporus* associated with white root rot disease of rubber tree (*Hevea brasiliensis*) in Malaysia. *Journal of Rubber Research*, *24*(1), 175–186. https://doi.org/10.1007/s42464-021-00083-x

Arena, M., Auteri, D., Barmaz, S., Bellisai, G., Brancato, A., Brocca, D., Bura, L., Byers, H., Chiusolo, A., Court Marques, D., Crivellente, F., De Lentdecker, C., De Maglie, M., Egsmose, M., Erdos, Z., Fait, G., Ferreira, L., Goumenou, M., Greco, L., … Villamar-Bouza, L. (2017). Peer review of the pesticide risk assessment of the active substance propiconazole. *EFSA Journal*, *15*(7), e04887. https://doi.org/10.2903/j.efsa.2017.4887

Atan, S. (2015). *Integrated pest and disease management (IPDM): Our story* [Unpublished document]. Malaysian Rubber Board.

Balamurugan, S. (2014). *In vitro* antifungal activity of *Citrus aurantifolia* linn plant extracts against phytopathogenic fungi *Macrophomina phaseolina*. *International Letters of Natural Sciences*, *13*, 70–74. https://doi.org/10.18052/www.scipress.com/ILNS.13.70

Bharali, P., Saikia, J. P., Ray, A., & Konwar, B. K. (2013). Rhamnolipid (RL) from *Pseudomonas aeruginos*a OBP1: A novel chemotaxis and antibacterial agent. *Colloids and Surfaces B: Biointerfaces*, *103*, 502–509. https://doi.org/10.1016/j.colsurfb.2012.10.064

Borah, S. N., Goswami, D., Sarma, H. K., Cameotra, S. S., & Deka, S. (2016). Rhamnolipid biosurfactant against *Fusarium verticillioides* to control stalk and ear rot disease of maize. *Frontiers in Microbiology*, *7*, 1505. https://doi.org/10.3389/fmicb.2016.01505

Charles Oluwaseun, A., Julius Kola, O., Mishra, P., Ravinder Singh, J., Kumar Singh, A., Singh Cameotra, S., & Oluwasesan Micheal, B. (2017). Characterization and optimization of a rhamnolipid from *Pseudomonas aeruginosa* C1501 with novel biosurfactant activities. *Sustainable Chemistry and Pharmacy*, *6*, 26–36. https://doi.org/10.1016/j.scp.2017.07.001

Chen, C., Sun, N., Li, D., Long, S., Tang, X., Xiao, G., & Wang, L. (2018). Optimization and characterization of biosurfactant production from kitchen waste oil using *Pseudomonas aeruginosa*. *Environmental Science and Pollution Research*, *25*(15), 14934–14943. https://doi.org/10.1007/s11356-018-1691-1

de Freitas Ferreira, J., Vieira, E. A., & Nitschke, M. (2019). The antibacterial activity of rhamnolipid biosurfactant is pH dependent. *Food Research International*, *116*, 737–744. https://doi.org/10.1016/j.foodres.2018.09.005

Deepika, K. V., Ramu Sridhar, P., & Bramhachari, P. V. (2015). Characterization and antifungal properties of rhamnolipids produced by mangrove sediment bacterium *Pseudomonas aeruginosa* strain KVD-HM52. *Biocatalysis and Agricultural Biotechnology*, *4*(4), 608–615. https://doi.org/10.1016/j.bcab.2015.09.009

Durgeshlal, C., Sahroj Khan, M., Prabhat, S. A., & Aaditya Prasad, Y. (2019). Antifungal activity of three different ethanolic extract against isolates from diseased rice plant. *Journal of Analytical Techniques and Research*, *1*(1), 47–63. https://doi.org/10.26502/jatri.007

Farhana, A. H. K. F., Bahri, A. R. S., Thanh, T. A. V., & Zakaria, L. (2017). Morphological features of *Rigidoporus microporus* isolated from infected Malaysian rubber clones. *Malaysian Journal of Microscopy*, *13*(1), 17–23.

Go, W. Z., Wong, M. Y., Tan, G. H., Chuah, A. L., Salmiah, U., Soni, O., Wong, W. Z., Chin, K. L., & Chai, E. W. (2013). Occurence and characterisation of mycoflora in soil of different health conditions associated with white root rot disease in Malaysian rubber plantation. *Journal of Rubber Research*, *18*(3), 159–170.

Goswami, D., Borah, S. N., Lahkar, J., Handique, P. J., & Deka, S. (2015). Antifungal properties of rhamnolipid produced by *Pseudomonas aeruginosa* DS9 against *Colletotrichum falcatum*. *Journal of Basic Microbiology*, *55*(11), 1265–1274. https://doi.org/10.1002/jobm.201500220

Hadi, S. M. H. S. A., Zakaria, L., Sidique, S. N. M., Mahyudin, M. M., & Mohd, N. (2021). The potential of soluble silicon for managing white root disease in rubber (*Hevea brasiliensis*). *Australian Journal of Crop Science*, *15*(10), 1346–1354. https://doi.org/10.21475/ajcs.21.15.10.p3343

Hashim, I., & Chew, B. H. (1997). Effects of integrating *Trichoderma* and fungicide on control of white root disease of *Hevea* rubber. *Journal of Natural Rubber Research*, *12*(1), 43–57.

Ismail, H., & Azaldin, M. Y. (1985). Interaction of sulphur with soil pH and root diseases of rubber. *Journal Rubber Research Institute Malaysia*, *33*(2), 59–69.

Jayasuriya, K. E., & Thennakoon, B. I. (2007). Biological control of *Rigidoporus microporus*, the cause of white root disease in rubber. *Chiang Mai Journal of Science*, *46*(5), 850-866.

Jishma, P., Shad, K. S., Athulya, E., Sachidanandan, P., & Radhakrishnan, E. (2021). Rhizospheric *Pseudomonas* spp. with plant growth promotion and antifungal properties against *Sclerotium rolfsii* mediated pathogenesis in *Vigna unguiculata*. *Plant Biotechnology Reports*, *15*(4), 483–491. https://doi.org/10.1007/s11816-021-00687-0

Kaiser, C., Merwe, R. Van Der, Bekker, T. F., & Labuschagne, N. (2005). *In-vitro* inhibition of mycelial growth of several phytopathogenic fungi, including *Phytophthora cinnamomi* by soluble silicon. *South African Avocado Growers' Association Yearbook*, *28*(1), 70–74.

Knebel, C., Neeb, J., Zahn, E., Schmidt, F., Carazo, A., Holas, O., Pavek, P., Püschel, G. P., Zanger, U. M., Süssmuth, R., Lampen, A., Marx-Stoelting, P., & Braeuning, A. (2018). Unexpected effects of propiconazole, tebuconazole, and their mixture on the receptors CAR and PXR in human liver cells. *Toxicological Sciences*, *163*(1), 170–181. https://doi.org/10.1093/toxsci/kfy026

Md Noh, N. A., Mohd Salleh, S., & Yahya, A. R. M. (2014). Enhanced rhamnolipid production by *Pseudomonas aeruginosa* USM-AR2 via fed-batch cultivation based on maximum substrate uptake rate. *Letters in Applied Microbiology*, *58*(6), 617–623. https://doi.org/10.1111/lam.12236

Monnier, N., Cordier, M., Dahi, A., Santoni, V., Guénin, S., Clément, C., Sarazin, C., Penaud, A., Dorey, S., Cordelier, S., & Rippa, S. (2020). Semipurified rhamnolipid mixes protect *Brassica napus* against *Leptosphaeria maculans* early infections. *Phytopathology*, *110*(4), 834–842. https://doi.org/10.1094/PHYTO-07-19-0275-R

Monnier, N., Furlan, A., Buchoux, S., Deleu, M., Dauchez, M., Rippa, S., & Sarazin, C. (2019). Exploring the dual interaction of natural rhamnolipids with plant and fungal biomimetic plasma membranes through biophysical studies. *International Journal of Molecular Sciences*, *20*(5), 1009. https://doi.org/10.3390/ijms20051009

Nalini, S., & Parthasarathi, R. (2018). Optimization of rhamnolipid biosurfactant production from *Serratia rubidaea* SNAU02 under solid-state fermentation and its biocontrol efficacy against Fusarium wilt of eggplant. *Annals of*

Agrarian Science, *16*(2), 108–115. https://doi.org/10.1016/j.aasci.2017.11.002

Ndlovu, T., Rautenbach, M., Khan, S., & Khan, W. (2017). Variants of lipopeptides and glycolipids produced by *Bacillus amyloliquefaciens* and *Pseudomonas aeruginosa* cultured in different carbon substrates. *AMB Express*, *7*, 109. https://doi.org/10.1186/s13568-017-0367-4

Nicole, M. R., & Benhamou, N. (1991). Ultrastructural localization of chitin in cell walls of *Rigidoporus lignosus*, the white-rot fungus of rubber tree roots. *Physiological and Molecular Plant Pathology*, *39*(6), 415–431. https://doi.org/10.1016/0885-5765(91)90008-6

Noh, N. A., Salleh, S. M., Abdullah, A. A., & Mohd, A. R. (2012). Fed-batch cultivation of *Pseudomonas aeruginosa* USM-AR2 producing rhamnolipid in bioreactor through pulse feeding strategy. *International Proceedings of Chemical, Biological and Environmental Engineering*, *40*(34), 168-174.

Ogbebor, O. N., Adekunle, T. A., Eghafona, O. N., & Ogboghodo, A. I. (2015). *In vitro* and *in vivo* botanical control of *Rigidoporus microporus* (sw.) overeem of para rubber in Nigeria. *European Journal of Academic Essays*, *2*(3), 60–68.

Oghenekaro, A. O., Raffaello, T., Kovalchuk, A., & Asiegbu, F. O. (2016). *De novo* transcriptomic assembly and profiling of *Rigidoporus microporus* during saprotrophic growth on rubber wood. *BMC Genomics*, *17*(1), 234. https://doi.org/10.1186/s12864-016-2574-9

Prasetyo, J., Aeny, T. N., & Suharjo, R. (2009). The corelations between white rot (*Rigidoporus lignosus* L.) incidence and soil characters of rubber ecosystem in Penumangan Baru, Lampung. *Jurnal Hama dan Penyakit Tumbuhan Tropika*, *9*(2), 149–157.

Radzuan, M. N., Banat, I. M., & Winterburn, J. (2017). Production and characterization of rhamnolipid using palm oil agricultural refinery waste. *Bioresource Technology*, *225*, 99–105. https://doi.org/10.1016/j.biortech.2016.11.052

Rodesuchit, A., Suchatgul, S., Klaewklong, B., & Damnoi, S. (2012). Efficacy of fertilizers to control white root disease of rubber caused by *Rigidoporus microporus* at the early planting stages. *Rubber Thai Journal*, *72*(3), 62–72.

Satapute, P. P., & Kaliwal, B. B. (2015). *In vitro* toxicity screening of triazole fungicide propiconazole. *International Journal of Recent Scientific Research*, *6*(9), 6525–6528.

Satchuthananthavale, V., & Halangoda, L. (1971). Sulphur in the control of white root disease. *Journal Rubber Research Institute Ceylon*, *48*, 82–91.

Sha, R., & Meng, Q. (2016). Antifungal activity of rhamnolipids against dimorphic fungi. *The Journal of General and Applied Microbiology*, *62*(5), 233–239. https://doi.org/10.2323/jgam.2016.04.004

Shi, J., Chen, Y., Liu, X., & Li, D. (2021). Rhamnolipid production from waste cooking oil using newly isolated halotolerant *Pseudomonas aeruginosa* M4. *Journal of Cleaner Production*, *278*, 44–54. https://doi.org/10.1016/j.jclepro.2020.123879

Siddiqui, N., Middleton, C., Ribeiro, C., Atan, S., & Di Cola, A. (2017). Gel-based proteomic study for differential expression of *Hevea brasiliensis* root proteins in response to infection by soil fungus *Rigidoporus microporus*. *Acta Horticulturae*, *1152*(31), 229–234. https://doi.org/10.17660/ActaHortic.2017.1152.31

Skidmore, A. M., & Dickinson, C. H. (1976). Colony interactions and hyphal interference between *Septoria nodorum* and phylloplane fungi. *Transactions of the British Mycological Society*, *66*(1), 57–64. https://doi.org/10.1016/s0007-1536(76)80092-7

Sotirova, A., Avramova, T., Stoitsova, S., Lazarkevich, I., Lubenets, V., Karpenko, E., & Galabova,

D. (2012). The importance of rhamnolipid-biosurfactant-induced changes in bacterial membrane lipids of *Bacillus subtilis* for the antimicrobial activity of thiosulfonates. *Current Microbiology*, *65*(5), 534–541. https://doi.org/10.1007/s00284-012-0191-7

Soytong, K., & Kaewchai, S. (2014). Biological control of white root of rubber trees using *Chaetomium cupreum*. *International Journal of Agricultural Technology*, *10*(1), 93–103.

Vanavil, B., & Seshagiri Rao, A. (2018). Dual substrate fermentation using palm oil and glucose for production of eco-friendly biosurfactants using *P. aeruginosa* NITT 6L. *Indian Journal of Chemical Technology*, *25*(1), 101–105.

Wattanasilakorn, S., Sdoodee, S., Nualsri, C., Chuenchit, S., Meesawat, U., & Sopharat, J. (2017). Assessment of rubber clonal rootstocks for the tolerance of white root disease (*Rigidoporus microporus*) in Southern Thailand. *Walailak Journal of Science and Technology*, *14*(7), 549–561.

Yan, F., Xu, S., Chen, Y., & Zheng, X. (2014). Effect of rhamnolipids on *Rhodotorula glutinis* biocontrol of *Alternaria alternata* infection in cherry tomato fruit. *Postharvest Biology and Technology*, *97*, 32–35. https://doi.org/10.1016/j.postharvbio.2014.05.017

Yan, F., Xu, S., Guo, J., Chen, Q., Meng, Q., & Zheng, X. (2015). Biocontrol of post-harvest *Alternaria alternata* decay of cherry tomatoes with rhamnolipids and possible mechanisms of action. *Journal of the Science of Food and Agriculture*, *95*(7), 1469–1474. https://doi.org/10.1002/jsfa.6845

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Arbuscular Mycorrhizal Association with Rattan Species of the Belum-Temengor Forest Complex, Perak, Malaysia

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ABSTRACT

Rattan (*Calamus* spp.) is a high commercial value palm from the subfamily Calamoideae the primary source for cane in the well-developed rattan industry. Most studies on rattan have focused on its biodiversity, distribution, quality, strength, physical, mechanical, and morphological properties and genetics. Still, only a few have investigated the association of rattan with arbuscular mycorrhizal (AM) fungi. These mycorrhizal fungi are well known to play essential functions as promoting plant growth, maintaining plant community biodiversity and nutrient cycles in soil. This study aims to identify the established AM fungi community and their ecological interactions with *Calamus* spp. in the Belum-Temengor Forest Complex, Perak, Malaysia. *Calamus* spp. roots and their rhizospheric soil samples were collected from six sampling sites in the Belum-Temengor Forest Complex, one of the oldest rainforests in the world. The degree of mycorrhizal colonisation in *Calamus* spp. was evaluated using the grid lines method. At the same time, the AM fungi spore diversity in the rhizospheric soils were isolated using the wet sieving method and identified taxonomically analysed into different genera. *Calamus insignis* showed the highest degree of mycorrhizal colonisation amongst all the *Calamus* spp. present on the sampling sites. The AM fungi spores isolated from the rhizospheric soil from Belum-Temengor Forest Complex belonged to the genera *Acaulospora*, *Entrophospora*, *Gigaspora*, *Glomus*, and *Scutellospora*. *Glomus* was the most frequently found genus in all the sampling sites. This study is the first record of the AM fungal diversity found in the Belum-Temengor Forest Complex.

Keywords: *Calamus* spp., *Glomus* spp., mycorrhiza, rainforest, rhizosphere

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INTRODUCTION

Rattans originated from Southeast Asia are the primary source for cane in the well-developed rattan industry. Rattan belongs to the subfamily Calamoideae, has spiny stems and scaly fruits (Stiegel et al., 2011), and is economically and ecologically essential in Asian rainforests (Gentry, 1991). The state of Perak has 17.9 %; the second-highest percentage of whole rattan group in the Permanent Reserved Forests, Peninsular Malaysia, based on the Third National Forest Inventory (NFI-3), behind Pahang with 37.2% (Chin et al., 1994). Certain rattan species' stem flexibility has made them useful for matting, binding, and furniture production (Dransfield, 1992). It is one of the most popular materials for handicrafts besides being used as a construction material, such as temporary building or construction for fishing traps, baskets, mats, and ornaments (Afentina et al., 2020).

Rattans are important to tropical primary and secondary rainforests, but their evolution, diversity and phylogenetic relationships remain poorly understood (Dransfield, 1992). One rattan species, *Calamus manan*, locally known as Rotan Manau, has substantial commercial value because it has a large diameter stem (approximately up to 8 cm without sheath), able to grow up to 100 m length (Kusuma et al., 2011), and it is widely distributed in Southern Thailand, Malaysia, Sumatera, and Kalimantan (Mohamad, 1993). However, due to its high commercial value and despite its broad distribution range, Rotan Manau has been excessively harvested from nature (Mohamad, 1993), which resulted in severe population depletion; therefore, this species has been designated as 'vulnerable.' Another factor contributing to the severe population depletion of Rotan Manau is habitat destruction and degradation (Baillie et al., 2004). In general, rattan is facing challenges in which the resources produced by these species are under serious threat by the transformation of forests to agricultural and other land uses, as well as excessive exploitation of the remaining stocks in the forests (Hirschberger, 2011). The novelty of this paper is the association of indigenous mycorrhizal fungi with local specific rattans in Perak, Malaysia, for example, Rotan Manau. Besides having a high economic value, rattan is associated with mycorrhizal, which plays an important role in promoting rattan plant growth and health. Therefore, it is necessary to isolate and identify the indigenous mycorrhizal fungi associated with the indigenous Malaysian rattan, for example, Rotan Manau.

Rattans have been studied in various research areas, such as clarification of their quality, strength, physical, mechanical, and morphological properties, efficiency in using different species of rattan (Isnard, 2006; Isnard & Rowe, 2007; Mathew & Bhat, 1997; Sudarmonowati et al., 2004), their genetics (Ramesha et al., 2007; Sarmah et al., 2007; Sudarmonowati et al., 2004), and conservation (Lyngdoh et al., 2005). However, most rattan studies mainly focused on the distribution, diversity, and commercial value, but only a few have investigated their association with arbuscular mycorrhizal

(AM) fungi. Arbuscular mycorrhizal fungi are an essential component in the soil ecosystem, as they form a mutualistic symbiosis with more than 80% of terrestrial plant roots (Brundrett & Tedersoo, 2018; Heijden et al., 2015; S. E. Smith & Read, 2008). The AM symbiosis is believed to be fundamental for land colonisation by plants (Brundrett & Tedersoo, 2018). It is found in almost all plant species, including those with commercial value (de Moura et al., 2019). Furthermore, the AM fungi are the fungi from the phylum Glomeromycota, and they form a mutualistic symbiosis with the majority of vascular plants and some non-vascular plants (Peterson et al., 2004).

AM fungi play essential roles, and fundamental functions of several ecosystems (de Moura et al., 2019) processes ranging from maintaining plant biodiversity, plant development and growth, nutrient cycling, organic matter decomposition, absorption of water and nutrients, phosphate solubilisation, soil aggregation (Andrade Júnior et al., 2018; Silva-Flores et al., 2019; Wicaksono et al., 2018), and improving plant tolerance to different abiotic and biotic stresses (Ait-El-Mokhtar et al., 2019; Meddich et al., 2015). However, despite the contributions showed by AM fungi to plant communities, there is data paucity to elucidate rattans' ability to form a mutualistic symbiosis with AM fungi. Therefore, it is essential to isolate and identify the indigenous AM fungi associated with rattan trees in the Perak reserve forest, Malaysia and understand the symbiotic dynamics between rattans and AM fungi communities in their natural growing environment. Isolation, identification, and propagation of the AM fungi from the rhizosphere of wild rattans are important because it will help understand the symbiotic dynamic of rattan and AM communities on how AM help to enhance rattan growth and health. This knowledge will be fundamental for rattan management, cultivation, productivity, and reduction of production costs in the future. This study was conducted to identify the established AMF community and their ecological interactions with *Calamus* spp. in the Belum-Temengor Forest Complex, Malaysia.

MATERIALS AND METHODS

Study Sites and Sampling

Soil and roots samples of rattan species were collected from Temengor Forest Reserve and Royal Belum State Park, in the Belum-Temengor Forest Complex located in the Northern region of Malaysia, Hulu Perak, Perak. This forest complex is managed under the Perak State Parks Corporation. The temperature of the Belum-Temengor Forest Complex ranges from 20°C to 35°C depending on the time of year, and the mean monthly rainfall (annual) of the Belum-Temengor Forest Complex is 1,500–2,000 mm (Malaysian Meteorological Department [MET], n.d.). The sampling sites at Temengor Forest Reserve were Pulau Perhilitan (N 05°28'13.2" E 101°20'34.3"), Sungai No. 2 (N 05°26'48.5'' E 101°21'38.1''), and Sungai Rokan (N 05°29'47.1" E 101°18'14.8"). Meanwhile, the sampling sites at Royal Belum State Park were Sungai Kejar (N 05°48'28.4"

E 101°25'31.3''), Sungai Papan (N 05°37'54.7" E 101°24'11.8"), and Sungai Kooi (N 05°39'21.3'' E 101°24'18.6''). Root samples and 100 g of soils from the rhizosphere area were collected in three replicates for each rattan tree per site. These sampling sites were chosen due to various rattan species' availability and closely related soil types: clay loam, silty clay, silty clay loam, and sandy clay loam. The soil texture of these sampling sites was determined using the ball and ribbon test (Whiting et al., 2015) and the Feel Method as detailed by Thien (1979).

The sampling was conducted in a 50 m × 20 m plot for each site. Once the rattan species were identified, the root systems of *Calamus* spp. were shovelled carefully to ensure that the fine hairy roots collected did not mix up with other plant roots. Only the confirmed roots attached to the base of rattan were collected to prevent any mix up of roots that are not from rattan. The soil around the rattan's rhizosphere was collected to study the arbuscular mycorrhizal (AM) fungi diversity. The collected root samples were cleaned, cleared, and stored in 50% ethanol (Brundett et al., 1996) before being processed further at the Mycorrhiza Laboratory, School of Biological Sciences, Universiti Sains Malaysia.

Soil Analyses

For moisture content analysis, a 5 g sub-sample of soil from each sampling location was weighted, oven-dried overnight, and the final weight was determined. First, the soil pH was measured using a digital benchtop automatic pH meter; 5 g of dry soil was weighed and mixed with distilled water. Then, the pH meter probe was placed, and the reading was repeated thrice before obtaining the average reading. Next, a digital soil thermometer was used to measure the soil temperature on-site for each location. The probe of the digital soil thermometer was pushed 5 cm vertically into the ground, and the reading was repeated thrice before recording the average reading (modified from GLOBE, 2014).

Arbuscular Mycorrhizal Fungi Spore Isolation and Identification

The spores of AM fungi were isolated using wet-sieving, centrifugation, and filtration methods as described by Brundett et al. (1996) and were identified in Mycorrhiza Laboratory, School of Biological Sciences, Universiti Sains Malaysia. Approximately 100 g of collected soil samples were weighed based on a sampling location and mixed with water. The soil samples were sieved through three different sizes of sieves: 250 µm, 75 µm, and 45 µm were collected and transferred separately into 50 mL centrifugation tubes for the centrifugation process. The soil samples were centrifuged for 5 minutes at 2,000 rotations per minute (rpm) to separate the spores from the soil. The debris with supernatant was discarded, and the pellet was kept for the second centrifugation. After that, the pellet was suspended in 50% sucrose and was centrifuged for 1 minute at 2,000 rpm. A vacuum pump containing filter paper was used to filter the supernatant of

the 50% sucrose. The collected spores from all sieves were counted and collected under a stereomicroscope (Olympus Research Stereomicroscope System SZX16, Olympus, Japan) for the isolation and identification process.

The filter paper containing soil supernatant placed in the Petri dish was observed under a stereomicroscope (Olympus Research Stereomicroscope System SZX16, Olympus, Japan) to separate, count, and collect the spores from all sieves under 400x magnification. The AM fungi spores were taxonomically analysed into different genera based on their phenotypic characteristics, such as shape, size, arrangement, and colour (Oehl et al., 2011). The spores were then mounted on slides with Melzer reagent (1.5 g potassium iodide (KI), 0.5 g iodine crystals (I), and 20 g chloral hydrate ($C_2H_3Cl_3O_2$) were added to 20 mL distilled water and mixed until dissolved.) and observed under an Olympus BX41 Phase Contrast and Darkfield Microscope (Olympus, Japan). The spores' physical morphology image was captured using a camera attached to Olympus BX41 equipped with the software Cell A and saved. The AM fungi spores were taxonomically analysed into different genera based on their phenotypic characteristics, such as shape, size, arrangement, and colour (Oehl et al., 2011) according to the identification manual of VA mycorrhizal fungi (Schenk & Perez, 1990) and the identification keys from International Culture Collection of Vascular-Arbuscular Mycorrhizal Fungi website (INVAM biogeographical database: http//: invam.caf.wvu.edu).

Staining Mycorrhizal Roots and Determination of Root Colonisation Degree

For the staining process, the roots in 15% potassium hydroxide (KOH) (Sigma-Aldrich, Malaysia) were autoclaved for 20 minutes at 121°C and washed in 2% hydrochloric acid (HCL) (Sigma-Aldrich, Malaysia) for a few minutes, leading to the neutralisation of the roots. The roots then were kept in a universal bottle containing 0.05% Trypan blue (Sigma-Aldrich, Malaysia) with lactoglycerol (TBLG) and left overnight (Brundett et al., 1996; Schenk & Perez, 1990). Next, the stained roots were transferred to the universal bottles containing lactoglycerol [lactic acid:glycerol:water, 1:1:1 (volume/volume/volume)], which functioned to store the stained roots for further observation. The stained roots were randomly selected and observed under the stereomicroscope (Olympus Research Stereomicroscope System SZX16, Olympus, Japan) and were smashed carefully to locate the presence of vesicles and hyphae. The most abundant mycorrhizal area in the root was identified under a stereomicroscope (Olympus Research Stereomicroscope System SZX16, Olympus, Japan), selected, and cut before being transferred permanently on a glass slide to be observed further under Olympus BX41 Phase Contrast and Darkfield Microscope (Olympus, Japan) at a magnification of 20× and 40×. The best images showing mycorrhizal colonisation were captured using a camera attached to Olympus BX41 (Olympus, Japan) equipped with the software Cell A and saved.

The degree of AM fungi roots colonisation was calculated using the gridline intersection method (Brundett et al., 1996). The most common mycorrhizal colonisation degree measuring method utilised was the gridline intersect method (McGonigle et al., 1990). The 50 segments of cleared and stained roots (1 g) were spread out evenly on a 9 cm x 9 cm Petri dish with grid lines marked on the bottom of the dish with 1 cm × 1 cm squares. The vertical and horizontal grid lines were scanned under a stereomicroscope (Olympus Research Stereomicroscope System SZX16, Olympus, Japan). The presence and absence of AM infection were recorded at each point where the roots intersected a line. The root segments were re-spread and re-examined three times. The fraction of root length mycorrhizal and total root length was calculated using the derived conversion factor (Giovannetti & Mosse, 1980).

Data Analysis

Statistical analyses were performed using the Minitab 19 Statistical Software (Minitab, 2019), and each data point represented the mean of different groups. One-way analysis of variance (ANOVA) and Tukey test were used to examine the differences among AM root colonisation degree data. *P* values less than 0.05 were considered statistically significant.

RESULTS

Soil Moisture, pH, Temperature, and Physical Characteristics

Moisture, pH, temperature, and physical characteristics of soil collected from each sampling site were measured, and Table 1 shows the recorded measurement.

Arbuscular Mycorrhizal Colonisation

The percentage of the mean degree of arbuscular mycorrhizal colonisation in

Table 1
Moisture, pH, temperature, and physical characteristics of soil collected from six sampling sites of Calamus *spp. from Temengor Forest Reserve and Royal Belum State Park in the Belum-Temengor Forest Complex*

Site	Soil moisture (%)	Soil pH	Soil temperature (°C)	[3]Forming ball	[3]Forming ribbon	[3]Soil feel	Type of soil
[1]Pulau Perhilitan	31.58	6.4	25.4	Yes	Yes	No noticeable feel	Clay loam
[1]Sungai No.2	30.72	6.58	24.6	Yes	Yes	Smooth	Silty clay loam
[1]Sungai Rokan	41.84	6.58	24.5	Yes	Yes	Smooth	Silty clay
[2]Sungai Kejar	21.86	6.03	23.5	Yes	Yes	No noticeable feel	Clay loam
[2]Sungai Papan	23.69	6.21	24.1	Yes	Yes	No noticeable feel	Clay loam
[2]Sungai Kooi	21.8	6.82	24.5	Yes	Yes	Gritty	Sandy clay loam

Note. [1]Temengor Forest Reserve, [2]Royal Belum State Park, [3]Characterised based on a ball and ribbon test (Whiting et al., 2015), and Feel Method as detailed by Thien (1979)

Calamus spp. roots were significantly different (one-way ANOVA test, $p < 0.0001$) among the rattan species (Figure 1). *Calamus insignis* showed the highest percentage of AM colonisation degree, whereas *Calamus viridispinus* recorded the lowest percentage degree of AM colonisation compared to other *Calamus* species (Figure 1). Optical microscopy images revealed the structure of arbuscular mycorrhizal fungi, likely arbuscules, vesicles, and hyphal coils in the *Calamus* spp. roots (Figure 2 and Figure 3). *Paris* type of arbuscules was present in the *Calamus* spp. roots (Figure 2a and Figure 3c). The *Paris*-type is characterised by extensive intracellular hyphal coils and arbusculate coils in the root cortex (Cavagnaro et al., 2001).

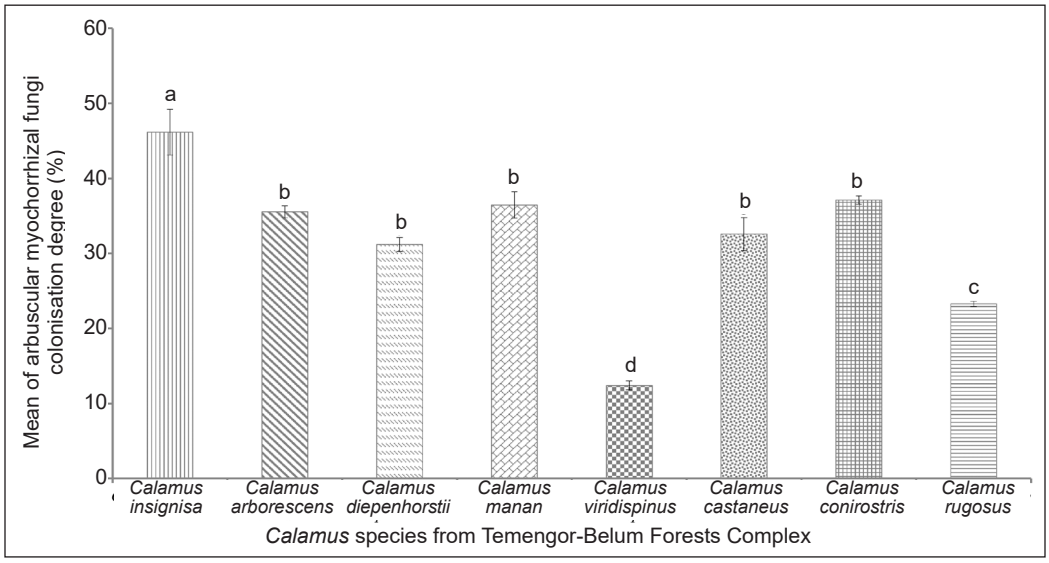

Figure 1. Degree of arbuscular mycorrhizal colonisation in *Calamus* spp. from Temengor Forest Reserve and Royal Belum State Park in the Belum-Temengor Forest Complex
Note. Bars represent mean value ± SE (n = 57). Bars with different alphabetical letters indicate the Tukey's test varies significantly at level $p < 0.05$

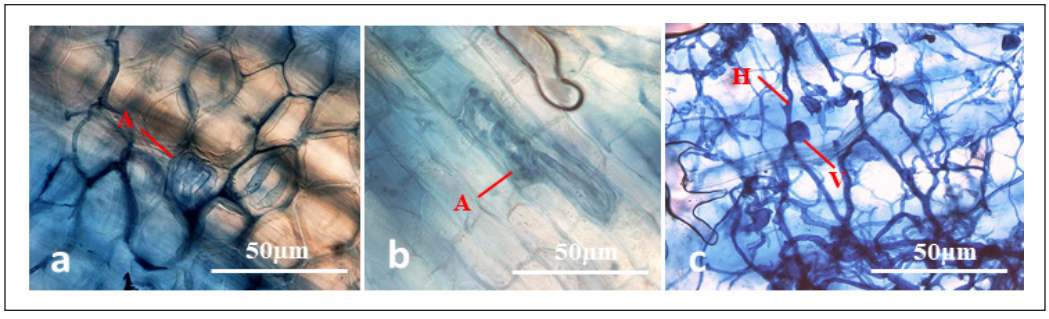

Figure 2. Mycorrhizal structures in the root of *Calamus* spp. from Temengor Forest Reserve in the Belum-Temengor Forest Complex (a-c) were observed under Olympus BX41 Phase Contrast and Darkfield Microscope. Optical microscopy images of *Calamus* spp. roots showed structures of arbuscules (A), vesicle (V), and hyphae (H). Bar scales = 50 µm

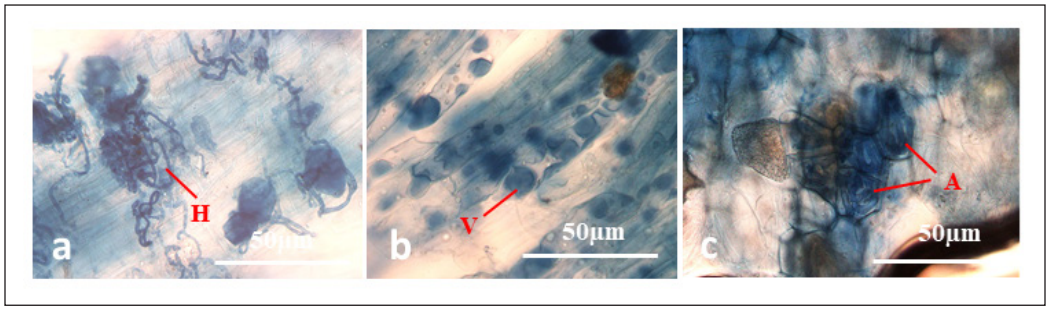

Figure 3. Mycorrhizal structures in the roots of *Calamus* spp. from Royal Belum State Park in the Belum-Temengor Forest Complex (a-c) were observed under Olympus BX41 Phase Contrast and Darkfield Microscope. Optical microscopy images of *Calamus* spp. root showed structures of arbuscules (A), vesicle (V), and hyphae (H). Bar scale = 50 µm

Arbuscular Mycorrhizal Spore Distribution

The arbuscular mycorrhizal fungi spores isolated from the rhizospheric soil samples from Belum-Temengor Forest Complex are *Acaulospora* sp., *Entrophospora* sp., *Gigaspora* sp., *Glomus* sp., and *Scutellospora* sp. (Figure 4 and Figure 5). Five genera of AM fungi spores were identified from all soil samples from six sampling sites at Temengor Forest Reserve and Royal Belum State Park in the Belum-Temengor Forest Complex (Table 2). Sungai No. 2, located in Temengor Forest Reserve, recorded the presence of all five genera of AM fungi spores, with a total count of AM spores is 226 (Table 2, Figure 5). Meanwhile, Pulau Perhilitan, also located in Temengor Forest Reserve, recorded the presence of only one AM fungi spore genus, *Glomus* sp., with a total count of AM spores is 95 (Table 2, Figure 4). *Glomus* sp. was isolated in every sampling site; meanwhile, *Gigaspora* sp. was isolated only from the soil sample in Temengor: Sungai No. 2 and Sungai Rokan, while *Entrophospora* sp. was isolated only from Sungai No. 2, Temengor and Sungai Kooi, Royal Belum State Park. Total count of arbuscular mycorrhizal spores in Royal Belum state park is higher compared

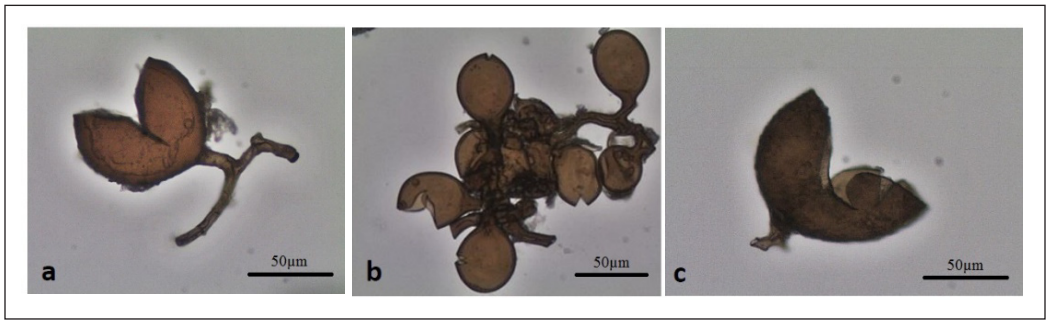

Figure 4. Arbuscular mycorrhizal spores. *Glomus* sp. (a-c) isolated from soil of Pulau Perhilitan, Temengor Forest Reserve in the Belum-Temengor Forest Complex observed under Olympus BX41 Phase Contrast and Darkfield Microscope. Bar scale = 50 µm

to Temengor Forest Reserve with 556 spores (Table 2). *Glomus* sp. is a relatively simple structure with globules shape, which develop thickened walls that may be multi-layered, brown, and usually with subtending hyphal attachment (Figure 4); *Acaulospora* sp. squashed spore of stained with Melzer's reagent showed orange outer wall later and dark purple stain in the innermost layer, the thin intermediate wall layer can be seen (Figure 5a) and multi-layer walls (Figure 5k). *Gigaspora* sp. is appeared to be a glomoid with a simple single structural wall (Figure 5b-c). *Scutellospora* sp. is formed singly in the soil, formed broadly ellipsoid, subglobose to oblong-shaped spores, the spore colour is brown to dark brown with a multi-layered wall (Figure 5i). Moreover, *Entrophospora* sp. is a spore with cicatrices formed; spore is globose to subglobose and has two walls: an outer spore wall and an inner wall (Figure 5j). *Glomus* sp. was isolated in every sampling site; meanwhile, *Gigaspora* sp. was isolated only from the soil sample in Temengor: Sungai No. 2 and Sungai Rokan, while *Entrophospora* sp. was isolated only from Sungai No. 2, Temengor and Sungai Kooi, Royal Belum State Park. The total count of arbuscular mycorrhizal spores in Royal Belum state park is higher compared to Temengor Forest Reserve, with 556 spores (Table 2).

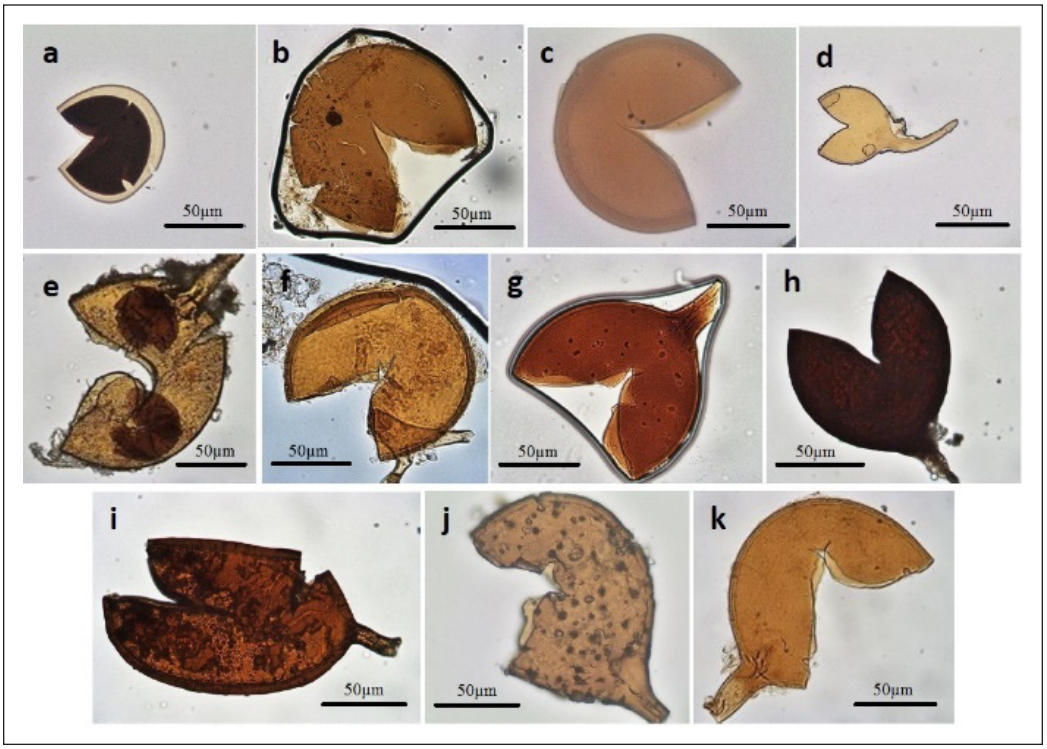

Figure 5. Arbuscular mycorrhizal spores. *Acaulospora* sp. (a), *Gigaspora* sp. (b-c), *Glomus* sp. (d-h), *Scutellospora* sp. (i), *Entrophospora* sp. (j), and *Acaulospora* sp. (k) observed under Olympus BX41 Phase Contrast and Darkfield Microscope. These spores are isolated from the soil of *Calamus arborescens* at Sungai No. 2, Temengor Forest Reserve in the Belum-Temengor Forest Complex. Bar scale = 50 µm

Table 2
The number of Calamus *spp. presence, mean degree of arbuscular mycorrhizal (AM) fungi colonisation (%), the total count of AM spores in 100 g soil and AM spore diversity and distribution from six sampling sites of* Calamus *spp. from Temengor Forest Reserve and Royal Belum State Park in the Belum-Temengor Forest Complex*

Sampling sites	Number *Calamus* sp. present per site	Mean degree of AM fungi colonisation (%) per site	Total count of AM spores (per 100 g soil)	Types of AM spore
[1]Pulau Perhilitan	2	46.18	95	*Glomus* sp.
[1]Sungai No. 2	2	34.93	226	*Acaulospora* sp., *Entrophospora* sp., *Gigaspora* sp., *Glomus* sp., and *Scutellospora* sp.
[1]Sungai Rokan	2	29.94	146	*Acaulospora* sp., *Gigaspora* sp., *Glomus* sp., and *Scutellospora* sp.
[2]Sungai Kejar	2	40.27	157	*Acaulospora* sp., *Glomus* sp., and *Scutellospora* sp.
[2]Sungai Papan	3	31.96	154	*Acaulospora* sp., *Glomus* sp., and *Scutellospora* sp.
[2]Sungai Kooi	3	27.01	245	*Acaulospora* sp., *Entrophospora* sp., *Gigaspora* sp., *Glomus* sp., and *Scutellospora* sp.

Note. [1]Temengor Forest Reserve, [2]Royal Belum State Park

DISCUSSION

Arbuscular Mycorrhizal Colonisation on Forest Rattan Root Samples

Rattans are known as climbing palms and are well used for cane and cane products due to their stem's flexibility. Scanty literature is available on the mycorrhizal study of rattans. The earlier study on AM fungi associated with rattan was conducted by Zakaria (1991), comprising preliminary investigations on growth dependency of *in vitro* micro-propagated *Calamus manan* on the AM fungi before transplanting to the field. Meanwhile, Gong et al. (1994, 1995) studied the colonisation and presence of the AM fungi in the rhizosphere soils of four rattan species (*Daemonorops margaritae*, *Calamus simplicifolius*, *Calamus tetradactylus*, and *Calamus tetradactyloides*). Finally, Marati and Devadiga (2018) studied the colonisation of AM fungi on *Calamus thwaitesii*, *Calamus nagabettai*, and *Calamus prasinus* and reported variation in the spore density and spore diversity in the rhizosphere soils. To our knowledge, the present study is the first attempt to investigate the colonisation of arbuscular mycorrhizal (AM) fungi in the roots of *Calamus insignis*, *Calamus viridispinus*, *Calamus arborescens*, *Calamus diepenhorstii*, *Calamus manan*, *Calamus castaneus*, *Calamus conirostris*, and *Calamus rugosus*. In addition, this study reports the AM and spore diversity in the Belum-Temengor forest complex. The AM fungi are able to form AM symbioses in

exchange for carbon with 80% of terrestrial plants roots and play a crucial role in soil fertility, assist the plant in uptakes and mobilisation of nutrients (e.g., phosphorus and nitrogen) and inducing changes in plant physiology and secondary metabolism (Cervantes-Gámez et al., 2016; Schweiger & Müller, 2015; Wipf et al., 2014), which indirectly makes AM fungi is important for plant health and establishment (Ban et al., 2017).

All *Calamus* spp. roots located in the Belum-Temengor Forest Complex were colonised by the AM fungi with different colonisation degrees (Figure 1). Compared to other species, *Calamus insignis* showed the significantly highest colonisation degree with 46.18%, and the lowest colonisation degree of 12.40% was demonstrated by *C. viridispinus* (Figure 1). It is because the primary root system of the rattan is fibrous, and the roots spread to 1 to 2 m in width and range from 50 to 60 cm depth. This root system is a favourable structure for AM colonisation. Therefore, this might explain why all the *Calamus* spp. found in the samplings site were able to form the AM colonisation. Furthermore, the soil physical characteristics from the sampling sites for both Temengor Forest Reserve and Royal Belum State Park were approximately the same, with soil moisture ranging from 21.80% to 41.84%, soil pH ranging from 6.03 to 6.58, and soil temperature ranging from 23.5°C to 25.4°C (Table 1). These properties provide a suitable condition for AM fungi colonisation and propagation.

The presence of arbuscular mycorrhizal structures like hyphae and vesicles; hyphal swellings in the root cortex contain lipids and cytoplasm (Reddy et al., 2013) arbuscules in the *Calamus* spp. roots indicate the ability of this plant to form the mycorrhizal association (Figure 2 and Figure 3). Arbuscules are important macro-and micronutrient and water exchange sites between the AM fungi and their symbiotic plants (Bonfante & Requena, 2011; Lee et al., 2012; Miransari, 2011). The type of arbuscules was formed in the *Calamus* spp. roots from the Belum-Temengor Forest Complex is the *Paris*-type (Figure 2 and Figure 3). *Paris*-type arbuscules are formed from small branches developed from the hyphal coil (Peterson et al., 2004). The hyphae grow from cell to cell without any intercellular phase, and in the whole cortex cell, the AM fungus forms large coils (Franken, 2010). *Paris*-type arbuscules seem to occur more frequently compared to the *Arum*-type (F. A. Smith & Smith, 1997).

Arbuscular Mycorrhizal Spore Distribution

The AM fungi spores isolated from the soil samples from Belum-Temengor Forest Complex were *Acaulospora* sp., *Entrophospora* sp., *Gigaspora* sp., *Glomus* sp., and *Scutellospora* sp. (Figure 4 to Figure 5). The distribution of AM fungi species depends on the environment, such as the microclimate factor, the biome's location, and the competition between the AM fungi species. The AMF communities are influenced by different soil types (Oehl

et al., 2010; Torrecillas et al., 2014), and the diversity of AM fungi and the types of AM fungi propagules depending on their taxonomic group are affected by soil texture (H. Zhao et al., 2017; Lekberg et al., 2007). From the soil collected from all the six sampling sites in the Belum-Temengor Forest Complex, the AM fungal genus *Glomus* sp. was found in all sites, followed by *Acaulospora* sp. and *Scutellospora* sp., which were equally present in all sites except Pulau Perhilitian, Temengor (Table 2). Meanwhile, *Gigaspora* sp. was isolated from Sungai No. 2 and Sungai Rokan, Temengor and Sungai Kooi, Royal Belum State Park sampling sites (Table 2). *Entrophospora* sp. was isolated only from Sungai No. 2, Temengor and Sungai Kooi, Royal Belum State Park (Table 2).

Gong et al. (2000) showed that *Glomus*, *Acaulospora*, and *Scutellospora* were the predominant genera found in rattan plantations, and *Glomus* sp. was found in all sampling sites. Lekberg et al. (2007) reported that AM fungi from Glomeraceae species are more prevalent in soils with higher clay content. Mahulette et al. (2021) also reported that soils with clay texture and pH near neutral are dominated by *Glomus* sp. It supports the presence of *Glomus* sp. in all sampling sites due to clay soil texture present in all sampling sites (Table 2) and would also probably because AM fungi from the Glomeraceae family are known to have different propagation strategies (Lekberg et al., 2007), which provided them with a greater adaptation to various soil situation (Vieira et al., 2020). Marati and Devadiga (2018) found that *Glomus* was the dominant genus of the AM fungi in the rhizosphere soils of all rattan trees. Meanwhile, Huang et al. (2020) demonstrated that *Glomus* spp. increased root length, projected area, surface area, and volume, and increased leaf photosynthesis rate, transpiration rate, and stomatal conductivity, reducing intercellular carbon dioxide (CO_2) concentrations and leaf temperature. Marinho et al. (2018) found that *Glomus* and *Acaulospora* were the most representative genera in tropical forests. *Glomus* and *Acaulospora* genera are known to adapt to a different environment (Loss et al., 2009), as evidenced by their global distribution (INVAM biogeographical database: http//: invam.caf.wvu.edu), and they are tolerant to a wide range of pH (Silva et al., 2007). These genera produce numerous small-diameter spores (Dandan & Zhiwei, 2007), which potentially make them the most representative genera in tropical forests. The ability to alter its germination pattern depending on the environment has made *Glomus* sp. the most available genus in the re-established and logged-over forest soil (Ong et al., 2012). Bever et al. (1996) reported that *Glomus* and *Acaulospora* species usually produce more spores than *Gigaspora* and *Scutellospora* species in the same environment due to the difference in development, and they required less time to produce spores (Hart & Reader, 2002; Piotrowski et al., 2004). It indirectly increased the domination of *Glomus* sp. in the soil (Gai et al., 2009) and revealed an excellent adaptation feature, which promotes its ability to survive in disturbed

soils (Muleta et al., 2008). *Glomus* and *Acaulospora* are the AM fungi genera commonly found worldwide in a broad range of ecosystems (Davison et al., 2015).

Hart and Reader (2002) reported AM fungi from Gigasporaceae species to have robust hyphae, and their mycelial growth is higher in sandy soils. It supports the presence of *Gigaspora* sp. in Sungai No. 2 and Sungai Rokan, Temengor and Sungai Kooi, Royal Belum State Park because of the sandy and silty soil textures present only in these locations. The distribution and expansion of AM fungi hyphae are influenced by the size of soil particles in which the mycelial growth is reported to be higher in sandy soils (coarse particles compared to fine particles (Kohler et al., 2016). Sandy soils with a high porosity could stimulate mycorrhizal colonisation by favouring root growth compared to clay soil, restricting root growth due to low porosity (Carrenho et al., 2007). The seasonality, dormancy, edaphic factors, host dependence, age of the host plants, and the sporulation abilities of arbuscular mycorrhizal fungi could also affect the distribution and abundance of AM fungi spores in the soils (Bever et al., 1996; Z. W. Zhao et al., 2003). Ong et al. (2012) reported in their study that *Gigaspora* was found only in the logged-over forest and not in the re-established forest. The broad diversity of plant species in Malaysian forests may contribute to the selection of this host by *Gigaspora* sp. and enhance its survivability (Keen Chubo et al., 2009; Z. W. Zhao et al., 2003).

CONCLUSION

This study revealed the ability of a significant commercial plant, rattans, to form a mutualistic symbiosis with arbuscular mycorrhizal (AM) fungi. *Calamus insignis* showed the highest percentage, whereas *Calamus viridispinus* recorded the lowest percentage of AM colonisation degree compared to other *Calamus* species. Five genera of the AM fungi were isolated from Sungai No. 2 and Sungai Kooi, four genera of AM fungi were isolated from Sungai Rokan, three genera of AM fungi were isolated from Sungai Kejar and Sungai Papan, and one genus of AM fungus was recorded in Pulau Perhilitan, which is a relatively small number of AM genera considering the high plant diversity in the sampling locations. *Glomus* sp. was the main distributed genera, followed by *Acaulospora* sp. and *Scutellospora* sp. Sungai Kooi, Royal Belum State Park, recorded the highest spore density, 245 spores per 100 g of soil. This study has shown that the rattans species in the Belum-Temengor Forest Complex do form the AM association. The rhizospheric soils of these rattans are present with the indigenous AM fungi constitution.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Afentina., McShane, P., & Wright, W. (2020). Ethnobotany, rattan agroforestry, and conservation of ecosystem services in Central Kalimantan, Indonesia. *Agroforestry Systems*, *94*(2), 639–650. https://doi.org/10.1007/s10457-019-00428-x

Ait-El-Mokhtar, M., Laouane, R. B., Anli, M., Boutasknit, A., Wahbi, S., & Meddich, A. (2019). Use of mycorrhizal fungi in improving tolerance of the date palm (*Phoenix dactylifera* L.) seedlings to salt stress. *Scientia Horticulturae*, *253*, 429–438. https://doi.org/10.1016/j.scienta.2019.04.066

Andrade Júnior, J. A. D., Souza, B. R. D., Souza, R. F., & Moura, J. B. D. (2018). Fixação de carbono em sistemas agroecológicos na região do Vale do São Patrício, Goiás [Carbon sequestration in agroecological systems in the region of the São Patrício Valley, Goiás]. *Científic@-Multidisciplinary Journal*, *5*(2), 85–98. https://doi.org/10.29247/2358-260x.2018v5i2.p85-98

Baillie, J. E. M., Hilton-Taylor, C., Stuart, S. N., & Zedan, H. (2004). *2004 IUCN Red List of Threatened Species: A global species assessment*. International Union for the Conservation of Nature and Natural Resources.

Ban, Y., Jiang, Y., Li, M., Zhang, X., Zhang, S., Wu, Y., & Xu, Z. (2017). Homogenous stands of a wetland grass living in heavy metal polluted wetlands harbor diverse consortia of arbuscular mycorrhizal fungi. *Chemosphere*, *181*, 699–709. https://doi.org/10.1016/j.chemosphere.2017.04.135

Bever, J. D., Morton, J. B., Antonovics, J., & Schultz, P. A. (1996). Host-dependent sporulation and species diversity of arbuscular mycorrhizal fungi in a Mown Grassland. *The Journal of Ecology*, *84*(1), 71-82. https://doi.org/10.2307/2261701

Bonfante, P., & Requena, N. (2011). Dating in the dark: How roots respond to fungal signals to establish arbuscular mycorrhizal symbiosis. *Current Opinion in Plant Biology*, *14*(4), 451–457. https://doi.org/10.1016/j.pbi.2011.03.014

Brundett, M., Bougher, N., Dell, B., Grove, T., & Malajczuk, N. (1996). *Working with mycorrhizas in forestry and agriculture (ACIAR monographs)*. Australian Centre for International Agricultural Research (ACIAR).

Brundrett, M. C., & Tedersoo, L. (2018). Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytologist*, *220*(4), 1108–1115. https://doi.org/10.1111/nph.14976

Carrenho, R., Trufem, S. F. B., Bononi, V. L. R., & Silva, E. S. (2007). The effect of different soil properties on arbuscular mycorrhizal colonization of peanuts, sorghum and maize. *Acta Botanica Brasilica*, *21*(3), 723–730. https://doi.org/10.1590/s0102-33062007000300018

Cavagnaro, T. R., Gao, L., Smith, F. A., & Smith, S. E. (2001). Morphology of arbuscular mycorrhizas is influenced by fungal identity. *New Phytologist*, *151*(2), 469–475. https://doi.org/10.1046/j.0028-646x.2001.00191.x

Cervantes-Gámez, R. G., Bueno-Ibarra, M. A., Cruz-Mendívil, A., Calderón-Vázquez, C. L., Ramírez-Douriet, C. M., Maldonado-Mendoza, I. E., Villalobos-López, M. N., Valdez-Ortíz, N., & López-Meyer, M. (2016). Arbuscular mycorrhizal symbiosis-induced expression changes in *Solanum lycopersicum* leaves revealed by RNA-seq analysis. *Plant Molecular Biology Reporter*, *34*(1), 89–102. https://doi.org/10.1007/s11105-015-0903-9

Chin, T. Y., Nor Akhiruddin, M., Samsuanuar, M., Yong, N., Hasnuddin, M. A., & Nasir, M. S. I. (1994). *Inventori Hutan Nasional Ketiga Semenanjung Malaysia* [The Third National Forest Inventory of Peninsular Malaysia]. Jabatan Perhutanan Semenanjung Malaysia.

Dandan, Z., & Zhiwei, Z. (2007). Biodiversity of arbuscular mycorrhizal fungi in the hot-dry valley of the Jinsha River, Southwest China. *Applied Soil Ecology*, *37*(1–2), 118–128. https://doi.org/10.1016/j.apsoil.2007.06.003

Davison, J., Moora, M., Opik, M., Adholeya, A., Ainsaar, L., Ba, A., Burla, S., Diedhiou, A. G., Hiiesalu, I., Jairus, T., Johnson, N. C., Kane, A., Koorem, K., Kochar, M., Ndiaye, C., Partel, M., Reier, U., Saks, U., Singh, R., . . . Zobel, M. (2015). Global assessment of arbuscular mycorrhizal fungus diversity reveals very low endemism. *Science*, *349*(6251), 970–973. https://doi.org/10.1126/science.aab1161

de Moura, J. B., de Souza, R. F., Junior, W. G. V., Lima, I. R., Brito, G. H. M., & Marín, C. (2019). Arbuscular mycorrhizal fungi associated with bamboo under Cerrado Brazilian vegetation. *Journal of Soil Science and Plant Nutrition*, *19*(4), 954–962. https://doi.org/10.1007/s42729-019-00093-0

Dransfield, J. (1992). The ecology and natural history of rattans. In *A guide to the cultivation of rattan* (pp. 27–33). Forest Research Institute Malaysia.

Franken, P. (2010). Molecular–physiological aspects of the AM symbiosis post penetration. In H. Koltai & Y. Kapulnik (Eds.), *Arbuscular mycorrhizas: Physiology and function* (pp. 93–116). Springer. https://doi.org/10.1007/978-90-481-9489-6_5

Gai, J. P., Christie, P., Cai, X. B., Fan, J. Q., Zhang, J. L., Feng, G., & Li, X. L. (2009). Occurrence and distribution of arbuscular mycorrhizal fungal species in three types of grassland community of the Tibetan Plateau. *Ecological Research*, *24*(6), 1345–1350. https://doi.org/10.1007/s11284-009-0618-1

Gentry, A. (1991). Distribution and evolution of climbing plants. In F. E. Putz & H. A. Mooney (Eds.), *The biology of vines* (pp. 3–49). Cambridge University Press.

Giovannetti, M., & Mosse, B. (1980). An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytologist*, *84*(3), 489–500. https://doi.org/10.1111/j.1469-8137.1980.tb04556.x

GLOBE. (2014). *GLOBE Soil temperature protocol*. https://www.globe.gov/documents/352961/353769/Soil+Temperature+protocol/87a3491a-25af-4123-9e0c-08f341bfc004

Gong, M. Q., Chen, Y., & Wang, F. Z. (1994). Study on VA mycorrhizae of rattan species. *Forest Research*, *7*(4), 359-363.

Gong, M. Q., Chen, Y., & Wang, F. Z. (1995). Successful inoculation on rattan seedlings with VA mycorrhizal fungus. *Forest Research*, *8*(3), 247–251.

Gong, M. Q., Wang, F. Z., & Chen, Y. (2000). Effectiveness of VA mycorrhizal fungi associated with rattan. In H. C. Xu, A. N. Rao, B. S. Zheng, & G. T. Yin (Eds.), *Research on rattans in China* (pp. 15-25). International Plant Genetic Resources Institute.

Hart, M. M., & Reader, R. J. (2002). Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. *New Phytologist*, *153*(2), 335–344. https://doi.org/10.1046/j.0028-646x.2001.00312.x

Heijden, M. G. A., Martin, F. M., Selosse, M., & Sanders, I. R. (2015). Mycorrhizal ecology and evolution: The past, the present, and the future. *New Phytologist*, *205*(4), 1406–1423. https://doi.org/10.1111/nph.13288

Hirschberger, P. (2011). *Global rattan trade: Pressure on forest resources, analysis and challenges*. http://www.unece.lsu.edu/.http://www.unece.lsu.edu/responsible_trade/documents/2013Mar/rt13_04.pdf

Huang, G. M., Zou, Y. N., Wu, Q. S., Xu, Y. J., & Kuča, K. (2020). Mycorrhizal roles in plant growth, gas exchange, root morphology, and nutrient uptake of walnuts. *Plant, Soil and Environment*, *66*(6), 295–302. https://doi.org/10.17221/240/2020-pse

Isnard, S. (2006). Biomechanics and development of rattans: What is special about *Plectocomia himalayana* Griff. (Calamoideae, Plectocomiinae)?. *Botanical Journal of the Linnean Society*, *151*(1), 83–91. https://doi.org/10.1111/j.1095-8339.2006.00524.x

Isnard, S., & Rowe, N. P. (2007). Mechanical role of the leaf sheath in rattans. *New Phytologist*, *177*(3), 643–652. https://doi.org/10.1111/j.1469-8137.2007.02308.x

Keen Chubo, J., Kian Huat, O., Md. Jais, H., Faiqoh Mardatin, N., & Muhamad Nik Abdul Majid, N. (2009). Genera of arbuscular mycorrhiza occurring within the rhizospheres of *Octomeles sumatrana* and *Anthocephalus chinensis* in Niah, Sarawak, Malaysia. *ScienceAsia*, *35*(4), 340-345. https://doi.org/10.2306/scienceasia1513-1874.2009.35.340

Kohler, J., Roldán, A., Campoy, M., & Caravaca, F. (2016). Unraveling the role of hyphal networks from arbuscular mycorrhizal fungi in aggregate stabilization of semiarid soils with different textures and carbonate contents. *Plant and Soil*, *410*(1–2), 273–281. https://doi.org/10.1007/s11104-016-3001-3

Kusuma, Y. W. C., Dodo., & Hendrian, R. (2011). Propagation and transplanting of manau rattan *Calamus manan* in Bukit Dua Belas National Park, Sumatra, Indonesia. *Conservation Evidence*, *8*, 19–25.

Lee, B. R., Muneer, S., Avice, J. C., Jung, W. J., & Kim, T. H. (2012). Mycorrhizal colonisation and P-supplement effects on N uptake and N assimilation in perennial ryegrass under well-watered and drought-stressed conditions. *Mycorrhiza*, *22*(7), 525–534. https://doi.org/10.1007/s00572-012-0430-6

Lekberg, Y., Koide, R. T., Rohr, J. R., Aldrich-Wolfe, L., & Morton, J. B. (2007). Role of niche restrictions and dispersal in the composition of arbuscular mycorrhizal fungal communities. *Journal of Ecology*, *95*(1), 95–105. https://doi.org/10.1111/j.1365-2745.2006.01193.x

Loss, A., Robles, A., Guilherme, A., Callegario, P., Ana Carolina, L., Otávio, R., Lima, M., Márcio, O., Ribeiro da, S., Eliane, M., Saggin, J., & Orivaldo, J. (2009). Atributos químicos do solo e ocorrência de fungos micorrízicos sob áreas de pastagem e sistema agroflorestal, Brasil [Chemical attributes of soil and occurrence of mycorrhizal fungi under areas of pasture and agroforest system, Brazil]. *Acta Agronómica*, *58*(2), 91–95.

Lyngdoh, N., Santosh, S. H., Ramesha, T., Nageswara Rao, M., Ravikanth, G., Narayani, B., Ganeshaiah, K. N., & Uma Shaanker, R. (2005). Rattan species richness and population genetic structure of *Calamus flagellum* in North-Eastern Himalaya, India. *Journal of Bamboo and Rattan*, *4*(3), 293–307. https://doi.org/10.1163/156915905774310016

Mahulette, A. S., Alfian, A., Kilkoda, A. K., Lawalata, I. J., Marasabessy, D. A., Tanasale, V. L., & Makaruku, M. H. (2021). Isolation and identification of indigenous Arbuscular Mycorrhizal Fungi (AMF) of forest clove rhizosphere from Maluku, Indonesia. *Biodiversitas Journal of Biological Diversity*, *22*(8). https://doi.org/10.13057/biodiv/d220863

Malaysian Meteorological Department. (n.d.). *Iklim Malaysia* [Climate of Malaysia].

https://www.met.gov.my/pendidikan/iklim/iklimmalaysia?lang=en

Marati, R., & Devadiga, S. (2018). Screening of the Arbuscular Mycorrhizae in the rhizosphere soils of three Rattan species of Charmady Kanpadi Reserve Forest of Karnakata, India. *International Journal of Life Sciences Research*, *6*(3), 312–317.

Marinho, F., da Silva, I. R., Oehl, F., & Maia, L. C. (2018). Checklist of arbuscular mycorrhizal fungi in tropical forests. *Sydowia*, *70*, 107–127. https://doi.org/10.12905/0380.sydowia70-2018-0107

Mathew, A., & Bhat, K. M. (1997). Anatomical diversity of Indian rattan palms (Calamoideae) in relation to biogeography and systematics. *Botanical Journal of the Linnean Society*, *125*(1), 71–86. https://doi.org/10.1111/j.1095-8339.1997.tb02247.x

McGonigle, T. P., Miller, M. H., Evans, D. G., Fairchild, G. L., & Swan, J. A. (1990). A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytologist*, *115*(3), 495–501. https://doi.org/10.1111/j.1469-8137.1990.tb00476.x

Meddich, A., Oihabi, A., Jaiti, F., Bourzik, W., & Hafidi, M. (2015). Rôle des champignons mycorhiziens arbusculaires dans la tolérance du palmier dattier (*Phoenix dactylifera*) à la fusariose vasculaire et au déficit hydrique [Role of arbuscular mycorrhizal fungi in date palm (*Phoenix dactylifera*) tolerance to Fusarium wilt and water deficit]. *Botany*, *93*(6), 369–377. https://doi.org/10.1139/cjb-2014-0249

Minitab. (2019). *Minitab®*. https://www.minitab.com

Miransari, M. (2011). Soil microbes and plant fertilization. *Applied Microbiology and Biotechnology*, *92*(5), 875–885. https://doi.org/10.1007/s00253-011-3521-y

Mohamad, A. (1993). *PROSEA - Plant Resources of Southeast Asia*. PROSEA. https://prota4u.org/prosea/view.aspx?id=36

Muleta, D., Assefa, F., Nemomissa, S., & Granhall, U. (2008). Distribution of arbuscular mycorrhizal fungi spores in soils of smallholder agroforestry and monocultural coffee systems in southwestern Ethiopia. *Biology and Fertility of Soils*, *44*(4), 653–659. https://doi.org/10.1007/s00374-007-0261-3

Oehl, F., Laczko, E., Bogenrieder, A., Stahr, K., Bösch, R., van der Heijden, M., & Sieverding, E. (2010). Soil type and land use intensity determine the composition of arbuscular mycorrhizal fungal communities. *Soil Biology and Biochemistry*, *42*(5), 724–738. https://doi.org/10.1016/j.soilbio.2010.01.006

Oehl, F., Sieverding, E., Palenzuela, J., Ineichen, K., & da Silva, G. A. (2011). Advances in *Glomeromycota* taxonomy and classification. *IMA Fungus*, *2*(2), 191–199. https://doi.org/10.5598/imafungus.2011.02.02.10

Ong, K. H., Chubo, J. K., King, J. H., Lee, C. S., Su, D. S. A., & Sipen, P. (2012). Influence of soil chemical properties on relative abundance of arbuscular mycorrhiza in forested soils in Malaysia. *Turkish Journal of Agriculture and Forestry*, *36*(4), 451–458. https://doi.org/10.3906/tar-1107-32

Peterson, R. L., Massicotte, H. B., & Melville, L. H. (2004). *Mycorrhizas*. NRC Research Press.

Piotrowski, J. S., Denich, T., Klironomos, J. N., Graham, J. M., & Rillig, M. C. (2004). The effects of arbuscular mycorrhizas on soil aggregation depend on the interaction between plant and fungal species. *New Phytologist*, *164*(2), 365–373. https://doi.org/10.1111/j.1469-8137.2004.01181.x

Ramesha, B. T., Ravikanth, G., Nageswara Rao, M., Ganeshaiah, K. N., & Uma Shaanker, R. (2007).

Genetic structure of the rattan *Calamus thwaitesii* in core, buffer, and peripheral regions of three protected areas in central Western Ghats, India: Do protected areas serve as refugia for genetic resources of economically important plants?. *Journal of Genetics*, *86*(1), 9–18. https://doi.org/10.1007/s12041-007-0002-2

Reddy, C. A., & Saravanan, R. S. (2013). Polymicrobial multi-functional approach for enhancement of crop productivity. *Advances in Applied Microbiology*, *82*, 53–113. https://doi.org/10.1016/b978-0-12-407679-2.00003-x

Sarmah, P., Barua, P. K., Sarma, R. N., Sen, P., & Deka, P. C. (2007). Genetic diversity among rattan genotypes from India based on RAPD-marker analysis. *Genetic Resources and Crop Evolution*, *54*(3), 593–600. https://doi.org/10.1007/s10722-006-0017-1

Schenk, N. C., & Perez, Y. (1990). *Manual for identification of VA mycorrhizal fungi* (3rd ed.). Synergistic Publications.

Schweiger, R., & Müller, C. (2015). Leaf metabolome in arbuscular mycorrhizal symbiosis. *Current Opinion in Plant Biology*, *26*, 120–126. https://doi.org/10.1016/j.pbi.2015.06.009

Silva, L. X. D., Figueiredo, M. D. V. B., Silva, G. A. D., Goto, B. T., Oliveira, J. D. P., & Burity, H. A. (2007). Fungos micorrízicos arbusculares em áreas de plantio de leucena e sábia no estado de Pernambuco [Arbuscular mycorrhizal fungi in areas of leucaena and sage plantation in the state of Pernambuco]. *Revista Árvore*, *31*(3), 427–435. https://doi.org/10.1590/s0100-67622007000300008

Silva-Flores, P., Bueno, C. G., Neira, J., & Palfner, G. (2019). Factors affecting arbuscular mycorrhizal fungi spore density in the Chilean Mediterranean-type ecosystem. *Journal of Soil Science and Plant Nutrition*, *19*(1), 42–50. https://doi.org/10.1007/s42729-018-0004-6

Smith, F. A., & Smith, S. E. (1997). Structural diversity in (vesicular)–arbuscular mycorrhizal symbioses. *New Phytologist*, *137*(3), 373–388. https://doi.org/10.1046/j.1469-8137.1997.00848.x

Smith, S. E., & Read, D. (2008). The symbionts forming arbuscular mycorrhizas. In S. E. Smith & D. Read (Eds.), *Mycorrhizal symbiosis* (3rd ed., pp. 13–41). Academic Press. https://doi.org/10.1016/B978-012370526-6.50003-9

Stiegel, S., Kessler, M., Getto, D., Thonhofer, J., & Siebert, S. F. (2011). Elevational patterns of species richness and density of rattan palms (Arecaceae: Calamoideae) in Central Sulawesi, Indonesia. *Biodiversity and Conservation*, *20*(9), 1987–2005. https://doi.org/10.1007/s10531-011-0070-8

Sudarmonowati, E., Mogea, J. P., Hartati, N. S., Hong, L., & Rao, V. R. (2004). Morphology and genetic variation of manau rattan (*Calamus manan* Miq.) in Sumatra, Indonesia. *Journal of Bamboo and Rattan*, *3*(2), 123–137. https://doi.org/10.1163/156915904774195124

Thien, S. J. (1979). A flow diagram for teaching texture-by-feel analysis. *Journal of Agronomic Education*, *8*(1), 54–55. https://doi.org/10.2134/jae.1979.0054

Torrecillas, E., del Mar Alguacil, M., Roldán, A., Díaz, G., Montesinos-Navarro, A., & Torres, M. P. (2014). Modularity reveals the tendency of arbuscular mycorrhizal fungi to interact differently with generalist and specialist plant species in gypsum soils. *Applied and Environmental Microbiology*, *80*(17), 5457–5466. https://doi.org/10.1128/aem.01358-14

Vieira, L. C., da Silva, D. K. A., Escobar, I. E. C., da Silva, J. M., de Moura, I. A., Oehl, F., & da Silva, G. A. (2020). Changes in an arbuscular mycorrhizal fungi community along an environmental gradient. *Plants*, *9*(1), 52. https://doi.org/10.3390/plants9010052

Whiting, D., Card, A., & Wilson, C. (2015). *Estimating soil texture*. https://cmg.extension.colostate.edu/Gardennotes/214.pdf

Wicaksono, W. A., Sansom, C. E., Eirian Jones, E., Perry, N. B., Monk, J., & Ridgway, H. J. (2018). Arbuscular mycorrhizal fungi associated with *Leptospermum scoparium* (mānuka): Effects on plant growth and essential oil content. *Symbiosis*, *75*(1), 39–50. https://doi.org/10.1007/s13199-017-0506-3

Wipf, D., Mongelard, G., van Tuinen, D., Gutierrez, L., & Casieri, L. (2014). Transcriptional responses of *Medicago truncatula* upon sulfur deficiency stress and arbuscular mycorrhizal symbiosis. *Frontiers in Plant Science*, *5*, 680. https://doi.org/10.3389/fpls.2014.00680

Zakaria, M. (1991). Preliminary studies on growth dependency of *in vitro* micropropagated *Calamus manan* on VA mycorrhiza (VAM) prior to transplanting to the field. *RIC Bulletin*, *10*(1), 6–7.

Zhao, H., Li, X., Zhang, Z., Zhao, Y., Yang, J., & Zhu, Y. (2017). Species diversity and drivers of arbuscular mycorrhizal fungal communities in a semi-arid mountain in China. *PeerJ*, *5*, e4155. https://doi.org/10.7717/peerj.4155

Zhao, Z. W., Wang, G. H., & Yang, L. (2003). Biodiversity of arbuscular mycorrhizal fungi in a tropical rainforest of Xishuangbanna, southwest China. *Fungal Diversity*, *13*, 233–242.

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Feed Intake, Growth Performance and Digestibility of Nutrients of Goats Fed with Outdoor-Grown Hydroponic Maize Sprouts

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ABSTRACT

The study aimed to determine the effects of feeding outdoor-grown hydroponics maize sprouts (HMS) on the growth performance and digestibility of nutrients in goats. Three treatment groups (n = 5), group T1 (control), were fed 500 g concentrate, and Napier grass; T2, 500 g concentrate, and HMS, while T3 had sole feeding of HMS using a completely randomized design. The results showed that HMS had a better feed nutritive composition with the lower concentration of indigestible fibre (P<0.05) and higher concentration of crude protein (12.28%) compared to Napier grass (7.22%) (P<0.05). Goats in T1 and T2 fed with concentrate had a higher average daily gain (ADG) of 79 g/day and 48 g/day rivalling goats fed with HMS (44 g/day) (P<0.05). Feed conversion ratio (FCR) was significantly better (P<0.05) in goats in T1 and T2 compared to T3. Goats in groups T1 and T2 showed significantly higher dry matter digestibility (69.27% and 63.95%, respectively) and crude protein digestibility (71.89% and 72.28%, respectively) compared to group T3. Sole feeding of HMS exerted a minor impact on growth performance in the animals. However, the HMS could potentially replace the conventionally planted sprouts in conjunction with commercial concentrate to improve the growth performance of the small ruminants.

Keywords: Average daily gain, feed intake, hydroponic maize sprouts, Napier grass, nutrient digestibility

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INTRODUCTION

In Malaysia, fodder is currently being utilized as a significant component in the ruminant diet. Consistency in the quality and production of high-quality fodder is fundamental for a successful animal production system. The Napier grass (*Pennisetum purpureum* Schumach.), a perennial tropical grass in the Poaceae family, is currently the most used fodder in dairy and feedlot production in livestock management (Halim et al., 2013). It is the most prevalent forage species due to its high nutritive value and is quickly established through stem propagation (Wijitphan et al., 2009). However, this feeding method is labour-intensive, which requires sowing, earthing up, fertilizing, weeding, and harvesting. Furthermore, the yield and quality of Napier grass can be affected by cultivar selection (Halim et al., 2013), type and rate of fertilizer (Fauzi & Soetanto, 2020), plant density (Mukhtar et al., 2003), and lastly, the cutting management, such as cutting frequency (Mukhtar et al., 2003), cutting intervals (Jusoh et al., 2014), and cutting height (Wijitphan et al., 2009).

The hydroponics production reduced the expenses of farmers since minimal space is required with a vertical production system. The optimum harvesting period of hydroponic maize fodder is 7–10 days (Naik et al., 2013). The crude protein (CP) content of maize seed is significantly increased after seven days of sprouting (ranging from 8.60%–13.57%) (Naik et al., 2012). A numeric increase in CP, neutral detergent fibre (NDF), water-soluble carbohydrate (WSC), and ether extract (EE) concentration of 1.8%, 16.1%, 17.1%, and 1.8%, respectively, was observed in the 7-day sprouted barley compared with the barley grains (Hafla et al., 2014). Various commercial hydroponic fodder producers report yields of 6–10 times with dry matter (DM) content ranging from 6.4% to 20%, although trial yields range from 5–8 folds (Sneath & Mclntosh, 2003). Hydroponic maize fodder yields of 5–6 times on a fresh basis (1 kg seed produces 5–6 kg hydroponic maize fodder) and DM content of 11%–14% are common (Naik et al., 2013). Besides, hydroponic maize fodder contains higher CP, EE, and nitrogen-free extract (NFE) contents; lower crude fibre (CF), total ash (TA), and acid insoluble ash (AIA) contents than the conventional maize fodder (Naik et al., 2012). For the 7-day growth sprout samples, maize have reported nutrient profiles of DM ranging from 12.4% to 25.0%, CP from 13.3% to 13.8%, CF from 6.4% to 14.8%, EE from 3.3% to 3.6%, NFE from 60.7% to 75.3%, TA from 1.8% to 3.8%, and AIA from 0.3% to 0.6% when reported on a DM basis.

Most smallholder goat farms practice improper feeding regimes due to poor knowledge and information, resulting in lower growth and reproductive performance of the goats, feed consumption, and production (Ghani et al., 2017). Moreover, the consumption of mutton in Malaysia substantially increased. Nevertheless, the reports on the effect of hydroponic fodder on the growth performance and growth hormone profile of goats, particularly

in Malaysia, are scarce. Reviews have shown that hydroponic fodder is alternative to green fodder for animals. However, developing low-cost devices for hydroponic fodder production using locally accessible materials on different livestock categories requires more focus. This study aims to develop a low-cost device for hydroponic fodder, which plays the role of a new goat feeding system in achieving successful and profitable goat farming.

METHODS

This study was conducted at Wawasan Manis Sdn. Bhd., a breeding farm located at Lendu, Melaka, from November 2018 to February 2019. Chemical composition was analysed at Nutritional Laboratory, Department of Animal Science, Faculty of Agriculture, UPM.

Animal Welfare

This study was carried out according to the guiding principle stated in the Code of Practice for The Care and Use of Animals for Scientific Purposes, Universiti Putra Malaysia (The Institutional Animal Care and Use Committee [IACUC]).

Open-Air Hydroponic System

A used cattle pen-sized 35 ft width and 27 ft length were sheltered with polyethene sunshade netting to protect green fodders from heat before installing the open-air hydroponic fodder growing system (Figure 1). The cattle pen was also fenced with galvanized welded iron wire mesh (bottom) and zinc sheet (top) as a protective measurement from rodents. Two water tanks were used as the water storage system: Tank 1 was fed by clean tap water, and Tank 2 was fed by water from an outlet at the bottom of Tank 1 (Figure 2). Two centrifugal pumps (Model CPM-158, AC 200–240 V ~50 Hz, Victa™, Malaysia) connected to Tank 2 pumped water to a filter and then to the water channels (Figure 3). Polyethene pipes sized 25 mm were used as water-conducting networks from tanks, centrifugal pumps, filters, and water channels. The water channels drained water into eleven water ducts of polyethene pipes sized 16 mm. The water flow of five ducts was drained from pump 1 and another six ducts from pump 2. The water ducts were spaced 2 ft apart, and plastic misting spray nozzles were placed at 2-ft intervals along these water ducts.

Figure 1. A used cattle pen was transformed into the open-air hydroponic green fodder growing system

Figure 2. A two-tanks system was applied to ensure a continuous supply of water

Figure 3. The water-conducting network from Tank 2, two centrifugal pumps and filters

Preparation of Feeding Treatment

Napier grass was used as a control group as it is commonly fed to goats. Napier grass was already planted at this farm and fertilized with goat manure. The grass was harvested daily from the pasture (8–10 weeks old) at about 1.0–1.5 m height (Zailan et al., 2016). A fuel-driven chopper machine was used to chop the Napier grass to about 3–5 cm. However, the maize sprouts will be hydroponically grown, as reported by Morgan et al. (as cited in Naik et al., 2015, p. 3). The maize grains were grown through an outdoor hydroponic sprouts production unit (Figure 4). Polyethene trays sized 52.0 cm length × 32.0 cm width × 2.5 cm height were used as the hydroponic trays. Each tray was manually drilled to make holes at the base to drain excess water from irrigation. Maize grains were cleaned with several

piles of washing (at least three times) to remove the darkened or damaged seed and floating particles. Next, maize grains were treated with 0.1% hypochlorite solution (or bleach) for 20 minutes. Then, maize grains were washed again with water to remove excess bleach. The washed grains were covered with wet cotton cloths for 12–24 hours. Each hydroponic tray was spread with 1 kg sprouted grain with 1–1.5 cm layer thickness. All maize grains were irrigated using overhead mist ten times a day. Each irrigation session lasted for 1 minute, set by a digital timer. The sprouts of maize were harvested on Day 7 (Naik et al., 2015). There was no supplemental light used for this hydroponics system due to the high availability of sunlight at this hydroponic site. It is because photosynthesis is not dominant for the metabolism of the seedlings until the end of the fifth day when the chloroplasts are activated (Sneath & Mclntosh, 2003).

A commercial concentrate was purchased from Nutri Vet Trading (livestock feed supplier located in Negeri Sembilan) and used as the daily diet for the Control group (T1) and T2 in the morning. The chemical composition of the commercial concentrate is summarized in Table 1 (Association of Official Analytical Chemists [AOAC], 1990).

In a completely randomized design, a total of fifteen male Boer crossbred goats with a mean age of 178.4 days old with an average initial body weight of 18.8 kg were allotted to different single pent sized 3.5 ft width × 4.3 ft length on a raised slatted floor, which is equipped with water nipple. The goats were randomly grouped into three treatments, which consist of Treatment 1 (T1) fed with 100% fresh Napier grass and concentrate as Control group, Treatment 2 (T2) with 100% HMS and concentrate, and Treatment 3 (T3) with 100% HMS only, respectively. The concentrate (Table 1) was fed at the 500 g/goat rate in the morning (0830 h) daily.

Figure 4. The hydroponic maize sprouts, which were grown for seven days

Table 1

Chemical composition of commercial concentrate for T1 and T2

Parameters	Dry matter basis (%)
Dry matter (DM)	80.80±0.74
Crude protein (CP)	16.24±0.10
Organic matter (OM)	88.78±0.52
Neutral detergent fibre (NDF)	46.91±0.61
Acid detergent fibre (ADF)	22.53±0.13
Acid detergent fibre (ADL)	5.78±0.22
Hemicellulose	24.38±0.68
Cellulose	16.75±0.23
Ash	11.22±0.52

Feed Intake and Growth Performance

During the 13 weeks of the feeding trial, all fresh fodder was offered *ad libitum* in the evening (1630 h) at approximately 10% (fresh basis) of the live weight of the goats, which was approximately 20% above the observed intakes during the adaptation period. The pens and troughs are cleaned every day before offering a feed. All animals had free access to water. The goats were fed with treatments for two weeks of adaptation before data collection. Feed intake was determined on a subsequent day by weighing the remnants and subtracting them from the feed offered. A representative sample from the feed was obtained for chemical composition analysis. During the feeding trial, the live weight (LW) was measured weekly before the morning feed was served. The average of LW gain daily was determined by dividing the differences of the initial and final LW with the duration of the feeding trial (91 days). Feed conversion ratio (FCR) was calculated as follows:

$$\text{FCR} = \frac{\text{Feed intake}}{\text{Weight gain}}$$

Total Collection Digestibility Trials

During the last week of the feeding trial, a 5-day digestion trial was conducted on all experimental animals. The representative feed samples (200 g each) were taken from each treatment group to determine the nutrient composition. In addition, approximately 100 g of faecal sample was collected directly from the anus of each animal and kept in a sealed bag. The faecal samples were then taken to Plant Biochemistry and Animal Physiology Laboratory, Department of Biology, Faculty of Science, UPM for oven drying at 60 °C for 48 hours. The dried samples were ground, sieved through a 1-mm screen, and packed in an air-tight container to be used

for chemical analysis. The apparent nutrient was determined as follow:

$$\text{Nutrient digestibility (\%)} = \frac{\text{Nutrient voided in the faeces}}{\text{Nutrient intake}} \times 100$$

Analytical Procedure

Feed and faecal samples were oven-dried at 60 °C for 48 hours, ground to pass a 1-mm mesh screen sieve and stored for chemical analysis. The nutritive values were analysed by Near-infrared Spectroscopy (NIRS) (NIRS™ DS2500, FOSS, Denmark) with additional calibration from the fodder samples analysed using standard laboratory procedure. The amount of CP was determined (N × 6.25) (AOAC, 1990). The NDF and ADF were analysed using FiberCap 2023 System (FOSS, Denmark) (International Organization for Standardization [ISO], 2008). Feed intake was measured on a subsequent day by weighing the remnants and subtracting them from the feed offered. A representative sample from the remnants was obtained for chemical composition analysis.

Statistical Analysis

All data were examined using IBM SPSS version 22.0 for Windows and presented as the mean ± S.E.M. Statistical significance was established at $P<0.05$. The chemical compositions between two green fodders were compared using the independent sample *t*-test. While the collected data of growth performance, feed intake and apparent digestibility were analysed using the analysis of variance (ANOVA) test. The least significant difference (LSD) test determined the significance of mean differences among the treatments.

RESULTS AND DISCUSSION

Chemical Composition Between Conventional Fodder and Hydroponic Maize Sprouts

The total chemical composition in green fodders is summarized in Table 2. Napier grass presently denoted a higher DM but a lower CP composition than HMS ($P<0.05$). On the other hand, Napier grass showed significantly higher OM content, 90.99%, compared to 88.85% in HMS. Besides, higher NDF, ADF, and ADL compositions were significantly higher in Napier grass ($P<0.05$).

DM indicates the available amount of nutrients to the animal in a diet. Livestock voluntarily consume a certain amount of DM per day (measured in kg/day) to maintain health, growth, and production (Naik et al., 2014). The values of DM reported by Lounglawan et al. (2014) as 18.93% in Napier grass harvested at 10 cm height on day 60th, Ghani et al. (2017) as 13.04% in which Napier grass was harvested manually at the age of 8 weeks (Rahman et al., 2014) as 20.79% were lower than

the present study, 26.01%. In the present investigation, the DM content in HMS was 24.75%, lower than Napier grass because of high moisture content. However, this value was higher than the results reported by Gebremedhin (2015) and Naik et al. (2014) in HMS as 18.30% and 18.48%, respectively. Higher DM content in HMS was reported if harvested at an older age and cultivated in a nutrient solution. A study conducted by Thadchanamoorthy and Pramalal (2012) reported higher DM content as 26.07% in 10 days old HMS, while Adebiyi et al. (2018) noted 25.00% DM content in 7-days old HMS, which was cultivated with nutrient solution.

The CP content presently denoted in HMS was 12.28% higher than the Napier grass (P<0.05). CP in feeding stuff includes the true protein containing amino acids and non-protein nitrogenous compounds, such as amides. HMS was preferred as a source of quality forage for livestock because it has a high protein (Ndaru et al., 2020). It is encouraging to compare the findings of this study with the results by Vennila (2018), who recorded 10.55% CP content of HMS with an 8-days growth period but lower than the CP content reported by several studies as ranged from 13.30%–16.54% (Adebiyi et al., 2018; Kide et al., 2015; Naik et al., 2012, 2013, 2014; Thadchanamoorthy & Pramalal, 2012). The minor differences in minor results are probably due to the variety and quality of seed used, light intensity, quality of the irrigation water, and germination time (Kaouche-Adjlanea et al., 2016). A study showed that hydroponic maize fodder would have higher CP content (24.07 g/100g) when harvested on the eighth day (Islam et al., 2016). However, the current study produced outdoor HMS exposed to high humidity, unlike those grown in a greenhouse. High humidity is one of the promoting factors of mould growth at the mat of roots when the length of the growth period increases. Generally, a concentration of 6–8% CP in the basal forage is a threshold for a response by ruminant livestock to nitrogen (N) supplements (Mathis et al., 2000). The CP content of Napier grass investigated in the present study was 7.22%. The value was higher than the findings obtained by Zailan et al. (2016) in common Napier grass harvested at an interval of 8 weeks as 6.44% and Ghani et al. (2017) as 3.88%. The higher value of CP was reported by Rambau et al. (2016) in Napier grass leaves harvested at intermediate stage (8 weeks) as 140.4 g/kg and Halim et al. (2013) in common Napier grass as 9.79%, Lounglawan et al. (2014) as 8.87%, Bayble et al. (2007) as 14.13% in Napier grass. The increment in enzymatic activities of nutrients can improve the CP content in hydroponic maize fodder (Naik et al., 2013).

Napier grass showed significantly higher OM (90.99%) content due to the application of fertilizer after each cutting (P<0.05). The value of total ash (11.22%) observed in the HMS of the present study is higher than the results reported by Naik et al. (2013) as a range of 1.75–3.80%. The total ash content rises on account of the mineral absorption by roots throughout the sprouting phase. HMS had higher palatability due to

the younger harvesting age that showed a decrease in the number and size of cell walls for the synthesis of structural carbohydrates (Bayble et al., 2007). The comparable crude fibre content was reported by a few studies in HMS as a range of 9.33–14.10% (Gebremedhin, 2015; Kide et al., 2015; Naik et al., 2013). The higher value of crude fibre was reported by Adebiyi et al. (2018) as 14.77%, and lower values were also reported by Thadchanamoorthy and Pramalal (2012) as 8.21% and Naik et al. (2014) as 6.37%.

Although Napier grass showed a higher DM composition, the lower value of the CP justifies the need for HMS as alternative green fodders. In small ruminants, the amount of protein is more important than the quality of protein (Valente, 2016). In addition, the significantly lower NDF, ADF, and ADL in HMS represent lower indigestible fibres, which predict HMS as more acceptable green fodder to animals.

Table 2

Chemical composition of green fodder

Parameters (% of DM)	Conventional Napier green fodder	Hydroponic maize sprouts
	n = 32	n = 32
DM	26.01±0.38^{b}	24.75±0.20^{a}
CP	7.22±0.09^{a}	12.28±0.25^{b}
OM	90.99±0.10^{b}	88.85±0.35^{a}
NDF	75.66±0.24^{b}	64.02±0.45^{a}
ADF	48.92±0.38^{b}	17.90±0.51^{a}
ADL	43.55±0.32^{b}	3.49±0.40^{a}
Hemicellulose	26.80±0.60^{a}	46.09±0.92^{b}
Cellulose	5.38±0.66^{a}	14.41±0.70^{b}
Ash	8.93±0.15^{a}	11.22±0.38^{b}

Note. DM = Dry matter; CP = Crude protein; OM = Organic matter; NDF = Neutral detergent fibre; ADF = Acid detergent fibre; ADL = Acid detergent lignin; n = Number of samples
All analyses are mean ± standard error of means (S.E.M.)
Means with different superscript letters in a row are significantly different (P<0.05)

Feed Intake and Growth Performance

Table 3 shows the effect of experimental diets on the feed intake of goats. The performance in average weight gain (ADG) was highly significant in the control group, T1 (79 g/day), compared to T2 and T3. The higher performance in ADG by animals fed with Napier grass and 500g concentrate could be due to the higher DM content to supply necessary nutrients for growth. The

ADG was similar (P>0.05) among the T2 and T3, with a mean value of 48 g/day and 44 g/day, respectively. This result can be considered good performance, considering the long feeding period since it was higher than the one as 37.74 g/day of growing goats (Gebremedhin, 2015). It was in line with the concept of Naik et al. (2014) pointed out that hydroponic fodder is a good source of nutrients and have a grass juice component that helps livestock perform better. Similar results were also reported by Eshtayeh (2004) in lactating Awassi ewes fed the hydroponic barley and hydroponic with olive cake at a rate of 15, and 25% exhibited significant body weight gain. In addition, male calves fed green hydroponic fodder observed 200 g/day body weight gain (Fazaeli et al., 2011). However, Farlin et al. (1971) found no difference between cattle given sprouted grain and cattle fed non-sprouted grain.

The daily dry matter intake, DMI (g/day), for different treatment groups, T1, T2, and T3, was compiled and presented in Table 3. In the present investigation, the average daily DMI was observed to be 315.64 g/day in treatment group T1, 179.19 g/day in treatment group T2, and 513.81 g/day in treatment group T3, respectively. As the statistical result conveyed, the daily DMI (g/day) was found significantly higher in treatment group T3 in comparison with the other treatment groups. The higher daily DMI (g/day) observed in treatment group T3 was due to the higher amount of HMS offered since no concentrate was given for this treatment group. Raeisi et al. (2018) reported that DMI increased with diet containing 7%, 14%, and 21%, respectively of hydroponic barley fodder fed to male sheep. The DMI of control group T1 showed 136.45 g/day more than the one in T2 may be due to the high-water content hydroponic fodders may have made them bulky, limiting the DMI of animals (Fazaeli et al., 2011). The present finding was comparable with the reports of Fazaeli et al. (2011) as they observed 6.6 kg DMI/day in male calves fed as control (roughage + concentrate) and 7.2 kg DMI/day in male calves supplemented 22.8% barley hydroponic fodder in their daily ration. Naik et al. (2014) denoted 8.85 kg/day in milking cows fed hydroponic maize fodder. According to a recent study that used hydroponic fodder as the sole feed, the DMI (% of body weight) of hydroponic maize fodder was 1.11%, lower than the recommended 2.6% to 2.8% for adult sheep maintenance (Ansari et al., 2019). In addition, some studies indicate a decrease in the DM intake of the animals when hydroponics fodder is fed (Farghaly et al., 2019). However, some reports recommended that 30% CP of kid starters be replaced with hydroponic maize fodder without affecting growth and DMI (Shyama et al., 2016).

The average total weight gain (TWG) of animals for the experiment is presented in Table 3. The TWG achieved 7.20, 4.40, and 4.00 kg in T1, T2, and T3, respectively. At the same time, the average total feed intake of each treatment group was recorded as 110.50, 65.8, and 188.7 kg in T1, T2, and T3, respectively. Statistically, the total body

weight gain of animals in T2 and T3 were at par, but T3 had significantly lower TWG than the control group (T1). In addition, the value of total feed intake in T3 was significantly higher than T1 and T2. Thus, the FCR value of T3 was 49.42 (P>0.001) compared to 14.93 in T1 and 16.62 in T2. T3 was the practice of solely feeding HMS to the animals, which did not fulfil the required production attributes unless combined with dry fodder to promote utilization (Prasad et al., 1998). A previous study showed a higher daily gain of goats fed with sprouted barley on agriculture by-products (48.13–49.43 g/day) (Helal, 2015). Although the difference of FCR values between T1 and T2 is insignificantly different, this implied that the amount of green fodder required for 1.0 kg weight gain (kg) in the goats fed Napier grass was insignificantly minimum, followed by T2. However, the diet in T2 able to attain a similar average weight gain (g/day) as comparable to T1 with lower daily feed intake. The hydroponic sprout mat is entirely edible and highly nutritious; the animals ingested the entire mat, including roots and green shoots, as it is a living food and no waste. It was supported by the concept of Eshtayeh (2004), who reported that the high DMI observed among the Awassi ewes could be due to the high palatability of hydroponic barley. In addition, the age of the animal, sexual state, the composition of body weight gain, feed digestibility, and energy density can all affect feed efficiency (Lage, 2012; Mazon et al., 2017).

Table 3

The animal feed intake of goats fed with the experimental diets

Parameters	Types of diet			*P*
	T1	T2	T3	
	n = 5	n = 5	n = 5	
Total feed intake (kg)	110.5±29.57^{a}	65.8±5.48^{a}	188.7±27.28^{b}	0.009
Daily feed intake (kg/day)	1.21±0.32^{a}	0.72±0.06^{a}	2.07±0.30^{b}	0.009
Dry matter intake (DMI) (g/day)	315.64±84.64^{ab}	179.19±14.97^{a}	513.81±74.07^{bc}	0.012
Total weight gain (kg)	7.20±1.12^{b}	4.40±0.71^{a}	4.00±0.71^{a}	0.046
Average daily gain (g/day)	79±0.12^{b}	48±0.01^{a}	44±0.01^{a}	0.046
Feed conversion ratio (FCR)	14.93±2.18^{a}	16.62±2.92^{a}	49.42±4.78^{b}	0.000

Note. T1 = Fresh Napier grass and 500g concentrate; T2 = HMS and 500g concentrate; T3 = HMS only; n = Number of samples

All analyses are mean ± standard error of means (S.E.M.)

Means with different superscript letters in a row are significantly different (P<0.05)

Apparent Digestibility

Figure 5 shows the effect of dietary treatments on nutrient digestibility in goats after feeding with different treatments. The CP digestibility of T1 (71.89%) and T2 (72.28%) were at par with each other and significantly higher than the results of T3 (52.34%). The increasing CP digestibility (P>0.05) observed in the T2 treatment groups could be attributed to the higher CP intake compared to the T1 treatment group due to highly soluble protein and amino acids following the enzymatic transformations throughout the early stages of plant growth. The hydroponic maize sprouts with lower harvesting age were rich in grass juice that could be a good source of nutrients for bacterial rumen activity, leading to increased nutrient digestibility (Finney, 1983). Similar results were reported by Naik et al. (2014), who found that digestibility of CP was 72.46% in milking cows fed hydroponic maize fodder. The average DM digestibility values in experimental goats kept on treatment T1 was 69.27%, 63.95% in treatment T2 and 35.09% in treatment T3. The average digestibility of DM in treatment T1 and T2 were significantly higher than treatment T3. Naik et al. (2014) pointed out the digestibility of DM as 65.39% in milking cows fed hydroponic maize fodder. In the present investigation, goats fed with HMS (T3) only showed the lowest digestibility (%) of CP and DM, comparable with T1 and T2 that had a concentrate-based diet. However, the apparent digestibility of DM and CP is higher in hydroponic barley fodder as 87.14% and 95.87%, respectively (Tawfeeq et al., 2018). The difference in the digestibility of the nutrients might be due to the difference in the quantity of hydroponics maize fodder fed to the experimental animals (Gebremedhin, 2015).

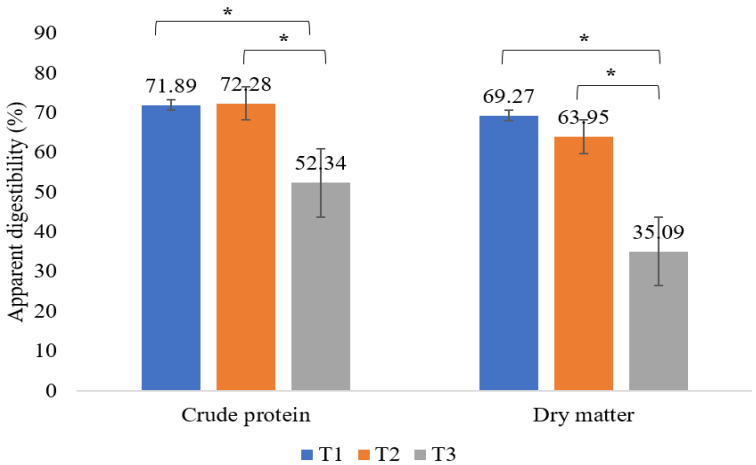

Figure 5. Apparent digestibility (%) of crude protein and dry matter for the different treatment groups
Note. T1 = Fresh Napier grass and 500g concentrate; T2 = HMS and 500g concentrate; T3 = HMS only
All analyses are mean ± standard error of means (S.E.M.)
* Indicates that the results have significant difference (P<0.05)

CONCLUSION

Sole feeding of hydroponic maize sprouts exerted a minor impact on growth performance in the animals. However, the HMS could potentially replace the conventionally planted sprouts in conjunction based on the insignificant difference in feed intake and average daily gain between the treatment groups that were offered with commercial concentrate in this study.

The open-air hydroponic system could become a solution among the smallholder farmers for its simple design and inexpensive cost compared to the customised or high-tech hydroponic system. The outdoor-grown HMS is highly palatable due to its tenderness, and younger harvesting age possesses lower indigestible fibres (including NDF and ADF) compared to conventional fodder. The highly nutritious HMS is fed as alternative green fodder because of the improved CP content. Although the dry matter of HMS was significantly lower than Napier grass, this shortcoming could be overcome by adding dry fodder or concentrate to the livestock diet.

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REFERENCES

Adebiyi, O. A., Adeola, A. T., Osinowo, O. A., Brown, D., & Ng'ambi, J. W. (2018). Effects of feeding hydroponics maize fodder on performance and nutrient digestibility of weaned pigs. *Applied Ecology and Environmental Research*, *16*(3), 2415-2422. https://doi.org/10.15666/aeer/1603_24152422

Ansari, S. T., Ravi, A., Ramana, J. V., & Raju, G. G. (2019). Voluntary intake and plane of nutrition of sheep fed hydroponic fodder varieties. *Indian Journal of Animal Research*, *53*(8), 1054-1058. https://doi.org/10.18805/ijar.B-3610

Association of Official Analytical Chemists. (1990). *Official methods of analysis* (15th ed.).Association of Official Analytical Chemists.

Bayble, T., Melaku, S., & Prasad, N. K. (2007). Effects of cutting dates on nutritive value of Napier (*Pennisetum purpureum*) grass planted sole and in association with Desmodium (*Desmodium intortum*) or Lablab (*Lablab purpureus*). *Livestock Research for Rural Development*, *19*(1), 120-136.

Eshtayeh, I. F. A. (2004). *A new source of fresh green feed (hydroponic barley) for Awass sheep* [Doctoral dissertation, An-Najah National University]. DSpace. https://hdl.handle.net/20.500.11888/7668

Farghaly, M. M., Abdullah, M. A., Youssef, I. M., Abdel-Rahim, I. R., & Abouelezz, K. (2019). Effect of feeding hydroponic barley sprouts to sheep on feed intake, nutrient digestibility, nitrogen retention, rumen fermentation and ruminal enzymes activity. *Livestock Science*, *228*, 31-37. https://doi.org/10.1016/j.livsci.2019.07.022

Farlin, S. D., Dahmen, J. J., & Bell, T. D. (1971). Effect of sprouting on nutritional value of wheat in cattle diets. *Canadian Journal of Animal Science*, *51*(1), 147-151. https://doi.org/10.4141/cjas71-020

Fauzi, M. M., & Soetanto, H. (2020). Effects of nitrogen and sulphur fertilization on the production and nutritive values of two elephant grass cultivars at two different harvesting times. In *IOP Conference Series: Earth and Environmental Science* (Vol. 478, No. 1, p. 012082). IOP Publishing. https://doi.org/10.1088/1755-1315/478/1/012082

Fazaeli, H., Golmohammadi, H. A., Shoayee, A. A., Montajebi, N. & Mosharraf, Sh. (2011). Performance of feedlot calves fed hydroponics fodder barley. *Journal Agricultural Science Technology*, *13*(3), 365-375.

Finney, P. L. (1983). Effect of germination on cereal and legume nutrient changes and food or feed value: A comprehensive review. In C. Nozzolillo, P. J. Lea, & F. A. Loewus (Eds.), *Mobilization of reserves in germination: Recent advances in phytochemistry* (Vol. 17, pp. 229-305). Springer. https://doi.org/10.1007/978-1-4684-1167-6_12

Gebremedhin, W. K. (2015). Nutritional benefit and economic value of feeding hydroponically grown maize and barley fodder for Konkan Kanyal goats. *IOSR Journal of Agriculture and Veterinary Science*, *8*(7), 24-30.

Ghani, A. A. A., Shahudin, M. S., Zamri-Saad, M., Zuki, A. B., Wahid, H., Kasim, A., Salisi, M. S., & Hassim, H. A. (2017). Enhancing the growth performance of replacement female breeder goats through modification of feeding program. *Veterinary World*, *10*(6), 630-635. https://doi.org/10.14202%2Fvetworld.2017.630-635

Hafla, A. N., Soder, K. J., Brito, A. F., Rubano, M. D., & Dell, C. J. (2014). Effect of sprouted barley grain supplementation of an herbage-based or haylage-based diet on ruminal fermentation and methane output in continuous culture. *Journal of Dairy Science*, *97*(12), 7856-7869. https://doi.org/10.3168/jds.2014-8518

Halim, R. A., Shampazuraini, S., & Idris, A. B. (2013). Yield and nutritive quality of nine Napier grass varieties in Malaysia. *Malaysian Journal of Animal Science*, *16*(2), 37-44.

Helal, H. G. (2015). Sprouted barley grains on olive cake and barley straw mixture as goat diets in Sinai. *Advances in Environmental Biology*, *9*(22), 91-102.

International Organization for Standardization. (2008). *Animal feeding stuffs - Determination of acid detergent fibre (ADF) and acid detergent lignin (ADL) contents*. https://www.iso.org/standard/43032.html

Islam, R., Jalal, N., & Akbar, M. A. (2016). Effect of seed rate and water level on production and chemical analysis of hydroponic fodder. *European Academic Research*, *4*(8), 6724-6753.

Jusoh, S., Alimon, A. R., & Kamiri, M. S. (2014). Agronomic properties, dry matter production and nutritive quality of guinea grass (*Megathrysus maximus*) harvested at different cutting intervals. *Malaysian Journal of Animal Science*, *17*(2), 31-36.

Kaouche-Adjlanea, S., Bafdelc, A. A. S. M., & Benhacined, R. (2016). Techno-economic approach to hydroponic forage crops: Use for feeding dairy cattle herd. *Journal of Applied Environmental and Biological Sciences*, *6*(3), 83-87.

Kide, W., Desai, B., & Kumar, S. (2015). Nutritional improvement and economic value of hydroponically sprouted maize fodder. *Life Sciences International Research Journal*, *2*(2), 76-79.

Lage, I. N. K., Paulino, P. V. R., Pires, C. V., Villela, S. D. J., de Souza Duarte, M., de Campos Valadares Filho, S., Paulino, M. F., Maia, B. A., Silva, L. H. P., & Teixeira, C. R. V. (2012). Intake,

digestibility, performance, and carcass traits of beef cattle of different gender. *Tropical Animal Health and Production*, *44*(2), 361-367. https://doi.org/10.1007/s11250-011-0030-z

Lounglawan, P., Lounglawan, W., & Suksombat, W. (2014). Effect of cutting interval and cutting height on yield and chemical composition of King Napier grass (*Pennisetum purpureum* x *Pennisetum americanum*). *APCBEE Procedia*, *8*, 27-31. https://doi.org/10.1016/j.apcbee.2014.01.075

Mathis, C. P., Cochran, R. C., Heldt, J. S., Woods, B. C., Abdelgadir, I. O., Olson, K. C., Titgemeyer, E. C., & Vanzant, E. S. (2000). Effects of supplemental degradable intake protein on utilization of medium-to low-quality forages. *Journal of Animal Scienc*e, *78*(1), 224-232. https://doi.org/10.2527/2000.781224x

Mazon, M. R., Carvalho, R. F., Pesce, D. M. C., Silva, S. D. L., Gallo, S. B., & Leme, P. R. (2017). Time on feedlot and sexual effects on animal performance and characteristics of lamb's meat. Acta Scientiarum. *Animal Sciences*, *39*(1), 103-109. https://doi.org/10.4025/actascianimsci.v39i1.32749

Mukhtar, M., Ishii, Y., Tudsri, S., Idota, S., & Sonoda, T. (2003). Dry matter productivity and overwintering ability of the dwarf and normal napiergrasses as affected by the planting density and cutting frequency. *Plant Production Science*, *6*(1), 65-73. https://doi.org/10.1626/pps.6.65

Naik, P. K., Dhuri, R. B., Karunakaran, M., Swain, B. K., & Singh, N. P. (2013). Hydroponics technology for green fodder production. *Indian Dairyman*, *65*(3), 54-58.

Naik, P. K., Dhuri, R. B., Karunakaran, M., Swain, B. K., & Singh, N. P. (2014). Effect of feeding hydroponics maize fodder on digestibility of nutrients and milk production in lactating cows. *Indian Journal of Animal Sciences*, *84*(8), 880-883.

Naik, P. K., Dhuri, R. B., Swain, B. K., & Singh, N. P. (2012). Nutrient changes with the growth of hydroponics fodder maize. *Indian Journal of Animal Nutrition*, *29*(2), 161-163.

Naik, P. K., Swain, B. K., & Singh, N. P. (2015). Production and utilisation of hydroponics fodder. *Indian Journal of Animal Nutrition*, *32*(1), 1-9.

Ndaru, P. H., Huda, A. N., Prasetyo, R. D., Shofiatun, U., Nuningtyas, Y. F., & Ndaru, R. K. (2020). Providing high quality forages with hydroponic fodder system. In *IOP Conference Series: Earth and Environmental Science* (Vol. 478, No. 1, p. 012054). IOP Publishing. https://doi.org/10.1088/1755-1315/478/1/012054

Prasad, R., Sehgal, J. P., Patnayak, B. C., & Beniwal, R. K. (1998). Utilization of artificially grown barley fodder by sheep. *The Indian Journal of Small Ruminants*, *4*(2), 63-68.

Raeisi, Z., Tahmasbi, R., Dayani, O., Ayatollahi Mehrgardi, A., & Tavassolian, I. (2018). Digestibility, microbial protein synthesis, rumen and blood parameters in sheep fed diets containing hydroponic barley fodder. *Journal of Livestock Science and Technologies*, *6*(1), 9-17. https://doi.org/10.22103/JLST.2017.10424.1211

Rahman, M. M., Abdullah, R. B., Wan Khadijah, W. E., Nakagawa, T., & Akashi, R. (2014). Feed intake and growth performance of goats offered Napier grass (*Pennisetum purpureum*) supplemented with concentrate pellet and soya waste. *Sains Malaysiana*, *43*(7), 967-971.

Rambau, M. D., Fushai, F., & Baloyi, J. J. (2016). Productivity, chemical composition and ruminal degradability of irrigated Napier grass leaves harvested at three stages of maturity. *South African Journal of Animal Science*, *46*(4), 398-408. https://doi.org/10.4314/sajas.v46i4.8

Shyama, K., Rajkumar, G., Rani, K. J., & Venkatachalapathy, R. T. (2016). Effect of feeding of hydroponic fodder maize as partial

protein supplement on growth performance in kids. *Journal of Indian Veterinary Association, Kerala*, *14*(3), 32-36.

Sneath, R., & McIntosh, F. (2003). *Review of hydroponic fodder production for beef cattle*. https://cpb-us-east-1-juc1ugur1qwqqqo4.stackpathdns.com/blogs.cornell.edu/dist/e/4211/files/2014/05/Hydroponicfodder-article-11wpnm0.pdf

Tawfeeq, J. A., Hassan, S. A., Kadori, S. H., Shaker, R. M., & Hamz, Z. R. (2018). Evaluation of feeding hydroponics barley on digestibility and rumen fermentations in Awassi lambs. *The Iraqi Journal of Agricultural Science*, *49*(4), 639.

Thadchanamoorthy, S., & Pramalal, V. J. C. (2012). *Evaluation of hydroponically grown maize as a feed source for rabbits*. http://agri.pdn.ac.lk/ext_staff/upload/publications/publication_249_dept_5_fbe225af2b33ebfaab9e85855d643937.pdf#page=17

Valente, T. N. P., da Silva Lima, E., dos Santos, W. B. R., Cesario, A. E. S., Tavares, C. A. J., & de Freitas, M. A. M. (2016). Ruminal microorganism consideration and protein used in the metabolism of the ruminants: A review. *African Journal of Microbiology Research*, *10*(14), 456-464. https://doi.org/10.5897/AJMR2016.7627

Vennila, C. (2018). Productivity, nutritive value, growth rate, biomass yield and economics of different hydroponic green fodders for livestock. *International Journal of Livestock Research*, *8*(9), 261-270. https://doi.org/10.5455/ijlr.20171013104959

Wijitphan, S., Lorwilai, P., & Arkaseang, C. (2009). Effect of cutting heights on productivity and quality of King Napier grass (*Pennisetum purpureum* cv. King Grass) under irrigation. *Pakistan Journal of Nutrition*, *8*(8), 1244-1250. https://doi.org/10.3923/pjn.2009.1244.1250

Zailan, M. Z., Yaakub, H., & Jusoh, S. (2016). Yield and nutritive value of four Napier (*Pennisetum purpureum*) cultivars at different harvesting ages. *Agriculture and Biology Journal of North America*, *7*(5), 213-219. https://doi.org/10.5251/abjna.2016.7.5.213.219

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Sequencing Analysis of Partial *N* gene of Feline *Morbillivirus* from Malaysia

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ABSTRACT

Feline morbillivirus (FeMV) is a new emerging virus of domestic cats categorized under the genus of *Morbillivirus*, associated with chronic kidney disease (CKD). The origin of the virus is yet to be determined, and whether it is caused by a spill-over event from wildlife or domestic cats remains speculative. Recombination event has been reported in FeMV isolate found in Japan; therefore, characterization of FeMV strains isolated in Malaysia (i.e., FeMV-Malaysia isolates) may provide some insight, thus adding some information on the viral evolution of FeMV. Therefore, this study aims to conduct a phylogenetic analysis and assess any genetic changes in the *N* gene of FeMV-Malaysia isolates. Through sequencing of *N* gene of seven isolates using three overlapping primer sets, the sequences spanning approximately 1.5kb of FeMV-*N* gene were obtained. DNA sequencing, nucleotide sequences, amino acid residues alignments, and phylogenetic analysis were performed. A nucleotide sequence alignment was also performed to compare the isolates obtained from two previous studies. From the alignment mentioned above, there were 19 variable sites of which there were absence of amino acid changes except for isolate UPM210 at position 806 and isolate UPM315 at position 823. Furthermore, protein alignment was done to compare FeMV-Malaysia isolates with FeMV strains from other countries, along with other morbillivirus-related isolates. From one of the conserved regions located within the *N* gene, similar amino acid sequences were detected across different morbilliviruses. Lastly, from the phylogenetic tree, it was illustrated that all partial FeMV-*N* gene Malaysia isolates sequenced in this study were clustered together in the same clade whereby these FeMV-*N* genes Malaysia isolates shared a

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common ancestor with isolates from Japan (SS3, MiJP003, ChJP073) and Thailand (Thai-U16, CTL16. CTL43).

Keywords: Feline morbillivirus, partial *N* gene, sequencing analysis

INTRODUCTION

Feline morbillivirus (FeMV) is a non-segmented, negative-sense, single-stranded RNA morbillivirus that was first discovered in Hong Kong in 2012, and it has been speculated to cause chronic kidney disease (CKD) in domestic cats (Woo et al., 2012). It forms a distinct species under the genus *Morbillivirus*, with nucleotide identities less than 80% of the known paramyxoviruses. Similar to other paramyxoviruses and morbilliviruses, the genome organization of FeMV follows the rule of six, where it efficiently replicates when there are six nucleotides in length. In addition, the gene organization of FeMV (3'-*N*-*P*/*V*/*C*-*M*-*F*-*H*-*L*-5') is similar to that of other morbilliviruses.

Nucleoprotein (N), which is translated from the *N* gene, plays a crucial role in the transcription of the virus (Sourimant & Plemper, 2016). The RNA-dependent RNA polymerase (RdRp) will synthesize the viral RNA only when encapsidated by the N protein as a template. Both polymerase-associated phosphoprotein (*P*) and large (*L*) genes, which are involved in all polymerase activities, are responsible for forming the RdRp complex and the N protein. In addition, two glycoproteins, which are the fusion (F) and haemagglutinin (H) proteins, make up the outer layer or envelope of the virus that connects to the matrix (M) protein surrounding the ribonucleoprotein (Rima et al., 2019).

Genetic changes in a virus could be identified by performing sequences alignment of the nucleotide or amino acid residue. Genetic changes of a virus may include mutation and recombination (Fleischmann, 1996). Compared to DNA viruses, RNA viruses hold a higher mutation due to a lack of proofreading function in their replication enzymes. Recombination of FeMV within *F* and *H* genes has reported that the FeMV-Japan strains (MiJP003) could probably originate from recombination between two FeMV parental strains that are closely related to Japan (ChJP073) and Hong Kong (776U) isolates (Park et al., 2014). Hence, any genetic changes that might have occurred in the *N* gene of FeMV-Malaysia isolates could be investigated through this work.

MATERIALS AND METHODS

Sample for Sequencing Analysis

Samples for sequencing analysis (n = 7) were obtained as part of two studies to determine the molecular prevalence of FeMV in Malaysia and the development of TaqMan-based reverse-transcriptase polymerase chain reaction (RT-PCR) assay targeting the FeMV-*N* gene (Makhtar et al., 2021; Mohd Isa et al., 2019). Briefly, the urine sample was collected from cats either by manual compression or cystocentesis. The collected urine was then placed into a

sterile sample collection bottle and stored at 4°C prior to processing. The debris in the urine was removed by centrifugation at 2,320 × *g*, where the supernatant was obtained, mixed at a ratio of 1:1 with RNAlater® solution (Ambion, USA), and stored at –20°C prior to RNA extraction.

Total RNA Extraction

Total RNA extraction for collecting urine samples was performed by using the Direct-zol™ RNA MiniPrep Plus Kit according to the manufacturer's recommendation (Zymo Research, USA). Briefly, the TRI Reagent® solution was added into each sample at a ratio of 3:1 and mixed thoroughly for 5 min. Next, an equal volume of ethanol (95%–100%) was added to the mixture and vortexed thoroughly. The mixture was then transferred into a Zymo-Spin™ IIICG Column attached to a collection tube and centrifuged at 12,000 × *g* for 1 min. The flow-through was discarded, and the column was transferred into a new collection tube. Next, a volume of 400 µL of Direct-zol™ RNA PreWash was added into the column and centrifuged at 12,000 × *g* for 1 min, upon which the flow-through was discarded. This step was repeated, followed by the addition of 700 µL of RNA Wash Buffer to the column and centrifugation at 12,000 × *g* for 2 min. The column was then transferred into an RNase-free tube, followed by the elution step repeated twice by adding a volume of 20 µL of RNase-Free Water to the column matrix and centrifuged at 12,000 × *g* for 1 min.

cDNA Synthesis

Extracted RNA was converted into cDNA prior to conventional RT-PCR assay. The cDNA synthesis was conducted using the SensiFAST™ cDNA Synthesis Kit (Bioline, United Kingdom) following the suggested protocol: 25°C for 10 min in primer annealing step, 42°C and 48°C each for 15 min in reverse transcription step, and 85°C for 5 min in inactivation step. The cDNA templates were stored at -20°C prior to conventional RT-PCR analysis.

FeMV-*N* Gene Amplification by RT-PCR and Sequencing of *N* Gene

Conventional RT-PCR was performed using MyTaq™ Red Mix (Bioline, United Kingdom) on the seven FeMV-positive urine samples (UPM23, UPM52, UPM53, UPM210, UPM231, UPM305, and UPM315). RNase-free water (Promega, USA) was used as a negative control in each PCR run. Three different primers were used to obtain the full sequence of the FeMV-*N* gene from Malaysian isolates (Figure 1). The first primer set (1F_16 and 1R_8) covering the early region of the *N* gene was designed using the PrimerQuest tool from Integrated DNA Technologies (IDT) website based on the alignment sequences from Thailand (Thai-U16), China isolates (M252A), and Japan isolates (ChJP073, MiJP003, and SS3) (Table 1). The second (FN-2F and FN-2R) and third (FN-3F and FN-3R) primer sets targeting the middle and end regions of the *N* gene in this study, respectively, were published primers (Park et al. (2014). The amplification

was performed in a thermal cycler using different annealing temperatures for each primer set (Table 2). Amplified PCR product was analyzed on 1.5% agarose gels and visualized using a UV transilluminator (Syngene, United Kingdom). The PCR products were then purified and sequenced on an ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, USA) utilizing both forward and reverse primers described in this study (Table 1).

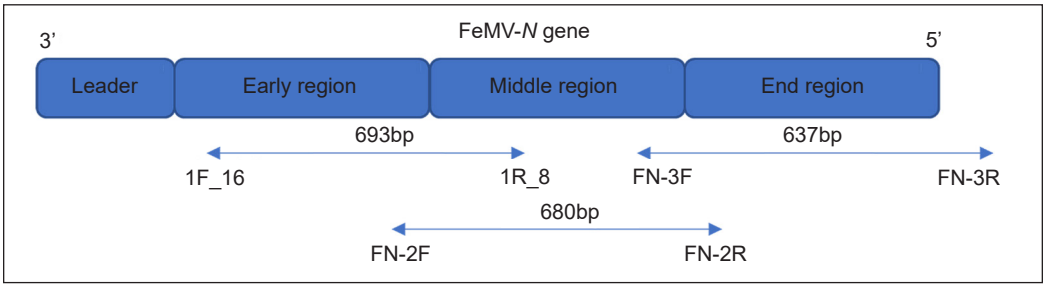

Figure 1. Schematic diagram on *N* gene primer design. Three different sets of overlapping primers were designed to obtain a ~1.5kb sequence of the *N* gene. Both primers set FN-2 (FN-2F and FN-3R) and FN-3 (FN-3F and FN-3R) were obtained from a previous study by Park et al. (2014), whereas 1F-16 and 1R-8 were newly designed primer sets

Table 1
Primer sequences were used to amplify three different regions targeting the N *gene*

Region	Primer	Sequence (5'-3')	Product size (bp)	Source
Early region	1F_16 1R_8	CTGAAATCACTTGCCGCATTTA TGCCACCATGAATCGTCTTAT	693	This study
Middle region	FN-2F FN-2R	GTTAGCTTAGGATTTGAGAACCC CACCATCTCTTGACCAAGTCT	680	(Park et al., 2014)
End region	FN-3F FN-3R	GCTATGGAGTTATGCCATGGG GTTGTGAACCTTGAGGTCCTAAG	637	

Table 2
PCR condition for three different primer sets targeting the N *gene*

Step	Temperature	Time	Cycle
Initial denaturation	95°C	1 min	1×
Denaturation	95°C	15 sec	35×
Annealing	54°C(1F_16 and 1R_8) 58°C(FN-2F and FN-2R) 58°C(FN-3F and FN-3R)	1 min	
Extension	72°C	1 min	
Final extension	72°C	5 min	1×
Hold	12°C	∞	1×

Phylogenetic Analysis

The sequences from all seven isolates were aligned and trimmed using the MEGA X (version 10.0.05) software. Then, all sequences were compared with all available FeMV sequences in National Center for Biotechnology Information (NCBI) nucleotide databases using nucleotide basic local alignment search tool (BLAST). Partial sequences of *N* gene FeMV-Malaysia isolates were subsequently subjected to phylogenetic tree construction, along with the other FeMV-*N* gene sequences available in the GenBank using the MEGA X software (Table 3). The phylogenetic tree was constructed using the Maximum Likelihood method with bootstrap values of 1,000 replicates based on the general time-reversible (GTR) model. The construction of the phylogenetic tree also included other distantly-related viruses under the genus *Morbillivirus*, which were the canine distemper virus (CDV), peste des petits ruminants virus (PPRV), rinderpest virus (RPV), dolphin morbillivirus (DMV), phocine distemper virus (PDV), and measles virus (MeV) as an outgroup comparison (Sakaguchi et al., 2014; Sieg et al., 2019).

Table 3
Viral strains included for phylogenetic tree construction

Species	Isolate	Year	Location	Accession number
FeMV	761U	2012	Hong Kong	JQ411014
FeMV	776U	2012	Hong Kong	JQ411015
FeMV	M252A	2012	China	JQ411016
FeMV	OtJP001	2014	Japan	AB924120
FeMV	MiJP003	2014	Japan	AB924121
FeMV	ChJP073	2014	Japan	AB924122
FeMV	SS1	2014	Japan	AB910309
FeMV	SS2	2015	Japan	LC036586
FeMV	SS3	2015	Japan	LC036587
FeMV	US1	2015	USA	KR014147
FeMV	Thai-U16	2017	Thailand	MF627832
FeMV	PIUMA/2015	2015	Italy	KT825132
FeMV	Tremedino	2018	Italy	MK088516
FeMV	Pepito	2018	Italy	MK088517
FeMV	Capitan Harlock	2018	Italy	MK188746
FeMV	Nerina	2018	Italy	MK188747
FeMV	Pedro	2018	Italy	MK188748
FeMV	Sheryl	2018	Italy	MK188749
FeMV	Rossino	2018	Italy	MK188750
FeMV	Trezampe	2018	Italy	MK188751
FeMV	Claudio	2018	Italy	MK188752
FeMV	Tris	2018	Italy	MK188753
FeMV	Lilly	2018	Italy	MK188754

Table 3 (*continue*)

Species	Isolate	Year	Location	Accession number
FeMV	TV17	2017	Germany	MG563820
FeMV	Gordon	2018	Germany	MK182089
FeMV	TV25	2018	Germany	MK182090
FeMV	ZRU293/17	2018	South Africa	MH813465
FeMV	CTL16	2019	Thailand	MN164531
FeMV	CTL43	2019	Thailand	MN164532
CDV	CDV SY	2012	China	KJ466106
PPRV	SnDk11I13	2013	Africa	KM212177
RPV	Kabete O	1996	Africa	NC006296
DMV	631IMM5031711	2011	USA	KU720625
PDV	PDV/USA 2006	2006	USA	KY629928
MeV	Ichinose-B95a	1998	USA	NC001498

RESULTS

DNA Sequencing Analysis and Basic Local Alignment Search Tool (BLAST) of *N* Gene

BLAST search analysis of partial *N* gene of all seven FeMV-Malaysia isolates revealed high similarities with 29 FeMV isolates from other countries (81%–99%). All sequences have been submitted in the GenBank under accession numbers MN264638-MN264642, MN792827, and MN792828 (Table 4). In addition, high similarities were observed between FeMV-Malaysia isolates (98%–99%) and FeMV isolates originated from Asian countries, such as Japan (LC036587), and Thailand (MN164531) (Table 5).

Table 4
Genome submission to NCBI

Sample ID	Accession number
UPM23	MN264638
UPM52	MN264639
UPM53	MN264640
UPM210	MN264641
UPM231	MN264642
UPM305	MN792827
UPM315	MN792828

Pairwise Alignment on *N* gene of FeMV-Malaysia Isolates

Multiple alignments of FeMV-Malaysia isolates detected in this study were performed to assess any significant nucleotide difference. There were 19 variable sites observed out of 962 among the seven FeMV-*N* genes Malaysia isolates. However, none of these sites resulted in different amino acid residues except for isolate UPM210 and isolate UPM315 at positions 806 and 823, respectively (Figure 2). For UPM210, nucleotide change at position 806 resulted in amino acid serine compared to asparagine in other isolates. In contrast, amino acid valine was detected as a result of nucleotide change at position 823 for UPM315 isolate compared to amino acid isoleucine among the other six FeMV-Malaysia isolates.

Table 5
*Summary of BLAST search for partial FeMV-*N *gene sequences*

Sample ID/ Accession no.	Identities similarity X/Y (%)	Accession number for reference strain	Remark for reference strain
UPM23 MN264638	1595/1608 (99%)	LC036587	Feline morbillivirus *N*, *P*/*V*/*C*, *M*, *F*, *H*, *L* genes for nucleocapsid protein, phosphoprotein, matrix protein, fusion protein, hemagglutinin, protein, RNA polymerase, complete CDS, strain: SS3
UPM52 MN264639	1567/1584 (99%)	LC036587	Feline morbillivirus *N*, *P*/*V*/*C*, *M*, *F*, *H*, *L* genes for nucleocapsid protein, phosphoprotein, matrix protein, fusion protein, hemagglutinin, protein, RNA polymerase, complete CDS, strain: SS3
UPM53 MN264640	1563/1578 (99%)	MN164531	Feline morbillivirus isolate CTL16, complete genome
UPM210 MN264641	1565/1580 (99%)	MN164531	Feline morbillivirus isolate CTL16, complete genome
UPM231 MN264642	1603/1618 (99%)	MN164531	Feline morbillivirus isolate CTL16, complete genome
UPM305 MN792827	1547/1564 (99%)	LC036587	Feline morbillivirus *N*, *P*/*V*/*C*, *M*, *F*, *H*, *L* genes for nucleocapsid protein, phosphoprotein, matrix protein, fusion protein, hemagglutinin, protein, RNA polymerase, complete CDS, strain: SS3
UPM315 MN792828	1587/1607 (99%)	MN164531	Feline morbillivirus isolate CTL16, complete genome

Note. X: Total nucleotide similarities between FeMV-*N* gene Malaysia isolates and FeMV-*N* gene of reference strains in GenBank; Y: Total nucleotide of FeMV-*N* gene of reference strains in GenBank; CDS: Coding DNA sequence

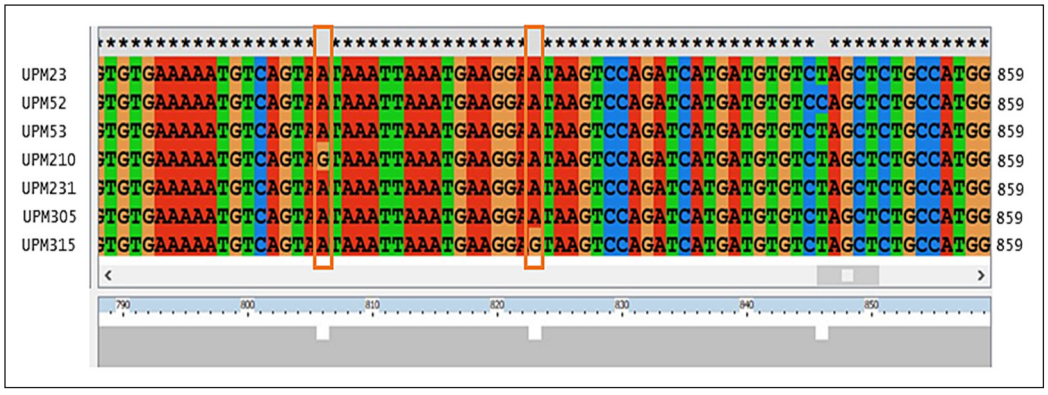

Figure 2. Multiple alignments of nucleotide detected in *N* gene from FeMV-Malaysia isolates. Boxes indicate nucleotide differences for UPM210 and UPM315 at positions 806 and 823, respectively, among the sequenced FeMV-Malaysia isolates. The position of the nucleotides is at the bottom, while the number of nucleotides for each isolate is presented to the right of each isolate. The asterisk (*) symbol represents identical nucleotides among the isolates

Nucleocapsid Protein Analysis

In this work, 320 amino acids were successfully encoded, compared with 519 amino acids that should be encoded when a full *N* gene is sequenced. One highly conserved sequence motif among paramyxoviruses from Sequence 3 was indicated in the red box in Figure 3. Comparison between amino acid residues for FeMV-Malaysia isolates and other FeMV isolates, along with species from the same genus of *Morbillivirus*, demonstrated consistent conserved amino acid residues (as underlined in Figure 3) in all species included in the alignment. In addition, the amino acid residue PPRV and RPV at position 328 was detected as alanine, whereas in other isolates, the amino acid at the same position was serine. Due to the nucleotide differences, wherein PPRV and RPV, nucleotide GCC was detected, compared with other isolates whereby nucleotide TCT was detected.

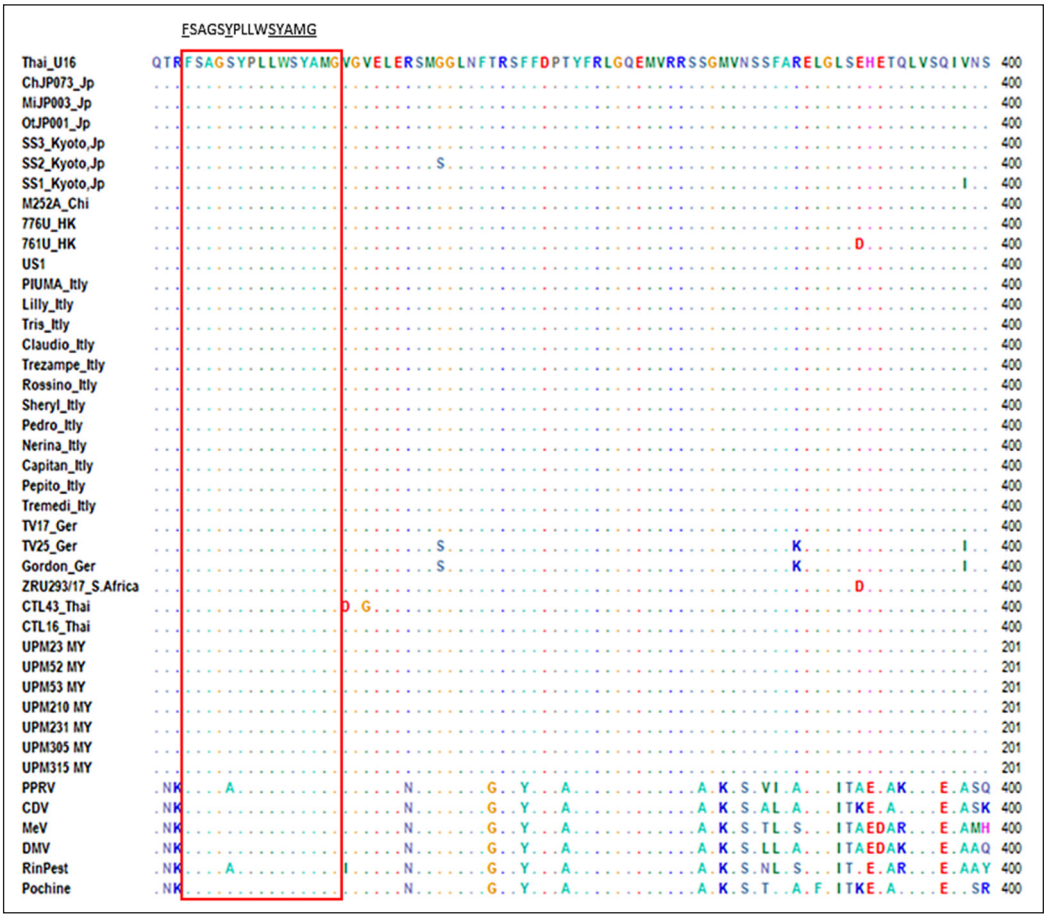

Figure 3. Multiple alignments of N protein amino acid residues of FeMV and other morbilliviruses sequences. Box indicates conserved motifs in paramyxoviruses. Amino acid residue numbers for each protein isolate are presented to the right of each isolate. Dots denote the identical amino acid residues between each protein isolate

Phylogenetic Tree Analysis of Partial FeMV-*N* Gene Sequences of Malaysia Isolates

Phylogenetic analysis was conducted to review the evolutionary relationship between isolates from different countries. All partial FeMV-*N* gene Malaysia isolates (UPM210, UPM231, UPM305, and UPM315) showed >99% identical sequence with the isolates detected in the previous study: UPM23, UPM52, UPM53; thus, these seven isolates were clustered together within the same clade (Figure 4). In addition, FeMV-Malaysia isolates showed a close relationship to isolates from Thailand (Thai-U16, CTL16, and CTL43) and China (M252A) with 98% nucleotide similarities while sharing 98%–99% nucleotide similarities with three of

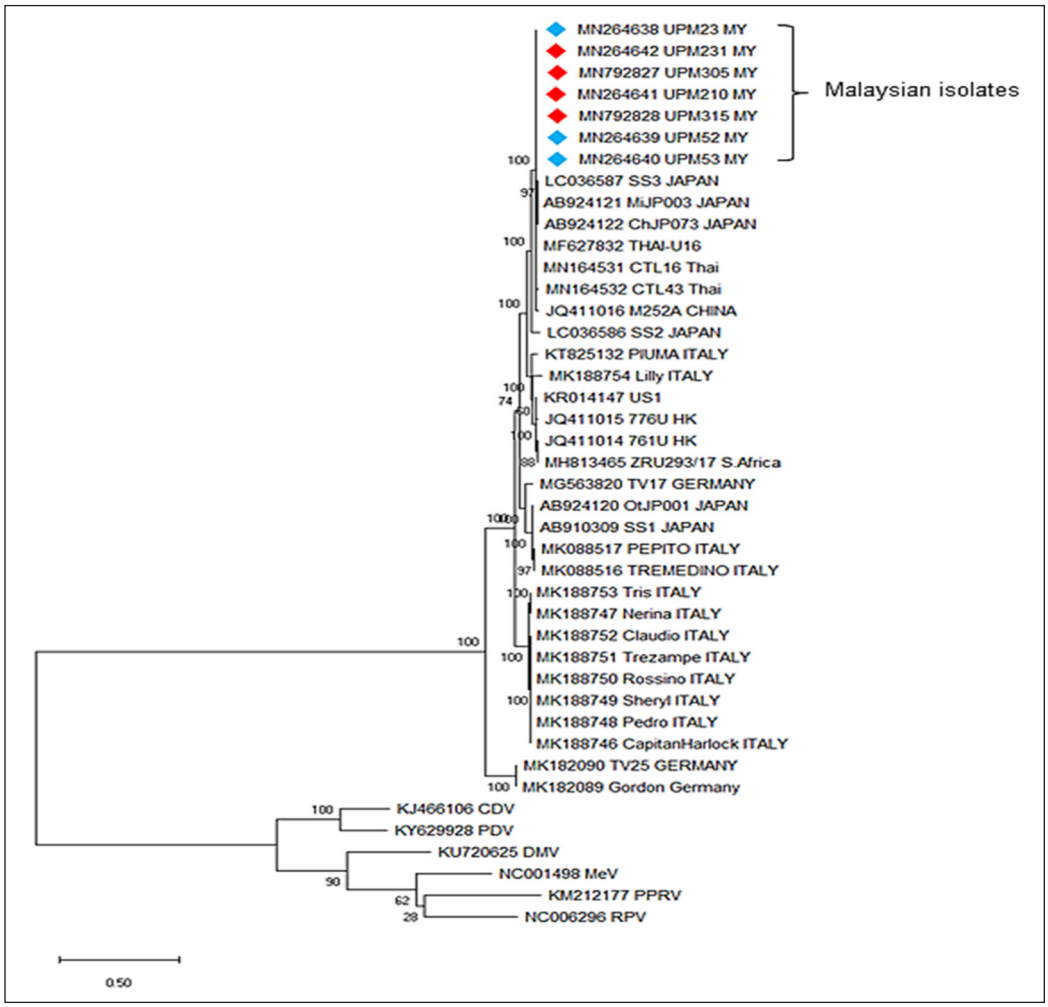

Figure 4. Phylogenetic analysis based on partial FeMV-*N* gene sequences of Malaysia isolates. FeMV-*N* gene Malaysia isolates detected in this study, which clustered together, are represented by red diamond shape (♦) (Makhtar et al., 2021) and blue diamond shape (♦) (Mohd Isa et al., 2019). The evolutionary analysis was constructed using the maximum likelihood method based on the general time-reversible model using MEGA X (version 10.0.05) software. The scale represents the number of substitutions per site

Japan isolates (SS3, MiJP00, and ChJP073). However, the SS2 isolate from Japan showed only 94% nucleotide similarities with Malaysian isolates, of which it formed a different branch within the phylogenetic tree. Furthermore, comparing with BLAST search of nucleotide similarity result, it also can be noted that isolates (SS3 and CTL16) that recorded >98% similarity with FeMV-*N* gene Malaysia isolates detected in this study were closely related to each other, illustrating a consistent finding with the outcome from BLAST search. In addition, another genotype of FeMV, FeMV genotype 2 (FeMV-GT2), established its cluster with two other isolates: TV25 and Gordon. All 29 FeMV isolates available in GenBank also formed a distinct clade separated from the outgroup isolates, comprising other members of the genus *Morbillivirus*.

DISCUSSION

Feline morbillivirus has been implicated as the cause of interstitial nephritis associated with chronic kidney disease in cats; however, further evidence is needed to prove Koch's postulate of FeMV infection (de Luca et al., 2021). Nevertheless, many countries have reported the occurrence of FeMV among cats; therefore, the importance of this virus should not be undermined. Upon successful detection of FeMV among cats in Malaysia, further characterization of these FeMV isolates is warranted. However, an attempt to obtain a full sequence of the FeMV-*N* gene from this study had been unsuccessful, as none of the primer sets designed could amplify the leader region of the *N* gene. Nevertheless, BLAST search and sequencing analyses of partial *N* gene were performed on seven positive samples from previous studies: UPM23, UPM52, UPM53, UPM210, UPM231, UPM305, and UPM315 (Makhtar et al., 2021; Mohd Isa et al., 2019). Furthermore, a BLAST search showed a high similarity percentage between isolates obtained from a study by Makhtar et al. (2021) with previously detected FeMV-Malaysia isolates, as the samples were collected within the same geographical area. From the BLAST result of 29 FeMV isolates from other countries, a high similarity (98%–99%) was observed with FeMV isolates from China, Japan, and Thailand, indicating that FeMV isolates from Malaysia have been circulating in the Asian region. Besides that, the factor of Asian climate may also play a role in transmission and adaptation of the virus, which could contribute to the high similarity percentage among the isolates from Asian countries, compared with that from other countries (Wu et al., 2016).

Based on the nucleotide alignment of the partial *N* gene of the seven FeMV-Malaysia isolates, two different amino acid substitution site was detected, each one within isolate UPM210 and UPM315. This amino acid difference might be due to the position of the nucleotide where it lies at the structurally variable region of the N_{TAIL} domain, which comprises approximately 120 to 150 amino acid residues (Communie et al., 2014). This finding is also in agreement with a study on FeMV-N protein by Woo et al. (2012), whereby at both positions (806 and 823), amino acid substitutions were

detected among the FeMV isolates from China and Hong Kong. Even though RNA virus does frequently undergo mutation by approximately one mutation per virus genome copy to combat the host immune response, the mutation usually would not interfere with the essential virus function, and if it does, it will result in positive or negative selection, which leads to a superior mutation that enables the virus to survive despite the replication of the host immune response (Fleischmann, 1996). On the other hand, if the mutation damages the virus's essential function, a negative selection will occur, whereby the mutation will be deleted. Nevertheless, due to the redundancy of genetic code, most mutations are neutral, demonstrating no changes towards protein function, as the amino acid remains unchanged. However, to assess any mutation for the FeMV-Malaysia isolates, whole genome sequencing should be done to compute the pairwise alignment of FeMV isolates. This study focused only on N protein, so calculating the percentage of nucleotide similarities for the other five open reading frames (ORFs) for FeMV could not be accomplished. Based on the phylogenetic analysis, even though whole genome sequencing was not done, it is noteworthy that the FeMV-Malaysia isolates formed a distinct clade with the previously introduced new genotype of FeMV, FeMV-GT2, which was detected in Germany (Sieg et al., 2019).

Nucleoprotein (N) comprises of the N_{CORE} which is the N-terminal moiety covering the first 400 amino acids of N protein and the N_{TAIL} domain which is the C-terminal moiety (Thakkar et al., 2018). N_{CORE} is responsible for RNA encapsidation, a prerequisite for viral RNA synthesis. Amino acid analyses identified conserved motifs within the *Paramyxovirinae*, which are Region 1, 172-QxW(I,V)xxxK(A,C) xT-184; Region 2, 268-FxxT(I,L)(R,K) Ω(G,A) (L,I,V)xT-280; and Region 3, 323- FxxxxYPxxΩSΩAMG-339, where x indicates any amino acid residue, Ω indicates an aromatic amino acid residue, and either one of the residues in parentheses (Morgan, 1991). On the other hand, the interaction of N and P proteins is mediated by the N_{TAIL} region of approximately 120 to 150 amino acids of the structurally variable region (Communie et al., 2014). During the initial stage of infection, the RNA-dependent RNA polymerase (RdRp) complex will only recognize and synthesize viral RNA, which is encapsidated by N protein as a template (Sourimant & Plemper, 2016). Although the *N* gene is quite conserved compared to the *F* and *H* genes, given the importance during early infection, the characterization of the *N* gene may give further insight into the newly emerging FeMV infection in cats.

However, from the three highly conserved regions of the N protein, only one conserved region was obtained in this study. The conserved sequence (amino acid residue at a position between 324–328) showed a similar amino acid residue in alignment with 29 other FeMV isolates from different countries, consistent with a previous study (Woo et al., 2012). Moreover, within the conserved Region 3, a different amino acid residue, alanine (A), was detected in RPV and PPRV at position

328, whereas other isolates resulted in serine (S) amino acid residue. However, there was little information on whether the amino acid difference played a significant role in RNA binding and forming the helical nucleocapsid; hence, there is a need for future *in vitro* study elucidating the effects of amino acid changes towards the nucleocapsid role. In addition, the full N protein sequence analysis is warranted by designing a primer set that targets the leader region.

CONCLUSION

Sequences of ~1.5kb from seven FeMV-Malaysia isolates were obtained through conventional RT-PCR detection from selected urine samples. A BLAST search of the partial *N* gene of FeMV-Malaysia isolates (UPM23, UPM52, UPM53, UPM210, UPM231, UPM305, and UPM315) revealed to have had a high percentage of nucleotide similarities with isolates from Japan (SS3) and Thailand (CTL16). From phylogenetic analysis, the seven FeMV-Malaysia isolates are clustered together with other Asian countries, such as Thailand (Thai-U16, CTL43, CTL16) and Japan (SS3, MiJP003, ChJP073). In addition, nucleotide alignment of the seven FeMV-Malaysia isolates detected 19/962 variable sites, of which only two isolates, UPM210 and UPM315, had amino acid substitution. From the alignment of amino acid residues of FeMV-isolates and other morbilliviruses, consistent amino acid residues were observed in one of the conserved regions in morbilliviruses. Therefore, to assess for any genetic change, it is suggested to perform sequence alignments and phylogenetic analysis from other genes, especially on *F* and *H* genes, which are more heterogeneous and prone for recombination.

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REFERENCES

Communie, G., Ruigrok, R. W., Jensen, M. R., & Blackledge, M. (2014). Intrinsically disordered proteins implicated in paramyxoviral replication machinery. *Current Opinion in Virology*, *5*(1), 72-81. https://doi.org/10.1016/j.coviro.2014.02.001

de Luca, E., Sautto, G. A., Crisi, P. E., & Lorusso, A. (2021). Feline morbillivirus infection in domestic cats: What have we learned so far?. *Viruses*, *13*(4), 683. https://doi.org/10.3390/v13040683

Fleischmann, W. R. (1996). Viral genetics. In S. Baron (Ed.), *Medical microbiology* (4th ed., p. 43). University of Texas Medical Branch.

Makhtar, S. T., Tan, S. W., Nasruddin, N. A., Abdul Aziz, N. A., Omar, A. R., & Mustaffa-Kamal, F. (2021). Development of TaqMan-based real-time RT-PCR assay based on *N* gene for the quantitative detection of feline morbillivirus. *BMC Veterinary Research*, *17*(1), 128. https://doi.org/10.1186/s12917-021-02837-6

Mohd Isa, N. H., Selvarajah, G. T., Khor, K. H., Tan, S. W., Manoraj, H., Omar, N. H., Omar, A. R., & Mustaffa-Kamal, F. (2019). Molecular detection and characterisation of feline morbillivirus

in domestic cats in Malaysia. *Veterinary Microbiology*, *236*, 108382-108382. https://doi.org/10.1016/j.vetmic.2019.08.005

Morgan, E. M. (1991). Evolutionary relationships of *Paramyxovirus* nucleocapsid-associated proteins. In D. W. Kingsbury (Ed.), *The Paramyxoviruses* (pp. 163-179). Springer. https://doi.org/10.1007/978-1-4615-3790-8_5

Park, E. S., Suzuki, M., Kimura, M., Maruyama, K., Mizutani, H., Saito, R., Kubota, N., Furuya, T., Mizutani, T., Imaoka, K., & Morikawa, S. (2014). Identification of a natural recombination in the *F* and *H* genes of feline morbillivirus. *Virology*, *468-470*, 524-531. https://doi.org/10.1016/j.virol.2014.09.003

Rima, B., Balkema-Buschmann, A., Dundon, W. G., Duprex, P., Easton, A., Fouchier, R., Kurath, G., Lamb, R., Lee, B., Rota, P., Wang, L., & Ictv Report, C. (2019). ICTV virus taxonomy profile: *Paramyxoviridae*. *Journal of General Virology*, *100*(12), 1593-1594. https://doi.org/10.1099/jgv.0.001328

Sakaguchi, S., Nakagawa, S., Yoshikawa, R., Kuwahara, C., Hagiwara, H., Asai, K. I., Kawakami, K., Yamamoto, Y., Ogawa, M., & Miyazawa, T. (2014). Genetic diversity of feline morbilliviruses isolated in Japan. *Journal of General Virology*, *95*(7), 1464-1468. https://doi.org/10.1099/vir.0.065029-0

Sieg, M., Busch, J., Eschke, M., Böttcher, D., Heenemann, K., Vahlenkamp, A., Reinert, A., Seeger, J., Heilmann, R., Scheffler, K., & Vahlenkamp, T. W. (2019). A new genotype of feline morbillivirus infects primary cells of the lung, kidney, brain and peripheral blood. *Viruses*, *11*(2), 146. https://doi.org/10.3390/v11020146

Sourimant, J., & Plemper, R. K. (2016). Organization, function, and therapeutic targeting of the morbillivirus RNA-dependent RNA polymerase complex. *Viruses*, *8*(9), 251. https://doi.org/10.3390/v8090251

Thakkar, V. D., Cox, R. M., Sawatsky, B., da Fontoura Budaszewski, R., Sourimant, J., Wabbel, K., Makhsous, N., Greninger, A. L., von Messling, V., & Plemper, R. K. (2018). The unstructured paramyxovirus nucleocapsid protein tail domain modulates viral pathogenesis through regulation of transcriptase activity. *Journal of Virology*, *92*(8), e02064-17. https://doi.org/10.1128/jvi.02064-17

Woo, P. C., Lau, S. K., Wong, B. H., Fan, R. Y., Wong, A. Y., Zhang, A. J., Wu, Y., Choi, G. K., Li, K. S., Hui, J., Wang, M., Zheng, B. J., Chan, K. H., & Yuen, K. Y. (2012). Feline morbillivirus, a previously undescribed paramyxovirus associated with tubulointerstitial nephritis in domestic cats. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(14), 5435-5440. https://doi.org/10.1073/pnas.1119972109

Wu, X., Lu, Y., Zhou, S., Chen, L., & Xu, B. (2016). Impact of climate change on human infectious diseases: Empirical evidence and human adaptation. *Environment International*, *86*, 14-23. https://doi.org/10.1016/j.envint.2015.09.007

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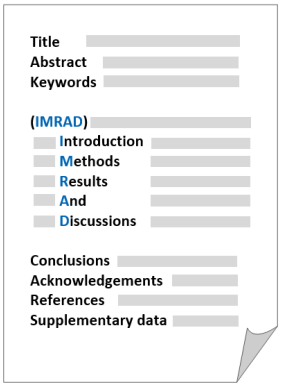

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Journal article with an article number	**Information prominent' (the author's name is within parentheses):** … (Bougnom et al., 2020) … Or **'Author prominent' (the author's name is outside the parentheses):** Bougnom et al. (2020) …	Bougnom, B. P., Thiele-Bruhn, S., Ricci, V., Zongo, C., & Piddock, L. J. V. (2020). Raw wastewater irrigation for urban agriculture in three African cities increases the abundance of transferable antibiotic resistance genes in soil, including those encoding extended spectrum β-lactamases (ESBLs). *Science of The Total Environment*, *698*, 134201. https://doi.org/10.1016/j.scitotenv.2019.134201
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	Newspaper	
Newspaper article – with an author	… (Morales, 2020) ... Or ... Morales (2020) ...	Morales, C. (2020, November 13). Scientists destroyed a nest of murder hornets. Here's what they learned. *The New York Times*. https://www.nytimes.com/2020/11/13/us/murder-hornets-us.html
Newspaper article – without an author	("Japan bird flu outbreak", 2020). OR "Japan bird flu outbreak" (2020) … Use a shortened title (or full title if it is short) in Headline Case enclosed in double quotation marks.	Japan bird flu outbreak spreads to farm in fourth prefecture. (2020, December 01). *The Straits Times*. https://www.straitstimes.com/asia/east-asia/japan-bird-flu-outbreak-spreads-to-farm-in-fourth-prefecture
	Dissertation/Thesis	
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Conference proceedings published as a book chapter	… (Kurbatova et al., 2019) … Or Kurbatova et al. (2019) …	Kurbatova, S. M., Aisner, L. Y., & Naumkina, V. V. (2019). Some aspects of the essence and legal regulation of agriculture digitalization as one of the priorities of modern state policy of agriculture development. In *IOP conference series: Earth and environmental science* (Vol. 315, No. 3, p. 032021). IOP Publishing. https://doi:10.1088/1755-1315/315/3/032021

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Online	… (Melanie et al., 2017) … Or Melanie et al. (2017) …	Melanie., Rustama, M. M., Kasmara, H., Sejati, S. A., Fitriani, N., & Madihah. (2017, October 25-26). *Pathogenicity of* Helicoverpa armigera *polyhedrosis sub culture virus (*Ha*NPV$_1$) on* Spodoptera litura *Fabricius* [Paper presentation]. Prosiding Seminar Nasional Penelitian dan Pengabdian pada Masyarakat (SnaPP) 2017 Sains dan Teknologi, Bandung, Indonesia. http://proceeding.unisba.ac.id/index.php/sains_teknologi/article/view/988/pdf
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