Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

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Pertanika Journal of Tropical Agricultural Science
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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.

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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.

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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.

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EDITORIAL OFFICE

JOURNAL DIVISION

Putra Science Park

1st Floor, IDEA Tower II

UPM-MTDC Technology Centre

Universiti Putra Malaysia

43400 Serdang, Selangor Malaysia.

Gen Enquiry

Tel. No: +603 9769 1622 | 1616

E-mail:

executive_editor.pertanika@upm.edu.my

URL: www.journals-jd.upm.edu.my

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ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS

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Contents

Foreword

Welcome to the third issue of 2023 for the Pertanika Journal of Tropical Agricultural Science (PJTAS)!

PJTAS is an open-access journal for studies in Tropical Agicultural 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 18 articles; three review articles and the rest are regular articles. The authors of these articles come from different countries namely Belgium, Egypt, Indonesia, Malaysia, Nigeria, Spain, Sri Lanka, Thailand and USA.

Tuan Zainazor Tuan Chilek and his team from Universiti Malaysia Terengganu isolated and identified the pathogenic bacteria in *kelulut* honey. Forty-eight samples of *kelulu*t honey (open and closed pot) and propolis were obtained from selected farms in Terengganu by focusing on *Heterotrigona itama*. Besides that, the swabbing technique was done on the wooden beehive of the *kelulut* to evaluate the environmental contamination. This study indicates that contamination of *kelulut* honey with *Bacillus cereus*, *Pseudomonas aeruginosa*, *Pantoea* spp., *Serratia plymuthica*, and *Staphylococcus aureus*, which may exist in the *kelulut* honey through food handlers, utensils, and the environment. The detailed information of this article is available on page 861.

A selected article entitled “Growth and Yield Comparison of Rice Plants Treated with Encapsulated *Trichoderma asperellum* (UPM 40) in Response to Drought Stress” evaluated the effects of encapsulated *Trichoderma asperellum* (UPM 40) on the growth and yield of rice plants planted in saturated and flooded soil conditions in response to drought stress. The drought stress was imposed by halting watering during early anthesis for 14 days and resumed afterward. They found out that applying 5 g encapsulated *T. asperellum* (UPM 40) to the rice plants would perform best in flooded soil conditions during drought stress. On the contrary, rice plants planted in saturated soil conditions without inoculation of the fungus did not perform as well. Full information of this study is presented on page 875.

A regular article entitled “Effect of Straw Compost (*Oryza sativa* L.) on Crop Production” analyzed the effectiveness of rice straw compost for intensified-rice cultivation by composting the rice straw from the previous planting season on the field (*in situ*) using the “Effective Microorganisms version 4” (EM-4), which contains *Lactobacillus* sp., *Rhodopseudomonas* sp., *Actinomycetes* sp., *Streptomyces* sp., yeast, and cellulose-decomposing fungus. The results show that the application of straw compost provides a

significant increase in dry grain weight, panicle length, and the number of grains per rice plant, as well as the C-organic, total N, and K levels in the soil. However, the treatment did not give significant results on the clumps number and rice grain weight. The further details of this study are found on page 1047.

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.

Chief Executive Editor
Prof. Ir. Dr. Mohd Sapuan Salit
executive_editor.pertanika@upm.edu.my

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Application of Salicylic Acid and Gibberellic Acid Increase Stem Cutting Growth of *Pennisetum purpureum* cv. Mahasarakham and *Pennisetum purpureum* x *Pennisetum americanum*

Amnat Phetsuwan[1]*, Narisa Kunpratum[1], Marootpong Pooam[1], Khanitta Somtrakoon[2] and Waraporn Chouychai[3]

[1]Department of Biology, Faculty of Science, Naresuan University, Phitsanulok 65000, Thailand
[2]Department of Biology, Faculty of Science, Mahasarakham University, Mahasarakham 44150, Thailand
[3]Department of Science, Faculty of Science and Technology, Nakhonsawan Rajabhat University, Nakhonsawan 60000, Thailand

ABSTRACT

The Napier grass continues to be a popular forage crop in Nakhonsawan Province and other areas in the northern and east-northern parts of Thailand. Increasing the growth and biomass of Napier grass growing in low-quality sandy soil will increase the economic value of this plant. The stem cutting of two cultivars of Napier grass, cv. Pakchong 1 (*Pennisetum purpureum* x *Pennisetum americanum*) and sweet grass (*Pennisetum purpureum* cv. Mahasarakham), were exposed to two plant growth regulators, gibberellic acid and salicylic acid, and two application methods, soaking for 24 hr and watering after inoculation into the soil, were studied. For one plant growth regulator test, the most appropriate concentration of gibberellic acid and salicylic acid were 0.01 and 100 mg/L, respectively, for the soaking and watering method. There was an interaction between the combination of plant growth regulator type and application method for both cultivars. The best stimulation effect for sweet grass was soaking with 100 mg/L salicylic acids only. The response of Napier grass cv. Pakchong 1 was different. Soaking with 0.01 mg/L gibberellic acid and watering with 0.01 mg/L gibberellic acid was suitable for increasing shoot and root length while soaking with 0.01 mg/L gibberellic acid and watering with 100 mg/L salicylic acid was suitable for increasing fresh and dry weight. This information will be useful for stimulating the growth of Napier grass in agricultural purposes.

Keywords: Cutting, gibberellin, Napier grass, salicylic acid, soaking

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E-mail addresses:
amnatp62@nu.ac.th (Amnat Phetsuwan)
narisak@nu.ac.th (Narisa Kunpratum)
marootpongp@nu.ac.th (Marootpong Pooam)
khanitta.s@msu.ac.th (Khanitta Somtrakoon)
waraporn.c@nsru.ac.th (Waraporn Chouychai)
*Corresponding author

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INTRODUCTION

Napier grass (*Pennisetum purpureum*) is a popular forage crop for economic ruminants in Thailand's central and northeastern parts. Napier grass has several advantages, such as high crude protein, high non-fiber carbohydrate, and low acid detergent fiber content, even though it grows in low soil quality. Also, feeding with sweet grass, the cultivar of Napier grass, decreases methane production from cows compared to feeding with rice straw (Mapato & Wanapat, 2018; Thongruang et al., 2021). Moreover, there are other effective applications of this grass. Napier grass was reported to be an effective choice for phytoremediation. Dwarf Napier grass has been reported to phytostabilize arsenic in soil (Chouychai & Somtrakoon, 2022; Kowitwiwat & Sampanpanish, 2020). This grass species is also reportedly used in the phytoremediation of petroleum hydrocarbon-contaminated soil (Ologidi et al., 2022). The fiber of Napier grass has been reported to be suitable for the paper industry (Daud et al., 2014; Reddy et al., 2014). Also, the lignocellulosic biomass of Napier grass is suitable for bioethanol production because of its low lignin content and relatively high mass per year and per area (Liong et al., 2013; Yasuda et al., 2014). Increasing Napier grass biomass in low soil quality will increase economic worth for all purposes.

The suitable cultivars and planting methods have been selected in many countries in Asia and Africa, but most of them study the number of nodes per stem cuttings (J⊘rgensen et al., 2010; Ramadhan et al., 2015). However, using plant growth regulators (PGRs) to induce stem cuttings growth is another interesting method to increase biomass and tolerate unsuitable conditions for growth. Many PGRs have been used to induce plant growth in this condition. The foliar application of 100 mg/L salicylic acid (SA) to three flax cultivars growing in sandy soil could increase plant growth, photosynthetic pigment, phenolic content, and seed yield (Dawood et al., 2019). Also, mung bean plants growing in sandy soil and grown from seed soaking with 50 mg/L gibberellic acid (GA_3) for 12 hr contained a high amount of photosynthetic pigment and indigenous auxin (El Karamany et al., 2019). The application method is also an important step toward success. For example, spraying 100 mg/L salicylic acid on tomato leaves before exposing them to salinity was better than spraying leaves after salinity exposure for enhancing root growth, leaf proline, and soluble sugar content, and this pretreatment in leaves was also better than immersing the tomato seedling root in the same concentration for 30 min before exposing to salinity (Souri & Tohidloo, 2019). For these reasons, the plant growth regulator's appropriate type, concentration, and application method should be studied first in each condition.

Napier grass cultivation in Nakhonsawan Province, Thailand, is increasing, especially in some districts that often suffer from drought conditions, such as Nongbua and Phaisali districts. However, the soil in some areas of the Nongbua district is sandy and low in nutrient elements, making it difficult to cultivate economic crops. If Napier grass could grow well in this area, this would increase farmers' income. The possibility of using plant growth regulators to

increase the growth of Napier grass growing in sandy soil was studied. The different concentrations and application methods of salicylic acid and gibberellic acid to different Napier grass cultivars growing in sandy soil were compared. There were two cultivars of Napier grass used in this study, Napier grass cv. Pakchong 1 (*Pennisetum purpureum* x *Pennisetum americanum*) and sweet grass (*Pennisetum purpureum* cv. Mahasarakham), a cultivar of dwarf Napier grass.

MATERIALS AND METHODS

Plant and Soil Preparation

Napier grass cv. Pakchong 1 (*Pennisetum purpureum* x *Pennisetum americanum*) and sweet grass (*Pennisetum purpureum cv*. Mahasarakham*)* were commercial stem cuttings from Nong Bo sub-district, Muang Ubon Ratchathani district, Ubon Ratchathani Province, Thailand. The five-month-old stem was cut to be similar to 24–26 cm/piece for Napier grass cv. Pakchong 1 and 6–8 cm/piece for sweet grass. These stem cuttings were soaked in water for seven days after being received before being used. The soil used in this experiment was collected from the Nongklab sub-district, Nongbua district, Nakhonsawan Province, Thailand. This soil was air-dried at 28–31°C for at least 24 hr to a constant weight and sent to analyze the chemical and physical properties at the Central Laboratory (Thailand) Co. Ltd., Khonkean, Thailand. This soil texture is sand (87.79% sand, 9.39% silt, and 2.82% clay) with low nitrogen (6.1 mg/kg dry soil) and organic matter (15.1 mg/kg dry soil). The soil pH was 5.67, and available phosphorus and total potassium were 13.65 and 19.66 mg/kg dry soil, respectively.

Experimental Design

Single Plant Growth Regulator. There were two PGRs used in this study, salicylic acid (SA, purity 99.5%, KEMAUS, Australia) and gibberellic acid (GA_3, purity 90%, Sigma-Aldrich, Germany), and two application methods, immersion, and watering, were tested in this study. The experimental design was completely randomized (CRD) with one factor, the different concentrations of each plant growth regulator. After rooting, stem cuttings of Napier grass cv. Pakchong 1 and sweet grass were soaked in 0, 10, 50, and 100 mg/L SA or 0, 0.01, 0.1, and 1.0 mg/L GA_3 for 24 hr before being transferred to soil. The pots used in this experiment were 11.43 cm in diameter, each containing 1 kg of dry soil. The cuttings were inoculated vertically into the soil. There was one stem cutting per pot, 5 pots per treatment, and each pot was watered daily at 20 ml/pot. Another experiment was done with stem cutting watered with 0, 10, 50, and 100 mg/L SA or 0, 0.01, 0.1, and 1.0 mg/L GA_3 as 20 ml/pot on day 0 of the experiment instead of immersion. All pots were cultured in the nursery and received natural sunlight in shaded conditions until the end of the experiment. The plants from each treatment were collected on day 20th after planting to determine the plant growth parameters, including the number of leaves and stems per plant, shoot length, root length, shoot

fresh weight, shoot dry weight, fresh root weight, and root dry weight. The ruler measured the length of the plant, and all plant weights were measured by digital balance. The root length/root dry weight and root dry weight/shoot dry weight ratios were calculated using the formulas described in Calvelo-Pereira et al. (2010) and Xu et al. (2018), respectively.

Combine Plant Growth Regulator. The experimental design was 3 x 3 factorial in CRD with 2 factors that were (1) soaking and (2) watering with different plant growth regulators. The plant growth regulators used in this experiment were 100 mg/L SA, 0.01 mg/L GA_3, and distilled water were used as controls. Each stems cutting of Napier grass cv. Pakchong 1 and sweet grass were soaked with 100 mg/L SA, 0.01 mg/L GA_3, or distilled water for 24 hr and combined with watering with 100 mg/L SA, 0.01 mg/L GA_3, or distilled water as 20 ml/pot on starting of the experiment. The details of the experiment are shown in Table 1. The cuttings were inoculated vertically into the soil. There was one stem cutting per pot, 5 pots per treatment, and each pot was watered daily at 20 ml/pot. The plants from each treatment were collected on day 30th after planting to determine the parameter described in the above experiment. The detail of the experimental design is shown in Table 1.

Table 1

The detail of the experimental design

Treatment	Soaking	Watering
1	Distilled water	Distilled water
2	Distilled water	100 mg/L SA
3	Distilled water	0.01 mg/L GA_3
4	100 mg/L SA	Distilled water
5	100 mg/L SA	100 mg/L SA
6	100 mg/L SA	0.01 mg/L GA_3
7	0.01 mg/L GA_3	Distilled water
8	0.01 mg/L GA_3	100 mg/L SA
9	0.01 mg/L GA_3	0.01 mg/L GA_3

Note. SA = Salicylic acid; GA_3 = Gibberellic acid

Statistical Analysis

One-way analysis of variance (ANOVA), two-way ANOVA, and least square difference (LSD) were used for variance analysis and pairwise comparison. One-way ANOVA was used for each plant growth regulator and method application in a single plant growth regulator experiment, while two-way ANOVA was used for combining plant growth regulator experiments.

RESULTS AND DISCUSSION

Effect of Single Plant Growth Regulator on Sweet Grass and Napier Grass cv. Pakchong 1

Exogenous salicylic acid soaking positively affected sweet grass's shoot and root growth. For example, the shoot length, shoot fresh weight, and shoot dry weight of cutting soaked in water were 13.7±0.25 cm, 6.3±0.28 g, and 3.3±0.13 g, respectively, while those of

cutting soaked in 100 mg/L salicylic acid were 37.5±20.13 cm, 11.1±0.47 g, and 4.4±0.22 g, respectively (Table 2). Soaking with salicylic acid dramatically increases the shoot length of sweet grass, and the highest shoot length was seen at a cut soaked in 100 mg/L salicylic acid. However, 10 mg/L salicylic acid could statistically induce more shoot fresh and dry weight of sweet grass than other treatments. The response of sweet grass root to exogenous salicylic soaking was as same as shoot, but the highest induction was found in treatment soaking with 100 mg/L salicylic acid for root length and fresh weight, while the highest induction for dry root weight was 50 mg/L salicylic acid. Watering with salicylic acid also increases sweet grass's shoot and root growth. The shoot length, shoot fresh weight, and shoot dry weight of cutting watered with water were 13.7±0.25 cm, 6.3±0.28 g, and 3.3±0.13 g, respectively, while those of cutting watered with 100 mg/L salicylic acid were 21.5±0.28 cm, 11.5±0.32 g, and 5.2±0.34 g, respectively (Table 2). Watering with 100 mg/L salicylic acid is the best concentration for shoot fresh and dry weight, root length, and root dry weight, while 50 mg/L salicylic acid is the best for shoot length and root fresh weight. The number of shoots and leaves per stem cutting of sweet grass soaked with salicylic acid was 1.0–1.4 shoots/cutting and 7–8 leaves/cutting, respectively. Slightly watering with salicylic acid increased the number of shoots per stem cutting to 1.2–2.0 shoots/cutting, but the leaves per cutting were the same number from cutting soaked with salicylic acid (Figure 1).

Exogenous gibberellic acid soaking also increases sweet grass's shoot and root growth. For example, the shoot length, shoot fresh weight, and shoot dry weight of cutting soaked in water were 20.8±0.26 cm, 8.3±0.37 g, and 3.9±0.35 g, respectively, while those of cutting soaked in 0.01 mg/L gibberellic acid were 24.6±0.52 cm, 13.7±0.23 g, and 6.6±0.22 g, respectively (Table 2). However, the low concentration of gibberellic acid (0.01 mg/L) was better than other concentrations for all growth of sweet grass. Increasing gibberellic acid concentration trended to decrease sweet grass growth statistically, especially for root growth. This trend of sweet grass responding to gibberellic acid was seen when watering to cutting. For example, the shoot length, shoot fresh weight, and shoot dry weight of cutting watered with 0.01 mg/L gibberellic acid were 27.4±0.29 cm, 14.0±0.50 g, and 6.1±0.51 g, respectively, while those of cutting soaked in 1.0 mg/L gibberellic acid were 24.9±0.28 cm, 12.8 ±0.36 g, and 5.7±0.38 g, respectively (Table 2). Soaking or watering with gibberellic acid to sweet grass cutting was the same trend as salicylic acid. However, the shoots and roots of cuttings receiving salicylic or gibberellic acid were more robust than those receiving only water (Figure 1). This trend was correlated with decreasing specific root length after exposure to a plant growth regulator that indicated a thicker root than the control (Table 2).

Table 2

Growth of sweet grass soaked or watered with various concentrations of plant growth regulators

	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Root-to-shoot ratio	Specific root length (m/g)
Salicylic acid-soaking Distilled water	13.7±0.25c	6.3±0.28c	3.3±0.13c	20.3 ±0.27d	1.7±0.16c	0.2±0.10c	0.07±0.03c	1.24±0.24a
10 mg/L	17.7±0.44b	11.7±0.50a	5.4±0.14a	26.4±0.43c	2.6±0.12b	1.3±0.01b	0.24±0.01b	0.21±0.01b
50 mg/L	31.9±0.47a	10.0±0.53b	4.5±0.23b	31.9±0.36b	2.8±0.19b	1.7±0.04a	0.39±0.02a	0.19±0.00b
100 mg/L	37.5±20.13a	11.1±0.47a	4.4±0.22b	36.6±0.38a	4.5±0.18a	1.3±0.05b	0.30±0.02b	0.28±0.01b
Salicylic acid-watering Distilled water	13.7±0.25c	6.3±0.28c	3.3±0.13c	20.3 ±0.27d	1.7±0.16c	0.2±0.10c	0.07±0.03b	1.24±0.24a
10 mg/L	20.6±0.21b	7.6±0.50b	3.5±0.24bc	22.1±0.54c	2.9±0.23b	1.5±0.03b	0.44±0.04a	0.15±0.01b
50 mg/L	25.2±0.97a	7.7±0.41b	4.0±0.16b	33.9±0.08b	4.6±0.42a	1.6±0.05b	0.40±0.03a	0.21±0.01b
100 mg/L	21.5±0.28b	11.5±0.32a	5.2±0.34a	38.7±0.48a	5.1±0.28a	2.1±0.16a	0.41±0.03a	0.19±0.02b
Gibberellic acid-soaking Distilled water	20.8±0.26c	8.3±0.37c	3.9±0.35c	28.2±0.30b	2.0±0.12c	0.2±0.07d	0.06±0.02c	1.73±0.41a
0.01 mg/L	24.6±0.52a	13.7±0.23a	6.6±0.40a	30.4±0.36a	4.7±0.21a	2.6±0.16a	0.40±0.04a	0.12±0.01b
0.1 mg/L	25.3±0.40a	13.1±0.60a	5.0±0.26b	28.0±0.26b	2.4±0.22c	1.4±0.09c	0.28±0.02b	0.21±0.01b
1.0 mg/L	22.4±0.56b	10.3±0.42b	4.9±0.31b	28.2±0.51b	3.4±0.13b	2.1±0.08b	0.44±0.04a	0.14±0.01b
Gibberellic acid-watering Distilled water	20.8±0.26d	8.3±0.37d	3.9±0.35b	28.2±0.30b	2.0±0.12c	0.2±0.07b	0.06±0.024c	1.73±0.41a
0.01 mg/L	27.4±0.29a	14.0±0.50a	6.1±0.51a	31.7±0.51a	4.1±0.26a	2.4±0.06a	0.39±0.034a	0.13±0.00b
0.1 mg/L	22.9±0.55c	10.2±0.43c	5.2±0.30a	27.8±0.38b	2.7±0.13b	1.5±0.09b	0.29±0.021b	0.18±0.01b
1.0 mg/L	24.9±0.28b	12.8±0.36b	5.7±0.38a	28.9±0.29b	3.7±0.20a	2.2±0.10a	0.39±0.015a	0.13±0.01b

Note. The different lower-case letters showed significant differences ($P<0.05$) between different plant growth regulator concentration

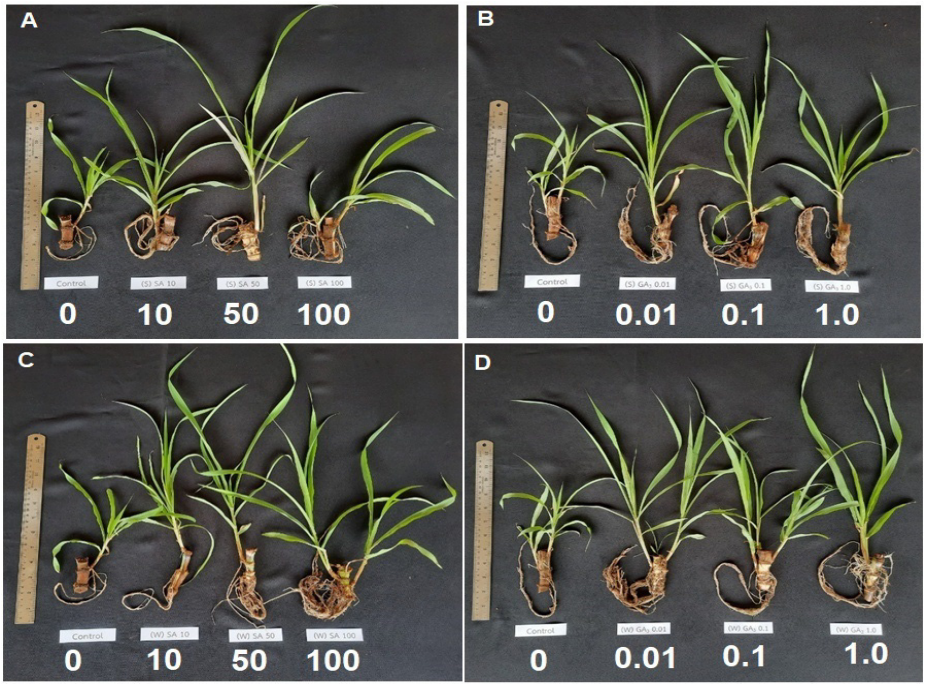

Figure 1. Shoot and root of Sweet grass soaking with various concentrations of SA (A) and GA_3 (B) or watering with various concentrations of SA (C) and GA_3 (D)

Note. SA = Salicylic acid; GA_3 = Gibberellic acid

Exogenous salicylic acid also soaking positively affected both shoot and root growth of Napier grass cv. Pakchong 1. For example, the shoot length, shoot fresh weight, and shoot dry weight of cutting soaked in water were 50.0±0.71 cm, 25.0±0.44 g, and 18.0±0.49 g, respectively, while those of cutting soaked in 100 mg/L salicylic acid were 51.8±0.49 cm, 27.5±1.91 g, and 23.3±0.38 g, respectively (Table 3). Soaking with 50 mg/L salicylic acid could increase shoot length and shoot dry weight of sweet grass, while only 100 mg/L salicylic acid could increase root length and weight of Napier grass cv. Pakchong 1. However, soaking with any concentration of salicylic acid could not induce the shoot fresh weight of Napier grass cv. Pakchong 1. Watering with salicylic acid did not stimulate Napier grass cv's shoot and root dry weight. Pakchong 1. Watering with 100 mg/L salicylic acid is the best concentration for shoot length and fresh weight, root length, and fresh weight. The shoot length, shoot fresh weight, root length, and root fresh weight of cutting watered with water were 50.0±0.71 cm, 25.0±0.44 g, 17.7±0.30 cm, and 1.73±0.03 g, respectively, while those of cutting watered with 100 mg/L salicylic acid were 59.6±0.63 cm, 29.0±0.95 g, 22.3±0.51 cm, and 2.48±0.06 g, respectively (Table 3). The number of shoots and leaves per

stem cutting of Napier grass cv. Pakchong 1 soaked with salicylic acid were 1.0 shoots/cutting and 7–8 leaves/cutting, respectively. The shoots and leaves per cutting of cutting watered with salicylic acid were the same as those from soaked salicylic acid (Figure 2).

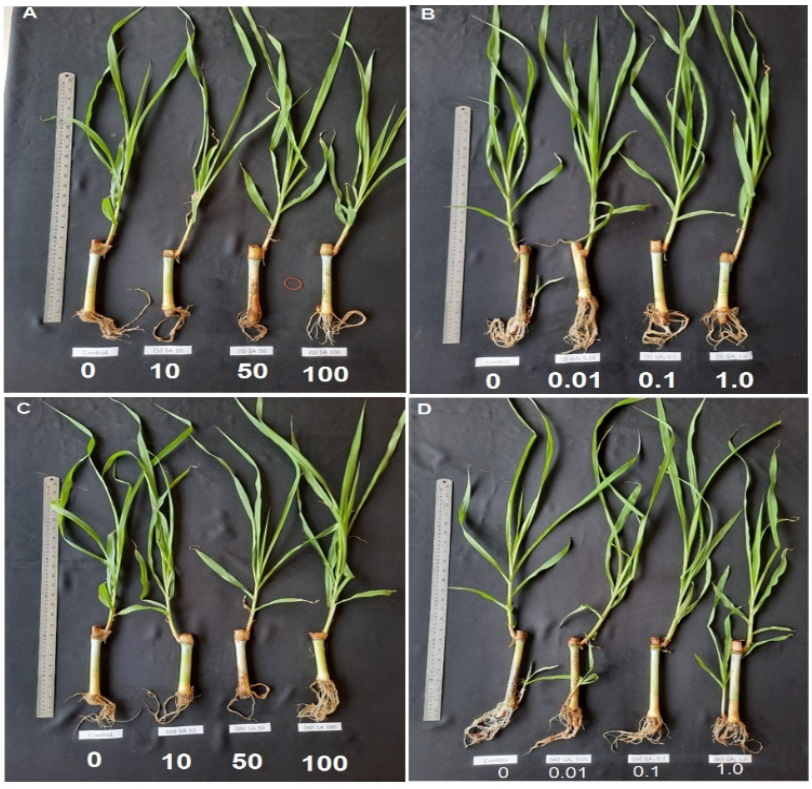

Figure 2. Shoot and root of Napier grass cv. Pakchong 1 soaking with various concentrations of SA (A) and GA_3 (B) or watering with various concentrations of SA (C) and GA_3 (D)

Note. SA = Salicylic acid; GA_3 = Gibberellic acid

Exogenous gibberellic acid soaking increases shoot and root growth of Napier grass cv. Pakchong 1. For example, the shoot length, shoot fresh weight, and shoot dry weight of cutting soaked in water were 52.9±0.64 cm, 24.4±0.92 g, and 20.1±0.28 g, respectively, while those of cutting soaked in 1.0 mg/L gibberellic acid were 66.6±1.50 cm, 29.5±0.79 g, and 23.6±0.47 g, respectively (Table 3). The high concentration of gibberellic acid (0.1-1.0 mg/L) could induce shoot growth, but the low concentration of gibberellic acid (0.01 mg/L) was better than other concentrations for root growth of Napier grass cv. Pakchong 1. The increase of gibberellic acid concentration trended to statistically decrease root growth of Napier grass cv. Pakchong 1. This trend of Napier grass cv. Pakchong 1 root responding to

gibberellic acid was seen when watering to cuttings but watering with gibberellic acid did not induce shoot length and root fresh and dry weight. For example, the shoot fresh weight, shoot dry weight, and root length of cutting watered with water were 24.4±0.92 g, 20.1±0.28 g, and 17.7±0.17 cm, respectively, while those of cutting watered with 0.01 mg/L gibberellic acid were 31.7±0.71 g, 25.1±0.44 g, and 18.2±0.42 cm, respectively (Table 3). Soaking or watering with gibberellic acid to Napier grass cv. Pakchong 1 cutting was the same trend as salicylic acid. However, the shoots and roots of cuttings receiving salicylic acid or gibberellic acid were the same as those receiving only water (Figure 2). This trend correlated with the non-significantly different specific root lengths after exposure to a plant growth regulator that indicated the same root size in all treatments (Table 3).

Salicylic acid has been reported to enhance plant growth under water-limit conditions, including plants in the Poaceae family. Bermuda grass (*Cynodon dactylon*) exposed to 1 mM salicylic acid ameliorate adverse effects of water deficit (40% available water) condition on plant growth (Taheri et al., 2017). Also, 0.75–1.50 mM salicylic acid increases the growth of *Lolium perenne* cv. "Numan" exposed to drought stress (Hosseini et al., 2015). However, 200 mg/L salicylic acid increased the growth of Zoysia grass (*Zoysia* sp.) in normal conditions (Beiraghdar et al., 2014) (or in normal conditions, 200 mg/L salicylic acid increased the growth of Zoysia grass [*Zoysia* sp.]) (Beiraghdar et al., 2014). The concentration of salicylic acid and gibberellic acid used in this study has been reported to enhance plant growth in pollution-contaminated soil. For example, foliar spray with 0.5 mM salicylic acid could enhance the growth of *Sorghum bicolor* in cromium (Cr)-contaminated soil (Sihag et al., 2019). Also, 1.0 mg/L gibberellic acid could increase the root fresh weight of *Luffa acutangular* growing in polycyclic aromatic hydrocarbon (PAH)-contaminated soil (Somtrakoon & Chouychai, 2022b), but the concentration used in this study was lower than the concentration appropriate to increase okra seedling growth (5–10 µM) in the presence of 100 mM sodium chloride (NaCl) (Yakoubi et al., 2019).

In this study, both types of grass were not exposed to drought conditions but only planted in sandy soil with low water-holding capacity and nutrient content. The results showed that the appropriate concentration of salicylic acid and gibberellic acid to enhance Sweet grass growth, especially root growth, was 100 and 0.01 mg/L, respectively. The best response of the root was the main point for plant growth regulator selection in this experiment because the robust root is necessary for water and nutrient uptake in low-quality soil. The application via soaking seemed to be better than watering sweet grass in this study. The application method often affects plant response to the plant growth regulator, but the best method trend to depend on plant species, type of plant growth regulator, and environmental conditions. For example, corn seed soaking in 0.1 M GA_3 or IAA

Table 3

Growth of Napier grass cv. Pakchong 1 soaked or watered with various concentrations of plant growth regulators

	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Root-to-shoot ratio	Specific root length (m/g)
Salicylic acid-soaking Distilled water	50.0±0.71b	25.0±0.44a	18.0±0.49b	17.7 ±0.30b	1.73±0.03b	0.60±0.02b	0.03±0.001b	0.30±0.01b
10 mg/L	53.4±0.51ab	25.6±0.33a	16.7±0.22c	17.6±0.19b	1.62±0.01c	0.38±0.01c	0.02±0.001b	0.46±0.021a
50 mg/L	60.4±1.29a	28.5±0.54a	22.2±0.44a	17.0±0.42b	1.77±0.02b	0.68±0.04b	0.03±0.002b	0.25±0.019b
100 mg/L	51.8±0.49ab	27.5±1.91a	23.3±0.38a	24.9±0.19a	2.14±0.05a	1.01±0.06a	0.04±0.003a	0.25±0.016b
Salicylic acid-watering Distilled water	50.0±0.71b	25.0±0.44b	18.0±0.49a	17.7 ±0.30bc	1.73±0.03b	0.60±0.02a	0.03±0.001a	0.30±0.01a
10 mg/L	53.6±0.24ab	26.2±0.52b	16.4±0.93a	18.7±0.34b	1.75±0.04b	0.67±0.03a	0.04±0.003a	0.29±0.017a
50 mg/L	52.6±0.93ab	27.2±0.63ab	17.2±0.25a	17.1±0.29c	1.63±0.02b	0.40±0.03a	0.02±0.002a	0.44±0.046a
100 mg/L	59.6±0.63a	29.0±0.95a	17.2±0.35a	22.3±0.51a	2.48±0.06a	0.70±0.17a	0.04±0.010a	0.69±0.422a
Gibberellic acid-soaking Distilled water	52.9±0.64b	24.4±0.92b	20.1±0.28c	17.7±0.17b	1.75±0.03b	0.66±0.03b	0.03±0.002b	0.27±0.014b
0.01 mg/L	61.4±1.53b	25.9±0.59b	19.5±0.36c	24.9±0.66a	2.49±0.09a	0.96±0.02a	0.05±0.002a	0.26±0.008b
0.1 mg/L	70.1±1.82a	28.8±0.40a	21.8±0.47b	18.0±0.42b	1.78±0.04b	0.56±0.04c	0.02±0.002c	0.33±0.023a
1.0 mg/L	66.6±1.50a	29.5±0.79a	23.6±0.47a	17.1±0.26b	1.68±0.03b	0.48±0.02d	0.02±0.001c	0.36±0.019a
Gibberellic acid-watering Distilled water	52.9±0.64a	24.4±0.92c	20.1±0.28c	17.7±0.17a	1.75±0.03a	0.66±0.03a	0.03±0.001a	0.27±0.014b
0.01 mg/L	50.0±5.96a	31.7±0.71b	25.1±0.44a	18.2±0.42a	1.78±0.03a	0.46±0.02b	0.02±0.000b	0.42±0.009a
0.1 mg/L	65.0±0.69a	32.6±0.67b	23.8±0.38b	16.5±0.58b	1.64±0.03b	0.36±0.02c	0.02±0.001b	0.45±0.039a
1.0 mg/L	56.1±1.76a	35.9±0.82a	25.9±0.62a	15.7±0.38b	1.65±0.04b	0.38±0.02c	0.02±0.001b	0.41±0.030a

Note. The different lower-case letters showed significant differences (P<0.05) between different plant growth regulator concentration

decreases Pb accumulation in plant tissue compared with 0.1 M GA_3 or IAA foliar spray when growing in 800 mg/kg lead (Pb) contaminated soil (Hadi et al., 2010). Seed soaking in 1 mg/L indole-3-butyric acid (IBA) increased shoot and root fresh weights of ridge gourd more than watering with 1 mg/L IBA when growing in 100 mg/kg phenanthrene and 100 mg/kg pyrene contaminated soil (Somtrakoon et al., 2022b). On the other hand, seed soaking with 0.01 mg/kg thidiazuron (TDZ) decreased shoot and root dry weight of corn growing in 46.8 mg/L hexachlorocyclohexane (HCH) contaminated soil when compared with corn watering with 0.01 mg/kg TDZ (Chouychai et al., 2015). The appropriate concentration of salicylic acid and gibberellic acid for the growth of Napier grass cv. Pakchong 1 was the same as sweet grass, 100 and 0.01 mg/L, respectively. This judgment was based on the best of plant root response also. There was more similarity between different application methods than sweet grass. However, the combination of plant growth regulator type and application method was studied further in the next experiment, with only the most appropriate concentration selected from this study.

Effect of Combination of Plant Growth Regulators on Sweet Grass and Napier Grass cv. Pakchong 1

Two factors of plant growth regulation application, soaking and watering, affected all Sweet grass growth traits. There was a significant interaction between soaking and watering with different plant growth regulators on all the growth traits of sweet grass. For the soaking factor, soaking with salicylic acid increases shoot length and shoot fresh weight, shoot dry weight, and root dry weight more than other plant growth regulators while soaking with gibberellic acid increases root-to-shoot ratio more than other factor levels. Also, watering with salicylic acid increased all growth traits of sweet grass more than other factor levels (Table 4).

The combination of plant growth regulators showed that the growth of sweet grass cutting soaked with salicylic acid is better than soaking with gibberellic acid or water. For all soakings with any plant growth regulator, sweet grass watering with salicylic acid or gibberellic acid increases root growth more than watering with water (Figure 3). Soaking with salicylic acid and watering with water increases sweet grass's shoot and root growth more than watering with other plant growth regulators. For example, the shoot and root length of sweet grass soaking with salicylic acid and watering with water were 48.8±0.41 and 33.8±0.41 cm, respectively, while the shoot and root length of sweet grass soaking with salicylic acid and watering with gibberellic acid were 42.4±0.70 and 30.8±0.70 cm, respectively (Table 5).

Two factors of plant growth regulation application, soaking and watering, affected all Napier grass cv. Pakchong 1 growth traits. Only specific root length did not affect by the soaking factor (or the soaking factor did not affect specific root length). There was a significant interaction between

soaking and watering with different plant growth regulators on all growth traits of Napier grass cv. Pakchong 1. For the soaking factor, soaking with salicylic acid increases shoot length, shoot fresh weight, shoot dry weight, and root dry weight more than other plant growth regulators, while soaking with gibberellic acid increases root fresh weight more than other factor levels. Also, watering with salicylic acid increased all growth traits of Napier grass cv. Pakchong 1 more than other factor levels (Table 6).

The response of Napier grass cv. Pakchong 1 to plant growth regulator application differed from sweet grass's application. The combination of plant growth regulators showed that the growth of Napier grass cv. Pakchong 1 cutting soaked with gibberellic acid, although watered with any plant growth regulator, is better than soaking with salicylic acid or water (Figure 4). The root's efficiency in producing shoot biomass increases when soaking with gibberellic acid, considering

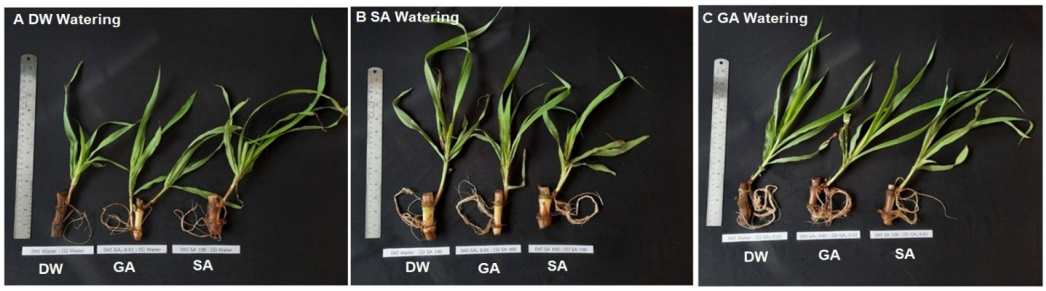

Figure 3. Shoot and root of Sweet grass watering with water (A) or 100 mg/L SA (B) and 0.01 mg/L GA_3 (C) in combination with water, 100 mg/L SA, and 0.01 mg/L GA_3 soaking

Note. SA = Salicylic acid; GA_3 = Gibberellic acid

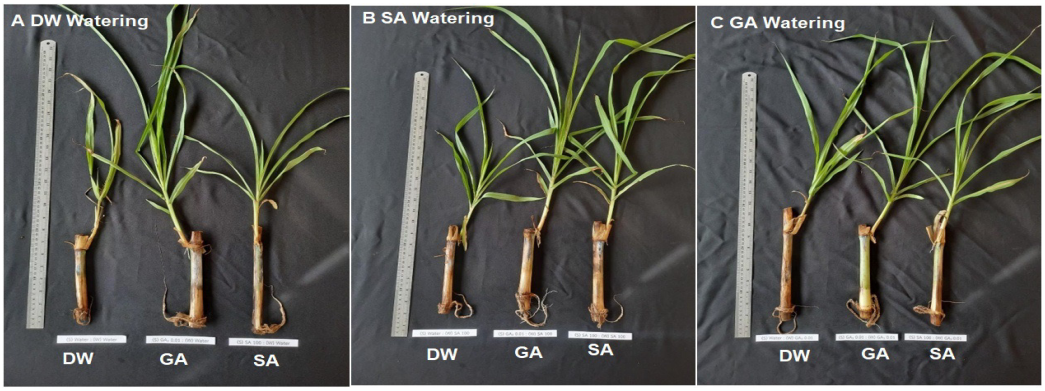

Figure 4. Shoot and root of Napier grass cv. Pakchong 1 watering with water (A) or 100 mg/L SA (B) and 0.01 mg/L GA_3 (C) in combination with water, 100 mg/L SA, and 0.01 mg/L GA_3 soaking

Note. SA = Salicylic acid; GA_3 = Gibberellic acid

Table 4

The main effect of plant growth regulators on sweet grass growth traits

	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Root-to-shoot ratio	Specific root length (m/g)
Soaked (factor 1)								
with water	40.3±0.38c	16.4±0.26b	10.0±0.32b	27.2±0.36b	2.0±0.10b	1.1±0.35b	0.13±0.01b	0.28±0.01a
with SA	45.7±0.38a	22.1±0.26a	16.4±0.32a	32.4±0.36a	2.7±0.10a	1.5±0.35a	0.10±0.01c	0.22±0.01b
with GA_3	43.3±0.38b	14.2±0.26c	6.5±0.32c	31.4±0.36a	2.1±0.10b	1.2±0.35b	0.19±0.01a	0.26±0.01a
F-test	**	**	**	**	**	**	**	**
Watered (factor 2)								
with water	41.9±0.38b	16.3±0.26c	10.6±0.32b	31.3±0.36a	2.4±0.10a	1.2±0.35b	0.15±0.01a	0.30±0.01a
with SA	45.7±0.38a	19.2±0.26a	12.7±0.32a	30.5±0.36a	2.2±0.10a	1.4±0.35a	0.12±0.01b	0.23±0.01b
with GA_3	41.7±0.38b	17.2±0.26b	9.6±0.32c	29.1±0.36b	2.2±0.10a	1.3±0.35b	0.15±0.01a	0.23±0.01b
F-test	**	**	**	**	ns	*	*	**
F-test Soaked x Watered	**	**	**	**	**	**	**	**

Note. Different lower-case letters show significant differences within each factor; ns, *, ** = Non-significance ($P>0.05$), statistical significance ($P<0.05$), and high statistical significance ($P<0.01$) of each factor, respectively; SA = Salicylic acid; GA_3 = Gibberellic acid

Table 5

Shoot and root growth of Sweet grass stimulated with various application methods of plant growth regulators (PGRs)

	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Root-to-shoot ratio	Specific root length (m/g)
Immerse with water Watered with water	32.2±0.73cC	7.5±0.16cC	3.52±0.26bC	24.0 ±0.35cC	1.29±0.13aC	0.57±0.04cC	0.09±0.018bB	0.19±0.04bB
Watered with SA	51.5±0.79aA	19.9±0.43bB	12.64±0.68aB	26.2±0.46bC	2.23±0.08aAB	1.52±0.08aA	0.12±0.009aB	0.17±0.010cB
Watered with GA_3	37.2±0.56bC	21.7±0.30aA	13.75±0.59aA	31.3±0.51aA	2.34±0.09aA	1.33±0.10bA	0.10±0.006aC	0.24±0.017aA
Immerse with SA Watered with water	48.8±0.41aA	26.7±0.75aA	21.58±0.94aA	33.8±0.41aB	3.44±0.29aA	1.85±0.04aA	0.09±0.005bB	0.18±0.003bC
Watered with SA	46.0±0.42bB	24.6±0.34bA	18.94±0.52bA	31.3±0.51bB	2.68±0.19aA	1.52±0.04bA	0.08±0.003bC	0.21±0.007bB
Watered with GA_3	42.4±0.70cB	15.0±0.56cB	8.57±0.47cB	30.8±0.70bA	2.04±0.20aA	1.20±0.04cA	0.14±0.008aB	0.27±0.017aA
Immerse with GA_3 Watered with water	44.8±0.37aB	14.7±0.42aB	6.60±0.24aB	36.2 ±0.41aA	2.34±0.12aB	1.28±0.06aB	0.20±0.013aA	0.28±0.012aA
Watered with SA	39.5±1.14bC	13.0±0.40bC	6.46±0.49aC	34.1±0.33bA	1.81±0.25aB	1.08±0.02bB	0.17±0.014aA	0.32±0.005aA
Watered with GA_3	45.5±0.39aA	14.9±0.45aB	6.57±0.46aC	23.9±0.48cB	2.18±0.19aA	1.28±0.08aA	0.20±0.022aA	0.19±0.013bB

Note. The different lower-case letters showed a significant difference between different PGR watering within the same PGR immersion; Different capital letters showed significant differences between different PGR immersions within the same PGR watering; SA = Salicylic acid; GA_3 = Gibberellic acid

Table 6

The main effect of plant growth regulators on Napier grass cv. Pakchong 1 growth traits

	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Root-to-shoot ratio	Specific root length (m/g)
Soaked (factor 1)								
with water	40.3±0.38c	16.4±0.26b	10.0±0.32b	27.2±0.36b	1.4±0.02c	1.1±0.35b	0.049±0.001a	0.24±0.01a
with SA	45.7±0.38a	22.1±0.26a	16.4±0.32a	32.4±0.36a	1.7±0.02b	1.5±0.35a	0.036±0.001b	0.24±0.01a
with GA_3	43.3±0.38b	14.2±0.26c	6.5±0.32c	31.4±0.36a	1.8±0.02a	1.2±0.35b	0.031±0.001c	0.25±0.01a
F-test	**	**	**	**	**	**	**	ns
Watered (factor 2)								
with water	41.9±0.38b	16.3±0.26c	10.6±0.32b	31.3±0.36a	1.4±0.02b	1.2±0.35b	0.040±0.001a	0.24±0.01b
with SA	45.7±0.38a	19.2±0.26a	12.7±0.32a	30.5±0.36a	1.8±0.02a	1.4±0.35a	0.039±0.001a	0.26±0.01a
with GA_3	41.7±0.38b	17.2±0.26b	9.6±0.32c	29.1±0.36b	1.8±0.02a	1.3±0.35b	0.036±0.001b	0.24±0.01b
F-test	**	**	**	**	**	**	**	*
F-test Soaked x Watered	**	**	**	**	**	**	**	**

Note. Different lower-case letters show significant differences within each factor; ns, *, ** = Non-significance ($P>0.05$), statistical significance ($P<0.05$) and high statistical significance ($P<0.01$) of each factor, respectively; SA = Salicylic acid; GA_3 = Gibberellic acid

Table 7

Shoot and root growth of Napier grass Pakchong 1 stimulated with various application methods of plant growth regulators

	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Root-to-shoot ratio	Specific root length (m/g)
Immerse with water Watered with water	55.8±0.37bC	18.6±0.36cC	11.4±0.65cC	13.2 ±0.94cC	1.1±0.03cC	0.54±0.03cB	0.05±0.00aA	0.24±0.02aA
Watered with SA	66.3±0.54aC	21.5±0.45bC	14.7±0.36bC	21.1±0.43aA	1.5±0.04bB	0.76±0.02bB	0.05±0.00aA	0.28±0.10aA
Watered with GA_3	66.4±0.60aC	24.1±0.70aC	18.0±0.32aB	17.1±0.48bB	1.8±0.04aA	0.84±0.02aA	0.05±0.00aA	0.20±0.01bA
Immerse with SA Watered with water	61.8±0.20cB	20.9±0.36cB	16.0±0.23cB	17.1±0.29bA	1.4±0.03cB	0.70±0.02bA	0.04±0.00aA	0.24±0.01aA
Watered with SA	71.2±0.58bB	30.5±0.86bB	24.2±0.35bB	21.5±0.27aA	1.9±0.05aA	0.82±0.02aAB	0.03±0.00bB	0.26±0.01aA
Watered with GA_3	74.0±1.09aB	34.5±1.16aB	25.4±0.22aA	17.9±0.29bB	1.7±0.04bB	0.76±0.02abB	0.03±0.00bB	0.23±0.01aA
Immerse with GA_3 Watered with water	86.0±0.89bA	35.1±0.99cA	23.6±0.24cA	15.5 ±0.50cB	1.7±0.01bA	0.70±0.02bA	0.03±0.00aB	0.22±0.01bA
Watered with SA	85.6±0.40bA	42.9±0.83aA	27.0±0.30aA	20.8±0.46bA	1.9±0.02aA	0.85±0.06aA	0.03±0.00aB	0.25±0.02abA
Watered with GA_3	88.2±0.97aA	37.3±0.52bA	25.5±0.37bA	23.1±0.24aA	1.8±0.04bA	0.82±0.02aAB	0.03±0.00aB	0.28±0.01aA

Note. The different lower-case letters showed a significant difference between different PGR watering within the same PGR immersion; Different capital letters showed significant differences between different PGR immersions within the same PGR watering

the root-to-shoot ratio (Table 7). Also, soaking with gibberellic acid and watering with gibberellic acid increased the shoot and root length of Napier grass cv. Pakchong 1 more than watering with other plant growth regulators. For example, the shoot and root length of Napier grass cv. Pakchong 1 soaked with gibberellic acid and watered with gibberellic acid were 88.2±0.97 and 23.1±0.24 cm, respectively, during the shoot and root length of Napier grass cv. Pakchong 1 soaked with gibberellic acid and watered with salicylic acid were 85.6±0.40 and 20.8±0.46 cm, respectively (Table 7). However, soaking with gibberellic acid and watering with salicylic acid increase the fresh and dry weight of Napier grass cv. Pakchong 1 more than watering with water and gibberellic acid. For example, the shoot and root fresh weight of Napier grass cv. Pakchong 1 soaked with gibberellic acid and watered with gibberellic acid were 37.3±0.52 and 1.8±0.04 g, respectively, during the shoot and root fresh weight of Napier grass cv. Pakchong 1 soaking with gibberellic acid and watering with salicylic were 42.9±0.83 and 1.9±0.02 g, respectively (Table 7).

The two cultivars of Napier grass respond to a combination of plant growth regulators in different ways. Salicylic acid soaking is appropriate to enhance the growth of sweet grass, but watering with salicylic acid or gibberellic acid after planting decreases the growth of sweet grass except at fresh root weight. The appropriate method for sweet grass increased the 513% of dry shoot weight when compared with treatment without plant growth regulator application. Napier grass cv. Pakchong 1 responds positively to gibberellic acid after soaking and watering with gibberellic acid again, increasing shoot and root length more than other treatments. However, watering with salicylic acid enhanced the fresh and dry weight of Napier grass cv. Pakchong 1 more than soaking with gibberellic acid only. The appropriate method for Napier grass cv. Pakchong 1 increased 137% of shoot dry weight and 58% of shoot length compared to treatment without plant growth regulator application. It is normally of gibberellic acid activity to increase the plant's internode length and shoot length (Graebe, 1987). Also, salicylic acid could increase the fresh weight of the plant. A Foliar spray of 0.01 mM salicylic acid on corn growing in acidic soil could increase the shoot fresh weight of corn (Somtrakoon & Chouychai, 2022a). Salicylic acid also increased the photosynthesis capacity of wheat exposed to heat stress via interaction with proline and ethylene metabolism (Khan et al., 2013). This result showed that it is possible to increase the biomass of Napier grass growing in sandy soil via soaking and watering with a plant growth regulator after planting. However, the physiological and biochemical responses within plant tissue should be studied further to explain the activity and interaction of salicylic acid and gibberellic acid in different cultivars.

CONCLUSION

The appropriate plant growth regulator to stimulate the growth of Napier grass

depends on its cultivar. Soaking the stem cutting with 100 mg/L salicylic acid is the only method appropriate to stimulate the length and weight of sweet grass. Soaking with 0.01 mg/L gibberellic acid and watering with 100 mg/L salicylic acid increases the weight of Napier grass cv. Pakchong 1, while soaking with 0.01 mg/L gibberellic acid and watering with 0.01 mg/L gibberellic acid, increases the length of this cultivar.

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Effects of Greywater Organomineral Liquid Fertilizer on the Growth, Yield Performance, and Proximate Composition of Chili (*Capsicum annum* L.)

Si Li Tan[1], Susilawati Kasim[1]*, Martini Mohammad Yusoff[2], Syaharudin Zaibon[1] and Sriharan Raguraj[1,3]

[1]Department of Land Management, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Crop Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[3]Soils and Plant Nutrition Division, Tea Research Institute of Sri Lanka, Talawakelle 22100, Sri Lanka

ABSTRACT

The production cost of chili in the fertigation system has increased recently due to the high cost of water-soluble fertilizers used in the system. Laundry greywater and biodegradable vegetable waste are rich in nutrients essential for plant growth. Thus, this research aims to investigate the effects of greywater organomineral fertilizer (OMF) on the chili plants' growth and yield performance in the fertigation system. The experiment was laid out in a completely randomized design under the rain shelter. OMF produced from laundry water and vegetable waste was applied with chemical fertilizer (CF) in different ratios, including 100% CF (T1, control), 75% CF + 25% OMF (T2), 50% CF + 50% OMF (T3), 25% CF + 75% OMF (T4), and 100% OMF (T5). Results showed that the combined use of CF and OMF produced non-significantly different chili plants from those solely treated by CF. Interestingly, chili plants treated with 50% CF and 50% OMF increased the yield by 4.71% compared to CF. Chili plants treated with 25% and 50% OMF showed non-significantly different plant height, stem diameter, plant dry weight, fruit number, and proximate composition of fruits over those treated with 100% CF. Solely application of OMF produced similar chili as CF in terms of fruit quality. The present study shows that plant performance and yield of chili were improved after the application of CF and OMF at a ratio of 50:50. It can be concluded that OMF has the potential to be used as an alternative for replacing 50% of chemical fertilizer in chili fertigation system without affecting its growth and yield.

Keywords: Chili, fertigation, greywater organomineral fertilizer, proximate composition, yield performance

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E-mail addresses:
silichen93@gmail.com (Si Li Tan)
susilawati@upm.edu.my (Susilawati Kasim)
martinimy@upm.edu.my (Martini Mohammad Yusoff)
syaharudin@upm.edu.my (Syaharudin Zaibon)
raguraj90@yahoo.com (Sriharan Raguraj)
*Corresponding author

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INTRODUCTION

Chili (*Capsicum annum* L.) is an economic crop from the Solanaceae family (Tripodi & Kumar, 2019). It is a global spice and vegetable used in various cuisines, especially in Asian countries like Malaysia, India, and Bangladesh (A. K. Singh et al., 2021). Chili cultivation generates high revenues for producers (Karungi et al., 2013). In Malaysia, red chili (Cili Kulai) is one of the main varieties produced on commercial farms. It is usually cultivated conventionally or in a fertigation system (in open or under shelter) at the lowland (Suhaimi et al., 2016). Malaysian red chili contains high value of vitamin C, calcium, iron, fiber, and protein (Fudholi et al., 2013).

According to the Department of Statistics Malaysia, local chili production cannot meet market demand despite its economic and cultural value. In 2020, a total estimation of 28,264 tonnes of red chili was produced in Malaysia, about 54.75% less than those yielded five years back in 2016, which was 43,738 tonnes. More chili was imported from neighboring countries like China, India, and Thailand to meet local needs. The importation of chili in Malaysia rose from 49,069 tonnes in 2016 to 66,295 tonnes in 2020 (Department of Statistics Malaysia [DOSM], 2021; Sarobo, 2019).

The reduction in chili yield may cause by the instability of market value, diseases, expensive and low-quality inputs, and poor extension services (Arain, 2019). Nevertheless, the fertigation system was more financially feasible than conventional farming by increasing on-farm income and reducing weed control and fertilizer application management costs. The fertigation system provides continuous macro- and micro-nutrients to improve the growth and yield of chili plants on tin-mined land by about 130% compared to conventional nitrogen, phosphorus, and potassium (NPK) fertilizer (Subiksa et al., 2019). Balanced nutrition is the most crucial factor which ensures chili growth and yield performance. However, fertilizer cost becomes the major constraint among chili growers as water-soluble fertilizers such as potassium nitrate, monopotassium phosphate, calcium nitrate, and sulfate of potash have relatively costly compared to conventional fertilizers such as urea, diammonium phosphate, and muriate of potash (Rajput et al., 2007; Reddy & Hebbar, 2018).

On the other hand, the combined use of organic and inorganic fertilizers with biofertilizers successfully improved the plants' growth and obtained a better yield of chili (Stan et al., 2021). Organomineral fertilizer (OMF), formulated from mineral fertilizer and organic waste, is an alternative that provides the good attributes of organic and mineral fertilizer (Sá et al., 2017). The application of bio-organo mineral fertilizer enhanced chili plants' germination, growth, and yield (Nofiyanto et al., 2018). The integrated use of organic and mineral fertilizers on tomato plants significantly enhanced plant development and improved the number of clusters and fruits per plant (Tonfack et al., 2009). Furthermore, organomineral fertilizers can

potentially improve soil structure more than conventional organic fertilizers (Bautista et al., 2020). The application of OMF built up nutrient retention and structural stability of the soil and ensured the continued availability of nutrients by its organic constituents (Egbuchua & Enujeke, 2013).

The present study involved liquid OMF from commercial waste (laundry greywater) and agriculture residues (dried vegetable wastes). Laundry greywater (LGW) is wastewater from commercial and urbanized clothes-washing processes. LGW mainly comprises cations: calcium, magnesium, and potassium, as well as anions such as nitrate, sulfate, carbonate, and chloride, along with organic micropollutants (OMPs) raised from the detergents (Chan et al., 2014; Mohamed et al., 2014). LGW can be reused by diverting it into gardens and lawns without treatment (Jeppesen, 1996) as its salts and nutrient contents benefit plants (Misra et al., 2010). Furthermore, vegetable waste is a biodegradable material that includes rotten peels, shells, and scraped portions of vegetables or slurries (A. Singh et al., 2012). It is a potential organic amendment owing to its high nutrient content, such as fiber, nitrogen, potassium, phosphorus, and other nutrients, and its low hazardous pollutants (Kim et al., 2018; Matthews & Maruthaipillai, 2016; Morales et al., 2016).

Considering chili is one of the essential commodities in Malaysia, there is an apparent necessity to study the effects of OMF on chili production. Despite the nutrients in LGW and vegetable waste, the product is expected to have great potential in improving chili plants' performance from their gradual release of nutrients. Therefore, this study aims to evaluate the effects of greywater OMF on chili plants' growth and yield performance in the fertigation system.

MATERIALS AND METHODS

Pot Experiment

The pot experiment was conducted under a rain shelter that allowed full sunlight for plant establishment at Agrotechnology Section, Putra Agriculture Centre, Universiti Putra Malaysia (2°58'54.5"N, 101°42'53.9"E). Chili seeds (Sakata Hot Pepper F1 Hybrid SJ1-461, India) were sown in peat moss in the nursery for 24 days. The homogenous seedlings showing better growth were selected for transplanting into the polyethylene bags of size (0.4 m width x 0.4 m height) filled with cocopeat as cultivation media under a rain shelter. The liquid OMF formulated from vegetable waste and laundry greywater was applied to chili plants (*Capsicum annum* L.) using a drip irrigation system and liquid chemical fertilizer in different ratios.

Production of Organomineral Fertilizer

Liquid OMF was prepared by mixing grounded vegetable waste and LGW in a container with a cap at a ratio of 1:10 to obtain a biological suspension. The suspension was shaken using an orbital shaker at 180 rpm for 30 min and further incubated for 12 days at room temperature. Upon reaching the respective incubation period, the fermented suspension was

filtered to remove the remaining particles of the biological humus to obtain the liquid fertilizer (Rosidi, 2018). The composition of OMF is shown in Table 1.

Preparation of Chemical Fertilizer

The liquid chemical fertilizer was ready-made by Dsyira Enterprise (Malaysia). It consisted of Fertilizer A and Fertilizer B. The composition of each fertilizer is given in Table 2. Fertilizers A and B were stored in separate containers with lids under shade to prevent coagulation and precipitation of the elements. A similar amount of both CF was mixed gradually into a water tank to get a nutrient solution with the preferred electrical conductivity (EC) level. The nutrient solution was then given to the chili plants through a dripping system.

Experimental Design

The experiment was conducted in a completely randomized design (CRD), with ten replications per treatment. The treatments were 100% CF (T1, control), 75% CF + 25% OMF (T2), 50% CF + 50% OMF (T3), 25% CF + 75% OMF (T4), and 100% OMF (T5), prepared in volume basis. The treatment was given to plants by setting the EC of nutrient solution at 1.5 $mS.cm^{-1}$ at 1 to 3 weeks after transplanting (WAT), EC 2 mS/cm at vegetative phase (6-8 WAT), and EC 3 mS/cm at generative phase (8–12 WAT). Each nutrient tank was filled up with water every morning. Then, the treatment fertilizer (T1 to T5) was poured gradually and stirred thoroughly into the respective tank until the EC meter showed its appropriate reading, as stated above.

Table 1

Composition of organomineral fertilizer

Element	Concentration (mg/L)
Nitrogen	376.00
Phosphorus	55.20
Potassium	4,139.00
Calcium	848.67
Magnesium	273.32
Sodium	397.27
Manganese	2.99
Iron	14.20
Zinc	2.07

Table 2

Composition of chemical Fertilizer A and B

Element	Composition % (w/v)
Fertilizer A	
Calcium	15.50
Nitrate nitrogen	19.00
Iron	0.05
Fertilizer B	
Nitrate nitrogen	9.00
Available phosphate	7.00
Soluble Potash	37.00
Boron	0.09
Copper	0.05
Iron	0.50
Manganese	0.19
Molybdenum	0.01
Zinc	0.19

Growth Measurements

The plant height, stem diameter, root length, and dry weight of chili plants were determined at the end of the experiment. The root length was determined with measuring tape after removing it from the growing media and washing off the loose cocopeat. The plant samples were then oven-dried at 60°C for 72 hr to obtain dry weight.

Yield

Upon the maturity stage of plants, chili fruits were continuously harvested at weekly intervals from 8 to 12 WAT. At the end of the harvest, yield (12 WAT), fruit number and weight were determined.

Proximate Composition

The quality of fruits was determined by analyzing their respective proximate composition. Moisture content was determined by drying the fruits in an oven at 105°C for 24 hr. Ash content was measured by placing the sample in the muffle furnace at 450°C for 12 hr. The total nitrogen (N) content of the fruit was determined by the Kjeldhal method (Bremner, 1965). Protein content was determined by multiplying the N content by a conversion factor (6.25) (Akhand et al., 2021).

Crude Fiber Content

Approximately 2 g of fruit sample was added into a flask with 100 ml of 1.25% sulfuric acid (H_2SO_4) (Fisher Scientific, USA). The mixture was heated at 60°C for 35 min. The flask was swirled to prevent particles from adhering to the inner surface of the flask. Next, the sample was filtered and transferred into a flask with 100 ml of 1.25% sodium hydroxide (NaOH) (Fisher Scientific, USA). The flask was again heated at 60°C for 35 min. The sample was then filtered, dried, and transferred into a crucible. The weight of the dried residue with the crucible was taken. Later, the crucibles with samples were calcined in a furnace at 450°C for 4 hr. The weight of the crucible with ash was taken, and crude fiber content was determined (Akhand et al., 2021).

Fat Content

The chili fruit was dried in an oven at 102°C for 5 hr. About 5 g of dried chili was weighed into a thimble. A piece of cotton wool was plugged into the opening of the thimble. The thimble with the sample was inserted in a Soxhlet liquid extractor. Later, the weight of a round bottom flask was taken. Next, 90 ml of petroleum ether (Sigma-Aldrich, USA) was measured and added to the flask. The extraction unit was assembled, connected to a condenser, and placed over an electrothermal extraction unit. Extraction was continued for 8 hr. After extraction, the round bottom flask was detached and removed from the heat source. The flask was then placed in a steam bath to evaporate off the residual petroleum ether. The flask was heated in an oven set at 105°C for one hour and then cooled in a desiccator. The weight was recorded, and fat content (%) was calculated according to Nielsen (2017) methods.

Data Analysis

The collected data were analyzed by one-way analysis of variance (ANOVA) using Statistical Analytical System (SAS 9.4). The treatment means were compared by Tukey's test at a 5% significant level. All analyzed parameters were subjected to a principal component analysis in order to determine which parameters largely discriminated among the different ratios of OMF using Origin software (Version 2022, Origin Lab Corporation, USA).

RESULTS AND DISCUSSION

Plant Growth

Based on Figure 1A, chili plants in T1 showed the highest plant height (66.50 cm), while T4 recorded the lowest value (50.80 cm). However, plant heights of T1, T2, T3, and T5 were not significantly different. Khandaker et al. (2017) reported that chili Kulai plants grown without fertilizer recorded a height of 42.12 cm. Besides, Figure 1B indicates that the average stem diameter of plants in T4 was significantly lower (96 mm) than T2 but statistically comparable to the rest of the treatments. Hence, applying OMF and CF have improved the growth performance of chili plant height.

Results revealed no significant difference in the root length of chili plants under five different fertilizer treatments (Figure 1C). Root length is a crucial parameter in absorbing water and plant nutrients that essential for plant growth and

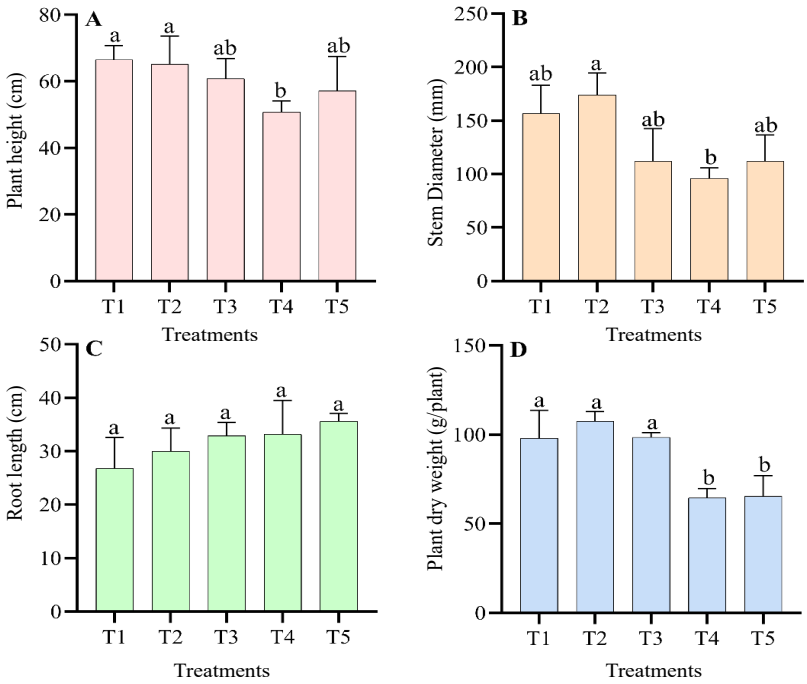

Figure 1. Effect of organomineral fertilizer (OMF) on plant height (A), stem diameter (B), root length (C), and plant dry weight (D). Vertical bars (standard deviation) with different letters are significantly different at $p \leq 0.05$ level according to Tukey's test. T1 = 100% CF; T2 = 75% CF + 25% OMF; T3 = 50% CF + 50% OMF; T4 = 25% CF + 75% OMF; T5 = 100% OMF

development (Prradhiepan et al., 2018). In this study, though no significant differences were observed among treatments in root length, plants grown under 100% OMF showed higher values.

In this experiment, the substitution of 50% recommended CF by OMF sustained all the growth parameters measured (Figure 1). Interestingly, 100% OMF showed no significant different in plant height, stem diameter, and root length to 100% recommended CF (Figures 1A, 1B & 1C). Our results are similar to the findings of Prradhiepan et al. (2018), where the combination of inorganic fertilizer and organic manure increased the root length of chili plants compared to the sole application of inorganic fertilizer (Semida et al., 2014). Further, OMF acts as a reservoir of nutrients, released slowly into substrate solution or directly to plant roots (Rady, 2012). It might be the reason which explains the higher root length in chili plants grown under 100% OMF.

However, plant dry weight was significantly lower in both 25% (T4) and 100% (T5) OMF treatments (Figure 1D) compared to the control (T1, 100% CF). As the concentration of OMF increased, the plant dry weight decreased. On the contrary, Oliveira et al. (2017) found that the dry weight of sorghum linearly increased with increasing doses of OMF. Plant growth could be attributed to different fertilizers' nutrient content encouraging vegetative growth (Atere & Olayinka, 2012). Hence, a single application of OMF could not provide enough nutrients to enhance the vegetative growth of chili plants.

The results show that the combined use of CF and OMF (25% and 50% OMF) produced chili plants with not significantly different plant height, stem diameter, and root length from those solely treated with CF and OMF. Studies revealed that mineral fertilizer is highly soluble, facilitating nutrient uptake. As a result, it promotes better crop growth during the early stages of plant development (Lourenço et al., 2013). Conversely, organic fertilizer and OMF have slow nutrient release characteristics because of their residual effect (Silva et al., 2015). As a consequence, the chili plants solely treated with OMF obtained lower plant dry weight.

Yield Performance

The results showed a significant difference in chili yield and fruit number among the treatments (Figures 2A and 2B). Chili plants under T1, T2, and T3 recorded non-significant yields, while T4 and T5 had lower yields.

The highest fruit number (145 fruits/plant) was observed in T2, which is non-significant to T1 and T3, while T4 and T5 recorded significantly lower fruit numbers. These results are similar to the finding of Olaniyi et al. (2010) in okra, where the highest fruit number and yield were recorded in the treatment with the combination of NPK fertilizer and OMF. Nevertheless, our results contradict the reported studies in which the combined use of chemical or inorganic fertilizer and organic manure enhanced the yield, fruit number, and average fruit weight of chili plants compared to a single application of

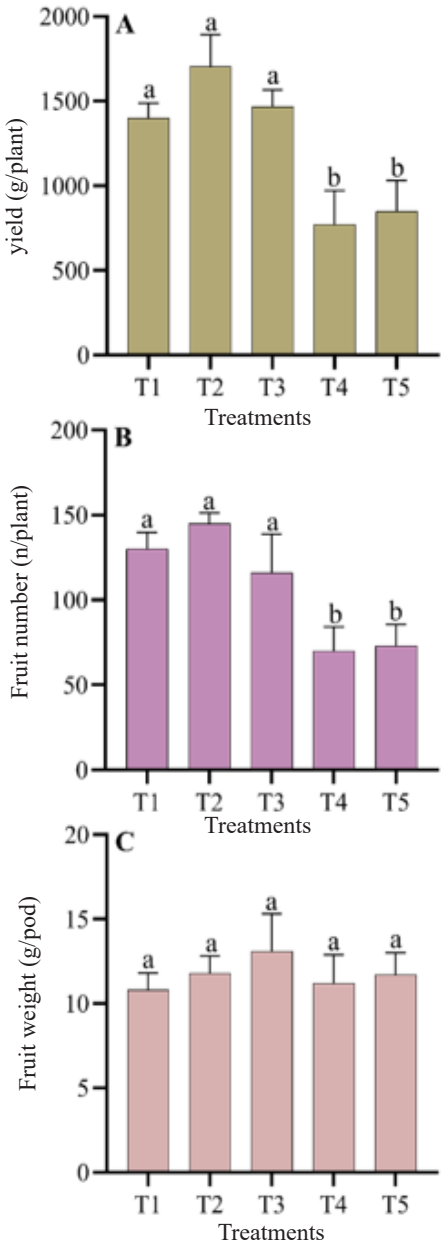

Figure 2. Effect of OMF on chili yield (A), fruit number (B), and fruit weight (C). Vertical bars (standard deviation) with different letters are significantly different at $p \leq 0.05$ level according to Tukey's test. T1 = 100% CF; T2 = 75% CF + 25% OMF; T3 = 50% CF + 50% OMF; T4 = 25% CF + 75% OMF; T5 = 100% OMF

each fertilizer (Prradhiepan et al., 2018; Stan et al., 2021). The use of OMF statistically improved the mean crop yields of forage maize, oilseed rape, and wheat more than conventional fertilizer (Deeks et al., 2013). However, OMF is sustainable due to its slow nutrients releasing nature throughout the plant growing period (Ogunlade et al., 2011). A previous study reported that N from compost was released eventually and made available evenly during OMF treatment for three consecutive years (Paul & Beauchamp, 1993). Nevertheless, it is important to note that, in annual crops, more than the amount of nutrients supplied by OMF may be needed to maintain a yield similar to chemical fertilizer.

Fruit weight showed no significant variation among treatments (Figure 2C). The results (Figures 2A and 2B) are similar to the previous studies in which the mean yield of winter wheat that received OMF treatment was approximately 20% lower than the crop yield from the conventional fertilizer plots (Deeks et al., 2013). However, a comparison of the weight of 1,000 grains of wheat showed no significant difference.

Proximate Composition

In this study, the composition of chili fruits to determine their quality and nutritional value is analyzed (Table 3).

Data revealed that the moisture content of chili samples in T2 was significantly lower than in other treatments. The moisture content was measured as it may have a marked impact on the taste, texture, appearance, shape, and weight of chili

Table 3

Effect of organomineral fertilizer on proximate composition of chili fruits

Treatment	Moisture content (%)	Ash content (%)	Nitrogen (%)	Protein (%)	Crude fiber (%)	Fat (%)
T1	87.77±0.28 ab	0.57±0.04 b	0.21±0.01	1.30±0.01	3.73±0.32 a	0.55±0.16
T2	86.29±1.22 b	0.01±0.01 c	0.21±0.01	1.33±0.02	2.31±0.21 b	0.55±0.08
T3	87.77±0.97 a	0.85±0.14 a	0.23±0.01	1.41±0.04	2.37±0.17 b	0.74±0.16
T4	87.92±1.18 a	0.85±0.10 a	0.23±0.01	1.43±0.07	2.36±0.15 b	0.45±0.10
T5	88.94±0.51 a	0.86±0.02 a	0.23±0.01	1.45±0.05	2.36±0.08 b	0.32±0.14
p-value	0.0738	<0.0001	0.1532	0.0368	0.0001	0.1014
F-value	2.98	41.96	2.69	6.09	19.09	2.59
CV (%)	1.142	15.65	4.41	4.37	9.31	31.71

Note. Columns with different letters are significantly different at $p < 0.05$ level. CV = Coefficient of variation; T1 = 100% CF; T2 = 75% CF + 25% OMF; T3 = 50% CF + 50% OMF; T4 = 25% CF + 75% OMF; T5 = 100% OMF

(Hurst et al., 2018). This result contradicts the finding that the sole application of mineral fertilizer on *Corchorus olitorius* (L.) recorded the significantly highest water content compared to those treated with the combination of organic poultry manure and mineral fertilizer (Ayinla et al., 2018).

The ash content of chili samples was measured to determine the number of minerals present within chili from each treatment (Bakkali et al., 2009). Based on Table 2, the ash content of chili samples increased significantly with increasing OMF. It might be caused by the increase of minerals received by chili plants under these treatments increased, as OMF produced from greywater and vegetable waste exhibits a wide range of minerals and elements (Chan et al., 2014; Matthews & Maruthaipillai, 2016).

The overall proximate composition of chili fruits collected from five treatments showed no significant differences in nitrogen, protein, and fat contents. The results were similar to the previous study that showed no differences in protein content in melon fruits among mineral and OMF treatments (Fernandes et al., 2003). It was reported that using organic poultry manure with inorganic fertilizer effectively combines the advantages of organic and inorganic fertilizers (Okunlola et al., 2011). Hence, it can be said that the OMF produced from vegetable waste and laundry water is compatible with commercial chemical liquid fertilizer, yielding a similar quality of fruits.

There was no significant difference observed in the fat content of chili samples. The characteristic of chili fruits having relatively low-fat content aligns with the findings that reported vegetables are not credible fat sources (Ejoh et al., 1996). Every 100 g of raw red chili is reported to contain 0.40 g of total fat (United States Department of Agriculture [USDA], 2019).

Principal Component Analysis

A comprehensive view of the growth attributes, yield, and proximate composition of chili fruit in response to different ratios of organomineral fertilizer was obtained using principal component analysis (PCA). Several scientific articles have demonstrated the effectiveness of PCA plotting for evaluating the growth enhancement, mineral nutrition, yield and quality traits of several crops (Colla et al., 2017; Raguraj et al., 2022; Rouphael et al., 2017, 2021). The first two principal components were associated with eigenvalues > 1 and explained 85.08% of the total cumulative variance, with PC1 and PC2 accounting for 64.12% and 20.96%, respectively (Figure 3). In this study, PCA scores presented in Figure 3 provide coordinated information on the growth, yield and proximate composition of chili fruit in relation to different ratios of OMF. The PC1 and PC2 score plot divides and classifies different ratios of OMF treatments into four groups. The upper right quadrant of the positive side of PC1 included 25 and 50% of OMF treatments and showed higher yield, fruit number, fruit weight, plant height, stem diameter, plant dry weight, and fat content in fruits. The lower right quadrant depicted 100% CF (Control) fertilizer treatment had higher crude fiber content. OMF 75% and 100% treatments were clustered on the negative side of the PC1. The PCA analysis conducted in the present study might provide the basis for a more in-depth investigation of the effects of different ratios of OMF treatments on the growth, yield and composition of chili fruit.

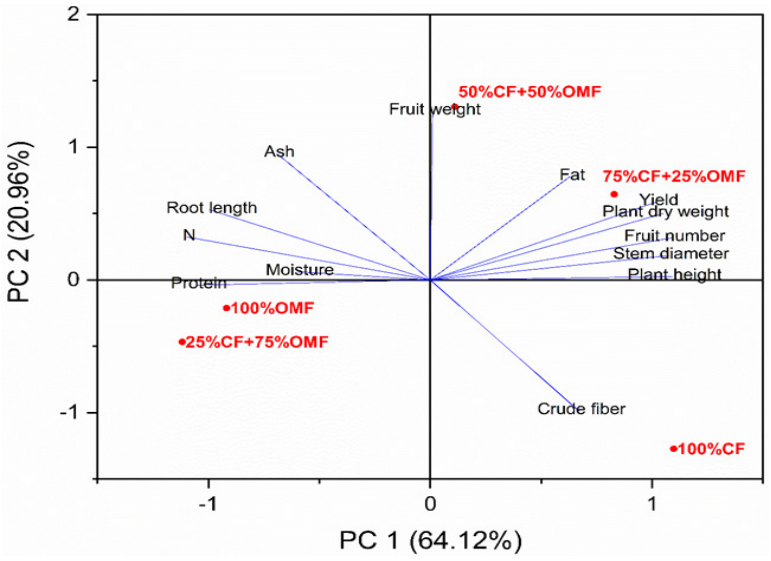

Figure 3. Principal component analysis biplot of growth attributes, yield parameters, and proximate composition of chili in response to different ratios of organomineral fertilizer

Note. CF = Chemical fertilizer; OMF = Organomineral fertilizer

CONCLUSION

Greywater liquid organomineral fertilizer is a potential material to reduce the need for commercial chemical liquid fertilizer in fertigation. The production of this fertilizer encourages nutrient recycling from urban waste into the agricultural ecosystem. Greywater organomineral fertilizer has a slow nutrient-releasing nature making them less effective in sole application. In this study, chili plants receiving 100% OMF showed comparatively lower growth and yield performance than chemical fertilizers. However, the combined use of chemical and organomineral fertilizers in chili produced yields and fruit quality similar to chemical fertilizers. Thus, it could significantly minimize chemical fertilizer costs and improve the sustainability of chili cultivation. Finally, it is suggested to use 50% of CF and 50% of OMF in the fertigation system to obtain better yield performance of chili plants.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Immunity Evaluation of Inactivated Newcastle Disease Virus Vaccine Inoculated at Different Doses in Day-old Specific-Pathogen-free Chicks

Siti Nor Azizah Mahamud[1,2], Tasiu Mallam Hamisu[1,3], Juan Luis Criado Rius[4], Shyong Wey Ong[4] and Abdul Rahman Omar[1,5]*

[1]Laboratory of Vaccines and Biomolecules, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, 16100 Kota Bharu, Kelantan, Malaysia
[3]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[4]HIPRA, Avenida La Selva 135, 17170 Amer, Girona, Spain

ABSTRACT

This research aims to evaluate the immunogenicity of different doses of HIPRAVIAR® BPL2 inactivated Newcastle disease virus (NDV) LaSota vaccine. Specific-pathogen-free day-old chicks were divided into 3 different groups, and each group was vaccinated subcutaneously with the vaccine dose of 0.1, 0.2, and 0.5 ml, respectively. Blood samples were collected to measure NDV-specific antibody titers using a hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA). The HI result showed that birds vaccinated with 0.5 ml HIPRAVIAR® BPL2 vaccine showed an increased statistically significant antibody titer compared to the other doses. Similarly, the ELISA result corroborated the HI finding. No significant difference between the results was detected when the antibody titers were measured using two ELISA kits, Biocheck CK116, and CIVTEST® AVI NDV. The percentage antibody-positive test based on HI amongst the different days post-vaccination showed that all the birds were positive from 28 to 42 days following vaccination with HIPRAVIAR® BPL2 0.5 ml (group D), whereas the highest percentage of antibody positivity were 80% and 70% at 42 days post-vaccination with HIPRAVIAR® BPL2 0.1 ml (group

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E-mail addresses:
norazizah.m@umk.edu.my (Siti Nor Azizah Mahamud)
tasiumallamhamisu@gmail.com (Tasiu Mallam Hamisu)
juan.criado@hipra.com (Juan Luis Criado Rius)
shyongwey.ong@hipra.com (Shyong Wey Ong)
aro@upm.edu.my (Abdul Rahman Omar)
*Corresponding author

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B) and HIPRAVIAR® BPL2 0.2 ml (group C), respectively. In conclusion, besides the difference in seroconversion, all the vaccine doses used had important levels of seroconversion and positivity.

Keywords: Antibody titer, ELISA, HI, inactivated vaccine, LaSota, seroconversion

INTRODUCTION

For decades, the poultry production in Malaysia has expanded steadily, in line with the establishment of feed mills, genetic improvement and veterinary services to meet the local demand and export trades (Abdurofi et al., 2017). However, the poultry industry in this region is constantly under threat by devastating diseases such as Newcastle disease (ND) and highly pathogenic avian influenza virus (HPAI) infection. ND is one of the highly contagious viral diseases causing economic losses in the poultry industry worldwide, causing a reduction in egg production, poor weight gain, morbidity, and mortality in infected chicken flocks (Alexander, 2000; Miller & Koch 2013). Other than poultry species, the disease also affects several species of wild birds such as owls, black swans, peacocks, and egrets (Shohaimi et al., 2015; Suarez et al., 2020; Mahamud et al., 2021). In addition, a study has shown wild migratory birds can act as reservoirs of virulent NDV, introducing the disease to commercial poultry farms (Naguib et al., 2022). Among the avian species, chickens are most susceptible to ND; ducks showed no clinical symptoms, while other waterfowls serve as natural reservoirs for the virus (Hines & Miller, 2012; Snoeck et al., 2013).

A single-stranded, negative-sense RNA virus causes Newcastle disease called Newcastle disease virus (NDV) or *Avian Orthoavulavirus 1* (AOAV-1) (formally called *Avian Avulavirus-1*) that belongs to the genus *Avulavirus* (ICTV, 2019; Dimitrov et al., 2019). The virus can be classified based on genotypes and pathotypes. The genotype classification is based on molecular characterization of the fusion (F) gene, where the virus can be different into 18 genotypes (genotypes I to XVIII) (Diel et al., 2012). Recently, Dimitrov et al. (2019) further refined the classification of NDV genotypes with the identification of several new genotypes (genotype I to XXIII). Presently, NDV genotype VII is causing the fifth panzootic that has spread rapidly across Asia and the Middle East (Miller et al., 2015; Dimitrov et al., 2019). The pathotype classification of the virus is based on biological characterization, where at least there pathotypes have been identified: the highly fatal velogenic strain, characterized by signs and lesions severely affecting the respiratory, gastrointestinal, and nervous systems; the moderately severe mesogenic strain; and the less severe lentogenic strain (Alexander & Jones, 2008, Jindal et al., 2010). The velogenic form of NDV caused the most significant disease in poultry species (Suarez et al., 2020). The virus is introduced into susceptible flocks through aerosols and fecal-oral routes (Samal, 2008).

Similar to many other poultry viral diseases, the key components in the control and prevention of ND at the farm level is to enhance biosecurity practices in combination with good flock health programs, which include vaccination program as a prophylactic measure (Miller & Koch, 2013; Dimitrov et al., 2017). Vaccines against ND have been developed since the early 1950's. Presently, poultry industries depend on live and inactivated ND vaccines to prevent economic losses due to morbidity and mortality resulting from virulent NDV infection (Gallili & Ben-Nathan, 1998). Since then, continuous research and development have been done to produce various forms of ND vaccines that have high potency against circulating strains in the farm. The commonly available and ND vaccines that have been used are inactivated or killed vaccines, live attenuated ND vaccines and recombinant ND vaccines using the fowlpox virus and herpesvirus of turkey vector vaccine (Boursnell, et al., 1990; Gergen et al., 2019), and genotype-match vaccine using reverse genetic technology (Dimitrov et al., 2017; Bello et al., 2020). Regardless type of ND vaccines used, the aim of vaccination is to immunize the vaccinated chickens to offer full protection against disease challenged. However, currently available ND vaccines are unable to elicit sterilizing immunity that is able to prevent the infection of NDV (Miller et al., 2007; Miller et al., 2009; Miller et al., 2013). Hence, effective control and prevention of ND require prudent farm management, biosecurity, and effective vaccines.

Presently, ND is endemic in many countries in Asia, including Malaysia, with velogenic NDV genotype VII continuously isolated from improperly vaccinated poultry flocks (Nooruzaman et al., 2022). Thus, there is a need to evaluate the post-vaccinal induced immune response in the vaccinated chickens. The flock health against ND is monitored based on the antibody profile tested with a rapid diagnostic assay such as HI or ELISA to determine the protective antibody level for each flock (Aldous et al., 2003; Czifra et al., 1998, Miller et al., 2007).

The objective of this study was to evaluate antibody response by vaccinated chickens following vaccination with different doses of HIPRAVIAR® BPL2, an inactivated vaccine using hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA) kits (Biocheck CK116 and CIVTEST® AVI NDV). Specifically, the effect of administering an off-label lower dose in younger animals, as the recommended full dose of 0.5 ml is difficult to apply in day-old chicks. The efficacy of vaccinations can best be evaluated with challenging experiments; however, these are expensive and time-consuming (Czifra et al., 1998). Consequently, serological tests such as the HI and ELISA are often used to assess protective response (Czifra et al., 1998).

MATERIALS AND METHODS

Vaccine

HIPRAVIAR® BPL2 (HIPRA, Spain) is an inactivated vaccine indicated to prevent

Table 1

Enzyme-linked immunosorbent assay reading and interpretations

CIVTEST® AVI NDV			BioCheck CK116 NDV		
SP value	NDV titer	Antibody status	SP value	NDV titer	Antibody status
≤0.185	0-219	-ve	<0.349	<1,158	-ve
0.185-0.234	219-317	Suspect	>0.350	>1,159	+ve
≥0.234	>317	+ve			
Cut-off titer: ≤ 317			Cut-off titer: ≤ 1,158		

NDV = Newcastle disease virus; SP = Sample to positive ratio value

ND by active immunization of birds. The vaccine comprises the inactivated LaSota strain virus (HI ≥ 1/16) formulated with liquid paraffin, administered by injecting subcutaneous 0.5 ml per bird.

Chickens

A total of 50 specific-pathogen-free (SPF) White Leghorn day-old chicks (DOCs) were used for the study (Malaysian Vaccine Pharmaceutical, Puchong, Selangor). They were raised at the Biologics Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The presence of maternally derived antibodies against NDV was rule-out by evaluating the blood of 10 randomly selected chicks. The chickens used for the study were negative against NDV. The birds were raised in a BSL-2 experimental animal facility, fed commercial feeds, and allowed to drink water *ad libitum*. The Institutional Animal Care and Use Committee at the Faculty of Veterinary Medicine approved the study protocol, UPM (reference no.: UPM/IACUC/AUP-R096/2015).

Experimental Design

The DOCs were randomly divided into four groups of 10 birds each. Birds in group A have injected subcutaneously with 0.2 ml of a sterile phosphate buffer saline (PBS) solution (1st BASE, Singapore) and thus used as control; birds in groups B and C were vaccinated subcutaneously with a dose of HIPRAVIAR ® BPL2 (HIPRA, Spain) of 0.1 and 0.2 ml, respectively. Birds in group D were vaccinated with the dose recommended by the manufacturer, 0.5 ml. The vaccinated groups were monitored for 42 days post-vaccination with weekly blood sampling to measure the antibody titer.

Hemagglutination (HA) and Hemagglutination Inhibition (HI) Tests

HI tested the harvested sera to determine the NDV-specific antibody titer. Hemagglutination (HA) and Hemagglutination Inhibition (HI) tests were performed using the NDV strain LaSota and a chicken erythrocytes suspension (Beard et al., 1975) following standard procedures (World Organisation for Animal Health

Table 2
NDV HI antibody titer following vaccination with different doses of HIPRAVIAR® BPL2 vaccine

Vaccine dose	Antibody titer, $\log_2$ (Geometric mean ± SD) at different days post-vaccination							Grand mean
	0	7	14	21	28	35	42	
A (PBS)	ND	1.0±0.0[a]	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1[abc]
B (0.1 ml)	ND	1.0±0.0	1.89±1.23	3.76±1.95	4.03±1.78[+]	4.53±1.70[+]	4.23±1.93[+]	3.24[*c]
C (0.2 ml)	ND	1.0±0.0	1.71±1.15	2.95±2.16	3.20±2.38	3.52±2.67	4.10±2.47[+]	2.75[*c]
D (0.5 ml)	ND	1.0±0.0[a]	2.46±1.83	5.55±1.34[+]	6.46±1.35[+]	7.37±0.70[+]	7.46±0.85[+]	5.05[*ab]

ND = Not detected; SD = Standard deviation; PBS = Phosphate buffer saline
[+]Chicks with a geometric mean antibody titer of $\geq 2^4 (\geq 16)$ were considered positive (seroconverted) (WOAH, 2012);
Values with different superscripts differ significantly at $p < 0.05$;
Values with * are significantly different at $p < 0.05$ when compared to the control;
Values with "a" are significantly different at $p < 0.05$ when compared to the HIPRAVIAR® BPL2 0.1 ml group at the same time point;
Values with "b" are significantly different at $p < 0.05$ when compared to the HIPRAVIAR® BPL2 0.2 ml group at the same time point;
Values with "c" are significantly different at $p < 0.05$ when compared to the HIPRAVIAR® BPL2 0.5 ml group at the same time point

[WOAH], 2012). Serum titers of 1:8 (2^3) or lower were considered negative for antibodies against NDV (WOAH, 2012).

ELISA

The antibody response against ND was evaluated using two commercially available indirect ELISA test kits, CIVTEST® AVI NDV kit (HIPRA, Spain) and BioCheck CK116 NDV (BioCheck, The Netherlands). The ELISA was conducted based on the respective manufacturer's protocol. The test was validated using the mean absorbance values (OD) of the positive and negative controls, and the results were interpreted based on the sample-to-positive ratio (SP) values (Table 1). Meanwhile, ELISA results were considered positive based on the recommended SP values of the kits (Table 1).

Statistical Analyses

The comparison between groups at different time points was conducted by the one-way ANOVA (analysis of variance) test, followed by the Tukey HSD (honestly significant different) post hoc test. The differences between groups showing *p*-values below 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The induction of antibody response correlates with the level of protection against ND (Bello et al., 2020). In a previous study, at least 95% of the SPF birds with detectable antibody titers in serum were protected against a challenge with the Herts strain of NDV (Maas et al., 1999). Therefore, this research was conducted to

Table 3

Number of chickens seroconverted based on HI test following vaccination with different doses of HIPRAVIAR® BPL2 vaccine

Vaccine dose	Number of HI NDV antibody positive chicks / Total number of chicks at different days post inoculation						
	0	7	14	21	28	35	42
A (PBS)	ND	0/10	0/10	0/10	0/10	0/10	0/10
B (0.1 ml)	ND	0/10	0/10	2/10	6/10	7/10	8/10
C (0.2 ml)	ND	0/10	0/10	1/10	6/10	6/10	7/10
D (0.5 ml)	ND	0/10	0/10	3/10	10/10	10/10	10/10

ND = Not detected; HI = Haemagglunation inhibition; NDV = Newcastle disease virus; PBS = Phosphate buffer saline

Table 4

Number of chickens seroconverted based on ELISA following vaccination with different doses of HIPRAVIAR® BPL2 vaccine

Vaccine Dose	Number of ELISA NDV antibody-positive chicks / Total number of chicks on different days post-vaccination											
	CIVEST® AVI NDV						BioCheck CK116 NDV					
	7	14	21	28	35	42	7	14	21	28	35	42
A (PBS)	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
B (0.1 ml)	0/10	1/10	8/10	8/10	9/10	8/10	0/10	1/10	9/10	9/10	9/10	9/10
C (0.2 ml)	0/10	0/10	7/10	7/10	7/10	7/10	0/10	1/10	7/10	7/10	7/10	7/10
D (0.5 ml)	0/10	1/10	10/10	10/10	10/10	10/10	0/10	2/10	10/10	10/10	10/10	10/10

Note. PBS = Phosphate buffer saline; ELISA = Enzyme-linked immunosorbent assay; NDV = Newcastle disease virus

Table 5

Detection of NDV-specific antibody titer by CIVTEST® AVI NDV ELISA following vaccination with different doses of HIPRAVIAR® BPL2 vaccine

Vaccine dose	Antibody titer (arithmetic mean) using CIVTEST® AVI NDV kit on different days post-vaccination							Grand mean
	0	7	14	21	28	35	42	
A (PBS)	7.8	0.9	0.0	0.0	0.0	0.0	3.0	1.67*^{abc}
B (0.1 ml)	7.8	1.2	115.6	2406.6	2160.1	3118.4	3122.7	1561.77*^{c}
C (0.2 ml)	7.8	1.6	49.9	1670.8	1784.7	3031.9	3763.1	1472.83*^{c}
D (0.5 ml)	7.8	2.7	176.9	4947.7	6171.3	7821.8	11224.8	4336.14*^{ab}

Note.
PBS = Phosphate buffer saline; ELISA = enzyme-linked immunosorbent assay; NDV = Newcastle disease virus
Chicks were considered positive based on the recommended SP value (>317) of the CIVTEST® AVI NDV ELISA kit;
Values with different superscripts differ significantly at $p < 0.05$;
Values with * are significantly different at $p < 0.05$ when compared to the control;
Values with "a" are significantly different at $p < 0.05$ when compared to the HIPRAVIAR® BPL2 0.1 ml group at the same time point;
Values with "b" are significantly different at $p < 0.05$ when compared to the HIPRAVIAR® BPL2 0.2 ml group at the same time point;
Values with "c" are significantly different at $p < 0.05$ when compared to the HIPRAVIAR® BPL2 0.5 ml group at the same time point

Table 6

Detection of NDV-specific antibody titer by BioCheck CK116 NDV ELISA following vaccination with different doses of HIPRAVIAR® BPL2 vaccine

Vaccine dose	Antibody titer (arithmetic mean) using CIVTEST® AVI NDV kit on different days post-vaccination							Grand mean
	0	7	14	21	28	35	42	
A (PBS)	156.0	43.0	17.0	15.0	44.0	28.0	61.0	52.0^{abc}
B (0.1 ml)	156.0	24.0	434.0	5287.0	6014.0	7197.0	5328.0	3491.43*^{c}
C (0.2 ml)	156.0	30.0	257.0	4636.0	5598.0	7015.0	6882.0	3510.57*^{c}
D (0.5 ml)	156.0	24.0	1262.0	12239.0	14520.0	13140.0	15501.0	8120.29*^{ab}

Note.
PBS = Phosphate buffer saline; ELISA = Enzyme-linked immunosorbent assay; NDV = Newcastle disease virus
Chicks were considered positive based on the recommended SP value (>1159) of the BioCheck CK116 NDV ELISA test;
Values with different superscripts differ significantly at $p < 0.05$;
Values with * are significantly different at $p < 0.05$ when compared to the control;
Values with "a" are significantly different at $p < 0.05$ when compared to the HIPRAVIAR® BPL2 0.1 ml group at the same time point;
Values with "b" are significantly different at $p < 0.05$ when compared to the HIPRAVIAR® BPL2 0.2 ml group at the same time point;
Values with "c" are significantly different at $p < 0.05$ when compared to the HIPRAVIAR® BPL2 0.5 ml group at the same time point

evaluate the immune response generated following inoculation of different doses of commonly used inactivated Newcastle vaccine to chicks. It was evaluated weekly during the entire life of a typical broiler chicken (42 days of life).

A previous study has reported that positive HI test results were a good indicator of immunity (Czifra et al., 1998). Our findings agree with Igwe et al. (2019), who reported that increased doses of the LaSota vaccine in broiler chickens significantly increased the antibody response to ND.

Based on the HI results, birds vaccinated with 0.5 ml HIPRAVIAR® BPL2 vaccine showed a statistically significant higher antibody response titer than the other doses (Table 2).

The sera were collected at 0, 7, 14, 21, 28, 35, and 42 days post-vaccination (dpv) to determine the HI NDV antibody titer. Pre-vaccination screening confirmed that the 1-day-old chicks were free of NDV HI antibodies. As shown in Table 2, the chicks from group A inoculated with PBS remained negative throughout the study. At the same time, treatment group D (0.5 ml) developed the highest HI antibody titer, followed by group B (0.1 ml) and group C (0.2 ml). Generally, the mean $\log_2$ HI NDV antibody titer for groups B, C, and D gradually increased from 7 to 42 dpv (Table 2). Group B HI NDV antibody titer increased from 1.89±1.23 to 4.23±1.93, group C increased from 1.71±1.15 to 4.10±2.47, and group D increased from 2.46±1.83 to 7.46±0.85. The percentage distribution of HI positivity amongst the different days post-vaccination showed that all the birds were positive at 28 days following vaccination with HIPRAVIAR® BPL2 0.5 ml, while 80 and 70% of the birds were positive at 42 days post-vaccination with HIPRAVIAR® BPL2 0.1 and 0.2 ml, respectively (Table 3).

HI NDV antibody titer in group B reached 80% seropositive birds with 4 $\log_2$ antibody titer. Group C showed similar results, reaching 70% seropositive birds with 4 $\log_2$ antibody titer. Group D showed excellent results with 100% seropositive birds with 7 $\log_2$ antibody titer. According to Mahamud et al. (2022), the mean HI antibody titer of 7 $\log_2$ following vaccination of SPF chickens with live ND vaccine can confer 100% protection against challenges with virulent NDV genotype VII. Further study involving challenge trials is required to confirm the protective immunity of the studied inactivated vaccine. However, in many Southeast Asia countries where ND is endemic, commercial broiler chickens were vaccinated with a combination of live and inactivated to induce vaccine-induced protective immunity (Dimitrov et al., 2017).

Besides HI titers, ELISA is another routine serological assay to determine ND vaccination profiles (Aldous et al., 2003; Miller et al., 2007; Mahamud et al., 2022). Previous studies have also evaluated the correlation of antibody titers detected using both assays in the detection of vaccine-induced immunity (Brown et al., 1990). In this study, the ELISA was performed using the CIVEST® AVI NDV and BioCheck CK116 NDV kit (Tables 4, 5, and 6). Both ELISA kits could detect 100% seropositive

birds in group D starting from 21–42 days post-vaccination. Furthermore, 90% of the birds were seropositive at 21–42 dpv with HIPRAVIAR® BPL2 0.1 ml using a BioCheck CK116 NDV kit (Tables 4 and 5). Although the antibody titer-positive birds were 80% at 21, 28, and 42 dpv with HIPRAVIAR® BPL2 0.1 ml after being tested with CIVTEST® AVI NDV kit, 90% of the birds, however, were positive at 35 days (Tables 4 and 6). When tested using both kits, there was a steady 70% positivity at 21–42 dpv of HIPRAVIAR® BPL2 0.2 ml (Table 4). This steady 70% positivity in this group is probably associated with poor vaccine administration, as the 30% negative birds did not seroconvert. Thus, there are no significant differences between the antibody titers measured using Biocheck CK116 and CIVTEST® AVI NDV kits, even though there is some variation in the percentage coefficient of these ELISA kits. The variations are probably associated with the inherent differences in the assay on the quality and quantity of the NDV antigen and other reagents used (Mao et al., 2022).

The serological method used in this study showed Group B detected 80% (HI), 80% (CIVEST® AVI NDV), and 90% (BioCheck CK116 NDV) seropositive birds following vaccination with 0.1 ml of HIPRAVIAR® BPL2. On the other hand, group C could only reach 70% seropositivity detected using all methods. Meanwhile, group D shows the highest seropositivity at 100% using all serological methods.

CONCLUSION

The study showed birds vaccinated with the HIPRAVIAR® BPL2 vaccine at different doses, thus significantly seroconverted compared to the control group. The variation of antibody titer detection using serological methods such as HI and ELISA of two different kits does not affect the seropositivity of the birds. Group B reached 80% (HI), 80% (CIVEST® AVI NDV), and 90% (BioCheck CK116 NDV) seropositivity. Group C reached 70% seropositivity detected using all methods. Meanwhile, using all serological methods, group D reached the highest seropositivity at 100%. However, groups B and C were only able to achieve 4 $\log_2$ antibody titer compared to group D 7 $\log_2$, which is usually associated with protective immunity. Further study involving challenge trials is required to confirm the protective immunity of the studied inactivated vaccine. The titers detected in the groups indicate the ability of the vaccine to generate a dose-dependent seroconversion as group D, 0.5 ml, had the higher seroconversion compared the groups B, 0.1 ml, and C, 0.2 ml. The negative seroconversion on HI in some of the samples from Groups B and C indicates a challenge to vaccine application in a lower dose administration as some birds did not seroconvert later.

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The Effect of Different Lignocellulose Biomass-based Substrates on the Enhancement of Growth, Yield, and Nutritional Composition of Grey Oyster Mushrooms

Noor Hafizoh Saidan, Mohd Shahrul Ridzuan Hamil, Khomaizon Abdul Kadir Pahirul Zaman, Nik Nur Azwanida Zakaria, Norsyafiera Syuhada Fadzil and Kharul Azmi Muazzam Abdul Rahman*

Faculty of Agro-Based Industry, Universiti Malaysia Kelantan, 17600 Jeli, Kelantan, Malaysia

ABSTRACT

The local agricultural industry has produced a lot of biomass waste from agro-based materials, which contain much lignocellulose that can be used as substrates for oyster mushrooms (*Pleurotus ostreatus*) cultivation. This study aims to compare the effect of different lignocellulose biomass-based substrates on oyster mushrooms' growth, yield, and nutritional composition. Three different substrates (cassava peel, sugarcane bagasse, and sawdust) were prepared, and the growth response was observed and measured. The fastest mycelial colonization was achieved from sugarcane bagasse after 29 days of cultivation. Oyster mushrooms grown with sugarcane bagasse substrate showed the highest carbohydrate, fiber, and energy content with 10.70%, 7.70%, and 52.00 kcal, respectively, compared to mushrooms grown with other substrates. The biological efficiency of sugarcane bagasse is also comparable to sawdust. Thus, it can be concluded that sugarcane bagasse has a high potential to be used as an alternative biomass-based substrate for cultivating *P. ostreatus* with enhancement on the growth, yield, and nutritional composition.

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E-mail addresses:
hafizoh.s@umk.edu.my (Noor Hafizoh Saidan)
shahrul.ridzuan18@gmail.com (Mohd Shahrul Ridzuan Hamil)
khomaizon@umk.edu.my (Khomaizon Abdul Kadir Pahirul Zaman)
azwanida@umk.edu.my (Nik Nur Azwanida Zakaria)
syafierafz94@gmail.com (Norsyafiera Syuhada Fadzil)
kharul.am@umk.edu.my (Kharul Azmi Mu'azzam Abdul Rahman)
*Corresponding author

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INTRODUCTION

Grey oyster mushroom (*Pleurotus ostreatus*), which belongs to the family Pleurotaceae, is widely cultivated in the global market as they contain high protein and carbohydrates,

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multivitamins, minerals, and folic acid that are good for health (Sanchez, 2010). The mineral content of mushrooms, such as calcium, salt, potassium, phosphorus, and folic acid, exceeds that of fish meat, making them a good source for improving bloodstream circulation and preventing anemia (Kalac, 2013). Furthermore, due to the low carbohydrate, calorie, and salt content of mushrooms, they are safe to take by patients with kidney and cardiac problems. Apart from the traditional nutrients, they are also considered functional and valuable for nutraceutical, pharmaceutical, and cosmetic products (Morris et al., 2016). In Malaysia, about 1,000 tonnes of this mushroom is grown annually for the local and export markets (Amin et al., 2014). *Pleurotus ostreatus* possess medicinal values such as antioxidant (Mitra et al., 2013), antibacterial (Vamanu, 2012), antidiabetic (Ghaly et al., 2011), antitumor (Devi et al., 2013), and antihypercholesterolic (Deepalakshmi & Sankaran, 2014) potentials.

In the natural environment, *P. ostreatus* grows on decaying substances, requiring carbon, nitrogen, and micro minerals as their nutritional sources. *Pleurotus* mushroom species are the most efficient species at decomposing a complex lignocellulose substrate from agricultural waste using lignocellulosic enzymes into a simpler compound as their nutrition source (Kumla et al., 2020). *Pleurotus ostreatus* is easy to cultivate and can thrive at various temperatures. Furthermore, they require a short growth time and are less susceptible to diseases and pests (Tesfaw et al., 2015). A high percentage of fruiting bodies harvested from suitable growth substrates will increase profitability using low-cost cultivation technology (Baysal et al., 2003).

Sawdust has become the main substrate in oyster mushroom cultivation (Rizki & Tamai, 2011). The typical lignocellulose components in sawdust are cellulose (39%), hemicellulose (29%), lignin (28%) and ash (4%) (Petchpradab et al., 2009). Although proven as an effective substrate to date, the continual usage of sawdust could potentially contribute to the reduction of wooded areas and might lead to a shortage of the material in the future. Additionally, the high demand for sawdust from rubber palm has caused a spike in its price, which burdened mushroom growers (Marlina et al., 2015). Lignocellulose biomass-based materials from agricultural waste such as cassava peels, sugarcane bagasse, cottonseed hulls, rice, and wheat straws contain high amount of carbon and nitrogen, which makes them suitable candidates for mushroom substrates (Verma et al., 2013). In Malaysia, a vast amount of unused lignocellulose agricultural waste such as cassava peel and sugarcane bagasse are available, and they are frequently thrown in dump sites, burned, or left to decay in the field resulting in environmental pollution (Tesfaw et al., 2015). Cassava is regarded as one of the most commercially important crops in Malaysia, growing either for fresh consumption or for industrial processing as tapioca flour. In cassava processing, a considerable quantities of cassava peel are generated where the thickness of peel

is about 1 ± 4 mm and it may consist of 10 to 13% of total dry matter in cassava root (Ezekiel et al., 2010). Cassava peel is high in energy and nutritional value, with a substantial amount of cellulose, hemicellulose and lignin content. It is more suitable for mushroom cultivation substrate than rice and wheat straw since it contains a lower ash concentration (Baah et al., 2011). With a projected boost in cassava production, waste production is expected to rise considerably. Although cassava peels can be used as livestock feed, the large numbers produced and the remoteness of many areas where processing occurs result in a lot of waste.

Sugarcane has been identified as one of the plants with the most excellent bioconversion efficiency of acquiring sunlight via photosynthesis, able to fix approximately 55 tons of dry matter on each hectare of land every year (Betancur & Pereira, 2010). The sugarcane stalk is divided into two parts, an interior pith containing most of the sucrose and an exterior containing lignocellulosic fibers. During the sugar production process, the stalk is pulverized for sucrose extraction and this process generates a substantial amount of residue (around 240 kg of bagasse with 50% moisture per tons of sugarcane) containing pulverized rind and pith fibers (Dias et al., 2009). The bagasse of sugarcane is made up of complex lignocellulose components such as cellulose (33-36%), hemicellulose (28-30%), and ash (4%), which is almost identical to the commercial base medium, sawdust (Betancur & Pereira, 2010). Any lignocellulose-containing agricultural by-product can be utilized as a viable substrate for *P. ostreatus* cultivation.

Utilizing lignocellulose waste for sustainable oyster mushroom cultivation is the best way for waste-to-wealth transformation that can solve an environmental pollution issue caused by agricultural waste and generate high profit from the production of high quality, large quantity, and nutritional oyster mushrooms. Additionally, using agricultural biomass can prevent mushroom growers from spending too much money on purchasing expensive sawdust raw materials. The present study aims to compare the effect of using different agricultural waste materials as substrates on the growth, yield, and nutritional composition of *P. ostreatus* oyster mushrooms.

MATERIALS AND METHODS

Spawn and Lignocellulose Substrates Preparation

Spawn of *P. ostreatus* were obtained from C & C Mushroom Cultivation Farm Sdn. Bhd, Johore, Malaysia. Fresh cassava peel, sugarcane bagasse, and sawdust were obtained from local food processing factories and sawmills in Kelantan, Malaysia. Cassava peels and sugarcane bagasse were cut into smaller pieces, dried, and ground into powder using a grinder. A total of 5 replicates were prepared for each different media using the following formula as described by Fasehah and Shah (2017):

Substrate : Rice bran : Calcium carbonate (100 : 10 : 1) (1)

A total of 3 kg of each media was weighed separately and put in a container, followed by the addition of 300 g of rice bran and 30 g of calcium carbonate. The mixture was then mixed thoroughly prior to the addition of water (80%) to the total weight of the mixture. The media was mixed properly until the water was absorbed. After that, the media was placed in a polyethylene bag (6 cm × 12 cm), compressed, and closed with polyvinyl chloride (PVC) necks, which were covered with a cap and sterilized at 100°C for 8 hr in a steamer.

After sterilization, the bags were left to cool down, followed by inoculation with 5 g of the spawn. The inoculated bags were then incubated at room temperature (25°C) for approximately 45 days. After incubation, the bags were shifted to a cropping room, where the bags were placed on a horizontal shelf. The room temperature was maintained at 25°C with 80–85% humidity by spraying water on the floor periodically.

Mycelium Growth and Yield Analysis

The mycelium growth of each substrate (n = 5) was measured every three days starting after the inoculation process until they filled the bags using a measuring tape (Marlina et al., 2015). Four flushes of mushrooms were harvested for each substrate bag. The growth from the inoculation stage to the first harvest and total harvesting time was observed and recorded. The stipe length, thickness, cap diameter, and the number of fruiting bodies from every flush were also measured and recorded. The mushroom's total yield (Y) and biological efficiency (BE) were calculated at the end of the harvesting period. BE refers to the weight (g) of fruiting bodies ratio for each dry weight (g) of substrates, and it was expressed in percentage (C. H. Liang et al., 2019). The equations used to calculate both BE and Y are shown below:

$$BE = \frac{MFW}{SDW} \times 100 \quad (2)$$

$$Y = \frac{MFW}{SDW} \quad (3)$$

where,
MFW = Mushroom fresh weight (g)
SDW = Substrate dry weight (g)

Meanwhile, the analysis for moisture, ash, protein, carbohydrate, dietary fiber, and energy content of mushrooms from each substrate was determined using the method as described by AOAC Method 991.43 (AOAC International, 2005).

Statistical Analysis

The data were subjected to analysis of variance (ANOVA), and the significant differences were determined using Duncan's multiple range test ($p < 0.05$) using SPSS (version 22.0).

RESULTS AND DISCUSSION

Mycelial Growth

Three different substrates, viz. cassava peel, sugarcane bagasse, and sawdust, were

investigated to determine the daily growth responses of *P. ostreatus*, and the result is shown in Table 1. No growth was observed during the first two days of inoculation, whereas the first growth was recorded from day three of inoculation for all the tested substrates. There is a significant mycelial growth after 7 days of inoculation with sugarcane bagasse recorded the fastest growth (4.49 ± 0.11 cm), followed by cassava peel (4.28 ± 0.25 cm) and sawdust (4.22 ± 0.07 cm). There is a significant mycelial growth at day 3 of inoculation with sugarcane bagasse recorded the fastest growth (1.84 ± 0.28 cm), followed by cassava peel (1.23 ± 0.10 cm) and sawdust (0.83 ± 0.27 cm). Mycelial growth serves as an essential initial phase in mushroom cultivation as it aids in the colonization of substrate and permit mushroom growers to determine the optimum growth of mushroom (Pokhrel et al., 2013). Additionally, the rapid growth of mushroom mycelium can prevent contamination from other competing bacteria and fungi (Jonathan et al., 2008).

From day 12, sawdust showed the fastest mycelial growth with 5.66 ± 0.02 cm, followed by sugarcane bagasse and cassava peel (5.60 ± 0.11 and 5.49 ± 0.17 cm), respectively. Mycelial growth at day 30 was measured at 10.13 ± 0.03 and 10.12 ± 0.04 cm for sawdust and sugarcane bagasse, respectively. However, cassava peel's slowest mycelial growth was recorded as substrate at 7.79 ± 0.22 cm. Mycelial growth is a preliminary step that creates suitable internal conditions for fruiting. Thus, the outstanding growth of mycelium is a vital factor in mushroom cultivation (Zubairi et al., 2022). Mushroom primarily requires nitrogen for optimal growth. Sawdust contains a high amount

Table 1

Effect of different substrates on mycelial growth of oyster mushrooms

Days of mycelial growth	Cassava peel (cm)	Sugarcane bagasse (cm)	Sawdust (cm)
1	-	-	-
2	-	-	-
3	1.23 ± 0.10^{b}	1.84 ± 0.28^{a}	0.83 ± 0.27^{a}
5	3.11 ± 0.06^{a}	3.45 ± 0.07^{a}	1.76 ± 0.34^{b}
7	4.28 ± 0.25^{a}	4.49 ± 0.11^{a}	4.22 ± 0.07^{a}
12	5.49 ± 0.17^{a}	5.60 ± 0.11^{a}	5.66 ± 0.02^{a}
16	7.08 ± 0.08^{b}	6.87 ± 0.31^{b}	7.96 ± 0.07^{a}
21	7.28 ± 0.03^{b}	8.32 ± 0.19^{a}	8.43 ± 0.33^{a}
25	7.43 ± 0.05^{b}	9.53 ± 0.11^{a}	9.47 ± 0.09^{a}
30	7.79 ± 0.22^{b}	10.12 ± 0.04^{a}	10.13 ± 0.03^{a}

Note. Superscripts with different letters across the rows are significantly different ($p < 0.05$)

of nitrogen (1.68%), which makes it easy for the mycelium to colonize the bag (Tabi et al., 2008). Oyster mushrooms grown on cassava peel showed the slowest colonization due to the low availability of nitrogen in the substrate compared to other substrates (Burns et al., 2012). Nitrogen is a vital element in mushroom growth used in cellular functions and numerous metabolic activities, especially in synthesizing proteins and enzymes. This element is utilized abundantly for mycelial growth during fructification. Generally, the nitrogen content in mycelia is in the range of 3–6%. Thus, nitrogen scarcity in the substrate during the fructification stage could lead to a reduction in the mushroom yield (Upadhyay et al., 2002).

Yield and Physical Characteristics

The colonization period, first harvest day, and morphological characteristics of mushrooms cultivated with different substrates are shown in Table 2. Total colonization of the substrates was completed between 29–51 days of incubation. The day for the first harvest of mushrooms was between 42–64 days, depending on the substrate used. The fastest colonization period (29 ± 0.58 days) and first harvest period (42 ± 0.58 days) were recorded from sugarcane bagasse and sawdust. There were no significant differences recorded for total colonization and the first harvest period for sawdust between sawdust and sugarcane bagasse. However, cassava peel took significantly longer for colonization and first harvest harvest as compared to sugarcane bagasse and sawdust with 51 ± 0.58 and 64 ± 0.58 days, respectively.

There was a significant difference in the cap diameter and stipe thickness of *P. ostreatus* grown on different substrates. The widest cap diameter was 10.10 ± 1.95 cm for sugarcane bagasse substrate, and the smallest was 6.70 ± 0.72 cm from

Table 2

Effect of different substrates on colonization period, first harvest, and morphological characteristics of the fruiting body of the oyster mushroom

Substrate	Total colonization period (day)	First harvest (day)	Cap diameter (cm)	Stipe length (cm)	Stipe thickness (cm)	No. of fruiting bodies	Weight (g)
Cassava peel	50.67 ± 0.58^{a}	64.33 ± 0.58^{a}	6.70 ± 0.72^{b}	11.57 ± 1.17^{ns}	0.80 ± 0.10^{c}	4.67 ± 2.52^{ns}	30.70 ± 6.46^{ns}
Sugarcane bagasse	29.33 ± 0.58^{b}	42.33 ± 0.58^{b}	10.10 ± 1.95^{a}	11.17 ± 2.08^{ns}	1.73 ± 0.25^{a}	5.33 ± 6.66^{ns}	34.20 ± 2.75^{ns}
Sawdust	29.67 ± 0.58^{b}	43.33 ± 0.58^{b}	7.83 ± 0.51^{ab}	11.83 ± 2.89^{ns}	1.23 ± 0.12^{b}	8.33 ± 2.08^{ns}	50.03 ± 5.32^{ns}

Note. Superscripts with different letters within the column are significantly different ($p < 0.05$); ns = Not significant

P. ostreatus grown with cassava peels. The cap diameter for sawdust was 7.83 ± 0.51 cm and was not significantly different from sugarcane bagasse and cassava peel. The stipe thickness was the highest from sugarcane bagasse with 1.73 ± 0.25 cm, followed by sawdust (1.23 ± 0.12 cm) and cassava peel (0.80 ± 0.10 cm). The thickness measurements significantly differed among all the mushroom samples grown with different substrates. On the contrary, there was no significant difference in stipe length on all the substrates. Our result was different compared to previous study by Elsisura et al. (2022), whom reported 100% sawdust showed the longest stipe (19.45 cm). Mushroom size depends on the types of substrates used. Cellulosic, hemicellulosic, and lignin materials could be a physical barrier that led to the difficulty in breaking them down without lignin-degrading enzymes. Additionally, ecological factors such as compactness or compressibility also play an essential role in affecting the height of the stipe and diameter of the pileus (Sanjel et al., 2021), which explain different stipe lengths obtained from the current and previous study.

The number of fruiting bodies harvested from the different substrates is shown in Figure 1. The highest number of fruiting bodies and mushroom weight were recorded from sawdust with 8.33 ± 2.08 and 50.03 ± 5.32 g, followed by sugarcane bagasse (5.33 ± 6.66 and 34.20 ± 2.75 g) and cassava peel (4.67 ± 2.52 and 30.70 ± 6.46 g), respectively. The differences in both parameters, however, were not statistically significant. Different substrates provide different nutrients composition for mushroom growth. Averagely, sawdust and sugarcane bagasse substrates produced mushrooms with better physical characteristics than cassava peel substrates. It is probably due to the cassava peel's low nitrogen content (Burns et al., 2012), a crucial element in mushroom growth (Naraian et al., 2009). The current finding is supported by S. Ahmed (1998), who emphasized that oyster mushroom growth was best achieved

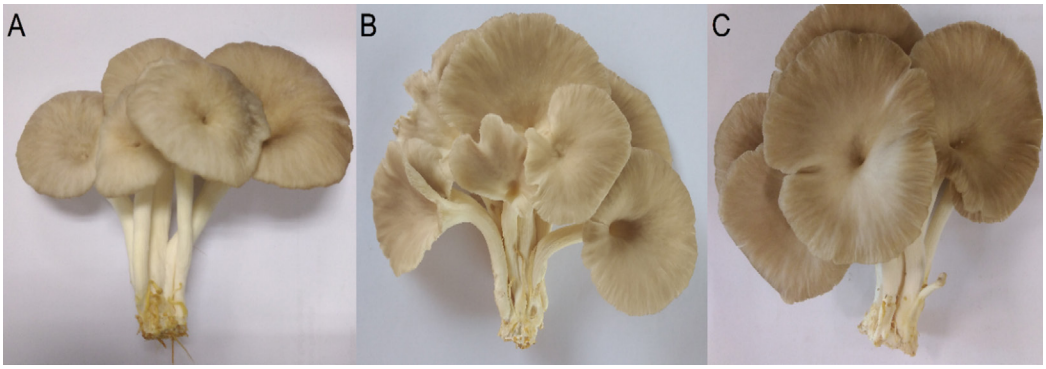

Figure 1. Fruiting bodies of *Pleurotus ostreatus* mushroom grown on different substrates: (A) cassava peels; (B) sugarcane bagasse; and (C) sawdust, respectively

using sawdust and sugarcane bagasse as substrates compared to other agricultural wastes. Sawdust and sugarcane bagasse are lignocellulosic agricultural wastes comprising three major components, lignin, cellulose, and hemicellulose, which could serve as a promising substrate for mushroom cultivation. *Pleurotus* species possess extensive enzyme systems that can degrade complex organic compounds efficiently. The degraded materials would then be utilized to produce mushroom fruiting bodies (Grimm & Wösten, 2018; Sanchez, 2009).

The characteristics of fruiting bodies that were recorded for 5 days prior harvest is shown in Figure 1 whereas the mean growth for mushroom pinhead on different substrates is presented in Table 3. On the first day, there was no significant difference in cap diameter and stipe length between the three substrates cultivated. Stipe thickness for mushrooms grown on sugarcane bagasse was significantly higher compared to cassava peels. There was no significant difference in stipe thickness between cassava peels and sawdust. Cap diameter and stipe thickness for sugarcane bagasse were the highest (8.64 ± 1.49 and 1.60 ± 0.24 cm) on day 3 of pinhead growth. The same parameters were significantly lower in cassava peels, with 5.43 ± 0.60 and 0.63 ± 0.11 cm, respectively. Cap diameter and stipe thickness for mushroom grown on sawdust were not significantly different as compared to sugarcane bagasse. According to Gunde-Cimerman and Cinerman (1995), the diameter range for the oyster mushroom caps is 5 to 25 cm during maturity, and the results obtained in the current study are still within that range. On the other hand, the stipe length of mushrooms grown on all substrates ranged from 9.10 to 9.98 cm, but the difference was insignificant. On day 5 prior to harvesting process,

Table 3

Effect of different substrates on the pinhead growth of oyster mushroom

Time	Substrate	Cap diameter (cm)	Stipe length (cm)	Stipe thickness (cm)
1 day	Cassava peel	1.09 ± 0.06^{d}	4.59 ± 0.43^{c}	0.51 ± 0.17^{c}
	Sugarcane bagasse	1.08 ± 0.52^{d}	4.28 ± 0.52^{c}	1.28 ± 0.27^{ab}
	Sawdust	1.05 ± 0.07^{d}	3.95 ± 0.26^{c}	0.89 ± 0.04^{c}
3 days	Cassava peel	5.43 ± 0.60^{c}	9.44 ± 1.16^{b}	0.63 ± 0.11^{c}
	Sugarcane bagasse	8.64 ± 1.49^{ab}	9.10 ± 2.49^{b}	1.60 ± 0.24^{ab}
	Sawdust	6.38 ± 0.58^{b}	9.98 ± 3.31^{b}	1.16 ± 0.12^{b}
5 days	Cassava peel	6.70 ± 0.72^{b}	11.57 ± 1.17^{a}	0.80 ± 0.10^{b}
	Sugarcane bagasse	10.10 ± 1.95^{a}	11.17 ± 2.08^{a}	1.73 ± 0.25^{a}
	Sawdust	7.83 ± 0.51^{ab}	11.83 ± 2.89^{a}	1.23 ± 0.12^{ab}

Note. Superscripts with different letters within the column are significantly different ($p < 0.05$)

it was observed that sugarcane bagasse and sawdust have significantly larger cap diameter (10.10 ± 1.95 and 7.83 ± 0.51 cm) and stipe thickness (1.73 ± 0.25 and 1.23 ± 0.12 cm), respectively compared to cassava peels. Cassava peels recorded smaller cap diameter and less thickness, with 6.70 ± 0.72 and 0.80 ± 0.10 cm, respectively. The stipe length for mushrooms grown on cassava peel, sugarcane bagasse, and sawdust were 11.57 ± 1.17, 11.17 ± 2.08, and 11.83 ± 2.89 cm, respectively, but the differences were not significant. The fruiting body of the oyster mushroom involving the stipe length, pileus width, and stipe girth varies depending on the types and mixtures of agricultural wastes used as substrates in mushroom cultivation. Different agricultural waste combinations produced different sizes of mushrooms whereas single agricultural waste composition produced uniform sizes (Chukwurah et al., 2013).

For each of the substrates tested in the current study, four flushes mushroom flushes were harvested for each of the substrates tested in the current study, and the result of its yield and BE is shown in Table 4. All substrates produced the highest yield during first flush. The yield was the highest in the first flush of all substrates. The study revealed that the yield for sawdust (131.69 ± 13.25 g) was significantly higher compared with the other two substrates. The mushroom yield was not significantly different between sugarcane bagasse and cassava peels. BE of mushroom cultivated on sawdust (43.87 ± 4.41%) was significantly higher compared to sugarcane bagasse and cassava peels (30.90 ± 1.40% and 26.86 ± 4.05%), respectively. The results of this study were similar to the study conducted by Kortei et al. (2014) and Z. C. Liang et al. (2011). Z. C. Liang et al. (2011) reported BE of oyster mushrooms grown on grass plant stalks ranging from 39.55 to 58.33%, whereas Kortei et al. (2014) reported that BE for oyster mushroom grown on various mixtures of cassava peel substrates were in the range of 26.0–42.4%. BE measures substrate conversion efficiency in mushroom cultivation, representing the ratio of harvested yield to the substrate's dry weight. Depending on the types of agricultural waste

Table 4

Effect of different substrates on yield and biological efficiency (BE) of oyster mushroom

Substrate	1st flush (g/bag)	2nd flush (g/bag)	3rd flush (g/bag)	4th flush (g/bag)	Total yield (g/bag)	BE (%)
Cassava peel	30.70 ± 6.46^{b}	20.56 ± 4.02^{b}	16.49 ± 0.92^{b}	12.87 ± 0.87^{b}	80.62 ± 12.15^{b}	26.86 ± 4.05^{b}
Sugarcane bagasse	34.20 ± 2.75^{b}	22.48 ± 4.33^{b}	18.97 ± 1.89^{b}	17.07 ± 1.74^{a}	92.72 ± 4.19^{b}	30.90 ± 1.40^{b}
Sawdust	50.03 ± 5.32^{a}	36.77 ± 5.67^{a}	25.85 ± 1.15^{a}	19.04 ± 1.27^{a}	131.69 ± 13.25^{a}	43.87 ± 4.41^{a}

Note. Superscripts with different letters within the column are significantly different ($p < 0.05$)

substrate used in mushroom cultivation, the BE percentage varied among the substrate (Girmay et al., 2016).

Nutritional Analysis

The nutritional composition of mushrooms cultivated on different substrates is shown in Table 5. Moisture content is a crucial analysis to determine the mushroom quality. Low moisture can cause textural changes in mushrooms (shrinkage) and loss of weight, which will affect the economic value of the produce. High moisture content will favor microbial growth and discoloration (Singh et al., 2010). The findings revealed that moisture content was significantly higher in sawdust substrates (89.00 ± 0.36%) than in cassava peels and sugarcane bagasse. There was no significant difference between cassava peels and sugarcane bagasse. This result is in line with the study conducted by Hoa et al. (2018), which reported that the moisture content was 86.95–92.45% for substrates containing sugarcane bagasse and sawdust. Similar moisture content (80.00–92.50%) was also reported for *Pleurotus* species grown on different lignocellulose waste (S. A. Ahmed et al., 2009).

Ash content analysis measures the mineral content in the mushrooms (Alam et al., 2007). Ash content plays a significant role in mushrooms' nutritional and physiochemical properties. Thus, determining the ash content is very important to ensure that no hazardous minerals are present in the mushroom and that it is safe to consume. Based on the current study, ash content was significantly lower in sugarcane bagasse with 7.16 ± 0.05% compared to sawdust. Both cassava peels and sawdust showed higher ash content but no significant different. The present result was slightly lower compared to previous study, which reported that ash content for *P. ostreatus* cultivated on different substrates were ranging from 7.32-7.83% (Kortei et al., 2014). On the other hand, our results were higher than previous study by Hoa et al. (2018), which reported ash content ranging from 5.90 to 7.10%.

Table 5

Effect of different substrates on the nutritional composition of oyster mushroom

Substrate	Moisture (%)	Ash (%)	Carbohydrate (%)	Protein (%)	Fat (%)	Fiber (%)	Energy (kcal/100 g)
Cassava peel	87.56 ± 0.27^{b}	7.20 ± 0.02^{ab}	6.40 ± 0.03^{c}	2.90 ± 0.03^{a}	NA	3.30 ± 0.04^{c}	37.00 ± 0.177^{c}
Sugarcane bagasse	88.01 ± 0.48^{b}	7.16 ± 0.05^{b}	10.70 ± 0.06^{a}	2.20 ± 0.02^{c}	NA	7.70 ± 0.02^{a}	52.00 ± 0.17^{a}
Sawdust	89.00 ± 0.36^{a}	7.25 ± 0.02^{a}	9.50 ± 0.08^{b}	2.40 ± 0.04^{b}	NA	5.80 ± 0.02^{b}	48.00 ± 0.26^{b}

Note. Superscripts with different letters within the column are significantly different ($p < 0.05$); NA = Not available

There was a significant difference in the nutritional composition of carbohydrates, protein, fiber, and energy for all mushrooms cultivated with the three substrates. Mushrooms cultivated on sugarcane bagasse showed the highest carbohydrate, fiber, and energy content with 10.70 ± 0.06%, 7.70 ± 0.02%, and 52.00 ± 0.17 kcal/100 g, respectively. On the other hand, protein content in mushrooms grown with cassava peels was significantly highest, at 2.90 ± 0.03%, compared to other substrates. The study indicated that oyster mushrooms grown on different substrates were rich in carbohydrates, fiber, protein, and energy but had very low-fat content, making them excellent for healthy and low-calorie diets. Sugarcane bagasse contains cellulose and sucrose, which are easily absorbed for the growth of oyster mushrooms (Rajapakse et al., 2007). It, in turn, resulted in the high nutrient content of mushrooms grown on the substrate. The result showed lower carbohydrate content in all the tested substrates compared to the study conducted by Sharma et al. (2013) and Sopanrao et al. (2010), with the carbohydrates amount ranging from 30.24–42.26% and 50.50–55.33%, respectively. In another study, carbohydrate, protein, and fiber content in oyster mushrooms grown on rubber sawdust were reported to be 3.54, 4.00, and 17.27%, respectively (Rashidi &Yang, 2016). Mushrooms are a potential source of dietary fiber due to the presence of non-starch polysaccharides. A high-fiber diet can reduce the risk of heart disease and diabetes (Khan et al., 2008). In the present study, it can be suggested that the mushrooms grown with sugarcane bagasse produce high-quality and nutritious fruiting bodies comparable to mushrooms grown with the commercial substrate, sawdust.

CONCLUSION

Determination of chemical and nutrient composition, especially for the substrate used in commercial cultivation of oyster mushrooms, is essential as it affects mycelium colonization and fruiting body development. The results of the present study showed that sugarcane bagasse and cassava peels have the potential to be substitute materials for sawdust in the cultivation of oyster mushrooms. Mycelial colonization was faster on sugarcane bagasse during the first harvest period and the mushroom showed the highest carbohydrate, fiber, and energy content (10.70%, 7.70%, and 52.00 kcal/100 g, respectively). The physical characteristics of mushrooms grown on sugarcane bagasse are also similar to those grown with commercial sawdust substrates. It can be concluded that sugarcane bagasse is a promising alternative substrate for cultivating high-quality oyster mushrooms and can save on the cost of purchasing sawdust. In return, the profit margin of mushroom growers can be increased, and at the same time, it can attract indigenous farmers to venture into the business of mushroom farming by utilizing local agricultural wastes that are available throughout the year.

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Field and Laboratory Detection of Clove Sumatra Disease Caused by *Ralstonia syzygii* subsp. *syzygii* in Java, Indonesia

Tri Joko*, Arzaq Prabantoro Yuantomoputro, Restu Indrawati, Alan Soffan and Siti Subandiyah

Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Flora Street No. 1, Bulaksumur, Yogyakarta 55281, Indonesia

ABSTRACT

The significant decrease in clove production in Indonesia is mainly due to the Sumatra disease caused by bacterial *Ralstonia syzygii* subsp. *syzygii*. It is necessary to manage the disease broadly based on disease detection in the field and the laboratory. This study aims to determine the technique for detecting the distribution pattern of Sumatra disease using geographic information systems and validate the presence of *R. syzygii* subsp. *syzygii* in clove plant tissues by molecular analyses based on the endoglucanase gene. This research was conducted by acquiring aerial photos using uncrewed aerial vehicles processed using photogrammetric techniques to produce geographic information system outputs as a thematic map of the clove Sumatra disease distribution pattern. The plant samples were collected for molecular analysis of the pathogens causing clove Sumatra disease in the laboratory. DNA extraction was performed and amplified by polymerase chain reaction (PCR) using UGMRss-specific primers followed by Sanger sequencing. The aerial photo images showed that the distribution of clove Sumatra disease has a random pattern, which tends to spread between healthy and diseased plants. Furthermore, the PCR and phylogenetic analyses revealed that all symptomatic clove plant samples (leaves, twigs, and roots) were positively infected by *R. syzygii* subsp. *syzygii*.

Keywords: Endoglucanase, geographic information system, polymerase chain reaction, *Ralstonia syzygii* subsp. *syzygii*, uncrewed aerial vehicle

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E-mail addresses:
tjoko@ugm.ac.id (Tri Joko)
arzaqprabantoro@mail.ugm.ac.id (Arzaq Prabantoro Yuantomoputro)
restuindrawati88@gmail.com (Restu Indrawati)
alan.soffan@ugm.ac.id (Alan Soffan)
sitisubandiyah@ugm.ac.id (Siti Subandiyah)
*Corresponding author

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INTRODUCTION

Clove (*Syzygium aromaticum* L.) is a native Indonesian spice plant with many benefits. It can be used as a cooking spice, traditional medicine, pharmaceutical material, and raw material for the food industry. Haro-González et al. (2021) reported that clove plants contain essential oils found in all plant parts (roots, stems, leaves, and flowers), which have many biological activities, including antibacterial, antifungal, insect repellent, and antioxidant properties. Clove oil is traditionally used as a flavoring and antibacterial agent in food. However, many factors are causing the reduction of clove production, one of which is the infection by a bacterial pathogen, *Ralstonia syzygii* subsp. *syzygii* causing Sumatra disease (Roberts et al., 1990).

Sumatra disease was reported for the first time on Sumatra Island, making it known as Sumatra disease (Waller & Sitepu, 1975). At present, clove Sumatra disease has been reported in several clove plantations in Indonesia (Dwimartina et al., 2017). Clove plants infected with Sumatra disease will exhibit symptoms such as leaf drop and wilting die-back of the plant shoot, beginning with the branches at the top followed by the leaves withering. The leaf drop is followed by shoot death due to the blockage of xylem vessels by the pathogens in plant roots and stems (Bennett et al., 1987). Sumatra disease symptoms generally appear after a long incubation period (Danaatmadja et al., 2009). The development of clove plantations to date has not yet experienced a recovery, with production still fluctuating from around 60,000 to 100,000 tonnes each year (Pratama & Darwanto, 2019). According to Bennett et al. (1985), losses due to Sumatra disease have been estimated to have 10–15% annual production losses. However, since 1996 clove production has decreased rapidly, and the average clove yield loss was approximately 40% yearly.

The effective control measures of the clove Sumatra disease are still not yet satisfied, even though insecticide application to its insect vector can suppress disease spread in a short time (Hartati et al., 1991). Likewise, applying biocontrol agents on diseased clove plants reduced the population of *Hindola striata* and the percentage of plant damage and stimulated the production of young leaf shoots (Mardiningsih et al., 2020).

Detecting Sumatra disease is an important key in making disease management decisions. Detection can be conducted directly in the cultivation area by observing the incidence of the disease and its distribution pattern, or it can be done in the laboratory to determine the cause of the disease. Observing disease incidence in a wide area requires more effort, time, and cost. Therefore, implementing an uncrewed aerial vehicle (UAV), complemented with geographic information systems (GIS), is potentially suitable. Detection in the field will provide current information regarding disease conditions existing in an area. On the other hand, detection in the laboratory is also needed to validate the causes of plant diseases. The polymerase chain reaction (PCR) technique is widely used to detect

plant diseases. This technique is quite sensitive for detecting and identifying plant pathogens (Joko et al., 2019).

This study aims to determine the distribution pattern of Sumatra disease in a wide area by applying UAV technology and GIS. Furthermore, this study also aims to validate the presence of *R. syzygii* subsp. *syzygii* in some clove plant tissues using the PCR technique.

MATERIALS AND METHODS

Study Sites

The research was conducted in a clove plantation in Kalisidi Village, West Ungaran District (coordinates 110°21'47,287"E and 7°8'20,471"S), Central Java Province, and at the Laboratory of Plant Pathology, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia. The location insets were made to identify the location of the research area. The observation area's location and the study's administrative borders were determined using satellite images. Using satellite imagery in the location inset provides wider spatial information so conditions around the land can be known. The satellite images were obtained through a data mining process against the freely available imagery available in the SASPlanet software (https://bitbucket.org/sas_team/sas.planet.bin). The satellite images obtained were then overlaid with polygon vectors to show the location of the observation block. The polygon vector was then saved in shapefiles (shp.) format, resulting in an observation block shape (Figure 1).

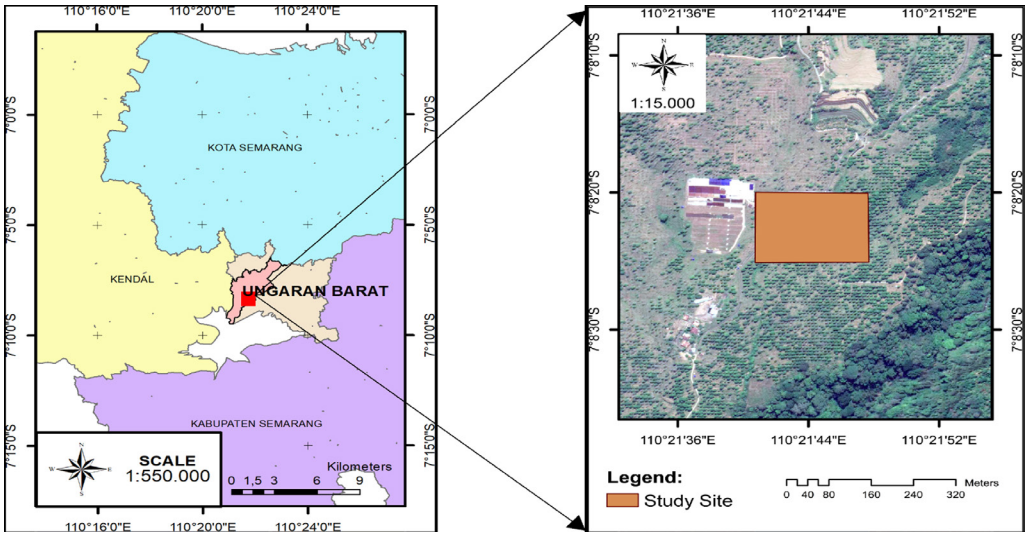

Figure 1. The location insets of the study sites located in Kalisidi Village, West Ungaran District, Central Java Province, Indonesia

Acquisition of Aerial Photography and Photogrammetry

Aerial photography was taken to observe the incidence of Sumatra disease using the UAV. Aerial photo images were obtained using the DJI Phantom 3 Standard quadcopter type UAV (China), with the flight on autopilot following the planned flight plan. The main purpose of flight planning was to obtain navigational data to be transferred to the UAV vehicle. The flight plan was set using the Pix4D Capture software on the smartphone and connected to the remote control (Hung et al., 2019). The Pix4D Capture application created a flight plan to create a 2D map model with an 80% overlap ratio, a camera tilt angle of 60°, and a flight altitude of 80 m (Figure 2). The coverage of aerial photos to be observed was 3 ha. Each flight took 7 min 30 s and produced 69 images. Each image has coordinated data processed using photogrammetry software, namely Agisoft Metashape (Ribeiro-Gomes et al., 2016). Through the Agisoft Metashape application, images previously acquired were processed for alignment. Processing using the Agisoft Metashape application produced a georeferenced (orthomosaic) photo mosaic.

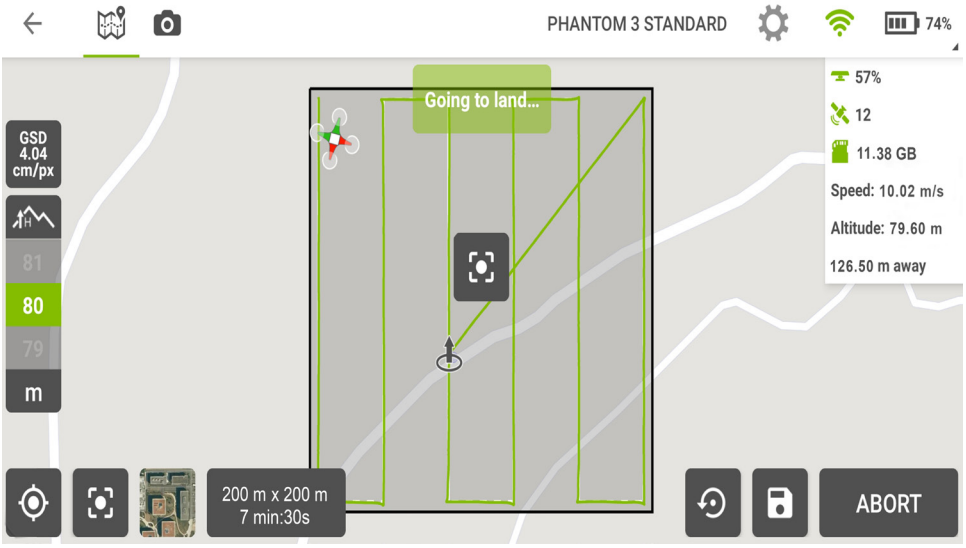

Figure 2. Uncrewed aerial vehicle (*autopilot*) flight plan using the Pix4D Capture application

Digitation on Screen

The photos merged using Agisoft Metashape software were exported to Tag Image File Format (TIFF) referenced with the World Geodetic System (WGS 1984) and Universal Transverse Mercator (UTM Zone 49S) Coordinate System. A coordinate system is a number used to indicate the location of a surface/space, including datums and map projections. The orthophoto results were

processed with on-screen digitization, which converts raster data from remote sensing into vector data accompanied by attributes or additional information about the related object (Figure 3). The digitization process was performed using the ArcGIS 10.8 application.

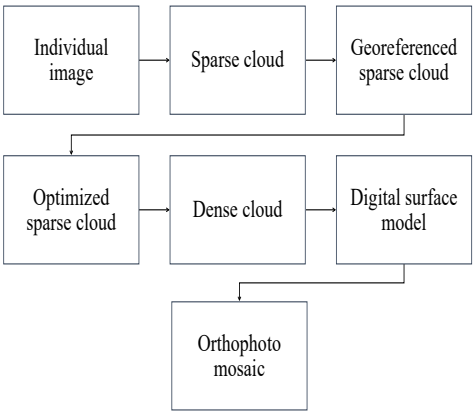

Figure 3. The steps in the process of merging individual photos into an orthophoto mosaic using Agisoft Metashape software referring to Ludwig et al. (2020) with some adjustments

The initial step in the on-screen digitizing process was to identify the area that would be the subject of observation. Because photo mosaic images are frequently less distinct or hazy at the margins, polygon features covering an area of three hectares must be used to establish the area of interest/ region of interest (ROI). Once the ROI is known, the incidence of the disease and the distribution pattern of Sumatra disease are identified. The identification process was conducted using a visual assessment technique, referring to the characteristics of the symptoms of Sumatra disease. Identification was performed by marking the individual clove plants, both sick and healthy, appearing on the photo mosaic (orthomosaic). Each clove tree was marked using a point vector in the image. Zooming to a scale of 1:150 in the defined area allowed the conditions of the plants to be visible. Healthy clove plants appeared completely green (Figure 4a), while diseased clove plants showed signs of shedding marked in gray (Figure 4b). The gray color in the aerial photo image of the clove plant represents the part of the twig seen as a result of the leaves drop. Healthy and diseased clove plants were marked with green and red points, respectively. The marking results were made into a geographic information system as a thematic map of the distribution pattern of Sumatra disease with a scale of 1:3500 (Figure 4c).

Based on the results of visual marking, the number of diseased and healthy clove plants was calculated using the features in ArcGIS 10.8 software, namely the data attribute. The percentage of the incidence of Sumatran disease was calculated using the following formula:

$$\text{Disease incidence (\%)} = \frac{\textit{Number of infected plants units}}{\textit{Total number of units assessed}} \times 100\%$$

(Widyaningsih et al., 2019)

Plant Materials Sampling

For molecular detection of Sumatra disease, plant samples were taken for laboratory testing. Plant samples were collected using the purposive sampling technique on plants with symptoms of Sumatra disease and

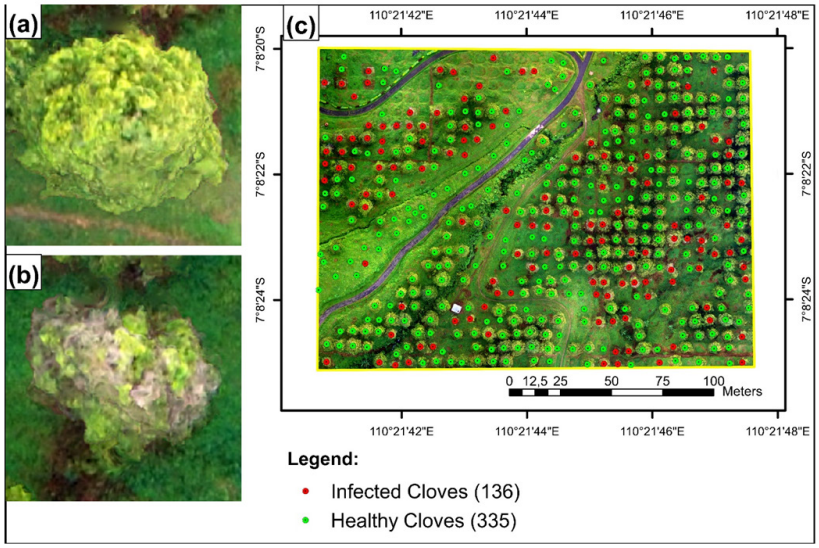

Figure 4. The process of identification of clove plants using aerial photography with a magnification (zooming) to a scale of 1:150 (a) healthy clove plants; (b) diseased clove plants; and (c) map for identification of symptoms of Sumatra disease (scale 1:3500)

healthy plants as control. The plant parts included the clove plant's twigs, leaves, and roots with three replicates. The samples were then brought to the laboratory and stored in a refrigerator.

Polymerase Chain Reaction (PCR) Amplification

DNA extraction was carried out using the ZymoBIOMICS™ DNA Mini Kit (USA) following the protocol contained in the Mini Kit. A total of 0.15 g of samples of twigs, leaves, and roots were added with liquid nitrogen and then crushed with a sterile mortal gun. Furthermore, the detection of *R. syzygii* subsp. *syzygii* in plant tissue samples was conducted by DNA amplification using the PCR technique. The extracted DNA was amplified using a specific primer designed by Trianom et al. (2018), namely UGM RssF: 5'-GCTCACCATCGCCAAGGACAGCG-3' and UGM RssR: 5'-TTCGATCGAACGCCTGGTTGAGC-3'. The PCR reaction process was run using a Bio-Rad T100 machine (Germany) with the following reaction composition: GoTaq Green master mix (Promega) (5 µl); UGMRss-F primer (1 µl); UGMRss-R primer (1 µl); and DNA (3 µl). The mixtures were mixed in a microtube and homogenized gently. Amplification by PCR machine was performed at 96°C for 5 min for initial denaturation, followed by 30 cycles of a denaturation process at 94°C for 15 s, an annealing process at 59°C for 30 s, and an extension process at 72°C for 30 s, which was then proceeded with the final extension at 72°C for 10 min.

The amplification results were analyzed to observe DNA fragments through

electrophoresis using agarose gel in 1× TBE (Tris-HCl, boric acid, EDTA) buffer at 70 V for 45 min. DNA fragments were measured using a 1 kb DNA ladder marker (Promega, USA). The gel was then placed in an electrophoresis machine with an electrophoresis buffer, and the machine was run at 70 V for 45 min. After completion, the gel was immersed in ethidium bromide (EtBr) solution for 20 min. The gel was observed under ultraviolet (UV) light using a UV transilluminator (Optima, Japan) to observe the DNA bands formed (Navitasari et al., 2020).

Sequencing and Phylogenetic Analysis

The PCR results were then sent to a DNA sequencing service provider (1st BASE, Malaysia) to determine the nucleotide sequence. Sequencing results were analyzed using the Basic Local Alignment Search Tool (BLAST) program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to be compared with the type strains references through the data mining process at GenBank. Then the similarity analysis was conducted by making a phylogenetic chart using the Mega 11 program. The phylogenetic tree was arranged using the maximum likelihood (ML) tree method (Rahma et al., 2020).

RESULTS AND DISCUSSION

Distribution Pattern of Sumatra Disease Using Geographic Information System

Regular monitoring of the incidence of Sumatra disease on cultivated land is one of the epidemiological approaches to prevent the expansion of the infected area. The UAV used to monitor the health condition of clove plants can acquire aerial photos of clove land and produce high-resolution aerial photo data. In this observation, the UAV's acquisition of merged aerial photos (photomosaic) can identify healthy and diseased clove trees (Figure 5). Although it looks blurry due to the overlapping process between photos, the individual clove plants can still be seen clearly. Healthy clove plants showed denser shoots and green leaves, while the diseased clove plants showed a leaf drop. The grey color in the aerial photo image indicates leaf drops in the diseased clove plants. The grey color represents the color of the twigs uncovered by leaves. Plant marking was conducted with the point vector feature in the ArcGIS 10.8 program after the aerial shot image of healthy and diseased clove plants could be recognized.

Figure 5. Raster display of aerial images on clove plantation land

The success of clove plant identification is highly dependent on the resolution of the photo used. The spatial characteristics of the aerial photo image of clove plantation conditions can be seen in the raster display (Figure 5). A raster is a data structure that represents the arrangement of color pixels. Caution is needed to identify individual clove plants, especially those still young because their small size makes them look biased when viewed from aerial photo images. A larger zoom is required for clearer observation of the young tree to avoid biases.

Identifying and mapping healthy and diseased plants is needed to monitor disease development and distribution of disease incidence, assess the impact of decreasing productivity, determine possible treatments to extend plant life, and estimate the replanting time (Ouyang et al., 2020). The distribution pattern and incidence of Sumatra disease were determined using spatial analysis based on photomosaic raster data. Based on the results of identification and marking with point vector features, the data were used as a geographic information system containing geographic reference data. Geographic reference data will be useful for direct confirmation in the field. The distribution pattern of Sumatra disease can be seen without direct contact with the object of observation through the identification and marking of the diseased and healthy clove plants using a GIS (Figure 6).

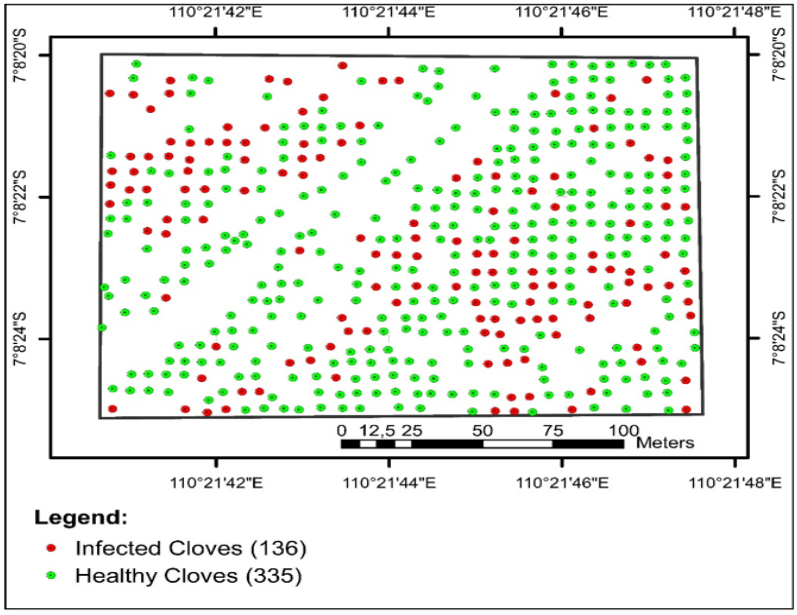

Figure 6. Distribution pattern and incidence of Sumatra disease in clove plantations

Note. Scale 1:3500; Coordinate System: WGS 1984 UTM Zone 49S; Projection System: WGS 1984; Datum: WGS 1984; Unit: Meters

The Sumatra disease identified with symptoms was randomly distributed and tended to jump between healthy and diseased plants. This result follows Bennett et al. (1985), explaining that the distribution pattern of Sumatra disease on land is the jump-spread pattern. Jump-spread patterns allow the disease to spread quickly in all directions to either highlands or lowlands, even across rivers. This distribution pattern indicates that the distribution of the disease is influenced by the presence of insect vectors, namely *Hindola fulva* and *H. striata*. The Sumatra disease can spread up to several kilometers from its primary source. Besides, there was secondary random distribution to trees adjacent to diseased trees (Eden-Green et al., 1992). This distribution pattern is known as the centrifugal spread. A similar distribution pattern of Sumatra disease is also found in lethal yellowing disease, which infects the phloem of coconut plants. The transmission of the lethal yellowing disease was characterized by a random distribution pattern, commonly known as the jump-spread pattern, carried by a planthopper insect vector (*Haplaxius crudus*). Infections in one or two trees will spread randomly around the infection center. It was explained that the jump-spread pattern indicates the presence of airborne transmission through insect vectors that can spread up to tens of kilometers from the primary infection (Tsai, 1980). Besides, the GIS technique can also be used to determine the percentage of the incidence of Sumatra disease in this study, which was 28.8%.

Visual Assessment Based on Disease Symptom

Clove plants' condition was determined by observing the disease symptoms visually. The symptoms of Sumatra disease observed included the leaves withering from the top of the shoot to the bottom (Figure 7). *R. syzygii* subsp. *syzygii* are classified as xylem-limited bacteria because it exclusively develops in the xylem vessel tissue. Wilting that occurs in symptomatic plants is one of the characteristics of symptoms due to bacterial pathogens that infect the xylem.

Figure 7. Symptoms of burning on the leaves that are still attached (on a circle)

The visual assessment by looking at the symptom is the easiest way to detect the early stage of wilting symptoms such as yellowing of leaves. Bacteria colonizing the xylem inhibit the supply of water and nutrients to other parts of the plant so that due to the blockage, the plant wilts and then dies. According to Bennett et al. (1985), leaf drop occurs suddenly from the tip of the lateral branch. The symptomatic part of the branch sheds its dead leaves (yellow), but some leaves are still attached and are generally yellowish (chlorosis). Over time,

the leaves turn brown as if exposed to heat (scorched).

Molecular Detection of *Ralstonia syzygii* subsp. *syzygii*

Molecular testing in the laboratory is an effort to validate disease detection in the field. Samples were collected from the research location and tested until they were visualized. Before the PCR was started, the first step was to extract the DNA of the clove plant. The DNA extraction results were amplified using PCR with specific primers designed based on the endoglucanase (*egl*) gene, followed by electrophoresis and DNA visualization. It was found that DNA extracted from various plant conditions could be amplified in the range of ~378 bp (Figure 8). This result means that the UGMRss-specific primer can only amplify the *egl* gene from *R. syzygii* subsp. *syzygii*. DNA of *R. syzygii* subsp. *syzygii* was detected in plant parts with mild, moderate, and severe symptoms. All three samples were at the same length of DNA base. It indicates that the bacteria infecting symptomatic plant tissues are caused by *R. syzygii* subsp. *syzygii*. As shown in Figure 8, the bacterial infection of *R. syzygii* subsp. *syzygii* is capable of causing damage on a mild, moderate, to severe scale. Molecular tests were also carried out on healthy clove plant tissues. Based on the results of DNA amplification, there was no bacterial DNA of *R. syzygii* subsp. *syzygii* in the tissue of healthy clove plants. Symptoms of wilt that commonly occur in Sumatra disease are not visible because there is no blockage of *R. syzygii* subsp. *syzygii* in the xylem tissue of the healthy plant.

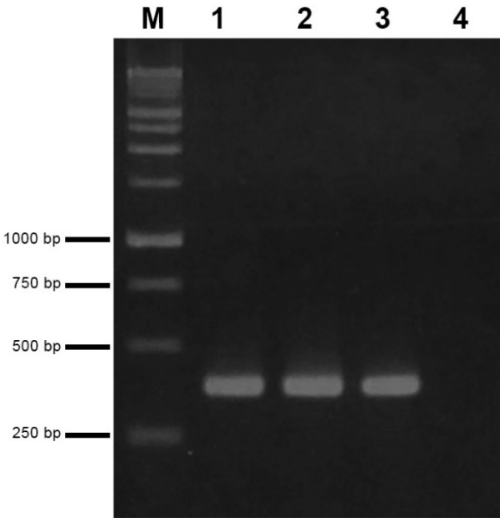

Figure 8. Visualization of DNA amplification results of *Ralstonia syzygii* subsp. *syzygii* of twig samples from various plant conditions by extraction using the zymo kit and specific primers UGMRss-F/UGMRss-R on 1% agarose gel

Note. M = DNA marker (1 kb ladder); Lane 1 = Samples of diseased twigs with severe symptoms; Lane 2 = Samples of healthy twigs from diseased plants; Lane 3 = Samples of diseased twigs with mild symptoms; Lane 4 = Samples of healthy twigs from healthy plants

Likewise, DNA extracted from various plant parts (leaves, twigs, and roots) can be amplified (Figure 9). It proves that *R. syzygii* subsp. *syzygii* are found in all parts of the plant. The presence of *R. syzygii* subsp. *syzygii* in some clove plant tissues indicates that *R. syzygii* subsp. *syzygii* can spread through vascular tissue. The extraction process involved xylem tissue from symptomatic plant tissue containing bacterial DNA of *R. syzygii* subsp. *syzygii* so

that it could be amplified through the PCR analysis. The results of DNA visualization showed that the DNA band was in the range of ~378 bp.

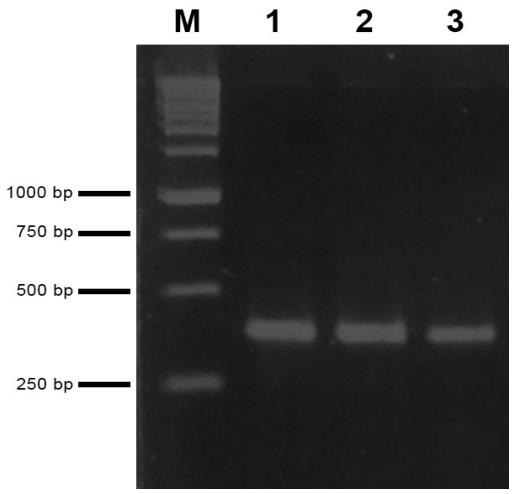

Figure 9. Visualization of DNA amplification results of *Ralstonia syzygii* subsp. *syzygii* from samples of several plant parts by extraction using the zymo kit and specific primers UGMRss-F/UGMRss-R on 1% agarose gel

Note. M = DNA marker (1 kb ladder); Lane 1 = Leaf samples; Lane 2 = Twig samples; Lane 3 = Root samples

UGMRss-specific primers can amplify about ~378 bp of *R. syzygii* subsp. *syzygii* DNA on clove plants. The use of specific primers based on the unique nucleotide sequence of *R. syzygii* subsp. *syzygii* was only able to detect DNA from the *egl* gene in *R. syzygii* subsp. *syzygii* (Trianom et al., 2019). The thickness of the DNA band varied from each sample. It is due to the differences in DNA concentration. Thick and light bands indicate a high concentration of DNA produced, while thin bands mean a low concentration of DNA produced.

Sequencing and Phylogenetic Analysis

Sequence analysis was conducted to confirm the PCR amplification data and to improve the data reliability. The edited nucleotide sequences of the *egl* gene were determined and deposited in GenBank under the accession numbers OK539688 (clove root), OK539686 (clove leaf), and OK539687 (clove twig). The results of DNA sequencing were used for phylogenetic analysis with those in GenBank (Figure 10). The phylogenetic analysis began by analyzing the kinship of *R. syzygii* subsp. *syzygii* on the BLAST results from the National Center of Biotechnology Information (NCBI) page. The results on the BLAST page revealed that the three samples namely leaves, twigs, and roots, had the highest similarity to the type of strain of *R. syzygii* subsp. *syzygii* (strain R001, accession number JF702320). The phylogenetic tree is divided into four clades, with samples taken from root, leaf, and twig tissue in the same node. As shown in Figure 10, the three samples are in the same clade as *R. syzygii* subsp. *syzygii* R001. It shows that the bacteria that infect clove plants in the roots, leaves, and twigs have the same relationship with the *R. syzygii* subsp. *syzygii* R001 type strain. Phylotypes in the same subgroup show similarities in pathogenicity abilities or are derived from the same source (Prakoso et al., 2022; Safni et al., 2014). The following are the results of the phylogenetic analysis using the ML Tree method with 1,000 bootstraps.

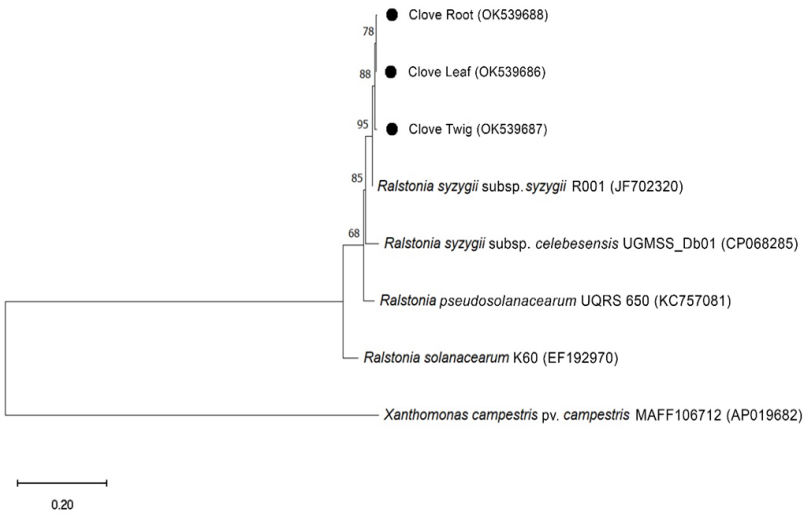

Figure 10. Phylogenetic tree of *Ralstonia syzygii* subsp. *syzygii* that infects cloves in root, leaf, and twig tissues with some proximity to strains of the genus *Ralstonia* based on the *egl* gene sequence using the maximum-likelihood method with 1,000 bootstrap times. *Xanthomonas campestris* pv. *campestris* sequences were used as an outgroup

Table 1

Percentage of nucleotide sequence homology of Ralstonia syzygii *subsp.* syzygii *with several strains of the genus* Ralstonia *and outgroup* Xanthomonas campestris *pv.* campestris *as comparison*

No.	Identity	1	2	3	4	5	6	7	8
1	Clove roots	ID							
2	Clove leaves	99.7	ID						
3	Clove twigs	99.1	99.1	ID					
4	*Ralstonia syzygii* subsp. *syzygii* R001 (JF702320)	99.1	99.4	98.5	ID				
5	*Ralstonia syzygii* subsp. *celebesensis* UGMSS_ Db01 (CP068285)	95.1	95.4	94.4	95.5	ID			
6	*Ralstonia solanacearum* K60 (EF192970)	89.2	89.6	88.5	90.4	88.3	ID		
7	*Ralstonia pseudosolanacearum* NCPPB 253	94.8	95.1	95.1	95.5	94.2	89.7	ID	
8	*Xanthomonas campestris* pv. *campestris* MAFF106712 (AP019682)	-23.1	-24.1	-21.8	-22.8	-27.6	-22.4	-22.4	ID

Based on the phylogenetic analysis, the DNA extracted from leaves, roots, and twigs are in the same class with a percentage of similarity between leaf and root samples of 99.7%, between roots and twigs of 99.1%, and between leaves and twigs by 99.1%. This result shows that there is a similarity in infecting bacteria. The similarity percentage between roots and twigs is 99.1%, 99.4%, and 99.5%, respectively. The value of the percentage of similarity indicates that the cause of Sumatra disease comes from the same group. According to Frank et al. (2008), the phylogenetic groups are different if there are more varied parts in a gene sequence. The phylogenetic analysis in this study also involved sequences from the genus *Ralstonia* as a comparison with the percentage of similarity between each species (Table 1). All strains used for comparison are the results of sequences obtained from the *egl* gene. According to Trianom et al. (2018), the sequences in the *egl* gene have good gene copy effectiveness, are easy to conserve, and are easy to amplify. In detection and monitoring efforts, *egl* gene sequencing analysis can be a method that can be used to determine the diversity of pathogens. The *Xanthomonas campestris* pv. *campestris* sequence was also used as an outgroup.

CONCLUSION

The application of UAV technology for aerial photo data acquisition and as a data source for making GIS thematic maps of Sumatra disease distribution patterns facilitated the detection of Sumatra diseases in a wide area. The distribution pattern of Sumatra disease was random and relatively jumped between healthy and diseased plants. Detection of *R. syzygii* subsp. *syzygii* causing Sumatra disease in cloves using the PCR technique with UGMRss-specific primers producing ~378 bp amplicon. In diseased clove plants, bacteria detected in all plant parts, including leaves, twigs, and roots, originate from single species of bacteria. The *R. syzygii* subsp. *syzygii* was also detected in asymptomatic branches in diseased clove individuals.

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In silico Comparative Analysis of Gene and Protein of Plant Lectins

Fathiya Khairiya[1], Fenny Martha Dwivany[1,2,3]*, Sony Suhandono[1], Sofia Safitri Hessel[1], Ima Mulyama Zainuddin[4] and Trina Ekawati Tallei[5]

[1]School of Life Sciences and Technology, Institut Teknologi Bandung, Jalan Ganesha No. 10, Bandung 40132, West Java, Indonesia
[2]Bali International Research Center for Banana, Badung 80361, Bali, Indonesia
[3]Bioscience and Biotechnology Research Center, Institut Teknologi Bandung, Bandung 40132, West Java, Indonesia
[4]Department of Biosystems, KU Leuven, Leuven 3000, Belgium
[5]Department of Biology, Faculty of Mathematics and Natural Sciences, Sam Ratulangi University, Manado 95115, North Sulawesi, Indonesia

ABSTRACT

Lectins are a family of proteins that can recognize and bind specific carbohydrates. Plant lectins play various roles in plant defense and can be utilized as insecticidal, antibacterial, antifungal, and antiviral agents. This study compares genes, proteins, and carbohydrate-binding motifs between 15 plant lectins using *in silico* methods. The lectin genes of *Artocarpus hypargyreus* Hance, *Hordeum vulgare* var. Betzes, *Triticum aestivum* L. cv. Marshall, *Galanthus nivalis* L., *Allium sativum* L., *Phaseolus vulgaris*, *Lens culinaris* subsp. *tomentosus*, *Robinia pseudoacacia*, *Glycine max*, *Cicer arietinum*, *Pisum sativum*, *Canavalia ensiformis*, *Amaranthus caudatus*, *Amaranthus hypochondriacus*, and *Musa acuminata* subsp. *malaccensis* were obtained from National Center for Biotechnology Information and Banana Genome Hub. The gene comparison results revealed different characteristics of the 15 plant lectin genes, with *A. hypargyreus* having the shortest lectin gene and *G. max* having the longest. Overall, the 15 plant lectin genes have 1–3 exons. Domain predictions revealed the presence of five domains: jacalin, chitin_bind_1, B_lectin, legume lectin, and agglutinin. Furthermore,

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E-mail addresses:
fenny@sith.itb.ac.id (Fenny Martha Dwivany)
fathiyakhairiya@students.itb.ac.id (Fathiya Khairiya)
sony@sith.itb.ac.id (Sony Suhandono)
hesselsafitrisofia@gmail.com (Sofia Safitri Hessel)
ima.zainuddin@kuleuven.be (Ima Mulyama Zainuddin)
trina_tallei@unsrat.ac.id (Trina Ekawati Tallei)
*Corresponding author

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there were 2 protein sequences from the jacalin domain, 2 protein sequences from the chitin_bind_I domain, 2 protein sequences from the B_lectin domain, and 4 protein sequences from the legume lectin domains that have complete carbohydrate-binding motifs compared to consensus motifs from literature. The data obtained from this study has not been previously reported and can be utilized for future lectin protein production with synthetic biology approaches. This method will allow scientists to obtain plant bioparts for lectin production using a heterologous system, even without plant samples.

Keywords: *In silico* comparative study, plant lectins, synthetic biology

INTRODUCTION

Lectins are a family of proteins with heterogeneous structures that can recognize and bind to specific carbohydrates. Lectins are found in various organisms, including bacteria, fungi, animals, and plants, and serve various functions. Lectins function in mediating biological processes such as cell-to-cell communication and cell migration. In addition, cell interactions with several types of lectins are known to cause cell agglutination or deposition of glycoconjugates and polysaccharides, which makes lectins in some organisms naturally able to act as agglutinins. Several studies discussed the results of conventional lectin isolation from various organisms and demonstrated that lectins in plants have higher protein yields than other lectin-producing organisms such as fungi and animals (Lam & Ng, 2011).

In plants, there are several lectin domains, including jacalin, chitin-binding, nictaba (*Nicotiana tabacum* agglutinin), *Galanthus nivalis* agglutinin (GNA), legume, hevein, amaranthin, and *Euonymus europaeus* lectin (EEL). The main function of plant lectins is to act as a defense system and to facilitate adaptation to abiotic and biotic stresses. Additional studies have also examined the function of plant lectins as insecticidal, antibacterial, antifungal, and antiviral agents (Tsaneva & Van Damme, 2020).

Several plant lectins, including legume, hevein, nictaba, and jacalin, have been shown in previous studies to have strong insecticidal activity against Lepidoptera, Coleoptera, and Diptera (Vandenborre et al., 2011). Plant lectins can act as antifungals by inhibiting pathogenic fungal growth (Coelho et al., 2017). Plant lectins with jacalin-related domains derived from bananas (*Musa acuminata*) are known to exhibit antiviral activity by recognizing glycans on the surface of the HIV-1 virus envelope, thereby inhibiting the entry of the virus into the host cell (Swanson et al., 2010). Another study showed that a *Lens culinaris*-derived plant lectin with a legume domain possesses antiviral activity against multiple SARS-CoV-2 variants (Wang et al., 2021). Another study demonstrated that lectins from *Archidendron jiringa* inhibited the growth of several bacteria, including *Bacillus subtilis* and *Staphylococcus* sp. (Charungchitrak et al., 2011).

A controversy in the utilization of lectins was reported in a publication by Singh et al. (2014), which is that wild-type banana lectin is a T-cell mitogen. A novel molecularly engineered lectin, H84T banana lectin (H84T), was reported to have real potential for clinical use against influenza by blocking the ability of the influenza virus to fuse with endosomes (Covés-Datson et al., 2020). The H84T was reported to be a potent inhibitor of the Ebola virus by blocking cellular entry and transcription/replication (Covés-Datson et al., 2019). Clinical use of lectins has been slowed due to concerns about their mitogenic activity toward immune cells. The H84T banana lectin was engineered by mutating a single amino acid, histidine, to threonine at position 84 to preserve the antiviral activity and lose the mitogenicity (Covés-Datson et al., 2021).

Importantly, *in silico* comparative analysis of plant lectin genes and proteins is required to characterize plant lectin sequences. In this case, *in silico* methods can be utilized in gene structure characterization and protein structure and function prediction. *In silico* methods have been widely used to characterize proteins and enzymes from various eukaryotic and prokaryotic species. Understanding the characteristics of lectin genes and proteins from various plant species is essential for developing more advanced lectin proteins. Parallel to this study, we also conducted an *in silico* analysis of the ability of wild-type and modified *M. acuminata* (H84T) and *M. balbisiana* lectins to inhibit the activity of the SARS-CoV-2 spike protein using molecular docking and molecular dynamics simulation. The results demonstrated that the modeled lectins and the SARS-CoV-2 RBD had good protein-protein interactions and strong binding (Hessel et al., 2022).

In addition to the study of plant lectins, methods for optimizing the production of lectins have also been investigated, and synthetic biology may offer a novel approach in this regard. The application of synthetic biology in agriculture, health, and pharmacy has been reviewed by Scott et al. (2015). However, this method has not yet been documented in plant lectin synthesis. Thus, the possibility remains open.

Therefore, this research employed an *in silico* method to compare plant lectin genes and proteins. A comparison of the carbohydrate-binding site motifs on plant lectins was also carried out to analyze the motifs related to their function in binding specific carbohydrates. This research's findings can serve as a reference for characterizing plant lectin sequences in the context of synthetic biology-based lectin protein production.

MATERIALS AND METHODS

Collection and Compilation of Plant Lectin Gene Sequences

Plant lectin gene sequences from 14 different plant species were obtained from the National Center for Biotechnology Information (NCBI) platform (https://www.ncbi.nlm.nih.gov/). The banana lectin gene was retrieved from the Banana Genome Hub platform (version 2) (https://banana-genomehub.southgreen.fr). The lectin gene sequences (accession/hit numbers)

acquired from 15 plants were *Musa acuminata* subsp. *malaccensis* (Ma09_t10460.1), *Artocarpus hypargyreus* Hance (KY924610.1), *Hordeum vulgare* var. Betzes (M29280.1), *Triticum aestivum* L. cv. Marshall (M25537.1), *Galanthus nivalis* L. (M55556.1), *Allium sativum* L. (U58948.1), *Phaseolus vulgaris* (AJ439715.1), *Lens culinaris* subsp. *tomentosus* (AJ421799.2), *Robinia pseudoacacia* (AB012633.1), *Glycine max* (NM_001250281.3), *Cicer arietinum* (XM_004509655.2), *Pisum sativum* (L11745.1), *Canavalia ensiformis* (AF308777.1), *Amaranthus caudatus* (AF401479.1), and *A. hypochondriacus* (AF143954.1).

Plant Lectin Protein Sequence Prediction

The prediction of 15 plant lectin protein sequences was made using the BLASTX program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?LINK_LOC=blasthome&PAGE_TYPE=BlastSearch&PROGRAM=blastx) on the NCBI web page. The principle of BLASTX is to compare the input gene sequences (nucleotides) with the protein sequence database in NCBI.

Prediction and Visualization of Plant Lectin Gene Structure

Structural prediction of the 15 plant lectin gene sequences was performed using the FGENESH+ online tool on the Softberry web page (http://www.softberry.com/berry.phtml?topic=fgenes_plus&group=programs&subgroup=gfs) using the similar protein-based gene prediction approach and GeneWise (version 2022) (https://www.ebi.ac.uk/Tools/psa/genewise/) (Xiong, 2006). The homologous protein sequences were obtained from the previous step's BLASTX results of protein sequences. Visualization of the structures was carried out using Illustrator for Biological Sequence (IBS) (version 1.0.3) (W. Liu et al., 2015). Following this, the genomes, exons, and introns sizes of the 15 plant lectin gene sequences were compared.

Plant Lectin Protein Phylogenetic Tree Construction

The MEGA-X (version 10.1.7) was employed to construct the phylogenetic tree of the 15 plant lectin protein sequences using the MUSCLE alignment method and the Maximum Likelihood parameter with a 1,000x bootstrap (Kamalesha et al., 2022). The phylogenetic tree construction was done to determine the clustering and evolutionary relationships of the 15 plant lectin protein sequences (Kumar et al., 2018).

Analysis of Plant Lectin Protein Domains and Motifs

Protein domains and motif analysis of plant lectins were performed using the Conserved Domain (CD)-Search Tool (version 2020) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) on the NCBI web page with an e-value threshold of 0.01 (Lu et al., 2020) and visualized using IBS. The Multiple Em for Multiple Elicitation (MEME)-Suite (version 5.4.1) (https://meme-suite.org/meme/tools/meme) (Bailey et al., 2009) was used for the prediction of plant lectin motifs. The parameter settings used were a minimum motif length

parameter of 10 amino acids (aa) and a maximum motif length of 50 aa, with other parameters remaining in the default settings (Dwivany et al., 2021; Kamalesha et al., 2022). Consensus motif detection and validation were performed using the InterPro online tool (version 88.0) (https://www.ebi.ac.uk/interpro/search/sequence/) (Mitchell et al., 2019). In addition, the motifs of carbohydrate-binding sites on plant lectin protein sequences were analyzed and compared to the consensus motifs from previous studies using multiple sequence alignment (MSA) with the MUSCLE method integrated into MEGA-X (version 10.1.7) (Kumar et al., 2018).

RESULTS AND DISCUSSION

In silico Characterization of Plant Lectin Genes

The lectin genes from 14 plant species and one banana species obtained from NCBI and Banana Genome Hub were used to identify the lectin type (Table 1).

Table 1

The identified lectin types from 15 plant species

No.	Plant species	Family	Accession number/Hits	Type of lectins
1	*Musa acuminata* subsp. *malaccensis*	Musaceae	Ma09_t10460.1	Lectin
2	*Artocarpus hypargyreus* Hance	Moraceae	KY924610.1	Lectin
3	*Hordeum vulgare* var. Betzes	Poaceae	M29280.1	Lectin
4	*Triticum aestivum* L. cv. Marshall		M25537.1	Wheat germ agglutinin isolectin D
5	*Galanthus nivalis* L.	Amaryllidaceae	M55556.1	Lectin GNA 2
6	*Allium sativum* L.		U58948.1	Mannose-specific lectin
7	*Phaseolus vulgaris*	Fabaceae	AJ439715.1	Lec4-B17 gene
8	*Lens culinaris* subsp. *tomentosus*		AJ421799.2	Lectin
9	*Robinia pseudoacacia*		AB012633.1	Lectin
10	*Glycine max*		NM_001250281.3	Lectin (LOC732576)
11	*Cicer arietinum*		XM_004509655.2	Seed lectin-like
12	*Pisum sativum*		L11745.1	Lectin (Blec4)
13	*Canavalia ensiformis*		AF308777.1	Con A
14	*Amaranthus caudatus*	Amaranthaceae	AF401479.1	Agglutinin
15	*Amaranthus hypochondriacus*		AF143954.1	Agglutinin

Several plant lectin types identified here were the amaranthins, the chitinase-related agglutinins, the GNA-related lectins, the hevein domain lectins, the jacalin-related lectins, and the legume lectins (Van Damme, 2022). The *in silico* comparative analysis of the lectin genes and proteins between these 15 plant species has not been reported yet.

The prediction and visualization of the 15 identified plant lectin genes by utilizing the FGENESH+ and IBS provided information on the length of the gene, exon, and poly-A signal (Figure 1).

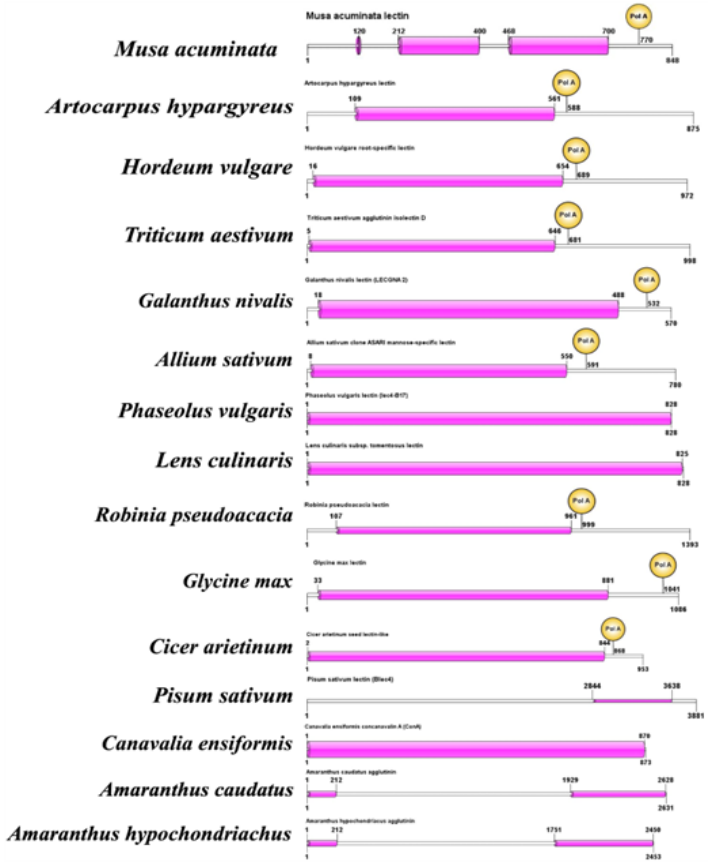


Figure 1. Gene structure of the 15 plant lectin genes

None of the 15 plant lectin gene sequences examined contained a transcription start site (TSS). As a result, the gene sizes were calculated starting from the first exon to the poly-A signal. A poly-A signal was found in nine plant lectin genes, but none were found in the six remaining genes. Therefore, the sizes of these six lectin genes were calculated from the first to the last exon. The lectin gene of *G. max*, measuring 1,009 bp, was the longest one. Furthermore, the shortest gene detected was

from *A. hypargyreus*, with a length of 480 bp. Plant lectin gene sequences ranged from 495 to 912 bp.

The number of exons in the lectin gene from 15 plants was also different; the highest number of exons detected was three in *M. acuminata*. Meanwhile, two exons were found in the lectin gene of *A. caudatus* and *A. hypochondriacus*. The remaining plant lectin genes had only one exon in each sequence. Table 2 displays the structural characteristics of the 15 plant lectin genes, including exon number and length, coding sequence (CDS) length, and mRNA length.

Table 2
Structural characteristics of the 15 plant lectin genes

No.	Plant species	Exon number	CDS length	mRNA length
1	*Musa acuminata* subsp. *malaccensis*	3	426	495
2	*Artocarpus hypargyreus* Hance	1	453	480
3	*Hordeum vulgare* var. Betzes	1	639	674
4	*Triticum aestivum* L. cv. Marshall	1	642	677
5	*Galanthus nivalis* L.	1	471	515
6	*Allium sativum* L.	1	543	584
7	*Phaseolus vulgaris*	1	828	828
8	*Lens culinaris* subsp. *tomentosus*	1	828	828
9	*Robinia pseudoacacia*	1	855	893
10	*Glycine max*	1	849	1,009
11	*Cicer arietinum*	1	843	867
12	*Pisum sativum*	1	755	755
13	*Canavalia ensiformis*	1	870	870
14	*Amaranthus caudatus*	2	912	912
15	*Amaranthus hypochondriacus*	2	912	912

The lectin gene sequences identified in *M. acuminata*, *A. caudatus*, and *A. hypochondriacus* occur at multiple locations in the genome. It indicates a duplication event in the lectin gene. One of the most important duplication events in the history of evolution and lectin expansion in the jacalin family occurred in the genome of *M. acuminata* (Van Holle & Van Damme, 2019).

Lectins are diverse in almost every aspect, including sequences, structures, binding site architectures, quaternary structures, carbohydrate affinities, and specificities. Not only that, their larger biological roles and potential applications

are wide as well (Van Damme, 2014). Another *in silico* comparative analysis study showed that the legume lectin sequences ranged from 700 to 900 bp. Hundreds of structures of plant lectins have been characterized, and legume lectins are the most extensively studied (Moraes Filho et al., 2017).

Until this day, lectins have been extensively studied in terms of their molecules and proteins due to various reports on their biomedical applications. However, proteomics and genomic analysis are still needed to explore various aspects of these proteins. More sequence information on lectins would be important for structural references and functional characterization, so more focus can be directed toward developing genetic engineering to produce recombinant lectins (Singh et al., 2014). Therefore, more information about different types of plant lectin genes and proteins from various species is required.

The 15 plant lectin proteins were characterized by identifying protein motifs and domains to predict protein classification, identify conserved sequences, and predict protein function (Table 3, Figure 2).

Table 3

Domain identification of 15 plant lectin proteins using the CD-search tool

No.	Lectin source	Identified domain
1	*Musa acuminata* subsp. *malaccensis*	Jacalin
2	*Artocarpus hypargyreus* Hance	
3	*Hordeum vulgare* var. Betzes	Chitin_bind_1
4	*Triticum aestivum* L. cv. Marshall	
5	*Galanthus nivalis* L.	B_lectin
6	*Allium sativum* L.	
7	*Phaseolus vulgaris*	Legume lectin
8	*Lens culinaris* subsp. *tomentosus*	
9	*Robinia pseudoacacia*	
10	*Glycine max*	
11	*Cicer arietinum*	
12	*Pisum sativum*	
13	*Canavalia ensiformis*	
14	*Amaranthus caudatus*	Agglutinin
15	*Amaranthus hypochondriacus*	

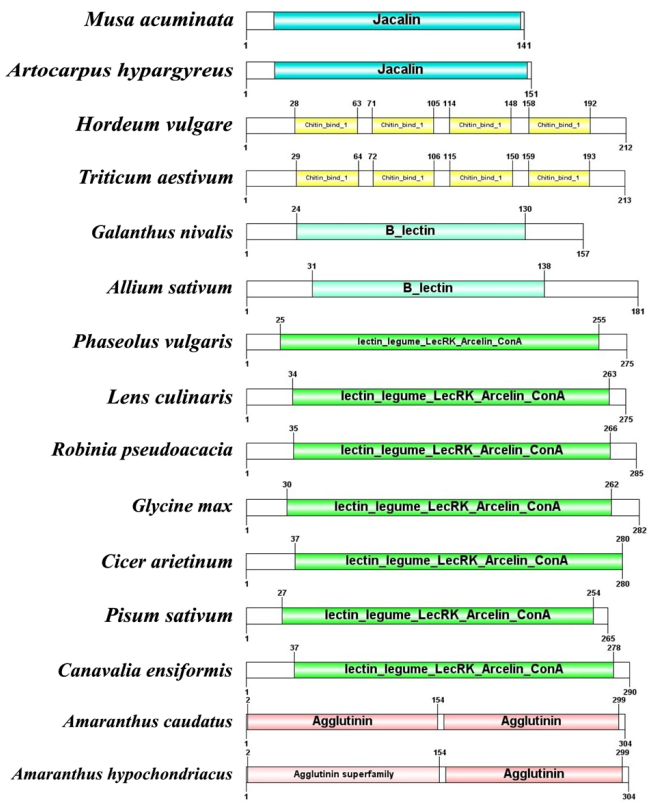


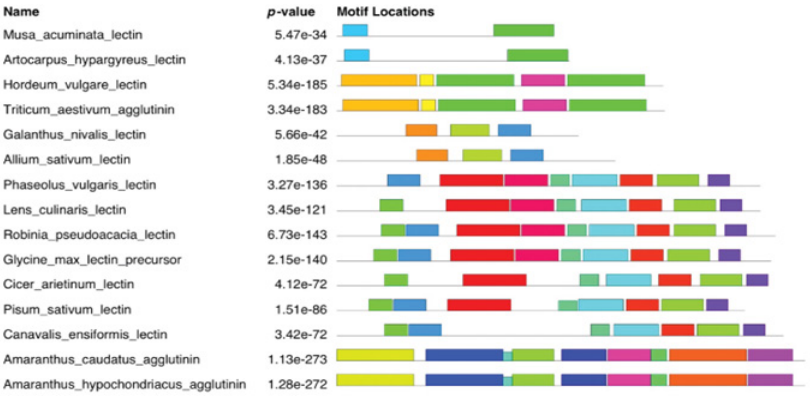
Figure 2. Visualization of domain positions on 15 plant lectin protein sequences

The results indicated that the location of the domains in the plant lectin protein sequences consisting of the same domain was quite uniform. In *M. acuminata* and *A. hypargyreus*, the jacalin domain was located between the 15 and 149 aa residues. In *H. vulgare* and *T. aestivum*, the four locations of the chitin_bind_1 domain were discovered in relatively consistent locations, specifically between the residues of 28–64 aa, 71–106 aa, 114–150 aa, and 158–193 aa. The B_lectin domain of *G. nivalis* and *A. sativum* was located between the 28 and 138 aa residues. The lectin_legume_LecRK_Arcelin_ConA domain, commonly called the "legume lectin domain", was located between 24 and 280 aa residues in seven species. Agglutinin domains were found in *A. caudatus* and *A. hypochondriacus* between residues 2–154 aa and 159–299 aa.

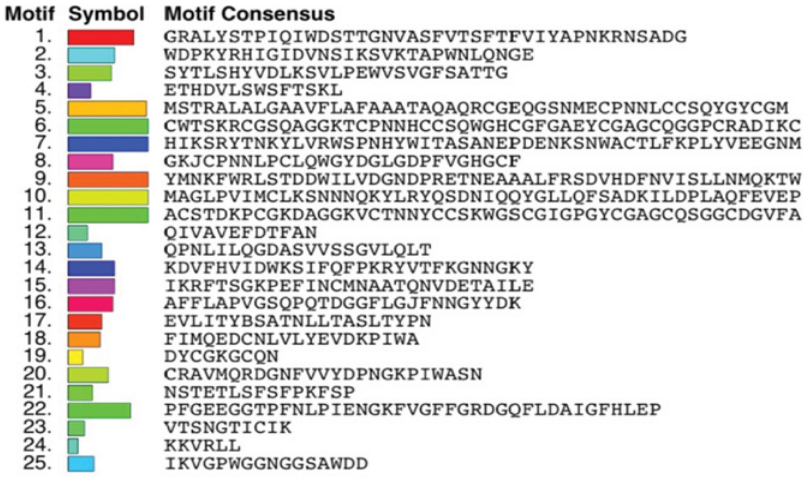
Identifying protein motifs from plant lectins yielded 25 consensus motifs (Figure 3). The results of the validation of these motifs are shown in Table 4.

The most important characteristic of lectins is a domain that can recognize and bind to carbohydrate structures specifically and reversibly (Peumans & Van Damme, 1995). Plant lectins can be classified based on the lectin domain, which consists of consensus motif sequences (Van Damme et al., 2008). A study showed that some lectin

domains are highly conserved in plants (Van Holle et al., 2017). Combinations of domains allow lectin domains to have new functions. The high diversity of domain architectures can make domains in lectins have more specialized roles (Dang & Van Damme, 2016).



(a)



(b)

Figure 3. A motif prediction of 15 plant lectin proteins: (a) location of predicted consensus motifs based on MEME-Suite, (b) 25 predicted consensus motif sequences

Table 4

Consensus motifs found in plant lectin protein sequences and InterPro detection results

No.	Consensus motif	InterPro detection
1	GRALYSTPIQIWDSTTGNVASFVTSFTFVIYAPNKRNSADG	Legume lectin

Table 4 *(Continue)*

No	Consensus motif	InterPro detection
2	WDPKYRHIGIDVNSIKSVKTAPWNLQNGE	Legume lectin
3	SYTLSHYVDLKSVLPEWVSVGFSATTG	Legume lectin
4	ETHDVLSWSFTSKL	-
5	MSTRALALGAAVFLAFAAATAQAQRCGEQGSNMECPNNLCCSQYGYCGM	Chitin-bd_1
6	CWTSKRCGSQAGGKTCPNNHCCSQWGHCGFGAEYCGAGCQGGPCRADIKC	Chitin-bd_1
7	HIKSRYTNKYLVRWSPNHYWITASANEPDENKSNWACTLFKPLYVEEGNM	Agglutinin
8	GKJCPNNLPCLQWGYDGLGDPFVGHGCF	-
9	YMNKFWRLSTDDWILVDGNDPRETNEAAALFRSDVHDFNVISLLNMQKTW	Agglutinin
10	MAGLPVIMCLKSNNNQKYLRYQSDNIQQYGLLQFSADKILDPLAQFEVEP	Agglutinin
11	ACSTDKPCGKDAGGKVCTNNYCCSKWGSCGIGPGYCGAGCQSGGCDGVFA	Chitin-bd_1
12	QIVAVEFDTFAN	-
13	QPNLILQGDASVVSSGVLQLT	-
14	KDVFHVIDWKSIFQFPKRYVTFKGNNGKY	-
15	IKRFTSGKPEFINCMNAATQNVDETAILE	Agglutinin
16	AFFLAPVGSQPQTDGGFLGJFNNGYYDK	-
17	EVLITYBSATNLLTASLTYPN	-
18	FIMQEDCNLVLYEVDKPIWA	Bulb-type _lectin
19	DYCGKGCQN	-
20	CRAVMQRDGNFVVYDPNGKPIWASN	Bulb-type_ lectin
21	NSTETLSFSFPKFSP	-
22	PFGEEGGTPFNLPIENGKFVGFFGRDGQFLDAIGFHLEP	Jacalin-like lectin
23	VTSNGTICIK	-
24	KKVRLL	-
25	IKVGPWGGNGGSAWDD	Jacalin-like lectin

Plant Lectin Phylogenetic Tree

According to the constructed phylogenetic tree shown in Figure 4, lectin proteins derived from *M. acuminata* and *A. hypargyreus* belong to the same clade, which exhibits a close relationship under their similar domains, namely, Jacalin. Then, *H. vulgare*, and *T. aestivum* species are also closely related in the tree, as indicated by the chitin_bind_1 domain that they possess. Furthermore, *G. nivalis* and *A. sativum* are positioned in the same clade, corresponding to the domain they both share, B_lectin. Two other species, *A. caudatus*, and *A. hypochondriacus*, form a clade according to their matching agglutinin domains.

Seven species, including *C. arietinum*, *P. sativum*, *C. ensiformis*, *L. culinaris*, *R. pseudoacacia*, *P. vulgaris*, and *G. max*, form a clade that demonstrates the close relationship of all seven lectin proteins. Consistently, each of the seven proteins shares a domain known as the legume lectin domain. In addition, it was observed that the legume lectin clade resulted from a different tree branching than the other species with the four other domains (jacalin, chitin_bind_1, B_lectin, and agglutinin). Legume lectin domains are mentioned in another study as being strongly conserved, which emphasizes their importance. The amaranthins, the chitinase-related agglutinins, the GNA-related lectins, the hevein domain lectins, the jacalin-related lectins, and the legume lectins are only found in vascular plants (Naithani et al., 2021).

The lectin genes in higher plants belong to the group of multi-copy genes. Duplication of genes plays an important role in the process of evolution and expansion of a gene (Van Holle et al., 2017). Tandem and segmental duplication are mechanisms for lectin gene expansion and play a role in the adaptation process of plants to various biotic and abiotic stresses (Jiang et al., 2010). Each plant lectin family shows a different evolutionary path as it is formed by full and partial gene duplication based on addition,

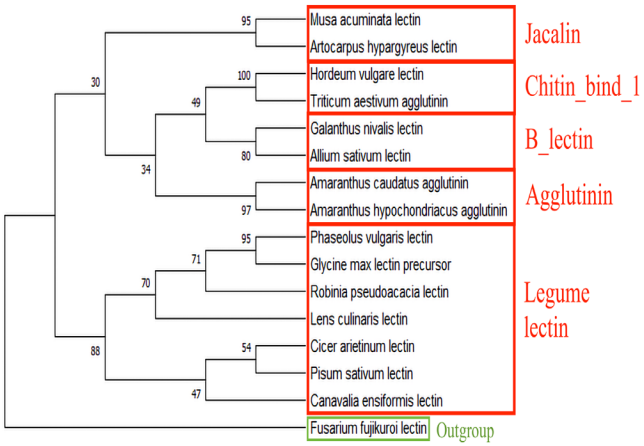


Figure 4. Phylogenetic tree of the 15 plant lectin protein sequences. The red boxes show the relationship between lectin sequences possessing the same domains

reduction, and/or recombination in each protein domain (Naithani et al., 2021).

In silico Analysis of Carbohydrate Binding Sites Motif on Plant Lectins

Carbohydrate binding motifs on plant lectin sequences serve as recognition sites for specific carbohydrates, which will later function as binding sites between lectin proteins and carbohydrates (monosaccharides to oligosaccharides). It is related to the function of lectin proteins in plants, such as their roles in pathogen defense mechanisms, abiotic stress responses, and the antiviral, antifungal, and antibiotic properties of plant lectins (Song et al., 2014).

Research on carbohydrate binding site motifs on legume lectins has previously been carried out by Cummings et al. (2017), which explained that there are 4 loops (consisting of loops A, B, C, and D) associated with carbohydrate binding sites. Below are the results obtained for CBS motif analysis of sequences with legume lectin domains.

As depicted in Figure 5, the key amino acid D132 (aspartic acid at position 132) in loop A contributes to establishing hydrogen bonds between the side chain and carbohydrate ligands (Katoch & Tripathi, 2021). It is denoted by a thick blue box containing a caret symbol (^). In addition, loop A consists of conserved amino acids, namely G133 (glycine in position 133) and F136 (phenylalanine in position 136), which are denoted by a red box and an asterisk symbol (*). Furthermore, loop B has G154 (glycine in position 154) as a key amino acid that forms hydrogen bonds. One of the key causes for preserving the structure of legume lectins is hydrogen bonding, which also happens to be one of the few factors that affect lectins' carbohydrate-binding specificity (Katoch & Tripathi, 2021). The MSA comparison reveals that in the same amino acid position, *P. sativum* and *C. ensiformis* contain key amino acids that are not consistent with previous research, specifically Q154 (glutamine in position 154) in *P. sativum* and R154 (arginine in position 154) in *C. ensiformis* (Cummings et al., 2017).

Loop C consists of several conserved amino acids, namely V185 (valine in the position of 185), E186 (glutamic acid in

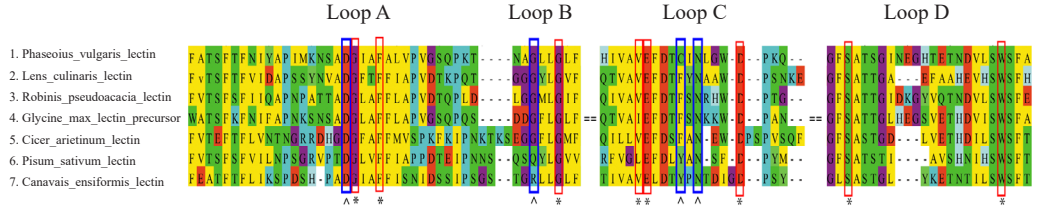


Figure 5. Comparison of carbohydrate-binding site motifs in plant lectin sequences with legume lectin domains based on the similarity of motifs in a previous study (Cummings et al., 2017)

Note. Red boxes and asterisk (*) symbols indicate conserved amino acids, while bold blue boxes and caret (^) symbols signify key amino acids that play a role in hydrogen bond formation

the position of 186), and D197 (aspartic acid in the position of 197), as indicated by a red box and an asterisk (*) in Figure 5. Nonetheless, according to the MSA results, there are two sequences with differences in the amino acid column/position where valine is the conserved amino acid. The *G. max* sequence has isoleucine in position 185, whereas the *P. sativum* sequence has a leucine in position 185. Loop C also has a conserved key amino acid, N192 (asparagine in position 192), which plays a role in hydrogen bond formation with the binding sugar's hydroxyl group through amide acid groups (Cummings et al., 2017). Furthermore, loop C also has key amino acids in the form of hydrophobic amino acids, including C190 (cysteine in position 190), F190 (phenylalanine in position 190), and V185 (valine in position 185). These hydrophobic amino acids may act as hydrophobic cavities that play a role in non-covalent interactions, although those interactions do not directly affect the carbohydrate-binding specificity of legume lectins (Katoch & Tripathi, 2021; Srinivas et al., 2000). Key amino acids are indicated by a bold blue box and a caret symbol (^). According to the MSA results, hydrophobic amino acids, part of the key amino acids, were not found in *P. sativum* and *C. ensiformis* lectin sequences. Instead, both sequences had a neutral amino acid, Y190 (tyrosine at position 190), instead of a hydrophobic one. The reason behind this is unknown, although several factors, such as random mutagenesis, may be the cause. Further structural analysis should be conducted to understand the effects of this substitution on the protein's structure. In loop D, the conserved amino acids were S285 (serine in position 285) and W303 (tryptophan in position 303), as indicated in Figure 5 by a red box and an asterisk (*).

The consensus motifs from this analysis were then compared to the reference literature (loops A-D), Cummings et al. (2017). Our findings revealed three sequences, namely *G. max*, *P. sativum*, and *C. ensiformis*, exhibiting a mismatch of key or conserved amino acids. However, the sequences from *P. vulgaris*, *L. culinaris*, *R. pseudoacacia*, and *C. arietinum* showed motif similarity with the referenced literature.

Similar to the previous study by Raval et al. (2004), the second domain, jacalin, was detected in two species with the same carbohydrate binding site motif. Jacalin carbohydrate binding site motifs are "GG", which forms a GG loop with said glycine amino acids positioned at 15 and 16, and "GXXXD" (with X representing any amino acid), which forms a binding loop in the position ranging through the amino acids 141 to 145 (Figure 6). Based on the MSA result, *M. acuminata* and *A. hypargyreus* consist of the jacalin domain consensus motif under the reference.

The GXXXD motif was considered a preferred characteristic in banana lectin (BanLec) sequences to be used and/or modified as a ligand-binding substance. The motif indicates ligand-binding sites (Covés-Datson et al., 2021; Meagher et al., 2005). The previously mentioned parallel study by Hessel et al. (2022) utilized this motif

Figure 6. Comparison of carbohydrate-binding site motifs in plant lectin sequences with jacalin domain based on the similarity of motifs found in a previous study (Raval et al., 2004). Jacalin's conserved motifs are "GG" and "GXXXD"

Note. The asterisk (*) symbol indicates conserved amino acids; "X" indicates any amino acid

as a selection parameter for banana lectin gene sequences. The sequences were then modeled and docked with the SARS-CoV-2 spike protein receptor-binding domain (RBD). The findings revealed an abundance of interactions between the two proteins and an adequate number of hydrogen bonds and salt bridges. These bonds accommodate the high binding affinities obtained from molecular docking analysis. It also showed that a single lectin excelled in the stability of interaction and binding with the SARS-CoV-2 spike protein RBD through molecular dynamics analysis.

The third domain, chitin_bind_1, is 43 residues long and was detected in two species that showed the same carbohydrate binding site motif as a previous study by Wright et al. (1991). This domain is frequently present in plant proteins that bind to N-acetylglucosamine and its polymers and is assumed to play a role in the recognition and binding of chitin subunits (Butler et al., 1991; Wright et al., 1991). It is known that chitin_bind_1 contains a motif of eight "C" or cysteine amino acids, which play a role in forming four disulfide bonds and are often found at the N-terminus (Wright et al., 1991). According to the MSA results, these eight "C" amino acid motifs were found in *H. vulgare* and *T. aestivum* species (Figure 7). The cysteine amino acids were positioned at the 30th, 39th, 44th, 45th, 51st, 58th, 62nd, and 67th amino acids, with the motif found being "CGXXXXXXXCXXXXCCSXXGXCGXXXXXCXXXCXXXXC", where X indicates any amino acid.

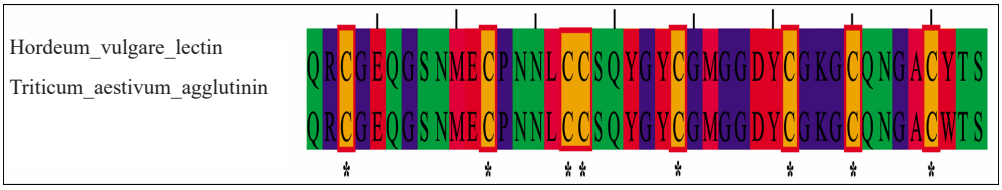


Figure 7. Comparison of carbohydrate-binding site motifs on plant lectin sequences with the chitin_bind_1 domain based on the similarity of motifs found in a previous study (Wright et al., 1991)

Note. The asterisk (*) symbol indicates conserved amino acids

These cysteine residues indicate that motifs in proteins containing the chitin_bind_1 domain are highly conserved. It is to be expected because the disulfide-rich domains of larger proteins, such as lectin and agglutinin, have a higher degree of sequence identity and are predicted to have descended from a common ancestor (Wright et al., 1991).

The B_lectin domain was found in two species that also possess the same carbohydrate binding site motif as the consensus of the B_lectin motif, according to a previous study by Zhao et al. (2017). B_lectin is known to have a conserved motif of QXDXNXVXY, where X represents any amino acid. Although the functionality of the motif is still in question, it confers the exclusive binding specificity of lectins containing the 5-amino acid motif (Pereira et al., 2015). This motif was detected in *G. nivalis* and *A. sativum* species, where it was found in the 56th through 64th and 88th through 96th amino acids of the aligned sequences with varying X amino acids (Figure 8).

The agglutinin domain is known to have no specific carbohydrate binding site. A study explained that the agglutinins in *Amaranthus* sp., more commonly known as amaranthin, assemble a binding site from a loop formed from two agglutinin subunits at the N- and C-terminals. This phenomenon is called a "head-to-tail arrangement", which will later form a binding site for carbohydrate binding. According to a comparison between the results and literature, *A. caudatus* and *A. hypochondriacus* are found to have the ability to assemble binding sites due to the presence of two agglutinin domain subunits in each lectin sequence of these two species, which allows the formation of a loop from a head-to-tail arrangement (Van Damme et al., 2008).

The analysis of the similarity or likeness of carbohydrate-binding site motifs in the 15 plant lectin protein sequences studied based on MSA revealed that 12 sequences matched the consensus of motifs found in the literature. The *G. max*, *P. sativum*, and *C. ensiformis* lectin sequences also exhibited a discrepancy between the consensus motif and the literature or incompleteness of carbohydrate-binding site motifs.

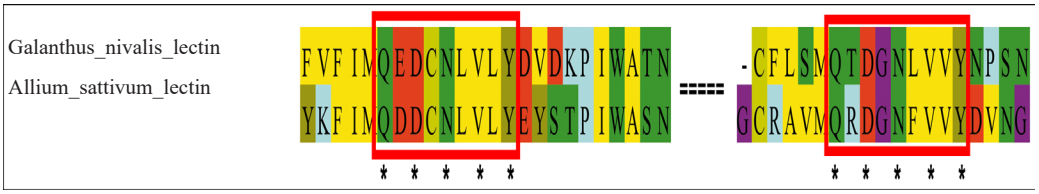


Figure 8. Comparison of carbohydrate-binding site motifs in plant lectin sequences with the B_lectin domain based on the similarity of motifs found in a previous study (Zhao et al., 2017). The conserved motif of B_lectin is "QXDXNXVXY"

Note. The asterisk (*) symbol indicates conserved amino acids; "X" indicates any amino acid

The Potential Roles of Plant Lectin

The five domains in the 15 plant lectin protein sequences have their respective functions or roles. The jacalin domain is a protein domain capable of binding monosaccharides and oligosaccharides with high specificity. At the beginning of its discovery, numerous jacalin domains were found in *Artocarpus* sp. (jackfruit), originating from the Moraceae family, but other studies have also found jacalin-related lectins in several plant families, such as the Asteraceae, Convolvulaceae, Musaceae, and Fagaceae families (Mann et al., 2001). Another study explained that the jacalin-related lectin domain plays roles in plant defense mechanisms, including disease resistance mechanisms, and is associated with abiotic stress signals (Esch & Schaffrath, 2017). In addition, previous studies have also shown that the jacalin-related lectin domain has a role in the transfer of proteins to the site of the pathogen attack by recognizing and binding to oligosaccharides that are typically present in pathogen infection processes (Esch & Schaffrath, 2017). Furthermore, other studies also explained that lectins derived from *M. acuminata* containing the jacalin domain could inhibit replication and block the entry of the HIV-1 virus (Swanson et al., 2010). Additionally, the modified lectin derived from *M. acuminata* (H84T) had inhibitory potential toward SARS-CoV-2 infection by blocking the binding of the SARS-CoV-2 spike protein to the ACE2 cellular receptor (Christodoulou et al., 2021). Lectins derived from *A. hypargyreus* are known to have the potential to act as immunomodulators by inducing T-lymphocyte activation (Zeng et al., 2019).

Chitin_bind_1, often found in the Poacea family, is a protein domain that recognizes and binds chitin subunits (type I). It also plays a role in plant defense mechanisms against pathogenic infections. A study showed broad-spectrum antifungal activity in plant chitinase proteins, which showed that the chitinase gene from *H. vulgare* could increase the fungal resistance of food crops (Kirubakaran & Sakthivel, 2007). In addition, results from other studies indicate the potential of lectins derived from *Triticum* sp. to be used as a therapeutic agent for leukemia (Ryva et al., 2019).

The next domain is B_lectin, which is a bulb-type mannose-specific lectin. The name "bulb-type lectin" corresponds to this type of lectin, which is often found in the bulb of a plant. This domain is found in several plant families, such as Amaryllidaceae and Aliaceae, and plays a role in the specific binding of mannose (Barre et al., 1997). B_lectin has the potential to be used as a natural insecticidal agent against specific receptors found on the insect midgut (Fitches et al., 2001).

The following domain is lectin_legume_LecRK_Arcelin_ConA, a combination of legume lectin, lectin-like receptor kinases, arcelin, concavalin A, and alpha-amylase. It is commonly known as legume lectin and is widely found in legumes in the Fabaceae family. This domain has a role in recognizing and binding specific glycoconjugates on cell surfaces and intracellular structures, where

they can serve as potential target molecules for development in agriculture, health, and pharmaceuticals (Lagarda-Diaz et al., 2017). In addition, legume lectins have shown potential to be developed as natural antifungal, antibacterial, and insecticidal agents for Coleoptera, Diptera, Lepidoptera, Hymenoptera, Isoptera, Neuroptera, and Homoptera, as well as possessing the ability to induce apoptosis in cancer cells (B. Liu et al., 2010; Gautam et al., 2018).

The last domain is agglutinin, a specific lectin that can agglutinate erythrocytes. Amaranthin has specific carbohydrate binding sites and can agglutinate A, B, and O red blood cells (Rinderle et al., 1989). In addition, agglutinins can inhibit the proliferation of tumor cells and are used as dyes for detecting human colon cancer through colonoscopy (Quiroga et al., 2015).

We then conducted a literature study concerning the production of lectin protein (yield) based on previous research. Table 5 lists the sources of plant lectin proteins in descending order of protein production and shows protein production and different organs that are the origin of lectins. Observation shows that the production of plant lectins through conventional methods varies widely from low (low yield) to high (high yield) categories. Plant lectin protein development can be carried out by synthetically utilizing its sequences as biological parts in producing plant lectin protein. Plant lectin protein sequences have the potential to be synthesized and further utilized in agriculture, health, and pharmacy by having information concerning the characteristics of each gene and the completeness of its carbohydrate binding site motifs.

The findings of this study show that several plant lectin sequences are potential candidates for utilization as biological parts by using the synthetic biology approach, especially in the coding sequence section. Some considerations for selecting the candidates include the completeness of the carbohydrate-binding site motif as well as the genetic characteristics such as CDS length (Table 2), which should be considered when producing lectin proteins through a synthetic biological approach because the longer the CDS, the more costly it will be compared to the shorter CDS. In addition, the completeness of the carbohydrate-binding site motif is also considered because it will affect the product proteins' ability to bind specific carbohydrates and their other roles as antifungal, antibacterial, antiviral, and insecticidal agents.

Candidate plant lectin sequences that can be utilized as biological parts for coding sequences include *M. acuminata*, *A. hypargyreus*, and *G. nivalis*. The first sequence is a lectin derived from *M. acuminata* with a CDS length of 426 bp, contains a jacalin domain, and has a complete carbohydrate binding site motif in the form of "GG" and "GXXXD" amino acids, according to a previous study (Raval et al., 2004). Banana lectin has shown potential as a candidate antiviral by inhibiting replication and blocking the entry of the HIV-1 virus (Swanson et al., 2010). In addition, previous studies

Table 5

Plant lectin sequences characteristics comparison

Species	Yield of lectin protein production (mg/g)	Protein domain	Completeness of carbohydrate-binding motifs	Organ of lectin origin
Amaranthus caudatus	1.600	Agglutinin	Two agglutinin subunits	Seed
Amaranthus hypochondriacus	1.280	Agglutinin	Two agglutinin subunits	Seed
Cicer arietinum	0.950	Legume lectin	Yes	Seed
Musa acuminata subsp. *malaccensis*	0.440	Jacalin	Yes	Fruit
Artocarpus hypargyreus Hance	0.400	Jacalin	Yes	Seed
Canavalia ensiformis	0.400	Legume lectin	No	Seed
Triticum aestivum L	0.300	Chitin_bind_1	Yes	Grain
Lens culinaris subsp. *tomentosus*	0.147	Legume lectin	Yes	Seed
Phaseolus vulgaris	0.130	Legume lectin	Yes	Seed
Hordeum vulgare var. Betzes	0.100	Chitin_bind_1	Yes	Grain
Galanthus nivalis L.	0.080	B_lectin	Yes	Tuber
Allium sativum L.	0.005	B_lectin	Yes	Tuber
Pisum sativum	0.005	Legume lectin	No	Seed
Robinia pseudoacacia	0.001	Legume lectin	Yes	Seed
Glycine max	0.001	Legume lectin	No	Seed

described the single amino acid-modified BanLec (H84T), which showed the potential for inhibiting SARS-CoV-2 infection by blocking the binding between the SARS-CoV-2 spike protein and the ACE2 cellular receptor (Christodoulou et al., 2021). Next is a lectin from *A. hypargyreus* with a CDS length of 453 bp and a jacalin domain with a complete carbohydrate binding site motif. Lectins from this species have potential as immunomodulators by inducing T-lymphocyte activation (Zeng et al., 2019).

The following candidate is a bulb-type lectin (B_lectin), a domain containing a lectin from *G. nivalis* with a CDS length of 471 bp. According to our reference

literature, this type of lectin has a complete carbohydrate binding site motif, which is "QXDXNXVXY", where X indicates any amino acid (Zhao et al., 2017). This lectin has shown potential as a natural insecticide with specific receptors in the insect midgut and exhibits antimetabolite activity in insects (Fitches et al., 2001).

CONCLUSION

This study compared the lectin gene lengths of 15 plant species. *Artocarpus hypargyreus* had the shortest lectin gene (480 bp), while *G. max* had the longest (1,009 bp). The length of the other plant lectin genes ranged from 495 to 912 bp. *Musa acuminata*, with three exons, had the most exons of any of the 15 lectin genes, while other plant lectin genes had 1-2 exons. The lectin proteins had five predicted domains: jacalin, chitin_bind_1, B_lectin, legume lectin, and agglutinin. Two protein sequences contained the jacalin domain; two contained the chitin_bind_1 domain; two contained the B lectin domain; and four contained the legume lectin domain. All domains' carbohydrate-binding site motifs were consistent with the consensus motifs found in the literature. The study results gave us gene, protein, and carbohydrate-binding motif comparison data from some lectin-producing plants, providing an understanding of future protein production, especially using a synthetic biology approach to obtain plant bioparts to develop more advanced lectin proteins.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Short-Term Effects of Bokashi Fertilizer with Reduced NPK Fertilization on Soil Fertility, Growth, and Yield of Rubber Trees

Auwalu Garba Gashua[1,4], Zulkefly Sulaiman[1,3]*, Martini Mohammad Yusoff[1], Mohd Yusoff Abd. Samad[2,3], Mohd Fauzi Ramlan[1] and Shafar Jefri Mokhatar[1]

[1]Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

[2]Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

[3]Institute of Plantation Studies, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

[4]Department of Agronomy, Faculty of Agriculture, Federal University Gashua, 631101, Yobe State, Nigeria

ABSTRACT

Rubber is currently the second major industrial crop in Malaysia after oil palm. The use of bokashi fertilizer (BF) on industrial crops is still not popular, and farmers rely mostly on chemical fertilizers (CFs) that are costly and hazardous to the environment. This research was conducted at *Hevea* plantation, Universiti Putra Malaysia, between August 2020–October 2021. The study was to assess the short-term effects of BF with reduced NPK fertilization on soil fertility, growth, and yield of rubber. Seven treatments (T) were involved. T1, T2, and T3 denote 4, 8, and 12 kg BF per pit. T4 was 1 kg NPK as control, whereas T5, T6, and T7 denote 4, 8, and 12 kg BF + 500 g NPK per pit, respectively. The variables observed included total nitrogen (TN%), total phosphorus (TP%), organic carbon (OC%), organic matter (OM%), exchangeable cations, microbial counts, tree girth, and dry rubber yield. The major findings indicated that applying 12 kg BF has raised the soil's TN%, OC%, and OM% by 165, 171.4, and 172.0%, respectively, compared to NPK control. Also, adding 4 kg BF + 500 g NPK has increased the soil's cation exchange capacity and TP% values by 107.8 and 42.9%, respectively, compared to the control. Adding sole bokashi increased the bacterial population by 22.2–133.3%. Rubber yield was better on trees treated with 12 kg BF, though

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E-mail addresses:
auwalu4real@gmail.com (Auwalu Garba Gashua)
zulkefly@upm.edu.my (Zulkefly Sulaiman)
martinimy@upm.edu.my (Martini Mohammed Yusoff)
myusoffas@upm.edu.my (Mohd Yusoff Abd. Samad)
fauzi@upm.edu.my / datodr.fauzi@gmail.com (Mohd Fauzi Ramlan)
shafarjefri@upm.edu.my (Shafar Jefri Mokhatar)
*Corresponding author

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this did not differ significantly from other treatments. Therefore, applying 12 kg BF or 4 kg BF + 500 g NPK can improve soil fertility and save costs from using inorganic fertilizer by at least 50%.

Keywords: Bokashi fertilizer, conservation pit, NPK, rubber trees, soil fertility

INTRODUCTION

British scientists brought the natural rubber tree to Malaysia in the nineteenth century, and it is now cultivated in several Asian countries (Abdulla & Arshad, 2017). Rubber (*Hevea brasiliensis*) is one of the major economic tree crops in Malaysia. Rubber is Malaysia's second largest agricultural commodity after oil palm (Ali et al., 2020). Most soils in the tropics are weathered and characterized by low levels of essential nutrients such as nitrogen, potassium, phosphorus, calcium, magnesium, sulfur, copper, zinc, and boron (Al Edrus et al., 2019). The tropical soils used to produce rubber are usually acidic and low in fertility, as caused by heavy leaching of nutrients and crop removal (Damrongrak et al., 2015). The conversion of undisturbed natural forests in the tropics for agricultural uses has resulted in the reduction of organic carbon, total nitrogen, and phosphorus, and water holding capacity of soils from 66.5% to 38.2% in disturbed agro-forest lands posing run-off and erosion dangers (Bohluli et al., 2015).

Rubber plantations usually receive intensive chemical fertilizations that are costly and detrimental to the environment to improve soil fertility and increase rubber productivity (Shabbir et al., 2021). Several tons of synthetic fertilizers added to soils are not fully utilized by crops but are lost through run-off or escape through atmospheric evaporation. It was reported that about 50% of nitrogen and up to 90% of phosphorus added to soils are lost through surface run-offs from croplands or escape into water bodies or the atmosphere, resulting in the emission of greenhouse gasses, pollution of the aquatic system, and the salinization of soils (Ye et al., 2020). Too much addition of synthetic fertilizer raises production costs and negatively impacts living organisms and other biological processes within the ecosystem (Guignard et al., 2017).

Synthetic fertilizer increases crop productivity, but its continuous application can greatly threaten the environment (Wahyuningsih et al., 2019). Soil quality is concerned with the physicochemical and biological properties which enable the better functioning of the ecosystem for human benefit (Carron et al., 2016). The abusive application and long-term usage of synthetic fertilizer can degrade soil quality, contaminate groundwater, and interfere with the good functioning of beneficial soil microorganisms (Hernandez et al., 2021).

The effect of fertilizer application on the yield of rubber has been poorly documented and contradictory and is yet to be fully understood (Chambon et al., 2018; Chotiphan et al., 2019; Tiva et al., 2016). Few studies were conducted to study the influence of organic amendments (OAs) on soil fertility status and the growth of rubber

in plantations (Huang et al., 2020). Similarly, the effects of OAs, such as bokashi, on soil fertility and the growth of rubber are yet to be fully understood (Huang et al., 2020). In addition, NPK fertilizer plays a crucial role in the growth and yield of a rubber tree, and the provision of adequate amounts of these nutrients is very vital for the performance of the crop (Al Edrus et al., 2019). Based on previous studies, using organic fertilizers (OFs) meets the requirements of agricultural sustainability by providing the physicochemical and microbiological properties needed in the soil for plant growth and soil health (Ansari et al., 2019; Hata et al., 2021; Pohan et al., 2019; Wijayanto et al., 2016). Also, using OAs and reducing the amounts of inorganic fertilizer is a cost-effective and environmentally friendly way of sustainable agriculture (Ning et al., 2017). However, the sole use or reliance on OFs may (sometimes) not provide the immediate nutrient requirements as they release nutrients very slowly, with just a fraction of nitrogen and other essential elements becoming readily available to plants in the initial year of application (Ning et al., 2017).

Therefore, using OFs with or without mineral fertilizer is a good management practice in agricultural production systems as it improves soil fertility and the quality of produced plants (Natsheh & Mousa, 2014). Nutrient management is the second driver of rubber yield (Vrignon-Brenas et al., 2019). In Malaysia, rubber is mostly grown on sloppy lands. The major problems of sloppy lands include soil erosion, fertilizer loss, and poor soil water storage (Mohsen et al., 2014). Hence, using OFs to replace or complement inorganic fertilizers can be viable in sustainable agriculture (Hernandez et al., 2021). Therefore, conserving the soil quality in plantations is of great importance for the proper functioning of the ecosystem (Wahyuningsih et al., 2019).

In plantations, pits sizing 1.8 m x 0.5 m x 0.6 m are usually dug between four trees under moderate slopes (Bohluli et al., 2015). Applying fertilizers in conservation pits serves to conserve nutrients and water. Rubber farmers are normally advised to add fertilizers in pits sizing 1-2 m x 0.6 m x 0.4 m between rows to absorb plant nutrients (Huang et al., 2020). Multipurpose conservation pits (MPCPs) (sometimes called 'pits' or 'silt pits') are an effective method of conserving soil water and plant nutrients (Moradidalini et al., 2011). Such pits are generally used to collect run-off water, trap down sediments, increase soil moisture, and reduce erosion and fertilizer losses (Bohluli et al., 2015). The silt pit is Malaysia's recommended soil and water conservation method (Sung et al., 2011).

Bokashi fertilizer (BF) can supply plants with nutrient requirements and improve soil fertility. The bokashi's ability to quickly decompose organic wastes (into useful OFs) makes it a promising waste management solution (Pohan et al., 2019). Several studies have indicated that BF has been used successfully to improve the growth, yield, and quality of crops and soils (Aung et al., 2019; Gashua et al., 2022;

Hoshino et al., 2021; Quiroz & Flores, 2019). Although CFs can provide plants with the nutrient requirements for their growth and development, these fertilizers do not have the same beneficial effects on the soil as OAs (Hernandez et al., 2021). It was reported that the availability and adequate use of organic and synthetic fertilizers are low, especially in large plantations (Apori et al., 2020). Therefore, this study aims to investigate the effects of BF with reduced NPK fertilization on soil fertility, growth, and rubber yield.

METHODS

Experimental Site and Materials Used

The major materials used include bokashi fertilizer, NPK blue ($N:P_2O_5:K_2O:MgO$ [12:12:17:2]) (ZINONG, China), MPCPs, and RRIM 3001 clone. This clone, 'Klon 1 Malaysia', is known for its high rubber productivity. This experiment was conducted at Ladang Hevea, Taman Pertanian University, Universiti Putra Malaysia. The trees were not of uniform girth size at the commencement of the study (August 2020). Before the commencement of this research, the fertilization practice in the plantation was NPK blue (12:12:17:2) at the rate of 1 $kg \cdot tree^{-1} \cdot yr^{-1}$, usually applied in 2 split doses, with the last dose applied four months before the start of this experiment. The tapping system practiced was ½S d/3 (tapped every 3 days).

Preparation of Bokashi Fertilizer

The BF was prepared using horse bedding waste, cow dung, and paddy husk charcoal in the 60, 20, and 20% ratios, respectively. These raw materials were treated with an effective microorganisms-4 (EM4) solution and molasses, as described by Gashua et al. (2022), and the BF was ready for use within 28 days.

Field Layout

Trees that were estimated to be above 50 cm girth size (1.5 m from ground level) were randomly selected with 4 trees in a rectangular arrangement forming one experimental unit, and the trees were tagged according to the design of the experiment. A multipurpose conservation pit (MPCP) was dug between every four trees for each treatment (except the control), with the MPCP oriented against the slope. Each pit was sized 1.0 m x 0.5 m x 0.3 m (0.15 m^3) in length, width, and depth, respectively (Figure 1). Each conservation pit was treated with 500 g of ground magnesium limestone (GML) and allowed for seven days before treatment application (Figure 2a). The control plots were also treated with 500 g GML before treatment application. Different rates of bokashi were then applied in the pits according to the treatment specifications (Figure 2b).

Treatments and Experimental Design

The experiment consisted of seven treatments, replicated three times, and laid out in a randomized complete block design (RCBD). The breakdown of the treatment combination is given in Table 1.

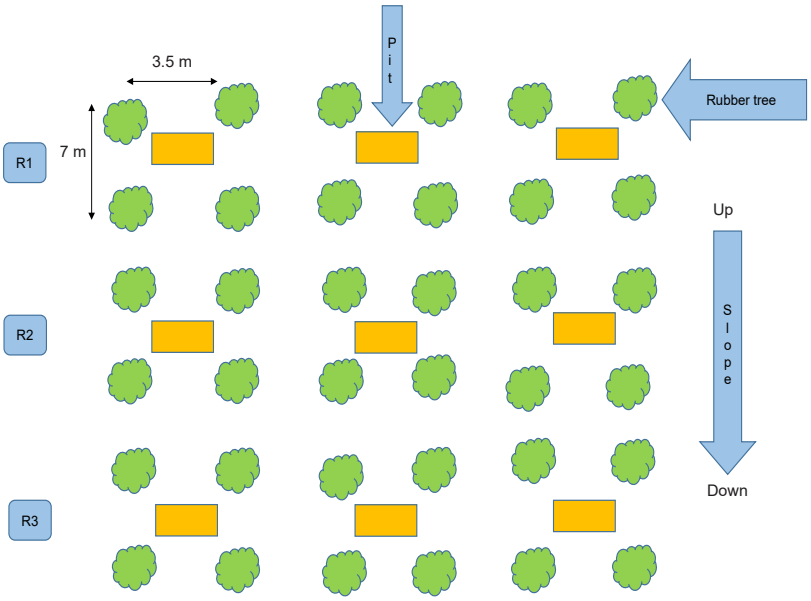

Figure 1. The layout of the field with pits dug in between four trees

Note. R = Replication

Table 1

Treatments and their descriptions

Treatment	Description
T1	4 kg bokashi + 500 g GML/pit
T2	8 kg bokashi + 500 g GML/pit
T3	12 kg bokashi + 500 g GML/pit
T4	Control 1 kg NPK blue (12:12:17:2) + 500 g GML surface application
T5	T1 + 500 g NPK blue/pit
T6	T2 + 500 g NPK blue/pit
T7	T3 + 500 g NPK blue/pit

Note. The NPK (500 g) in treatments 5, 6, and 7 were added 3 weeks after the commencement of the research. The depth of the pits used was within the rooting depth of fine roots of rubber (0–30 cm) that are responsible for the absorption of nutrients and water (Huang et al., 2020). Except for the control-T4, all other treatments were applied through the conservation pits; GML = Ground magnesium limestone

Figure 2. MPCPs (a) with GML and (b) bokashi applied

Note. GML = Ground magnesium limestone

Determination of Soil Chemical Properties

The soil samples 0–20 cm into the pits were collected before and after the experiment to determine the soil's chemical

properties. Before treatment application, nine samples, three from each replication, were randomly selected, composited, and subjected to chemical analysis. At the end of the experiment, the soil sample for each treatment and replication (except the control) was collected separately from 0–20 cm into the pits. Soil sample from the control treatment was also taken from the top 20 cm using a soil auger. The pH of the soil was determined in 1:2.5 (w/v) soil-water suspension and measured using a pH meter (Model HI 2211 pH/ORP meter, Hanna Instruments, Thailand). The EC was obtained in 1:5 (w/v) soil-water suspension using an EC meter (Model HI 2300 EC/TDS/NaCl meter, Hanna Instruments, Thailand). The leaching method determined the soil's cation exchange capacity and exchangeable bases in 1 N ammonium acetate buffered at pH 7 (Baruah & Barthakur, 1997). The first leachate was sent to the atomic absorption spectrophotometer (AAS) (PerkinElmer PinAAcle 900T, USA) for the determination of calcium (Ca), magnesium (Mg), and potassium (K) values. Leachate 3 was used to determine the cation exchange capacity (CEC) values using Auto Analyzer (AA) (LACHAT Instrument, QuickChem FIA+ 8000 series, USA). The soil's organic carbon and organic matter contents were obtained by the method of Walkley (1947). The total nitrogen was determined by Dumas's method using a carbon, nitrogen, and sulfur (CNS) analyzer (LECO TruMac® CNS, USA). The available N in the form of nitrate (NO_3^-) and ammonium (NH_4^+) ions were obtained from the distillation process. The aqua regia method (using hydrochloric acid [HCl] and nitric acid [HNO_3]) was employed, and the digests were sent to atomic absorption spectrophotometer (AAS) for determination of total P values. The available P was obtained using the Bray II method (Bray & Kurtz, 1945). The same analysis procedures were followed, collecting the samples from each treatment at the end of the experiment and analyzed separately.

Determination of Soil Microbial Properties

Soil samples were taken during the sampling for chemical analysis to evaluate the soil microbial properties. The soil samples were collected at a depth of 0-20 cm. The samples were collected before and after the experiment to evaluate bacterial and fungal counts. Potato dextrose agar (PDA) and nutrient agar (NA) were the two media used for fungi and bacteria, respectively. After the experiment, for each treatment, six grams of the mixed soil were placed in a 50 ml Falcon tube, and 15 ml distilled water was added and shaken for 24 hr to obtain the stock solution. Three serial dilutions were prepared. For each treatment, 9 ml of sterilized water was added into 15 ml Falcon tubes (seven treatments x three replications = 21 sterilized tubes). One milliliter of the stock solution was pipetted into each test tube containing 9 ml of sterile distilled water for the first serial dilution (10^1). From this (10^1), 1 ml was added to the next Falcon tube containing 9 ml of sterilized water (serial dilution number 2 or 10^2) and mixed thoroughly. Also, 1 ml was taken from this serial dilution number 2 and

added to the next Falcon tube designated as number 3, making serial dilution 3 (10^3). The same procedure was repeated for each treatment, and the samples were collected before treatment application. However, three samples from each of the three replications of composite soil samples were collected and bulked before treatment application. From each serial dilution, 100 µl was pipetted and gently spread on the media (PDA or NA, as the case may be), and three replications were made for each dilution. The Petri dishes (containing NA and the sample dilutions) were incubated for 24 hr at 30–32ºC to determine bacteria. For the fungi, the Petri dishes (containing PDA and the sample dilutions) were incubated for 72 to 96 hr at 25–27ºC (Akande & Adekayode, 2019). The microbial count for the bacteria and fungi was evaluated by determining the colony-forming unit (cfu). It was achieved by selecting the plates/dishes with the countable number of colonies (30–300). Then the total dilution factor and the volume of culture plated (100 µl or 0.1 ml) were used to compute the cfu. The results from serial dilution 3 (10^3) were finally considered to determine cfu. The population of bacteria and fungi in the soil was obtained after determining the cfu, as described by Akande and Adekayode (2019).

The cfu/ml was calculated using the formula:

$$\text{cfu/ml} = \frac{\text{Number of colonies} \times \text{Total dilution factor}}{\text{Volume of culture plated in ml}} \quad [1]$$

Assessment of Growth of Rubber Trees

The growth of rubber trees was assessed by measuring the trunk size using a tailor's measuring tape four times (including initial measurement) at four-month intervals. The measurement was taken 150 cm from the ground level (Fauzi et al., 2015). The increment in tree girth was calculated by subtracting the final girth from the initial. In contrast, the % girth increment was obtained by dividing the increment in tree girth by the initial girth multiplied by 100. The data were subjected to analysis of variance using the statistical analysis software (SAS 9.4 version, SAS Institute Inc., USA).

Rubber Yield

The rubber yield was initially collected as a cup lump yield. The tapping system was half-spiral, with 3 daily tapings and approximately eight monthly tappings. The use of ½S, d/3 is a recommended practice on high-yielding budded clones prone to tapping panel dryness (TPD) (Gates, 1979). The cup lump yield was later converted to dry rubber yield (Gonçalves et al., 2011). The yield data collected weekly were summed up and analyzed monthly (g·tree·month) over 7 months; September to December 2020 and August to October 2021. Finally, the dry rubber yield was expressed regarding land productivity in $kg \cdot ha^{-1} \cdot year^{-1}$ (Ali et al., 2020). Because the trees were not of uniform size across different treatments, yield computation was based on standardizing the initial tree size by taking the average tree size (75.8 cm) and assuming all trees were of the same

size across all treatments at the onset of the experiment. The rubber yield was obtained as given below.

$$\text{Yield}\ (\text{kg} \cdot \text{ha}^{-1} \cdot \text{year}^{-1}) = \frac{g/tree/trapping/year \times Tree\ stand/hectare \times Tapping\ days}{1{,}000} \quad [2]$$

Statistical Analysis

The collected data (excluding data on microbial count) were analyzed using analysis of variance (ANOVA) with statistical analysis software (SAS 9.4 version, SAS Institute Inc., USA), and significant means were compared using Tukey's honestly significant difference (HSD) test at 5%.

RESULTS

Soil Chemical Properties

Before the treatments, the soil was characterized by relatively low pH, EC, CEC, Ca, Mg, K, OC, OM, total N, NH_4^+-N, NO_3^--N, total P, and available P compared to the values obtained after the experiment. The initial soil values were 4.41, 0.04 mS/cm, 7.12 $cmol_{(+)}$/kg, 5.37 $cmol_{(+)}$/kg, 0.20 $cmol_{(+)}$/kg, 0.16 $cmol_{(+)}$/kg, 1.13%, 2.32%, 0.10%, 8.00 mg/kg, 4.00 mg/kg, 0.01%, and 2.00 mg/kg for pH, EC, CEC, Ca, Mg, K, OC, OM, total N, NH_4^+-N, NO_3^--N, total P, and available P, respectively (Table 2).

Most soil's chemical properties differed significantly at the end of the experiment (Table 2). The pH values differed significantly (p<0.05) between treatments, with the highest value (6.38) obtained from where 12 kg/pit of BF (T3) was added, and the lowest pH value (5.02) was obtained from the control-T4 (NPK at 1 kg per four trees, surface application) (Table 2).

Similarly, the EC values differed significantly (p<0.05) between the treatments, with the highest EC value (0.29 ms/cm) obtained in T3 and the lowest (0.11 ms/cm) in the control-T4 (Table 2). There was also a significant difference (p<0.05) in the CEC values between the treatments, with the highest CEC value (25.45 $cmol_{(+)}$/kg) obtained in T5 (4 kg bokashi + 500 g NPK per pit). The lowest CEC (12.25 $cmol_{(+)}$/kg) was obtained in the control-T4.

Significant differences were found in Ca and Mg values between the treatments, but K values did not differ significantly (p>0.05). The Ca highest value (14.61 $cmol_{(+)}$/kg) was obtained in T7 and can compare statistically with values obtained in T2, T3, and T6. The lowest Ca value (8.40 cmol(+)/kg) was obtained in the control-T4. However, Ca values in T1 and T5 were also low and statistically comparable with the control-T4. Magnesium (Mg) was highest in T2 (8.61 cmol(+)/kg) and lowest (4.12 cmol(+)/kg) in the control-T4 (Table 2).

The organic carbon (OC%) and organic matter (OM%) values differed significantly (p<0.05) between the treatments, with the respective highest values (5.13 and 8.84%) provided by T3. The lowest OC% and OM% (1.89 and 3.25%) were found in the control-T4 (Table 2). Similarly, a significant difference (p<0.05) was found in the total N (%) values between the treatments but not with NH_4^+-N or NO_3^--N (mg/kg). Total

N (%) was highest (0.53%) in T3 and lowest (0.15%) in T5. Total N values in the control-T4, T6, and T7 were also low and can compare statistically with T5 (Table 2).

The TP% values differed significantly (p<0.05) between treatments. The TP% was highest (0.20%) in T5 but lowest (0.14%) in the control treatment (Table 2). There was a marked rise in the levels of TP% in the soil with the increased rate of bokashi from 4–12 kg applied. The treatments' effect on the soil's available P content was insignificant.

Table 2

Soil chemical properties

Parameter	Before	T1	T2	T3	T4	T5	T6	T7
pH	4.41	5.54± 0.09 bc	6.24± 0.21 ab	6.38± 0.15 a	5.02± 0.12 c	5.65± 0.10 abc	6.08± 0.02 ab	5.92± 0.21 ab
EC (mS/cm)	0.04	0.26± 0.01 ab	0.2 ± 0.05 ab	0.29± 0.05 a	0.11± 0.01 b	0.18± 0.02 ab	0.15± 0.01 ab	0.20± 0.03 ab
CEC (cmol(+)/kg)	7.12	18.50± 2.80 ab	23.17± 0.81 ab	22.55± 3.15 ab	12.25± 0.55 b	25.45± 3.45 a	22.60± 0.90 ab	22.13± 1.71 ab
Ca (cmol(+)/kg)	5.37	8.42± 1.19 c	13.20± 0.68 ab	12.66± 1.65 ab	8.40± 1.16 c	10.34± 0.79 bc	12.97± 1.23 ab	14.61± 1.26 a
Mg (cmol(+)/kg)	0.20	7.46± 1.37 ab	8.61± 0.18 a	6.22± 0.01 ab	4.12± 0.69 b	5.49± 0.49 ab	7.38± 0.28 ab	6.51± 0.47 ab
K (cmol(+)/kg)	0.16	0.26± 0.03	0.25± 0.05	0.25± 0.05	0.18± 0.01	0.23± 0.05	0.27± 0.06	0.21± 0.02
OC (%)	1.13	2.74± 0.59 bc	4.61± 0.33 ab	5.13± 0.51 a	1.89± 0.20 c	3.18± 0.25 abc	2.95± 0.14 bc	3.35± 0.35 abc
OM (%)	2.32	4.72± 1.02 bc	7.94± 0.57 ab	8.84± 0.87 a	3.25± 0.34 c	5.47± 0.43 abc	5.08± 0.23 abc	5.77± 0.59 abc
TN (%)	0.10	0.33± 0.08 ab	0.32± 0.11 ab	0.53± 0.03 a	0.25± 0.05 b	0.15± 0.00 b	0.23± 0.02 b	0.25± 0.03 b
NH_4^+-N (mg/kg)	8.00	140.00± 0.01	280.00± 0.01	143.00± 0.00	230.00± 0.02	283.00± 0.00	125.00± 0.00	180.00± 0.00
$N0_3^-$-N (mg/kg)	4.00	97.00± 0.01	153.00± 0.01	90.00± 0.00	215.00 ± 0.00	273.00± 0.00	230.00± 0.01	163.00± 0.00
TP (%)	0.01	0.16± 0.01 bc	0.18± 0.01 ab	0.19± 0.01 ab	0.14 ± 0.01 c	0.20± 0.01 a	0.19± 0.01 ab	0.18± 0.00 abc
AP (mg/kg)	2.00	60.00± 0.00	127.00± 0.00	124.00± 0.00	59.00 ± 0.00	183.00± 0.04	176.00± 0.01	135.00± 0.00

Note. Means followed by the same letter(s) in a row are not significantly different (p>0.05) using Tukey's HSD ± standard error

T1 = 4 kg bokashi per pit; T2 = 8 kg bokashi per pit; T3 = 12 kg bokashi per pit; T4 = 1 kg NPK surface application; T5 = 4 kg bokashi + 500 g NPK per pit; T6 = 8 kg bokashi + 500 g NPK per pit; T7 = 12 kg bokashi + 500 g NPK per pit

Soil Microbial Counts

The soil bacterial and fungal counts before and after treatment application are given in Table 3. The sole bokashi treatments (i.e., T1–T3) gave the highest population of bacteria (1.10×10^5 – 1.37×10^5 cfu/ml) compared to 9.00×10^4 cfu/ml before treatment application. However, the bacterial count in the NPK treatment (control-T4) and T5, T6, and T7 were lower than the values obtained before the treatment application. The range of fungal populations obtained after treatments application in all treatments other than the control (3.33×10^3 – 4.67×10^4 cfu/ml) was far below the value obtained (5.00×10^4 cfu/ml) before treatment application with no trace of fungus in the NPK treatment (0.00×10^0 cfu/ml) (Table 3).

Table 3

Bacterial and fungal populations in the soil before and after treatment application

Treatment	Bacterial population (cfu/ml)			Fungal population (cfu/ml)		
	Before treatment	After treatment	% increase	Before treatment	After treatment	% decrease
T1	9.00×10^4	1.10×10^5	22.20	5.00×10^4	4.67×10^4	6.60
T2	9.00×10^4	2.10×10^5	133.30	5.00×10^4	4.00×10^4	20.00
T3	9.00×10^4	1.37×10^5	52.20	5.00×10^4	3.33×10^3	93.30
T4	9.00×10^4	3.00×10^4	-66.70	5.00×10^4	0.00×10^0	100.00
T5	9.00×10^4	5.33×10^4	-40.80	5.00×10^4	6.67×10^3	86.70
T6	9.00×10^4	2.73×10^4	-69.70	5.00×10^4	1.33×10^4	73.40
T7	9.00×10^4	1.20×10^4	-86.70	5.00×10^4	3.33×10^3	93.30

Note. T1 = 4 kg bokashi per pit; T2 = 8 kg bokashi per pit; T3 = 12 kg bokashi per pit; T4 = 1 kg NPK surface application; T5 = 4 kg bokashi + 500 g NPK per pit; T6 = 8 kg bokashi + 500 g NPK per pit; T7 = 12 kg bokashi + 500 g NPK per pit

Rubber Tree Growth

The growth of the rubber tree was monitored three times a year at a four-month interval. The treatments'effect showed no significant difference in the girth size increment across the data collection periods. However, some growth in terms of girth increment was noticed between the treatments over the three periods of data collection from the initial tree sizes before treatment application. The highest increment in tree girth (5.25%) was observed in T6 and the lowest (3.38%) in control T4 over 12 months.

Rubber Yield

The dry rubber yield was obtained between September-December 2020 and between August-October 2021. The mean monthly dry yield ($g \cdot tree^{-1} \cdot month^{-1}$), the average yield ($g \cdot tree^{-1} \cdot tapping^{-1}$) (over seven months), and the estimated land productivity per year are presented in Table 5. There was no significant difference ($p>0.05$) in average dry rubber yield ($g \cdot tree^{-1} \cdot tapping^{-1}$), monthly rubber yield, and land productivity between the treatments.

Table 4

Trunk size (girth) of rubber tree

	Trunk size (cm)					
Treatment	Initial	Dec. 2020	Apr. 2021	Aug. 2021	Increment (cm)	% increment
T1	77.77±5.24	78.35±5.18	79.07±5.46	80.60±5.40	2.84±0.42	3.66±0.55
T2	68.25±2.48	68.74±3.03	69.41±2.97	70.86±2.99	2.61±0.52	3.78±0.64
T3	72.02±5.18	72.63±5.30	73.39±5.48	74.86±5.20	2.84±0.31	3.98±0.55
T4	82.32±10.69	83.90±10.94	83.90±10.94	85.04±10.77	2.72±0.24	3.38±0.39
T5	80.18±2.15	80.73±2.16	81.81±2.16	83.85±2.24	3.68±0.12	4.59±0.10
T6	76.62±4.08	77.33±4.10	78.65±4.10	80.59±3.89	3.98±0.73	5.25±1.07
T7	73.44±13.66	74.09±13.54	75.17±13.54	76.45±13.62	3.01±0.04	4.26±0.85

Note. T1 = 4 kg bokashi per pit; T2 = 8 kg bokashi per pit; T3 = 12 kg bokashi per pit; T4 = 1 kg NPK surface application; T5 = 4 kg bokashi + 500 g NPK per pit; T6 = 8 kg bokashi + 500 g NPK per pit; T7 = 12 kg bokashi + 500 g NPK per pit. The values within columns are means ± standard errors

Table 5

Monthly dry rubber yield, mean yield per tapping, and land productivity

	Year 2020				Year 2021			Mean yield	Land productivity
	September	October	November	December	August	September	October		
Treatment	$g \cdot tree^{-1} \cdot month^{-1}$							$g \cdot tree^{-1} \cdot tapping^{-1}$	$(kg \cdot ha^{-1} \cdot year^{-1})$
T1	469.03±102.50	604.17±42.83	822.00±133.64	834.50±125.21	259.33±27.73	324.77±28.94	395.70±30.87	66.23±8.28	2,225.70±277.78
T2	354.33±40.75	571.77±55.27	858.20±43.19	868.73±16.19	227.77±27.33	396.83±126.80	427.90±78.45	66.17±4.62	2,223.40±155.24
T3	424.17±71.28	588.73±45.66	984.80±151.33	914.03±85.17	284.27±81.45	292.90±43.04	316.50±49.03	68.10±6.99	2,289.30±235.06
T4	396.20±76.85	534.73±31.70	794.20±116.12	831.93±71.81	271.93±76.85	373.47±91.45	388.10±98.43	64.13±8.73	2,154.30±293.06
T5	489.90±57.16	583.47±24.85	703.00±30.09	665.60±2.04	231.13±19.49	321.27±30.07	384.40±7.94	60.33±1.97	2,027.30±65.46
T6	389.00±21.74	566.20±51.06	762.50±50.76	790.00±28.96	181.43±2.89	239.07±33.92	266.40±41.71	57.07±2.42	1,916.70±80.72
T7	330.07±63.03	585.17±27.99	947.60±137.76	915.40±92.04	218.30±21.46	337.73±121.18	443.90±199.65	67.45±11.47	2,266.90±385.45

Note. The tapping system used was ½S d/3, tapping days = 96 per year (i.e., 8 times/month), and the estimated tree stand/ha = 350
T1 = 4 kg bokashi per pit; T2 = 8 kg bokashi per pit; T3 = 12 kg bokashi per pit; T4 = 1 kg NPK surface application; T5 = 4 kg bokashi + 500 g NPK per pit; T6 = 8 kg bokashi + 500 g NPK per pit; T7 = 12 kg bokashi + 500 g NPK per pit. The values within columns are means ± standard errors

DISCUSSION

Treatments Effects on Soil Chemical Properties

The application of treatments improved most soil chemical properties studied. The plantation soil before treatment application was acidic (pH 4.4), typical of tropical soils where the rubber is grown. Most soils where the rubber is grown are strongly acidic, low in exchangeable bases but high in aluminum and manganese (Damrongrak et al., 2015). The soil condition was significantly improved by raising the pH level after applying treatments. The application of 12 kg bokashi (T3) has raised the soil pH by 27.10% compared to the application of NPK-control fertilizer (T4). Treatment with sole NPK fertilizer usually causes soil acidification (Roba, 2018). However, the control treatment had its pH raised from 4.41 to 5.02, probably due to liming with GML before applying the NPK treatment. Similarly, adding bokashi with or without NPK fertilizer in other treatments has also raised the soil pH by 10.40-21.10% compared to the NPK control (T4). A significant increase in soil pH was reported due to the combined application of organic manure and NPK fertilizer (Roba, 2018). Wijayanto et al. (2016) reported increased soil pH due to adding bokashi.

Most plants grow better in pH ranging from 6.0 to 6.5 because this is the range in which most plant nutrients are in their available forms (Damrongrak et al., 2015). T2, T3, and T6 provided a pH of 6.08 to 6.24. Similarly, the EC value was significantly improved following treatment application. The application of 12 kg bokashi has caused an increase in the soil EC value by 163.60% compared to the NPK-control (T4) (Table 2). Adding bokashi with or without NPK fertilizer in the other treatments has also increased the soil EC value by 36.40–136.40% compared to the control T4. Although the EC value of the soil increased significantly in the different treatments, the obtained values were not above the critical limit of 4.00 mS/cm (Angelova et al., 2013). The increase in soil CEC value by 107.80% was caused by applying 4 kg bokashi + 500 g NPK (T5) compared to the NPK-control (T4). The soil CEC was also raised by 51.00–87.40% in other bokashi or bokashi + NPK treatments compared with the control-NPK treatment (T4) (Table 2). When added to the soil, CFs like NPK can improve soil chemical characteristics, add plant nutrients, and promote crop growth and yield (Ubi et al., 2013). Singh et al. (2017) also reported that balanced NPK fertilizers considerably raised the soil's CEC when combined with farmyard manure or applied alone. A significant increase in soil CEC due to the addition of bokashi and NPK fertilizer was also reported by Lasmini et al. (2018). The application of bokashi with or without NPK has raised the soil CEC status from low (≤ 12 cmol(+)/kg) to moderate (12–25 cmol(+)/kg) levels of fertility (Hazelton & Murphy, 2016).

The application of 12 kg bokashi + 500 g NPK (T7) has significantly raised the soil Ca value by 73.9% compared with the application of NPK-control (T4) (Table 2). Adding bokashi with or without NPK

fertilizer in the other treatments increased the soil Ca value in other treatments by 0.24–59.80% compared to the control (T4). The application of 8 kg bokashi (T2) has significantly caused an increase in the Mg content in the soil by 109% compared with the application of NPK-control (T4) (Table 2). Adding bokashi with or without NPK fertilizer in the other treatments has also raised the soil Mg content by 33.30–81.10% compared to the NPK control. Although the application of bokashi has raised the potassium level in the soil, there was no significant difference in potassium values between the treatments. Potassium plays a vital role in osmoregulation in plants and functions to activate enzymes, especially those involved in photosynthesis and respiration (Pohan et al., 2019).

The application of 12 kg bokashi (T3) has resulted in an increase in the OC content by 171.40% (Table 2) compared to the application of NPK-control (T4). Adding bokashi with or without NPK fertilizer in the other treatments has also raised the soil OC levels by 45.00-143.90% compared to the NPK control. Similarly, the application of 12 kg bokashi (T3) has caused an increase in the soil OM content by 172% compared to the application of NPK-control (T4). The rise in soil OC with the addition of high doses of bokashi was caused by high OM contents in this organic amendment and was similar to the result reported by Angelova et al. (2013). Adding bokashi to soils improves organic matter, supplies essential nutrients such as NPK, and minimizes the leaching of nutrients (Murillo-Amador et al., 2015). Applying bokashi with or without NPK fertilizer has also increased the soil OM levels in the other treatments by 77.50–144.30% compared to the NPK control (T4). Adding bokashi with or without NPK fertilizer has increased the soil OC and OM contents compared to the control and their initial levels before treatment application.

The application of 12 kg bokashi (T3) significantly increased the TN% in the soil by 165.0% compared to the application of NPK-control fertilizer (T4). Applying bokashi with or without NPK fertilizer has also increased the soil's TN% levels in the other treatments by 15–60% compared to the NPK control (T4). Although the application of bokashi has raised the level of NH_4^+-N in the soil, there was no significant difference in NH_4^+-N values between the treatments. Ning et al. (2017) reported that the combined use of organic and CFs did not significantly affect the value of soil available nitrogen. Similarly, applying bokashi had improved the NO_3^--N in the soil a little, but such increment was not significantly different between the treatments (Table 2). However, Cai et al. (2018) reported an increase in soil N and P contents in treatments combining organic and mineral fertilizers compared to NPK treatments. The use of pits in rubber plantations has conserved N, P, and K in the range of 12 to 29, 6 to 13, and 27 to 62 kg/ha, respectively (George et al., 2007).

The application of 4 kg bokashi + 500 g NPK (T5) has raised the TP% value in the soil by 42.90% compared to the application of NPK-control (T4). Applying bokashi with or without NPK fertilizer has also raised

the soil's TP% by 14.30–35.70% compared to the NPK control (T4). Phosphorus is a constituent of nucleic acid and functions in plant metabolism (Pohan et al., 2019). Maass et al. (2020) have also reported an increase in phosphorus levels with increased rates of bokashi application. Several studies have indicated that the addition of OAs has improved soil fertility such as soil OM, total N, NO_3^--N, available phosphorus, and potassium (Guo et al., 2015; Huang et al., 2020; Long et al., 2015; Seleiman & Kheir, 2018).

Treatments Effects on Soil Microbial Counts

The abundance of microorganisms in soil may indirectly indicate soil fertility. The application of OAs has raised the soil's population of bacteria and fungi. The application of 8 kg BF (T2) gave the highest bacterial population of 2.10 x 10^5 cfu/ml (indicating an increase of 133% from the initial population). The bacterial population decreased by 66.70% with the addition of NPK fertilizer (Table 3). It was reported that the application of farmyard manure to the soil had raised the bacterial population (Kumari et al., 2014). It was also reported that the addition of OFs has caused an increase in soil OC thereby raising the microbial count in the soil (Nakhro & Dkhar, 2010). Similarly, the fungal populations were mostly highest in sole bokashi treatments, with the highest fungal count (4.67 x 10^4 cfu/ml) from the treatment containing 4 kg bokashi (T1), which was still below the initial fungal count of 5.00 x 104 cfu/ml. No trace of a fungal population was obtained from the control-T4. However, Sulok et al. (2021) reported higher bacterial and fungal counts in soil OAs containing composts and biochar in the second year of addition compared to the control (NPK fertilizer).

Adding OAs can provide an ideal environment for microorganisms to multiply (Sulok et al., 2021). The use of synthetic fertilizers over a long period resulted in a decrease in microbial population and activity in the soil (Hernandez et al., 2021). The soils treated with OAs showed greater bacterial and fungal abundance compared to the control and soils treated with NPK mineral fertilizer (Hernandez et al., 2021). Generally, microorganisms such as bacteria, fungi, algae, and actinomycetes usually play a vital role in decomposing organic matter and nutrient recycling, resulting in increased soil fertility (Aytenew & Bore, 2020).

Effects of Treatments on Rubber Growth

Girth size is important in determining rubber yield and quality (Sopheaveasna et al., 2019). However, the treatment effect was insignificant on the girth size of rubber trees over the periods of data collection (12 months). A similar result was reported when rubber was treated with different rates of NPK fertilizer; there was no significant difference in the tree girth size in the first two years of treatment application (Sopheaveasna et al., 2019). Twelve months after soil improvement, the lowest girth increment (of 3.38%) was obtained with

NPK fertilization, and the highest girth increments (4.59 and 5.25%) were obtained with the application of 4 and 8 kg BF + 500 g NPK, respectively. Increments in girth size above what was obtained from the control were observed with the application of bokashi or bokashi with reduced NPK fertilization (Table 4). It agrees with the result reported by Damrongrak et al. (2015) after soil improvement in the first year. Similarly, the result agrees with the findings of Abraham et al. (2015), who reported no significant difference in the girth size of immature rubber in the first year of the trial on integrated nutrient management. Despite having the largest average initial girth size, trees treated with NPK fertilizer provided the lowest girth increment of 3.38% at the end of the experiment compared to the other trees that received sole bokashi treatments or a combination of bokashi with reduced NPK fertilization with girth increment ranging 3.66–5.25%. The cumulative girth increment in trees that received sole bokashi treatments or a combination of bokashi with reduced NPK fertilizer was 3.66–5.25% compared to an increment of 3.38% in trees treated with only NPK fertilizer.

Effects of Treatments on Rubber Yield

The direct impact of fertilization on rubber yield has not been well documented and is yet to be fully understood (Chotiphan et al., 2019). The treatments did not significantly affect the dry rubber yield (Table 5). It is in line with the findings of Damrongrak et al. (2015), who reported no significant difference in dry rubber yield as affected by treatments involving chemical fertilizer, dolomite, and compost within six months of treatment application. Similarly, Tiva et al. (2016) reported that the first-year rubber yield did not differ significantly between NPK fertilizer treatments. Siti Naimah et al. (2015) also reported that the application rates and type of fertilizers (organic or inorganic) had no significant influence on the growth and yield attributes of yard-long bean (*Vigna unguiculata* subsp. *sesquipedelis* L. VERDC.).

Although there was no significant difference between the treatments in the monthly dry yield of rubber, mean yield ($g \cdot tree^{-1} \cdot tapping^{-1}$), as well as in the land productivity, the application of bokashi without NPK fertilizer (T1, T2, and T3) had considerably resulted in more rubber yield (2,226–2,289 $kg \cdot ha^{-1} \cdot year^{-1}$) compared to the yield of 2,154 $kg \cdot ha^{-1} \cdot year^{-1}$ obtained due to the addition of NPK fertilizer. It means that applying 4 to 12 kg BF has caused an increase in rubber yield by 3.30–6.30% compared to the yield due to NPK fertilizer, although the yield increase was not significant in the short term. However, reports show that Malaysian smallholders produce an average of 1,400 kg of rubber per hectare per year (Ali et al., 2020) compared to the theoretical yield of 7,000 to 12,000 kg per hectare per year (Paardekooper, 1989). This yield gap could be due to several factors, such as crop genetics, agronomic practices, and other environmental conditions (Ali et al., 2020). Also, such high yields were not a surprise because yields of 3,000 to 4,000 $kg \cdot ha^{-1} \cdot year^{-1}$ were reported among the earliest

plantations that came from unselected seeds in Nigeria (Giroh et al., 2013). Therefore, the clone used for this study (RRIM 3001) is potentially high yielding, especially with good management practices such as using BF in conservation pits.

RRIM 3001 is a new clone with a very high latex production potential of land productivity above 3,000 $kg \cdot ha^{-1} \cdot year^{-1}$ (Salisu et al., 2013). In Malaysia, palm oil yields were also increased through management practices such as the use of pits (Bohluli et al., 2015). Using conservation pits significantly influenced rubber growth and yield (George et al., 2007). In addition, the combined application of 12 kg BF + 500 g NPK (T7) has also increased dry rubber yield by 5.20% over the yield obtained due to NPK fertilization. Kakar et al. (2019) reported a significant increase in rice grain yield and grain quality from combining organic and inorganic fertilizers. Several studies have indicated that inorganic fertilizers, combined with OFs, are more useful to crops and the environment (Hernandez et al., 2021; Siti Naimah et al., 2015). Good management of rubber trees, as well as a favorable environment, could be responsible for good performance (Chotiphan et al., 2019). Again, the potential benefits of fertilization in rubber are only possible with intensification in the tapping system practiced. Because tapping is the major driver in latex generation, it may not be a surprise if no fertilization effect was seen in the short term because the same tapping system was employed across all treatments.

CONCLUSION

This study revealed two major findings. First, the study indicated that high rates of BF, such as 12 kg or 4 kg BF plus 500 g NPK applied in conservation pits, are sufficient to improve the soil fertility status in rubber plantations and cut the need for costly CFs by at least 50%. Second, the use of bokashi, NPK, or their combined application did not significantly affect the growth and yield of rubber within the short period of the study. Therefore, it is recommended that a similar experiment be conducted over at least two years to assess the maximum benefits of BF on the growth and yield of rubber.

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Pathogenic Bacterial Communities Isolated and Identified in Stingless Bee (*Kelulut*) Honey from Selected Farms in Terengganu

Noor Aimi Shazana Mohd Yusoff, Fisal Ahmad, Amir Izzwan Zamri, Shamsul Bahri Abdul Razak, Muhammad Fauzi Mahmud and Tuan Zainazor Tuan Chilek*

Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

ABSTRACT

In Malaysia, stingless bees can be categorised into two genera: *Melipona* and *Trigona*, known as "*kelulut*". The high demand for *kelulut* honey boosts the production of the honey industry. Previous studies reported that stingless bee (*kelulut*) honey and its products were contaminated with pathogenic bacteria during harvesting and processing. This research aims to isolate and identify the pathogenic bacteria in *kelulut* honey. Forty-eight samples of *kelulu*t honey (open and closed pot) and propolis were obtained from selected farms in Terengganu by focusing on a major stingless bee species available in Malaysia, *Heterotrigona itama*. In addition, the swabbing technique was done on the wooden beehive of the *kelulut* to evaluate the environmental contamination. The pathogenic bacteria were isolated using specifically selected agar, such as *Bacillus cereus* agar (for *B. cereus*), Baird-Parker agar (for *Staphylococcus aureus*), and MacConkey agar (for other pathogenic bacteria), which were confirmed through a biochemical test. All samples were analysed, and the results showed that *B. cereus* (7/48), *Pseudomonas aeruginosa* (10/48), *Pantoea* spp. (11/48), *Serratia plymuthica* (6/48), and *S. aureus* (9/48) were obtained in the samples. This study indicates that *kelulut* honey was contaminated with *B. cereus*, *P. aeruginosa*, *Pantoea* spp., *S. plymuthica*, and *S. aureus*. Isolated pathogenic bacteria may exist in the *kelulut* honey through food handlers, utensils, and the environment. Hence, the stakeholders should strictly follow good standard operating procedures and guidelines by the *kelulut* honey industry to prevent foodborne illness.

Keywords: Food handlers, foodborne illness, *kelulut,* pathogenic bacteria, stingless bee honey

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E-mail addresses:
p4405@pps.umt.edu.my (Noor Aimi Shazana Mohd Yusoff)
fisal@umt.edu.my (Fisal Ahmad)
amir@umt.edu.my (Amir Izzwan Zamri)
shamsul@umt.edu.my (Samsul Bahri Abdul Razak)
p4809@pps.umt.edu.my (Muhammad Fauzi Mahmud)
t.zainazor@umt.edu.my (Tuan Zainazor Tuan Chilek)
*Corresponding author

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INTRODUCTION

The stingless bee is a small bee from the species of *Trigona* or meliponine and is also known as the '*kelulut*' bee in Malaysia. They are known as stingless bees because these bees are incapable of stinging (Salatino et al., 2019). Two genera that can be used to classify stingless bees are *Melipona* and *Trigona* (Jalil et al., 2017). In Malaysia, two species of stingless bee that are reported to produce honey are *Heterotrigona itama* and *Geniotrigona thoracica*, which have different colours and sizes, and other common species to be found in *H. itama* (Shamsudin et al., 2019). The content of the honey produced by these two species differs depending on its botanical origin, floral source, environmental conditions, geographic location, and methods used to harvest and process the honey (Bakar et al., 2017; Shamsudin et al., 2019).

Kelulut plays an essential role in the economy and culture. Their products, such as honey, pollen, and propolis, have been used for revenue and profit for ages. The Aboriginal people of northern Australia greatly value the stingless bee honey as a food source, which is important to their social customs and ceremonies (Boorn et al., 2010). It is easier to regularly extract honey, pollen, and propolis because the *kelulut* cannot sting. Furthermore, Jalil et al. (2017) reported that stingless bees are more effortless to handle than honeybees, which often abandon their hive and are endangered by disease. *Kelulut* honey also has a unique sweetness assorted with a sour and acidic taste (Jalil et al., 2017).

The pathogenic bacteria cause the most significant national public concern are *Escherichia coli*, *Staphylococcus* spp., *Shigella* spp., *Streptococcus* spp., and *Bacillus* spp. that frequently associated with honeybees (Adadi & Obeng, 2017). The pollen, air, flowers, and digestive tracts of *kelulut* are among the sources of bacteria. Ngalimat et al. (2020) reported that humans, tools, containers, wind, and dust could all be the primary or secondary sources of bacterial contamination in bee products. Most bacterial species associated with *kelulut* colonies are *Bacillus*, *Streptomyces*, and *Lactobacillus* (Ngalimat et al., 2020). The pathogenic bacteria should be concerning because it will show the level of food hygiene, food handlers, and the farm.

This research aims to isolate and identify the pathogenic bacteria in the *kelulut* honey. A few research were done on the microbiological contamination of bee honey, or *kelulut* honey, and its other products, including pollen and propolis with *E. coli*, *Staphylococcus* spp., *Shigella* spp., *Streptococcus* spp., and *Bacillus* spp. (Adadi & Obeng, 2017). These bacteria can cause serious food poisoning if contaminated with honey products. In addition, there is limited data on pathogenic bacteria contamination and characterisation in *kelulut* honey available in Malaysia. Malaysian Standard (MS 2683:2017) for *kelulut* honey specification is issued to control the quality and safety of honey produced. Honey entrepreneurs are still poorly implementing the food supply chain concept to avoid contamination by pathogenic bacteria.

MATERIALS AND METHODS

Samples

The samples of *kelulut* honey and propolis were obtained from four selected farms (locations A, B, C, and D) in Kuala Terengganu and Kuala Nerus by focusing on major *kelulut* species available in Malaysia: *H. itama* species. Forty-eight samples of *kelulut* honey consisting of the open pot (OP), close pot (CP), and propolis (PP) were obtained from the selected farms for pathogenic bacteria analysis. The swabbing technique was done on the hive swab (HS) of the *kelulut* to evaluate the environmental contamination. These samples were transported to the laboratory in a chilled condition at 4°C before being analysed. The samples were analysed within 24 hr.

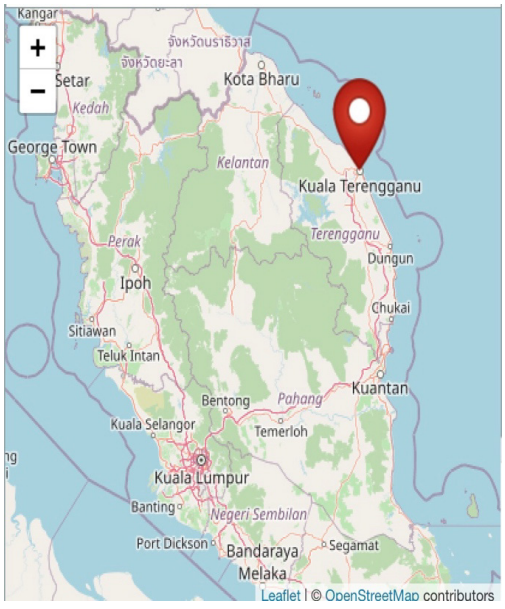

Figure 1. Maps of the region in Terengganu, where the samples were collected

Samples Location

The Google map provided the Global Positioning System (GPS) for the honey locations. Honey from *H. itama*, propolis, and hive swab samples were obtained from Kuala Terengganu, Terengganu (5°19'48.72"N 103°08'26.88"E) and Kuala Nerus, Terengganu (5° 21' 37.2882102"N 103° 1' 37.3284"E). Figure 1 shows the region maps in Terengganu where the samples were collected.

Isolation and Identification of Pathogenic Bacteria

Bacillus cereus agar (BCA, Oxoid International Ltd., United Kingdom), Baird-Parker agar (BPA, Oxoid International Ltd., United Kingdom), and MacConkey agar (non-selective agar, Oxoid International Ltd., United Kingdom) were used to isolate the *B. cereus*, *S. aureus*, and other pathogenic bacteria. Homogenous samples were prepared through a serial dilution, where 10 g of samples were obtained. Then, 90 ml of peptone water (Oxoid International Ltd., United Kingdom) was added as the first dilution. A total of 0.1 ml of each dilution were pipetted out, and then the spread plate method was applied for BCA, BPA, and MacConkey agar. Plates were incubated at 37°C for 24 to 48 hr, and then the morphology of colonies was observed. The bacterial cells are deposited at widely separated points on the surface of the medium and develop into colonies (Sanders, 2012). The positive colonies were confirmed using biochemical tests (Andrews & Hammack, 2022).

Swabbing Technique

The sterile cotton bud-tipped swab was moistened with 0.1% buffered peptone water (BPW) (Oxoid International Ltd., United Kingdom). Then, the swab head was gently pressed to remove the excess BPW. The stick was repeatedly swabbed on the wooden beehive surface area (10 cm x 10 cm). After swabbing, the swab head was immersed in 0.1% of BPW (Willes et al., 2013). The sample will be serially diluted up to 10^2 dilutions. The sample taken was conducted within 24 hr and continued for further analysis.

Analytical Profile Index (API) Technique

The API 20E system (Bio-Mérieux, Marcy l'Etoile, France) is a non-fastidious Gram-negative rod to identify Enterobacteriaceae was used in this study. Twenty microtubes make up this apparatus, which is filled with dehydrated substrates. The bacterial suspension used in these experiments was inoculated, reconstituting the media. The incubation process causes colour changes due to metabolism, which can occur naturally or be seen by adding chemicals. The API is used to identify the reactions after reading them in accordance with the table for reaction interpretation (Ashgar & El-Said, 2012).

Other Tests

Coagulase Test. Slide tests were conducted to detect bound coagulase. It causes a fast cell agglutination by immediately reacting with fibrinogen in plasma. The ends of a slide were given a drop of physiological saline. A piece of the isolated colony was emulsified in each drop using the loop straight wire to create two thick suspensions. One of the suspensions was given a drop of human or rabbit plasma, and it was gently mixed in within 10 s, a clumping of the organisms was observed (Leber, 2016).

Oxidase Test. In this study, the filter paper spot method was used. A well-isolated colony was selected using a loop from a new bacterial plate; then rubbed onto a small piece of filter paper. One or two drops of the 1% Kovács oxidase reagent (Sigma-Aldrich, USA) were applied to the organism smear. Then, a colour shift was noticed (Leber, 2016).

Catalase Test. The catalase test was conducted using the slide method. A small amount of colony development was transferred onto the surface of a clear, dry glass slide using a loop or sterilised wooden stick. Then, a drop of 3% hydrogen peroxide (H_2O_2, Sigma-Aldrich, USA) was added to the glass slide. The oxygen bubbles evolved were observed (Leber, 2016).

RESULTS

Selected samples were obtained from locations A, B, C, and D (Tables 1–4). Isolation using selected agar (BCA and BPA) which targeted pathogenic bacteria that commonly cause food poisoning, and non-selected agar (MacConkey agar) for suspected colonies were observed, characterised, and identified. Forty-eight

samples of *kelulut* honey comprised OP, CP, PP, and HS.

There are five strains of pathogenic bacteria obtained in the open pot honey, propolis, and hive swab from this study, which are *P. aeruginosa*, *B. cereus*, *S. aureus*, *Pantoea* spp., and *S. plymuthica*. Surprisingly, no pathogenic bacteria were found in the close-pot honey samples from all locations. The close pot honey was shielded and protected from any contamination by the pot's structure. Jalil et al. (2017) reported that honey is stored in a cerumen pot, which is used to mummify intruders and maintain a sterile environment inside the hive. However, in the open pot honey, 80% (10/12) of samples were contaminated with pathogenic bacteria (Tables 1–4). To our knowledge, no study has been done in open and closed-pot samples. In addition, propolis and hive swab samples were also contaminated with pathogenic bacteria with 100% (12/12) samples and 92% (11/12) samples, respectively (Tables 1–4). A few studies reported that *B. cereus*, *S. aureus*, and *Pantoea* sp. are found in honeybee and *kelulut* honey (Adadi & Obeng, 2017; Amin et al., 2020; Ngalimat et al., 2019; Pucciarelli et al., 2014).

In summary, out of twelve samples of propolis, three samples were contaminated with *S. aureus* (25%), five samples were detected with *P. aeruginosa*, three samples were identified with *B. cereus* and *Pantoea* spp., and two samples were confirmed with *S. plymuthica* strains. In addition, five out of twelve samples of the hive swab were contaminated with *P. aeruginosa*, two samples with *S. aureus*, six with *Pantoea* spp., and three with *S. plymuthica*. Lastly, four samples of open-pot honey contained *Pantoea* spp., six samples (50%) with *B. cereus*, and only two with *S. aureus*. Only *S. plymuhica* and *P. aeruginosa* were not found in the open-pot honey.

Table 1

Isolation and characterisation of selected samples obtained from location A

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
A	BCA	AOP3*	Blue green colonies	*Bacillus cereus*	Oxidase (+) Catalase (+)	Positive *B. cereus*
	BPA	APP1, APP2,	Black colonies with a clear zone	*Staphylococcus aureus*	Oxidase (+) Catalase (+) Coagulase (+)	Positive *S. aureus*
	MacConkey	AOP2	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.

Table 1 *(Continue)*

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
		APP1	Red, pink colonies	*Serratia plymuthica*	Oxidase (-) Catalase (+)	Positive *S. plymuthica*
		APP3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
		AHS1	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
A	MacConkey	AHS2	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
			Red, pink colonies	*Serratia plymuthica*	Oxidase (-) Catalase (+)	Positive *S. plymuthica*
		AHS3	Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*

Note. API = Analytical Profile Index; BCA = *Bacillus cereus* agar; BPA = Baird-Parker agar; OP = Open pot; CP = Close pot; PP = Propolis; HS = Hive swab

Table 2

Isolation and characterisation of selected samples obtained from location B

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
	BCA	BOP1	Blue, green colonies	*Bacillus cereus*	Oxidase (+) Catalase (+)	Positive *B. cereus*
	BPA	BOP2, BPP2	Black colonies with a clear zone	*Staphylococcus aureus*	Oxidase (+) Catalase (+) Coagulase (+)	Positive *S. aureus*
B	MacConkey	BPP1	Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
		BPP3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.

Table 2 *(Continue)*

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
B	MacConkey	BHS1	Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
		BHS3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.

Note. API = Analytical Profile Index; BCA = *Bacillus cereus* agar; BPA = Baird-Parker agar; OP = Open pot; CP = Close pot; PP = Propolis; HS = Hive swab

Table 3

Isolation and characterisation of selected samples obtained from location C

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
C	BCA	COP1, COP3, CPP1, CPP3	Blue, green colonies	*Bacillus cereus*	Oxidase (+) Catalase (+)	Positive *B. cereus*
	BPA	CPP2, CHS2	Black colonies with a clear zone	*Staphylococcus aureus*	Oxidase (+) Catalase (+) Coagulase (+)	Positive *S. aureus*
	MacConkey	COP2	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
		COP3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
		CPP1	Red, pink colonies	*Serratia plymuthica*	Oxidase (-) Catalase (+)	Positive *S. plymuthica*
			Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*

Table 3 *(Continue)*

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
C	MacConkey	CHS1	Red, pink colonies	*Serratia plymuthica*	Oxidase (-) Catalase (+)	Positive *S. plymuthica*
			Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
		CHS2	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
		CHS3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
		CHS4	Red, pink colonies	*Serratia plymuthica*	Oxidase (-) Catalase (+)	Positive *S. plymuthica*
			Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
		CHS5	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.

Note. API = Analytical Profile Index; BCA = *Bacillus cereus* agar; BPA = Baird-Parker agar; OP = Open pot; CP = Close pot; PP = Propolis; HS = Hive swab

Table 4

Isolation and characterisation of selected samples obtained from location D

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
D	BCA	DOP1, DOP2, DPP2	Blue, green colonies	*Bacillus cereus*	Oxidase (+) Catalase (+)	Positive *B. cereus*
	BPA	DOP2, DPP3, DHS3	Black colonies with a clear zone	*Staphylococcus aureus*	Oxidase (+) Catalase (+) Coagulase (+)	Positive *S. aureus*
	MacConkey	DOP3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.

Table 4 *(Continue)*

Sampling location	Agar	Isolated colonies	Characteristics colonies	API test	Other tests	Remarks
		DPP1	Colourless, flat, and smooth colonies	*Pseudomonas aeruginos*a	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
		DPP3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.
		DHS1	Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
D	MacConkey	DHS2	Red, pink colonies	*Serratia plymuthica*	Oxidase (-) Catalase (+)	Positive *S. plymuthica*
			Colourless, flat, and smooth colonies	*Pseudomonas aeruginosa*	Oxidase (+) Catalase (+)	Positive *P. aeruginosa*
		DHS3	Circle, smooth pink colonies	*Pantoea* spp.	Oxidase (-) Catalase (-)	Positive *Pantoea* spp.

Note. API = Analytical Profile Index; BCA = *Bacillus cereus* agar; BPA = Baird-Parker agar; OP = Open pot; CP = Close pot; PP = Propolis; HS = Hive swab

DISCUSSION

Pathogenic bacteria are usually found in the environment, such as soil, water, and air (Cavicchioli et al., 2019; Pandey et al., 2014). Abusing techniques from handling and the storage pattern before consumption can contribute to cross-contamination (Augustin et al., 2020). This statement is always true while handling food commodities, from the farm level to the customer throughout the supply chain. In this case, propolis and hive swab samples are the most contaminated with pathogenic bacteria at the farm level. According to Putri and Susanna (2021), the pollen, honeybee's digestive systems, and the environment (soil, dust, and air) are the primary sources of contamination, whereas food handlers, cross-contamination, harvesting equipment, buildings, and the environment are secondary sources.

Additionally, the presence of insects such as lizards, cockroaches, and ants might result in pathogenic bacteria that may cause the contamination of hive swab samples. Most food poisoning cases are caused by bacteria, which are from animal sources, and it has been discovered that pathogens can enter food supply chains through animal hosts, transporters, or inappropriate handling techniques (Kordiyeh, 2018).

Microorganisms in honey may affect the product's stability and hygienic quality (Erkan et al., 2017). However, it is quite challenging to eliminate the contamination of honey throughout the food supply chain. In contrast, good farming practices (GFP) and good manufacturing practices (GMP) can limit the secondary causes of honey contamination (Rivera-Gomis et al., 2019).

Naturally, open containers are exposed to bacteriological contamination. In this case, the open-pot honey was commonly exposed to environmental contamination. However, in this study, the open-pot honey samples were less contaminated with pathogenic bacteria than propolis and hive swab samples. The open-pot honey samples were less contaminated because the chemical transformation process of sugar by the bees is not complete yet. Hydrolyse the sucrose in the nectar into fructose and glucose by enzymes secreted by the worker bees is needed to maintain the sterile environment in the cerumen pot (Jalil et al., 2017). After collecting nectar, the bees released the enzyme invertase to break down the sucrose into a mixture of glucose and fructose that contribute to the function of antimicrobial properties (Hongu et al., 2017). Hence, the pathogenic bacteria were not found in all close-pot honey compared to open-pot honey because the process of breaking down sucrose is completed, the antimicrobial properties are well functioning, and the pot's structure protects against contamination.

Spore-forming bacteria from the genus *Bacillus* are commonly associated with stingless bee species (Ngalimat et al., 2019; Pucciarelli et al., 2014; Yaacob et al., 2018). A few studies reported that *B. cereus*, *S. aureus*, and *Pantoea* sp. were found in honeybee and *kelulut* honey in Malaysia (Adadi & Obeng, 2017; Amin et al., 2020; Ngalimat et al., 2019; Puciarealli et al., 2014). It is in line with this study; the pathogenic bacteria in the *kelulut* honey are *B. cereus*, *S. aureus*, and *Pantoea* species. Furthermore, pathogenic bacteria such as *P. aeruginosa*, *Serratia* sp., and *Pantoea* sp. were found in the hive swab samples.

A previous study by Akkaya et al. (2020), Feng and Hartman (1982), and Loir et al. (2003) discovered that *Clostridium botulinum*, *E. coli*, *P. aeruginosa*, and *S. aureus* spores contaminated the propolis. The contamination may come from dust in the air, the bees' gastrointestinal systems, pollens, bees' legs, and contaminated bee foods (Akkaya et al., 2020). Our results are similar to the studies mentioned above, where *P. aeruginosa* and *S. aureus* were found in the propolis samples.

These pathogenic bacteria can cause food poisoning. For example, *S. aureus* produces a fatal enterotoxin that frequently can cause pneumonia, wound infections, and nosomial bacteremia (Dinges et al., 2000; Tiemersma et al., 2004). Staphylococcal enterotoxins found in contaminated food can cause severe symptoms like vomiting, diarrhoea, and a high temperature with or without nausea and vomiting (Colombari et al., 2007). Moreover, *B. cereus* that invades the human body can cause bacteremia, pneumonia, and infection in the eye, central nervous system (CNS), and soft tissue

(Avashia et al., 2007; Gaur et al., 2001). Additionally, *Serratia* spp. are frequently found to be the source of illnesses such as meningitis, sepsis, and infections in the urinary tract, skin, bloodstream, and respiratory (Engelhart et al., 2003; Wu et al., 2013).

Good farming practices should be followed accordingly to prevent contamination at the farm level. The utensils used for harvesting and storage are expected to be free from contamination. Good practice by food handlers is compulsory to be followed. Personal hygiene is the key to preventing bacterial contamination through food handlers to avoid foodborne illness. The possibilities of contamination throughout the food supply chain of *kelulut* honey are shown in Figure 2.

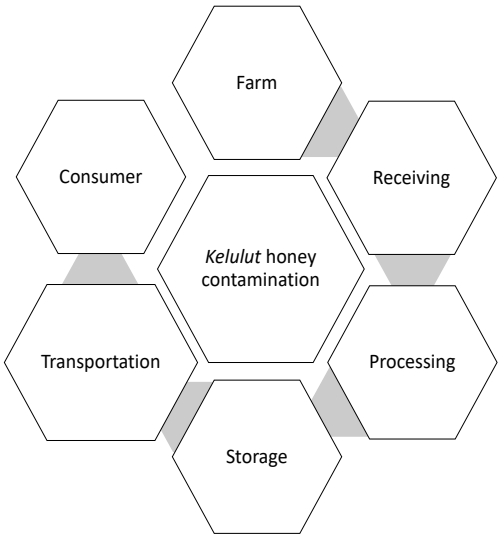

Figure 2. The possibilities of contamination throughout the food supply chain of *kelulut* honey

CONCLUSION

Pathogenic bacteria namely *B. cereus*, *S. aureus*, *Pantoea* sp., *P. aeruginosa* and *S. plymuthica* were isolated and identified in the *kelulut* honey, propolis and the wooden beehive samples. These pathogenic bacteria were confirmed with a few confirmation tests such as API 20E test, catalase test, oxidase test and coagulase test. *Kelulut* honey is a potential source of *B. cereus*, *S. aureus*, *Pantoea* sp., *P. aeruginosa* and *S. plymuthica* which may cause foodborne outbreaks. Every stakeholder should be responsible for the production of good quality *kelulut* honey to ensure that the industry is growing rapidly in the future.

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Growth and Yield Comparison of Rice Plants Treated with Encapsulated *Trichoderma asperellum* (UPM 40) in Response to Drought Stress

Iffatul Arifah Yusup[1], Martini Mohammad Yusoff[1]*, Mohd Razi Ismail[1], Zulkarami Berahim[2] and Fariz Adzmi[3]

[1]*Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2]*Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[3]*Institute of Plantation Studies, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

During low rainfall periods, rice plants often face drought stress, which would significantly affect rice yield. One of the methods to mitigate the problem is incorporating rice plants with fungi such as *Trichoderma*. This study evaluated the effects of encapsulated *Trichoderma asperellum* (UPM 40) on the growth and yield of rice plants planted in saturated and flooded soil conditions in response to drought stress. A randomized complete block factorial design was implemented with four replications and two factors. The first factor was encapsulated *T. asperellum* (UPM 40) concentration of 0 and 5 g. The second factor was the soil condition: saturated and flooded soil. The drought stress was imposed by halting watering during early anthesis for 14 days and resumed afterward. One of the significant interaction effects detected was on the relative water content of rice plants planted in flooded soil conditions and treated with *T. asperellum* (UPM 40), where the value was 78.51%, higher than the control of 72.09%, which showed the ability of the fungus to help rice plants alleviate detrimental effects of drought stress and delay the onset of adverse effects of drought stress. Thus, it contributed to the crop's simultaneous improvement in rice yield compared to untreated plants in saturated soil. Applying 5 g encapsulated *T. asperellum* (UPM 40) to the rice plants would perform best in flooded soil conditions during drought stress.

Keywords: Ceptometer, chlorophyll, photosynthesis, radiation, weight

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E-mail addresses:
iffatularifah@gmail.com (Iffatul Arifah Yusup)
martinimy@upm.edu.my (Martini Mohammad Yusoff)
razi@upm.edu.my (Mohd Razi Ismail)
zulkerami@upm.edu.my (Zulkarami Berahim)
farizadzmi@upm.edu.my (Fariz Adzmi)
*Corresponding author

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INTRODUCTION

Many regions struggle against drought in producing food crops (Isendahl & Schmidt, 2006). Drought is a global crisis that is severely affecting grain production and quality. On top of that, the inevitably increasing world population and global climate change are making it a lot worse (Hongbo et al., 2005). As a field crop, rice (*Oryza sativa* L.) is defenseless against water stress (Tao et al., 2006). Factors such as unpredictable, insufficient, and uneven rainfall patterns during the vegetative phase, reproduction phase, and ripening stage lead to drought, thus decreasing an estimated 50% of global rice production (Crosson, 1995). Along with the complexity of the drought itself, the way the plant responds to it is also complex. The plant will adopt numerous mechanisms to survive drought (Trethowan et al., 2002). Among them are increased plant water uptake, reduced water loss by enhanced diffusive resistance, and increased water uptake through prolific and deep root systems (Farooq et al., 2009).

There are known methods to enhance crop yield in response to water stress caused by drought, such as applying periodical water stress and potassium fertilization (Zain et al., 2014), application of exogenous hydrogen peroxide and salicylic acid (Sohag et al., 2020) and through genetic improvement (Sahebi et al., 2018). The outcome is encouraging but is limited by time and requires additional resources. A deeper understanding of plant drought tolerance's physiological and molecular basis is required to refine or possibly develop new and improved methods (Trethowan et al., 2002).

Evidently, *Trichoderma* is applied in many kinds of cereal, such as *Triticum aestivum* L., *Vigna radiata* (L.) R. *Wilczek* and *Sorghum bicolor* shows an improved drought response by plants (Kaur & Kumar, 2020; Shukla et al., 2014; Sugiharto et al., 2020). Favorable activities ascribed to the *Trichoderma*-plant interactions include induced disease resistance, plant growth promotion, and tolerance to abiotic stresses (Harman et al., 2004). The mechanisms triggered by *Trichoderma* in the adjustment of drought response are drought avoidance through morphological adaptations, drought tolerance through physiological and biochemical adaptations, and enhanced drought recovery (Malinowski & Belesky, 2000). Yield performance is dictated by the root size and the surrounding architecture, especially when water availability is limited. In cacao, root colonization by *Trichoderma* enhances the growth of roots and the entire plant, increasing plant productivity and yield of reproductive organs (Bae et al., 2009). Therefore, this study evaluated the effects of encapsulated *Trichoderma asperellum* (UPM 40) on the growth and yield of rice plants planted in saturated and flooded soil conditions in response to drought.

MATERIALS AND METHODS

The experiment was conducted in a glasshouse at Field 10, Rice Research Site, Faculty of Agriculture, Universiti Putra Malaysia (UPM), Serdang (3˚02'N, 101˚42'E, 31 m a. s. l). The soil for this

experiment was obtained from a rice field at Kuala Selangor. The soil type was silty clay with the classification Beriah Series, Inceptisol order, and subgroup Typic Endoaquept (Tan et al., 2017).

Unsterilized rice field soil from Kuala Selangor was utilized in this experiment to mimic real situations. The soil was packed into sacks before being transported to the glasshouse in Field 10, UPM. A quick soil test was done before planting to determine the soil pH and contents of total nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), and magnesium (Mg). The soil air-drying process was done by laying the soil on a plastic canvas inside the glasshouse for 14 days. The dried soil was then crushed into small particles and sieved. Fifteen kg of sieved soil was filled into plastic pails at size 37.5 cm × 31.5 cm. In total, there were 192 plastic pails. Those pails were then filled with water until reaching a similar level to the soil surface and stirred to obtain a muddy-soil texture. Approximately 34.5 g of dolomite (Perlis Dolomite Industries, Malaysia) was mixed thoroughly into the soil. The soil mixture-filled pails were left for 14 days before transplanting.

Preparation of *T. asperellum* (UPM 40) conidial suspension and encapsulation were done according to the methods described by Adzmi et al. (2012). In preparation for *T. asperellum* (UPM 40) suspension, for every 250 ml of encapsulated (UPM 40) liquid mixture, 20 plates of freshly matured *T. asperellum* (UPM 40) were needed. Each plate was filled with 10 ml of sterile distilled water. The surface of potato dextrose agar (PDA) media (Fisher Scientific, USA) in each plate was scraped lightly to harvest conidial suspension, which was needed for making *T. asperellum* (UPM 40) capsules. The conidial suspension was then poured into a centrifuge tube. Each tube contained 40 ml of conidial suspension from 4 plates of fresh matured *T. asperellum* (UPM 40). These filled tubes were centrifuged at 6,026 x g for 10 min. The supernatant was discarded, and only pellets were kept for the next stage. Ten ml of sterile distilled water was filled into the pellet-contained test tubes and shaken until no pellet stuck on their walls. An amount of 2.5 g montmorillonite clay (MMT, Sigma-Aldrich, USA) was dissolved into 250 ml sterile distilled water for 24 hr at room temperature. After 24 hr, 12.5 g of corn starch (R&M Chemicals, Malaysia) was added into the MMT solution and stirred for 1 hr. Following that step, 7.5 g of alginate (Fisher Scientific, USA) was added into the mixture and left to dissolve for 3 hr. After dissolving for 3 hr had completed, 7.5 ml of 30% glycerol (Acros Organics, USA) was added to the mixture. The conidial suspension was also added to the mixture. A 5-ml syringe without a needle was used to make the *T. asperellum* beads by pressing out the mixture through it and dropped into 0.5 M calcium chloride ($CaCl_2$) (Systerm Chemicals, Malaysia) precooled sterile aqueous solution under mild agitation. The beads were collected by sieving and washed with sterile distilled water. After that, they were left to air dry for 24 hr at 30 ± 2ºC. The viability of conidia in the beads was maintained in the 2.69×10^4 to 1.56×10^3 cfu/g range.

Rice seeds of the MR219 variety were obtained from Federal Land Consolidation and Rehabilitation Authority (FELCRA) Berhad Seed Center in Seberang Perak, Perak, Malaysia. The selection of rice seeds weighing approximately 0.02 g/seed was conducted to obtain the physical uniformity of the seeds used for the study. The seeds were then surface sterilized by Miché and Balandreau's method (2001). It was followed by soaking off the seeds in sterile distilled water for 24 hr and arranging the seeds on moist filter papers placed inside Petri dishes. The rice seeds were then left for 24 hr for germination.

Then the germinated rice seeds were transferred into a seedling tray and left to grow for 14 days to ensure uniform growth. On day 14, the rice seedlings were transferred into soil containing plastic pails. Each pail was planted with 3 rice seedlings. The pails were watered daily to field capacity for 7 days and flooded to 5 and 1 cm depth according to the treatments. Watering was halted at early anthesis for 14 days and as an implementation of drought stress. After 14 days, watering was resumed according to treatments until two weeks before harvesting.

The experimental design was a two-factorial randomized complete block design (RCBD) with 4 replications. The first factor was 0 and 5 g encapsulation of *T. asperellum* (UPM 40). The second factor implied drought stress in the two different soil conditions: saturated and flooded during the early anthesis stage. A 1 cm of standing water above the surface determined the saturated soil condition. As for flooded conditions, the plastic pails of soil were filled with water until there was 5 cm standing water above the soil surface.

The measured variables for the experiment: the total intercepted photosynthetically active radiation, radiation use efficiency (RUE), relative water content, total chlorophyll content, relative water content, maximum root area, root length, and root volume were recorded as physiological attributes. At the same time, the weight of 1,000 grains, harvest index, total yield per culm, spikelet weight per panicle, percentage of filled grains per panicle, and number of filled grains per panicle were recorded as yield attributes of the conducted experiment.

Physiological Attributes

Light Interception. Above and below photosynthetically active radiation (PAR) was recorded weekly using AccuPAR Ceptometer (Decagon Devices, USA) between 1200 and 1300 hr. The readings were taken three times for each replication, with an average reading recorded. The fraction of PAR was calculated using the following formula (Gallagher & Biscoe, 1978).

$$\mathbf{Fi} = \mathbf{1} - \frac{I_o}{I_t} + \times \mathbf{100}$$

where Fi is the fractional amount of radiation interception (%), *Io* is the measured incident PAR on the surface of the ground, and I_t is the radiant flux density on top of the canopy. The value of *Io* was measured at the vertical height level.

The total incident PAR was taken from daily incident solar radiation recorded by Serdang Meteorological Station. Fifty percent (50%) of the incident solar radiation received was taken as PAR (Monteith, 1972). The amount of intercepted PAR by the crop (Sa) was calculated by the following formula (Szeicz, 1974):

$$Sa = Fi \times Si$$

where Sa is the amount of intercepted PAR, Si is the total amount of incident PAR, and Fi is the fractional amount of radiation interception.

Radiation Use Efficiency (RUE)

The radiation use efficiency (RUE) was calculated as the slope of the linear relationship between accumulated crop biomass and accumulated intercepted PAR. The regression line was forced through the origin based on the assumption that no dry matter was produced when accumulated intercepted PAR was zero (Monteith, 1977).

Chlorophyll Content

The fully expanded leaf, normally found on the second or third leaf from the top of a rice plant, was taken on the 14th day after drought stress implied. The leaf samples were placed into paper bags and brought to the Physiology Laboratory, Department of Crop Science, UPM, for further analysis.

The fully expanded leaf of the main tiller of the rice plant was homogenized with 80% acetone. The absorption of the extracts at wavelengths of 663 and 645 nm was measured with a spectrophotometer. Chlorophyll *a*, chlorophyll *b*, and total chlorophyll content were determined in mg/g FW according to the formula given below (Arnon, 1949):

$$\text{Chlorophyll } a = 12.72A663 - 2.59A645$$
$$\text{Chlorophyll } b = 22.9A645 - 4.67A663$$
$$\text{Total chlorophyll} = 20.31A645 + 8.05A663$$

where A663 is the spectrophotometer reading at wavelength 663 nm, and A645 is the spectrophotometer reading at wavelength 645.

Data for relative chlorophyll content was taken by using portable chlorophyll meter (MINOLTA™ SPAD-502) (Konica Minolta, Japan) on top fully expanded leaf. The leaf chlorophyll content was taken by scanning five different points on the leaf and the data taken was averaged using the portable chlorophyll meter.

Relative Water Content (RWC)

Fully expanded rice leaves on the main tiller of the rice plants were snipped and kept inside paper bags. The RWC of leaf samples was measured at the Physiology Laboratory, Department of Crop Science, UPM.

The rice leaf tissues were weighed immediately to get the fresh weight (FW). The leaf tissues were rehydrated in water for 24 hr until they attained full turgidity, surface-dried, and reweighed to get the turgid weight (TW). Finally, the tissues were oven dried at 80°C for 48 hr (until constant weight) to obtain the dry weight (DW). The RWC was calculated using the equation below (Bhushan et al., 2007).

$$RWC = FW - DW / TW - DW \times 100 \quad (20)$$

where RWC = Relative water content (%), FW = Fresh weight, DW = Dry weight, TW = Turgid weight.

Photosynthesis

The rate of photosynthesis was recorded before and after drought treatment was imposed on the rice plants. Data were taken on fully expanded leaves on the main tiller of the rice plants. Each data was taken five times, and the average value was recorded. The data of photosynthesis rate were examined using an LI-6400XT Portable Photosynthesis System (LI-COR Inc., USA) between 0900 and 1000 hr.

Root Attributes

The roots of the rice plants were harvested after the final harvest. The roots were separated from the upper part of the crop. The roots were then cleansed thoroughly until no soil or other debris was on top.

Since the roots were condensed, they were divided into sections depending on the root size to enable them to be analyzed properly. Each part of the roots was floated in acrylic trays and arranged to reduce overlapping. The roots were placed on a transparent acrylic tray and filled with distilled water until submerged. The container was placed on the surface of the Epson Perfection V700 Photo scanner (Epson Malaysia Sdn. Bhd., Malaysia). The roots were analyzed using WinRhizo software (Regent Instruments Inc., Canada) for their length, surface area, and volume from the scanned image.

Yield Attributes

Weight of Grains. A thousand grains were counted from collected grains from all treatments and weighed on a digital balance.

Harvest Index. Harvest index (HI) was calculated as a ratio of crop yield to total above-ground crop biomass (Donald & Hamblin, 1976):

$$\mathbf{HI = GYBY \times 100}$$

where HI = Harvest index (%), GY = Grain yield, and BY = Dry biomass yield.

During the final harvest, the grains were harvested by separating the panicles from the rice plants. The panicles were kept inside paper bags and labeled for their treatment and replication. The rice plants without panicles were also harvested and kept inside paper bags with their respective labels. Those samples were weighed for fresh weight. The samples were kept inside an oven at 70°C for 48 hr and until constant weight was achieved. The dried grains and tillers were weighed.

Number of Filled Grains. Filled and unfilled grains were detached from the panicles and soaked in a 20% sodium chloride solution ($CaCl_2$) (Systerm Chemicals, Malaysia). The floated and sunk grains were separated and counted. The sunk grains were recorded as filled grains, and the floated grains were categorized as unfilled.

Percentage of Filled Grains. The percentage of filled grains was obtained by dividing the number of filled grains by

the total number of grains per panicle and multiplying by one hundred.

Statistical Analysis

The data were analyzed using the software SAS® 9.4 (SAS Institute, USA). Following the analysis of variance (ANOVA), differences among means were separated using the least significant difference (Fisher LSD) at $p \leq 0.05$.

RESULTS

Interaction between encapsulated *T. asperellum* (UPM 40) application and soil conditions was insignificant for total PAR (Table 1). No significant difference in total PAR between encapsulated *T. asperellum* (UPM 40) application and soil conditions was recorded. However, a significant interaction of RUE of rice plants treated with encapsulated *T. asperellum* (UPM 40) and different soil conditions was observed (Table 1) and presented in Figure 1. The relative water content of rice plants treated with encapsulated *T. asperellum* (UPM 40) in different soil conditions showed no significant interaction between the two factors. The relative water content of rice plants treated with encapsulated *T. asperellum* (UPM 40) was 78.51%, significantly higher than that of rice plants planted without encapsulated *T. asperellum* (UPM 40) at 72.09%. Rice plants planted in saturated soil conditions had significantly lower relative water content than those planted in flooded water conditions at 70.60 and 78.21%, respectively. Net photosynthetic rate of rice plants treated with encapsulated *T. asperellum* (UPM 40) was higher ($p < 0.05$) than the non-treated rice plants at 11.38 and 8.18 μmol CO_2/m^2 s. As for rice plants planted in different soil conditions, rice plants planted in flooded soil conditions recorded a higher net photosynthetic rate than in saturated conditions.

Table 1

Total photosynthetically active radiation (PAR), radiation use efficiency (RUE), and relative water content (RWC) of rice plants treated with encapsulated Trichoderma asperellum *(UPM 40) and planted in different*

Treatment	Total PAR (g/MJ)	RUE (g/MJ)	RWC (%)	Net photosynthetic rate ($\mu molCO_2 \cdot m^{-2} \cdot s^{-1}$)
Rate of encapsulated *T. asperellum* (UPM 40) (g) (T)				
0	$371.72^{a} \pm 8.47$	$0.09^{b} \pm 0.01$	$72.09^{b} \pm 1.90$	$8.18^{b} \pm 0.79$
5	$386.98^{a} \pm 4.04$	$0.38^{a} \pm 0.14$	$78.51^{a} \pm 2.07$	$11.38^{a} \pm 0.85$
Significance level	ns	***	**	**
Soil condition (S)				
Saturated	$372.08^{a} \pm 8.52$	$0.08^{b} \pm 0.01$	$70.60^{b} \pm 2.13$	$8.32^{b} \pm 0.95$
Flooded	$386.62^{a} \pm 4.21$	$0.39^{a} \pm 0.14$	$78.21^{a} \pm 2.07$	$11.25^{a} \pm 0.79$

Table 1 *(Continue)*

Treatment	Total PAR (g/MJ)	RUE (g/MJ)	RWC (%)	Net photosynthetic rate ($\mu molCO_2 \cdot m^{-2} \cdot s^{-1}$)
Significance level	ns	***	**	**
Interaction				
T × S	ns	***	ns	ns

Note. Means within column followed by the same letter are not significantly different by least significant difference, $p \geq 0.05$; * = Significant at $p \leq 0.05$; ** = Significant at $p \leq 0.01$; *** = Significant at $p \leq 0.001$; ns = Not significant

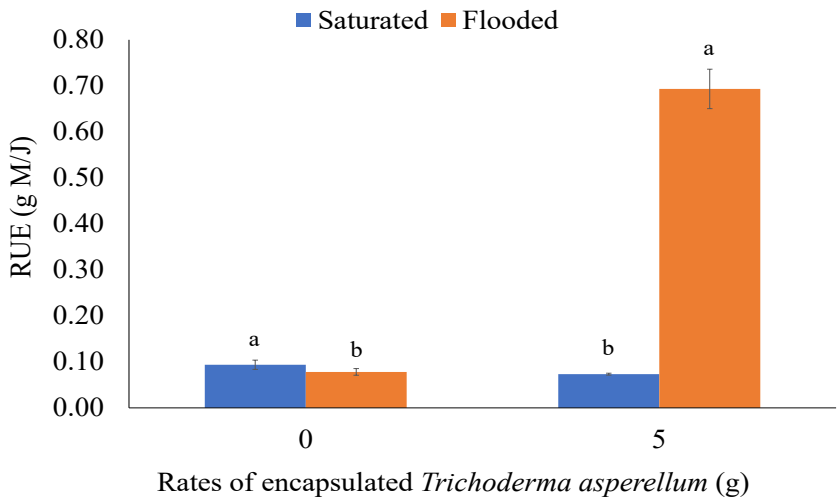

Figure 1. The RUE of rice plants in response to the application of *Trichoderma asperellum* (UPM 40) under saturated and flooded soil conditions

Note. Radiation use efficiency (RUE) followed by different letters indicate significance at the 0.001 probability level

No significant interaction was observed from Table 2 of the two factors on rice plants' total chlorophyll, and relative chlorophyll contents inoculated with *T. asperellum* (UPM 40) in different soil conditions. The total chlorophyll content of rice plants treated with 5 g encapsulated *T. asperellum* (UPM 40) was 6.98 mg/g, significantly higher than rice plants with 0 g treatment with 6.00 mg/g. It agreed with the result for the relative chlorophyll content of both treatments, with values of 45.45 and 41.20%, respectively. The total chlorophyll content of the rice plants planted in saturated soil conditions was 6.20 mg/g, significantly lower than those planted in flooded soil at 6.78 mg/g. The relative chlorophyll content of rice plants planted in flooded soil conditions also was significantly higher than that of saturated soil conditions, with values of 45.10 and 41.55, respectively.

Table 2

Chlorophyll a*, chlorophyll* b*, total chlorophyll, and relative chlorophyll content of rice plants treated with encapsulated* Trichoderma asperellum *(UPM 40) and planted in different soil conditions*

Treatment	Chlorophyll *a*	Chlorophyll *b*	Total chlorophyll mg/g	Relative chlorophyll content
Rate of encapsulated *T. asperellum* (UPM 40) (g) (T)				
0	$3.46^{b} \pm 0.23$	$2.54^{a} \pm 0.20$	$6.00^{b} \pm 0.21$	$41.20^{b} \pm 1.65$
5	$4.30^{a} \pm 0.11$	$2.68^{a} \pm 0.13$	$6.98^{a} \pm 0.18$	$45.45^{a} \pm 1.32$
Significance level	**	ns	**	*
Soil condition (S)				
Saturated	$3.89^{a} \pm 0.22$	$2.31^{b} \pm 0.11$	$6.20^{b} \pm 0.25$	$41.55^{b} \pm 1.64$
Flooded	$3.87^{a} \pm 0.30$	$2.91^{a} \pm 0.08$	$6.78^{a} \pm 0.27$	$45.10^{a} \pm 1.32$
Significance level	ns	**	***	
Interaction				
T × S	ns	ns	ns	ns

Note. Means within column followed by the same letter are not significantly different by least significant difference, $p \geq 0.05$; * = Significant at $p \leq 0.05$; ** = Significant at $p \leq 0.01$; *** = Significant at $p \leq 0.001$; ns = Not significant

Table 3 shows no significant interaction between the application of encapsulated *T. asperellum* (UPM 40) and soil conditions on the root area and root volume of cultivated rice plants. The root area of the rice plants was significantly affected by the application of encapsulated *T. asperellum* (UPM 40). Five (5) g applications of encapsulated *T. asperellum* (UPM 40) showed a higher root area produced by the rice plants at 2,281.80 cm^2 than the root area of rice plants planted without encapsulated *T. asperellum* (UPM 40) at 1,847.70 cm^2. Root area planted in flooded soil conditions was significantly higher than in saturated soil conditions, which were 2,402.50 and 1,726.90 cm^2, respectively. The root length of rice plants treated with and without encapsulated *T. asperellum* (UPM 40) significantly interacted with soil conditions (Figure 2). The root volume of rice plants treated with different rates of encapsulated *T. asperellum* (UPM 40) showed no significant interaction with different soil conditions. However, applying the encapsulated *T. asperellum* (UPM 40) on the rice plants caused the root volume to be significantly higher than the non-treated rice plants, with values of 298.90 and 202.28 cm^2, respectively. As for rice plants planted in different soil conditions, the root volume planted in saturated soil conditions was significantly lower than in flooded soil conditions. The values of the root volumes were 220.37 and 280.81 cm^2, respectively.

Table 3

Maximum root area, root length, and root volume produced by rice plants treated with encapsulated Trichoderma asperellum *(UPM 40) and planted in different soil conditions*

Treatment	Maximum root area (cm^2)	Root length (cm)	Root volume (cm^3)
Rate of encapsulated *T. asperellum* (UPM 40) (g) (T)			
0	$1847.70^b \pm 150.63$	$1366.70^b \pm 256.37$	$202.28^b \pm 17.54$
5	$2281.80^a \pm 193.56$	$3329.20^a \pm 117.70$	$298.90^a \pm 19.36$
Significance level	***	***	***
Soil condition (S)			
Saturated	$1726.90^b \pm 144.26$	$2018.40^b \pm 550.46$	$220.37^b \pm 24.10$
Flooded	$2402.50^a \pm 112.02$	$2677.50^a \pm 343.88$	$280.81^a \pm 25.89$
Significance level	**	**	**
Interaction			
T × S	ns	*	ns

Note. Means within column followed by the same letter are not significantly different by least significant difference, $p \geq 0.05$; * = Significant at $p \leq 0.05$; ** = Significant at $p \leq 0.01$; *** = Significant at $p \leq 0.001$; ns = Not significant

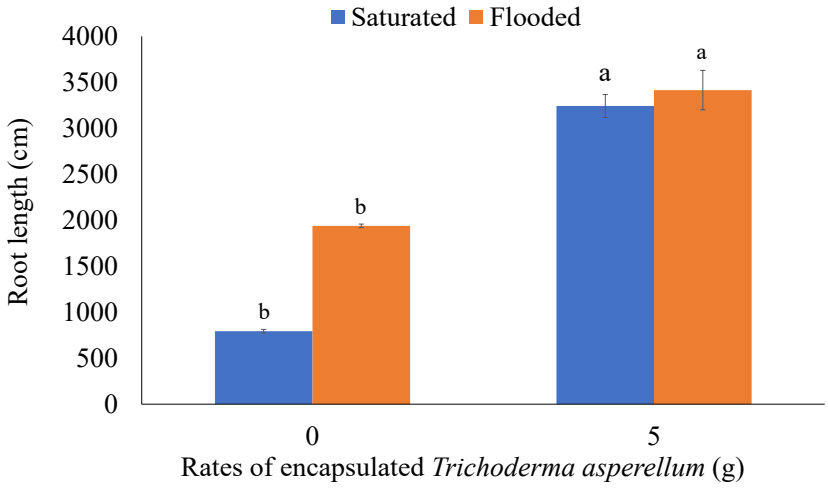

Figure 2. The root length of rice plants in response to the application of *Trichoderma asperellum* (UPM 40) under saturated and flooded soil conditions

Note. Root length followed by different letters indicate significance at the 0.05 probability level

Results in Table 4 show that the weight of 1,000 grains of rice plants treated with different rates of encapsulated *T. asperellum* (UPM 40) had no significant interaction with soil conditions. The 1,000 grains weight produced by the 5 g encapsulated

T. asperellum (UPM 40) treatment was significantly higher than the 0 g treatment, with 21.07 and 19.49 g, respectively. There was also a significant difference for 1,000-grain weight produced by rice plants planted in saturated and flooded soil conditions. The 1,000-grain weight planted in flooded soil was 21.75 g, higher than in saturated condition, 19.28 g. No significant interaction was found between rates of encapsulated *T. asperellum* (UPM 40) and soil conditions on the harvest index of rice plants. Rice plants treated with 5 g encapsulated *T. asperellum* (UPM 40) at 52.57% was significantly higher than 0 g treatment with 41.51%.

As for different soil conditions, the harvest index of rice plants planted in flooded soil conditions was significantly higher than in saturated soil conditions, with 52.00 and 42.08%, respectively. The total yield per culm of rice plants was independent of the effects of different rates of encapsulated *T. asperellum* (UPM 40) and soil conditions and showed that total yield was produced from rice plants treated with 5 g encapsulated *T. asperellum* (UPM 40) with 153.27 g, as compared to 0 g treatment encapsulated *T. asperellum* (UPM 40) with 147.78 g. The yield produced by rice plants planted in flooded soil conditions was significantly higher at 115.07 g than those planted in saturated soil conditions with 107.83 g.

Table 4

Weight of 1000 grains, harvest index, and total yield of rice plants per culm treated with different rates of encapsulated Trichoderma asperellum *(UPM 40) and planted in different soil conditions*

Treatment	Weight of 1000 grains (g)	Harvest index (%)	Total yield per culm (g)
Rate of encapsulated *T. asperellum* (UPM 40) (g) (T)			
0	19.49^{b}±0.41	41.51^{b}±1.96	147.78^{b}±1.58
5	21.07^{a}±0.49	52.57^{a}±2.91	153.27^{a}±0.80
Significance level	***	***	*
Soil condition (S)			
Saturated	19.28^{b}±0.32	42.08^{b}±1.93	107.83^{b}±2.22
Flooded	21.75^{a}±0.40	52.00^{a}±3.31	115.07^{a}±2.01
Significance level	***	***	**
Interaction			
T × S	ns	ns	ns

Note. Means within column followed by the same letter are not significantly different by least significant difference, $p \geq 0.05$; * = Significant at $p \leq 0.05$; ** = Significant at $p \leq 0.01$; *** = Significant at $p \leq 0.001$; ns = Not significant

There was significant interaction found between rates of encapsulated *T. asperellum* (UPM 40) and soil conditions of spikelet weight per panicle (Figure 3). Table 5 reveals no significant interaction between encapsulated *T. asperellum* (UPM 40) application and soil conditions for the percentage of filled grains per panicle. Rice plants treated with 5 g encapsulated *T. asperellum* (UPM 40) had a higher percentage of filled grains per panicle, which was 50.67%. Non-treated rice plants had a lower percentage of filled grains, 41.00%. Rice plants planted in flooded soil produced a significantly higher percentage of filled grains per panicle at 58.49% than those planted in saturated soil at 43.18%. The number of filled grains per panicle of the rice plants showed that the application of encapsulated *T. asperellum* (UPM 40) had significant interaction with soil conditions (Figure 4).

Table 5

Spikelet weight per panicle, percentage of filled grains per panicle, and number of filled grains per panicle of rice plants treated with encapsulated Trichoderma asperellum *(UPM 40) and planted in different soil conditions*

Treatment	Spikelet weight per panicle (g)	Percentage of filled grains per panicle (%)	Number of filled grains per panicle
Rate of encapsulated *T. asperellum* (UPM 40) (g) (T)			
0	$44.65^{b} \pm 2.65$	$41.00^{b} \pm 1.52$	$64.00^{b} \pm 3.80$
5	$60.98^{a} \pm 4.18$	$50.67^{a} \pm 1.31$	$83.50^{a} \pm 1.93$
Significance level	***	***	***
Water condition (S)			
Saturated	$45.53^{b} \pm 2.82$	$43.18^{b} \pm 2.39$	$67.67^{b} \pm 5.42$
Flooded	$60.10^{a} \pm 4.89$	$58.49^{a} \pm 2.05$	$79.83^{a} \pm 3.47$
Significance level	***	***	***
Interaction			
T × S	*	ns	*

Note. Means within column followed by the same letter are not significantly different by least significant difference, $p \geq 0.05$; * = Significant at $p \leq 0.05$; ** = Significant at $p \leq 0.01$; *** = Significant at $p \leq 0.001$; ns = Not significant

DISCUSSION

Relative water content (Table 1) shows the amount of solute available in the leaves of rice plants treated with encapsulated *T. asperellum* (UPM 40), which was less than rice plants planted without the fungal capsules after facing drought stress. According to Bhushan et al. (2007), relative

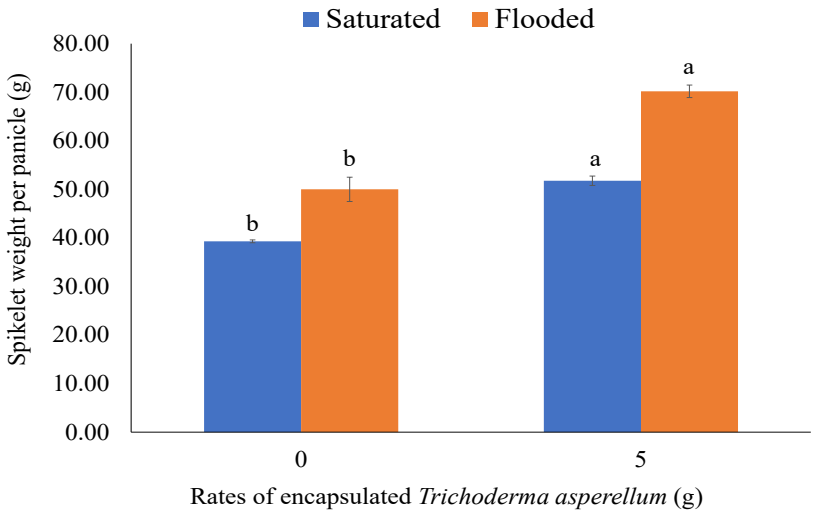

Figure 3. The spikelet weight per panicle of rice plants in response to the application of encapsulated *Trichoderma asperellum* (UPM 40) under saturated and flooded soil conditions

Note. Spikelet weight per panicle followed by different letters indicate significance at the 0.05 probability level

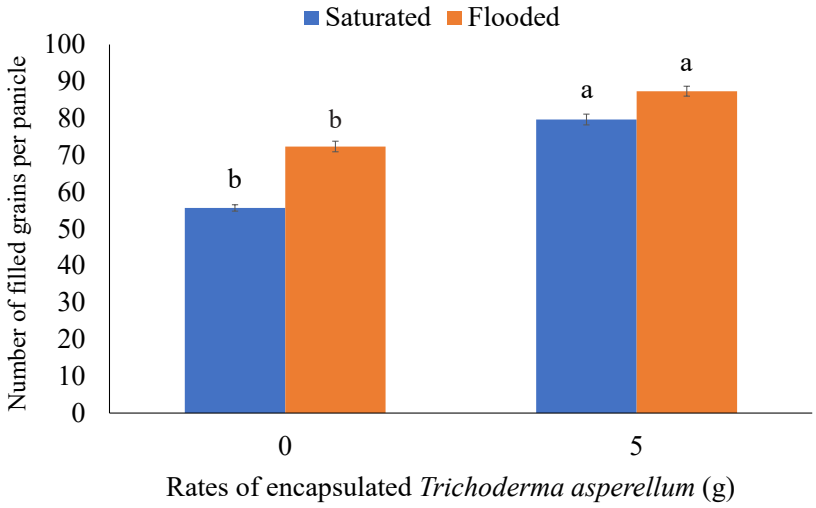

Figure 4. The number of filled grains per panicle of rice plants in response to the application of *Trichoderma asperellum* (UPM 40) under saturated and flooded soil conditions

Note. Number of filled grains per panicle followed by different letters indicate significance at the 0.05 probability level

water content is a term used to describe compatible solute accumulation like proline and an increase in cell membrane permeability causing ions and electrolyte leakage. It shows that the presence of *T. asperellum* (UPM 40) on the roots of the inoculated rice plants triggered the plants to accumulate the needed solutes or release

ions and electrolytes or both. Better RWC also led to a better net photosynthetic rate of the inoculated rice plants. However, since the RWC of non-inoculated rice plants was lower than the inoculated plants, the net photosynthetic rate was also lower, proving the higher drought stress level faced compared to the inoculated plants. Jayaweera et al. (2016) stated that lower performance of the net photosynthetic rate also influenced crop dehydration tolerance, including osmotic adjustment and cell membrane stability. Sanders and Ardnt (2012) reported that one of the plant's mechanisms to fight against drought stress is by accumulating osmotic proteins in the cells. An increase physiological processes in rice plants, such as net photosynthetic rate and water use efficiency, was a way *Trichoderma* spp. contributes to improving rice yield (Doni et al., 2014).

In addition, higher total chlorophyll content and relative chlorophyll content of the *T. asperellum* (UPM 40) inoculated rice plants in Table 2 also contributed to better net photosynthetic rate than the non-inoculated rice plants when exposed to drought stress. Rice seedlings were enhanced to become drought tolerant by *T. hamatum* 219-b by promoting greenness and chlorophyll content (Bae et al., 2009). Shukla et al. (2012) also reported the effect of drought stress on relative greenness (soil plant analysis development [SPAD] value), which was delayed from three to nine days. *Trichoderma harzianum* T 22 intensified leaf greenness through chlorophyll measurement, which provided further energy and carbon source for the development of maize plants (Harman, 2000). Another important evidence that *T. asperellum* (UPM 40) helped to enhance the growth of rice plants during drought was the maximum root area and root volume (Table 3) of the inoculated rice plants, which were found to be higher than the non-inoculated plants.

A study by Shukla et al. (2012) also found that during water cycle alteration, the presence of *Trichoderma* isolates increased the root and shoot length of the plants. The formation of more roots caused more water to be extracted into the soil during drought resulting in less stress held by the rice plants, bringing a higher net photosynthetic rate. Photosynthesis is an important process in cells to produce food to be stored in a sink for plants which for rice plants is the grains. An optimum photosynthesis rate may result in a satisfying yield. In this experiment, applying encapsulated *T. asperellum* (UPM 40) to rice plants resulted in a better net photosynthetic rate, believed to contribute to higher yield. In Tables 4 and 5, the weight of 1,000 grains, harvest index, total yield per culm, spikelet weight per panicle, percentage of filled grains per panicle, and number of filled grains per panicle showed the presence of *T. asperellum* (UPM 40) had significantly caused the yield attributes to be higher than the non-inoculated plants. Improved plant performance living under different biotic and abiotic stress was shown when treated with *Trichoderma* fungus (Mastouri, 2010).

Li et al. (2012) and Makino (2011) agreed that improving rice's physiological characteristics is needed to achieve high yields. A study by Charoenrak and Chamswarng (2016) also found that applying *Trichoderma* spp. to rice seedlings improved 1,000-grain weight by up to 1.65% compared to the untreated control. Similar findings were recorded by Chamswarng and Kumchang (2012), where wettable pellet formulation of *T. asperellum* isolate 01-52 caused the yield of rice vars. Pathum Thani 80 and Pin Kaset to increase. Khadka and Upkhoff (2019) also recorded that incorporating *Trichoderma* on rice seedlings under the System of Rice Intensification (SRI) produced a 26% yield increment compared to non-treated samples. These reports support the current research findings, where the involvement of encapsulated *T. asperellum* (UPM 40) could increase yield.

The yield attributes of rice plants planted in different soil conditions were also significantly different from each other (Tables 4 and 5). As in flooded soil conditions, more standing water caused less drought stress in the rice plants. Less water in saturated soil conditions caused a long drought stress period experienced by the rice plants. These events resulted in physiological and yield differences in both soil conditions. The situation caused less leaf senescence to occur, which led to the total biomass of the rice plants planted in flooded soil conditions than in saturated soil conditions. Chaves et al. (2003) explained that plants develop drought-coping mechanisms to complete their life cycle to avoid dehydration and combat drought stress. This circumstance was also described by Jamieson et al. (1995), where leaf senescence is the main cause of decreased radiation intercepted while causing biomass production to decrease. Bat-Oyun et al. (2011) reported that water stress caused the senescence of plants and brought a reduction in canopy photosynthetic capacity; such a situation also occurred in this experiment, where rice plants planted in saturated soil conditions had a lower net photosynthetic rate compared to flooded soil conditions.

Significant interactions existed between the *T. asperellum* treatment and soil conditions in RUE, root length, number of filled grains per panicle, and spikelet weight per panicle. The interactions were visible, where the listed attributes were higher when the rice plants were treated with encapsulated *T. asperellum* (UPM 40) and planted in flooded soil conditions than the non-inoculated rice plants planted in saturated soil conditions. However, RUE for inoculated rice plants planted in saturated soil conditions was lower than the non-inoculated rice plants. The rice plants focus on lengthening their roots to help increase the ability to look and absorb available water in the soil and to decrease drought stress held by the plants. As for roots increment rate of rice plants planted in flooded soil conditions from non-inoculated rice plants to inoculated rice plants was lower than in saturated soil conditions. *Trichoderma* root colonization promotes plant growth (Adams et al., 2007; Lynch, 2004). Spikelet weight per panicle of rice plants planted in

flooded soil conditions had a higher rate of increase from no application of encapsulated *T. asperellum* (UPM 40) to the presence of encapsulated *T. asperellum* (UPM 40) than in saturated condition (Figure 3).

The presence of the fungus encouraged rice plants planted in flooded soil conditions to improve their spikelet weight when faced with drought threats. As for the number of filled grains per panicle rate of increase from the non-inoculated *T. asperellum* (UPM 40) to the inoculated rice plants was higher when planted in saturated soil conditions than flooded soil conditions (Figure 4). The fungus facilitated the rice plants in producing a higher number of filled grains while combating drought stress. Producing more filled grains while having non-favorable conditions, which for this experiment was drought, was a way for the plants to ensure their legacy continues. A study by Asseng and van Herwaarden (2003) and Plaut et al. (2004) also stated that although drought stress caused an increase in the remobilization of assimilates to the grains, early senescence and reduction in grain filling also occurred.

CONCLUSION

During the drought season, the planting of rice plants in flooded soil conditions combined with the application of encapsulated *T. asperellum* (UPM 40) at 5 g was found to result in increased growth of the roots and yield of rice plants as indicated by higher spikelet weight per panicle and number of filled grains per panicle. On the contrary, rice plants planted in saturated soil conditions without inoculation of the fungus did not perform as well.

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Comparative Clinicopathological Changes Associated with Experimental *Streptococcus agalactiae* and *Streptococcus iniae* Cohabitation Infection in Red Hybrid Tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*)

Salleh Annas[1,2]*, Mohd Zamri-Saad[1,2], Md Yasin Ina-Salwany[2,3] and Mohammad Noor Azmai Amal[2,4]

[1]*Department of Veterinary Laboratory Diagnosis, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

[2]*Aquatic Animal Health and Therapeutics Laboratory (AquaHealth), Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

[3]*Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

[4]*Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

Streptococcus agalactiae and *Streptococcus iniae* are the two main pathogens causing streptococcosis in fish. This study compares the clinicopathological changes in red hybrid tilapia experimentally infected with *S. agalactiae* or *S. iniae*. A total of 180 tilapias were divided into six groups. Groups 1A, 2A, and 3A were inoculated intraperitoneally with sterile phosphate-buffered saline, *S. agalactiae*, and *S. iniae*. Fish of Groups 1A, 2A, and 3A were then immediately allowed to cohabitate with fish of Groups 1B, 2B, and 3B, respectively. All fish were observed at 6-hr intervals for 120 hr before surviving fish were euthanized. The spleen, liver, and brain samples were collected for bacterial isolation and histopathology. Clinical signs were developed at 72 hr in Groups 2A and 3A and 96 hr in Groups 2B and 3B. Group 2A showed the highest clinical score (P<0.05). Significantly (P<0.05), more cohabitating fish (Groups 2B) were infected by *S. agalactiae* compared to *S. iniae* (Group 3B) at 55.0±0.0 and 43.70±1.25%, respectively. The mortality rate was significantly (P<0.05) higher for Groups 2A and 2B than other groups. The gross lesions were significantly (P<0.05) more common in fish of Group 2A. Histopathologically, encephalitis was observed in fish infected

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E-mail addresses:
annas@upm.edu.my (Annas Salleh)
mzamri@upm.edu.my (Mohd Zamri-Saad)
salwany@upm.edu.my (Ina Salwany Md Yasin)
mnamal@upm.edu.my (Mohammad Noor Amal Azmai)
* Corresponding author

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with *S. iniae* of Groups 3A and 3B, while meningoencephalitis was observed in fish infected with *S. agalactiae* of Groups 2A and 2B. The findings suggest that *S. agalactiae* is more pathogenic than *S. iniae*, producing slightly different histopathological lesions in the brain.

Keywords: *Streptococcus agalactiae*, *Streptococcus iniae*, streptococcosis, tilapia

INTRODUCTION

Tilapia (*Oreochromis* sp.) is an important fish species in aquaculture (Amal et al., 2010). In 2018, the global production of farmed tilapia was 6.03 million tonnes (Miao & Wang, 2020). Tilapia grows rapidly, can survive in water of poor quality, is tolerable to environmental conditions, and is resistant to various diseases (Amal & Zamri-Saad, 2011; Ghozlan et al., 2018). However, tilapia is susceptible to streptococcosis, a disease caused by *S. agalactiae* and *S. iniae* (Evans et al., 2006).

Since the first occurrence of streptococcosis in rainbow trout (*Oncorhynchus mykiss*) in 1957 (Hoshina, 1958), this disease has continued to cause significant losses in the global aquaculture industry (Mishra et al., 2018). The economic losses are largely attributed to the high mortality and morbidity caused by streptococcosis. Mortality rates due to *S. agalactiae* infection can reach as high as 80% (Al-Harbi, 2016), while *S. iniae* infection can result in mortality rates up to 50% (Eldar et al., 1995). Streptococcosis has infected various species of freshwater, estuarine, and marine fish worldwide, including Africa, the Middle East, North and South America, Australia, as well as East, South, and Southeast Asia (Amal & Zamri-Saad, 2011). The estimated economic impact of *S. agalactiae* and *S. iniae* infections in tilapia was around USD 150 million annually in 2000 and further increased to USD 250 million annually in 2008, approximately 5.7 and 6.7% of the total global value of tilapia, respectively (Amal & Zamri-Saad, 2011). Later, it was estimated that losses due to streptococcosis in China in 2011 could be as high as USD400 million (M. Chen et al., 2012).

Both pathogens can result in similar clinical signs and lesions, including loss of orientation and erratic swimming, lethargy, dyspnea, exophthalmia, and congestion of visceral organs (C.-Y. Chen et al., 2007; Rahmatullah et al., 2017; Zamri-Saad et al., 2010). Field observations revealed that *S. agalactiae* infection usually resulted in high morbidity and acute mortality, while *S. iniae* infection resulted in chronic mortality (Chu et al., 2016; Jantrakajorn et al., 2014; Yuasa et al., 2008). This article compares the clinical signs, pattern and rate of mortality, and histopathological lesions in red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) following experimental infection by *S. agalactiae* and *S. iniae* using a cohabitation infection method.

MATERIALS AND METHODS

Animals

A total of 190 red hybrid tilapias of total length of 8.0±1.5 cm were bought from a commercial fish farm in Serdang, Selangor,

Malaysia. These fish were maintained in 12 aquaria (75 cm length × 45 cm height × 37 cm width) with a closed water system, where approximately 20% of the water was replaced every 24 hr. Throughout the study period, the recorded water dissolved oxygen was 6.48±0.90 mg/L, pH was 7.4±0.5, temperature was 27.80±1.50°C, and ammonia-nitrogen was 0.02±0.01 mg/L, as recorded by a handheld YSI meter (YSI, USA) and a handheld colorimeter (DR900, Hach Company, USA). All fish were fed twice daily with commercial feed at the rate of 2% body weight. They were acclimatized for 10 days prior to the experiment, during which time 10 fish were randomly subjected to necropsy to ensure that they were free from external parasites, as well as *Streptococcus* spp. by isolation and identification of *Streptococcus* spp. using polymerase chain reaction (PCR) (Rahmatullah et al., 2017). The Institutional approved the experimental procedure for Animal Care and Use Committee, Universiti Putra Malaysia (UPM/IACUC/AUP-U035/2018).

Preparation of Inoculums

Streptococcus agalactiae strain UPM1357 (accession number AF151357) and *S. iniae* strain UPM17 (accession number KT722586) that were previously isolated from outbreaks of streptococcosis were obtained from stock cultures (Rahmatullah et al., 2017; Syuhada et al., 2020). The isolates were cultured in brain heart infusion (BHI) broth (Merck, Germany) and incubated at 37°C for 18 hr. The inoculums were adjusted using serial dilutions and plate counts to achieve an infective dose of 1×10^7 cfu/ml.

Experimental Design and Clinical Evaluation

The remaining 180 fish were divided into six groups of equal size, each being replicated thrice. Groups 1A, 2A, and 3A received intraperitoneal injections of 0.03 ml of sterile phosphate-buffered saline (PBS) (Merck, USA), 3×10^5 cfu/ml of *S. agalactiae*, and 3×10^5 cfu/ml of *S. iniae*, respectively. All fish that were inoculated were tagged with tail sutures for identification purposes. Immediately after inoculation, fish in Groups 1B, 2B, and 3B were introduced to their respective aquaria to cohabit (Table 1). All fish were observed for clinical signs at 6-hr intervals for 120 hr. During the observation period, dead fish were collected for necropsy. At the end of the study, surviving fish were anesthetized using MS222 prior to euthanasia by pithing. The fish were then necropsied. Samples of the brain, liver, and spleen were collected to isolate and identify *S. agalactiae*

Table 1
Summary of the groups and the respective treatment

Groups	Treatments	Cohabitating groups
1A	0.03 ml of sterile PBS	1B
2A	0.03 ml of 3×10^5 cfu/ml of live *Streptococcus agalactiae*	2B
3A	0.03 ml of 3×10^5 cfu/ml of live *Streptococcus iniae*	3B

Note. PBS = Phosphate buffered saline

and *S. iniae*. Similar samples were also fixed in 10% neutral-buffered formalin and processed for histopathology.

During observation, the following clinical signs were noted: lethargy, anorexia, exophthalmia, corneal opacity, dyspnoea, abnormal swimming, and death. At each observation point, a clinical score was given to each group, where observation of each clinical sign was scored 1, while death was scored 4. The cumulative mortality rates were calculated and plotted over time.

Bacteriology

Swabs from the brain, liver, and spleen were placed on BHI agar (Merck, USA) containing 5% horse blood and incubated at 37°C for 18 hr. Initial identification of the cultured bacteria was based on the morphological characteristics of greyish-white, circular, and β-hemolysis of blood agar (Aisyah et al., 2015). Suspected colonies were subjected to identification using PCR. The bacterial DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, USA). The extracted DNA was subjected to PCR using forward primers 5′-ACG GAG TTA CAA AGG ACG AC-3′ and reverse primer 5′-AGC TCA GCC TTA ACG AGT AC-3′ for detection of *S. agalactiae*-specific 16S gene (Amal et al., 2012). For *S. iniae*, forward and reverse primers of 5'-CTAGAGTACACATGTAGCTAAG-3' and 5'-GGATTTTCCACTCCCATTAC-3', and forward and reverse primers of 5'-AAGGGGAAATCGCAAGTGCC-3' and 5'-ATATCTGATTGGGCCGTCTAA-3' were used for detection of 16S gene and lactate oxidase (*lctO*) gene, respectively (Rahmatullah et al., 2017).

Histopathology

The brain, liver, and spleen samples were fixed in 10% neutral-buffered formalin for 72 hr and processed using routine methods to isolate and identify bacteria. After embedding in paraffin, sections of 4 μm were obtained and stained with hematoxylin and eosin (R&M Chemicals, Malaysia) (Nurliyana et al., 2020). Histopathological lesions were evaluated by analyzing ten randomly selected fields at 200× magnification. Lesions for each sampled organ were identified before their severity was semi-quantitatively scored as previously described by Azzam-Sayuti et al. (2021) and Ulum et al. (2021), with minor modifications; score of 0 for normal tissue, a score 1 for tissue with ≤15% lesion, score 2 for tissue with 15-30% lesion, score 3 for tissue with 30-50% lesion, and score 4 for tissue with >50% lesion.

Data Analysis

Data on the clinical signs and cumulative mortality rates were analyzed using one-way analysis of variance (ANOVA), while the histopathology evaluations were analyzed using two-way ANOVA. The statistical analysis was done using the IBM® SPSS® Statistics (version 22).

RESULTS

Clinical Signs

After inoculation with *S. agalactiae* and *S. iniae*, the fish exposed to the pathogens

developed clinical signs observed from 72 hr until the end of the experiment. Similarly, the cohabitating fish in Groups 2B and 3B began exhibiting clinical signs starting from 96 hr until the end of the experiment. The clinical signs included lethargy, anorexia, exophthalmia, corneal opacity, dyspnoea, and abnormal swimming. Ten fish from Group 2A, five from Group 3A, and three from each Group 2B and 3B died without observable clinical signs. No clinical sign was observed in all control fish throughout the study period.

Group 2A, inoculated with *S. agalactiae*, had the highest clinical score of 8.9±1.7, significantly higher (P<0.05) than other groups. It was followed by the cohabitating Group 2B with a score of 7.5±0.5; Group 3A inoculated with *S. iniae* with a score of 6.6±0.7, and the cohabitating Group 3B with a score of 5.5±0.8. No significant difference (P>0.05) in the clinical score was observed between the cohabitating fish in Group 2B and those inoculated with *S. iniae* in Group 3A. Additionally, no significant difference (P>0.05) was observed between the fish inoculated with *S. iniae* in Group 3A and the cohabitating fish in Group 3B. A significant difference (P<0.05) was noted when 56.6±3.3% of the fish of Group 2B showed clinical signs compared to 43.3±3.3% of Group 3B, comparing the two cohabitating groups.

Fish in Group 2A inoculated with *S. agalactiae* experienced mortality starting from 78 hr at a rate of 23.3±3.3%. It was significantly higher (P<0.05) compared to other groups. On the other hand, mortality among the cohabitating Group 2B started at 90 hr at the rate of 6.7±3.3%. Similarly, fish of Group 3A that were inoculated with *S. iniae* showed mortality starting from 96 hr, while the cohabitating Group 3B started to show mortality from 102 hr. In all exposed Groups 2A, 2B, 3A, and 3B, the mortality rates consistently increased until the end of the experiment. At 120 hr, fish inoculated with *S. agalactiae* of Group 2A showed the highest cumulative mortality rate at 53.3±3.3%, followed by its cohabitating Group 2B at 36.7±6.7%, fish inoculated with *S. iniae* of Group 3A at 23.3±3.3%, and its comingling Group 3B at 20.0±0.0% (Figure 1). No mortality was observed in Groups 1A and 1B.

Bacterial Isolation and Identification

Streptococcus agalactiae was isolated and identified by PCR from all dead fish of Groups 2A and 2B, while *S. iniae* was isolated from Groups 3A and 3B. All survivors from cohabitating Group 2B were positive for *S. agalactiae* compared to 43.75% of survivors from cohabitating Group 3B that were positive for *S. iniae*. Bacterial isolation from all Groups 1A and 1B fish yielded no growth.

Gross and Histopathologic Lesions

All of the exposed fish in Groups 2A, 2B, 3A, and 3B showed gross lesions, including corneal opacity, peritonitis, and abdominal distension containing serosanguineous ascitic fluid, as well as discoloration and hemorrhages at the base of the fin (Figure 2). However, no lesions were observed in the control fish of Groups 1A and 1B. The

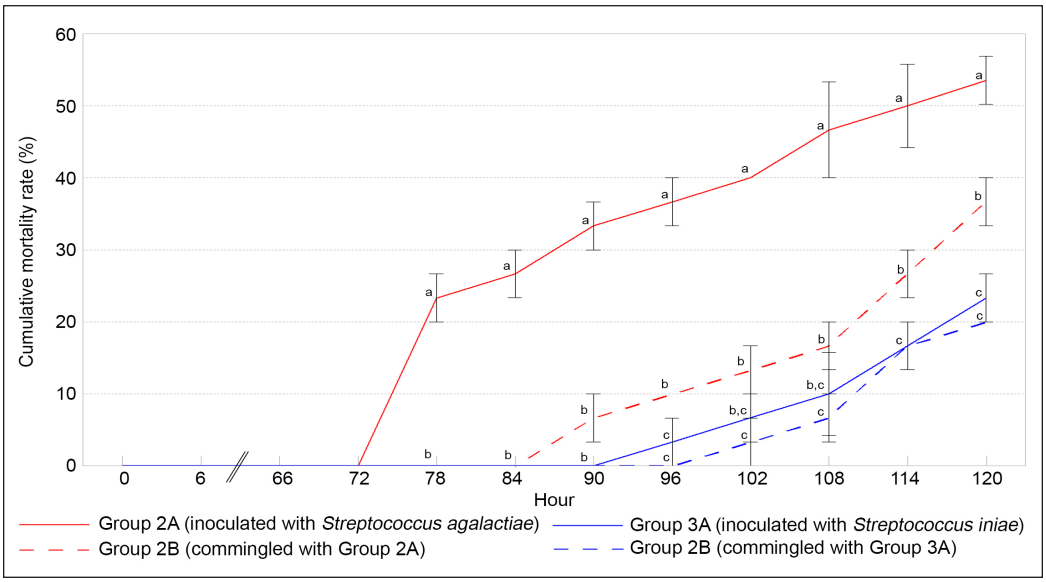
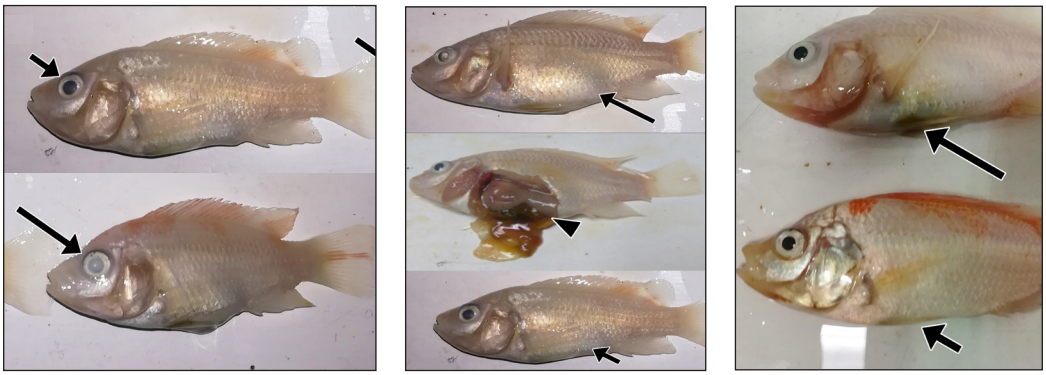


Figure 1. Mean cumulative mortality rates over time between the different treatment groups
Note. Different superscripts (^{a,b,c,d}) at each point of time indicate significant (P<0.05) differences between the groups



(a) (b) (c)

Figure 2. Gross lesions of tilapia infected by *Streptococcus*. (a) Corneal opacity (long arrow) in infected fish, compared to non-infected fish with a normal eye (short arrow); (b) Infected fish showing abdominal distention (long arrow), and ascites (arrowhead), compared to non-infected fish with normal abdomen; (c) Intraperitoneally infected fish showing a discoloration of the injection site (long arrow), compared to non-infected fish with a normal gross appearance

percentage of fish exhibiting gross lesions was significantly higher (P<0.05) in Group 2A (46.7±0.7%) compared to all other groups (Table 2). It is followed by the fish of Group 3A (17.8±4.0%). The lesion scores for these two groups were significantly higher (P<0.05) than the remaining groups. Approximately 7.8±4.0% and 4.4±1.8% of fish from the cohabitating Groups 2B and 3B exhibited gross lesions, respectively.

Four types of histopathological lesions were frequently observed in the brain of

the infected fish. These were congestion (Figure 3a), hemorrhage, encephalitis, and meningitis. All exposed fish showed congestion and hemorrhages of various internal organs. The histopathology lesion scorings indicated no significant difference (P>0.05) among the four infected groups. Fish exposed to *S. agalactiae* (Groups 2A and 2B) exhibited microglial infiltration in the brain (Figure 3b), and 70.00±4.71% of fish exposed to *S. iniae* (Groups 3A and 3B) also showed the same lesion (P<0.05).

Table 2
Percentage (±SEM) of fish from different treatment groups showing the gross lesions following infection by Streptococcus agalactiae *or* S. iniae

Groups	Treatments	Corneal opacity	Peritonitis	Fin base hemorrhage	Overall
1A	Sterile PBS	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{d}
1B	(cohabitated with 1A)	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{d}
2A	*Streptococcus agalactiae*	63.3±3.3^{a}	53.3±3.3^{a}	20.0±0.0^{a}	46.7±0.7^{a}
2B	(cohabitated with 2A)	23.3±3.3^{b}	0.0±0.0^{d}	0.0±0.0^{c}	7.8±4.0^{c}
3A	*Streptococcus iniae*	10.0±0.0^{c}	33.3±3.3^{b}	10.0±0.0^{b}	17.8±4.0^{b}
3B	(cohabitated with 3A)	3.3±3.3^{c,d}	10.0±0.0^{c}	0.0±0.0^{c}	4.4±1.8^{c}

Note. Different superscripts (^{a,b,c}) indicate significant differences (P<0.05) of the same column; PBS = Phosphate buffered saline; SEM = Standard error of the mean

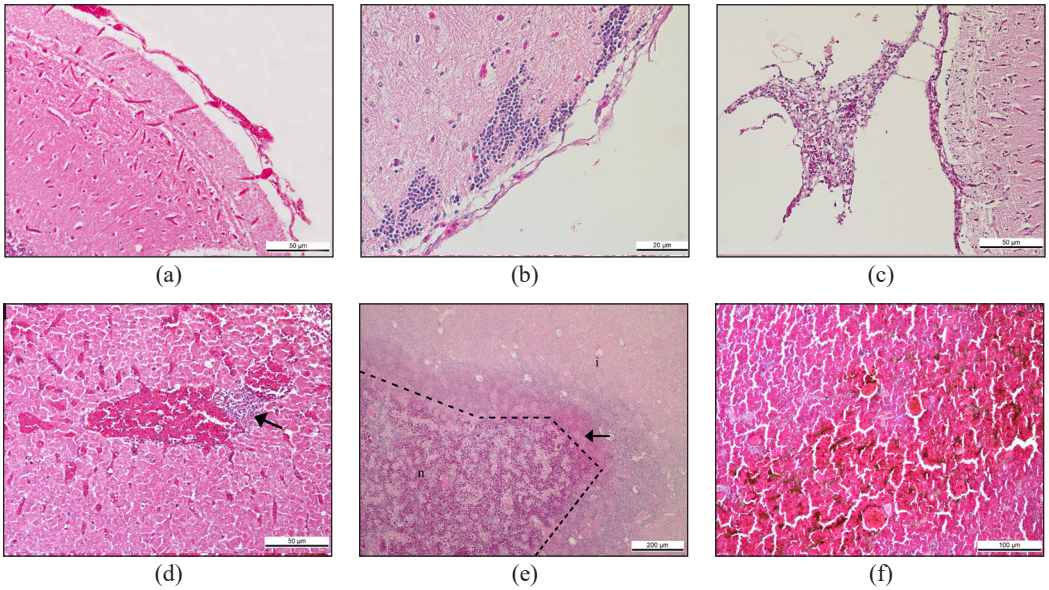


Figure 3. Histological lesions in organs of fish infected by *Streptococcus*. (a) Meningeal and cerebral congestion (bar = 50 µm, hematoxylin, and eosin [HE]); (b) Microglial cells in the brain (bar = 20 µm, HE); (c) Infiltration of inflammatory cells and congestion of meninges (bar = 50 µm, HE); (d) Congestion and infiltration of mononuclear cells (arrow) in the liver (bar = 50 µm, HE); (e) Spleen of fish showing normal (n) and infarcted (i) areas, accompanied by hemorrhage (arrows) (bar = 200 µm, HE); (f) Severe hemorrhage in the spleen (bar = 100 µm, HE)

Fish in Group 2A had the most severe encephalitis, significantly more severe (P<0.05) than Groups 3A and 3B, as revealed by encephalitis severity scores (Table 3). Meningitis (Figure 2c) was observed only in tilapia of Groups 2A and 2B infected with *S. agalactiae*, with moderate severity scores of 2.90±0.74 and 2.20±1.14, respectively. The overall histopathological lesion scores were significantly higher (P<0.05) in Groups 2A and 3A compared to Groups 2B and 3B.

The liver showed four types of lesions, namely congestion, hepatitis (Figure 2d), hemorrhage, and necrosis, which were observed in all infected fish, and there was no significant difference (P>0.05) in the number of fish affected by these lesions between the infected groups. Additionally, there was no significant difference (P>0.05) in the severity of histopathological lesions between Groups 2A, 2B, 3A, and 3B. The severity of all liver lesions was generally mild to moderate.

The spleen of the infected fish exhibited three types of lesions: congestion, infarction (Figure 3e), and hemorrhage (Figure 3f). Congestion and hemorrhages were found in all infected fish, whereas splenic infarction was observed in 85.00±3.33% of fish infected with *S. agalactiae* in Groups 2A and 2B and in all fish infected with *S. iniae* in Groups 3A and 3B. There was no significant difference (P>0.05) in the severity of all three types of spleen lesions among the infected groups.

DISCUSSION

Streptococcosis is long-known to be an important disease in fish. Many studies

Table 3
Histopathological lesion scores (± SEM) of brain, liver, and spleen in fish following infection by Streptococcus agalactiae *or* S. iniae

Organs	Lesions	Inoculated groups			Cohabitating groups		
		1A	2A	3A	1B	2B	3B
Brain	Congestion	0.10±0.32^{a}	3.00±0.667^{b}	2.10±1.20^{b}	0.22±0.44^{a}	3.00±0.667^{b}	1.40±0.84^{c}
	Hemorrhage	0^{a}	1.8±1.14^{b}	1.0±0.67^{b}	0^{a}	1.7±1.16^{b}	0.7±0.82^{b}
	Encephalitis	0^{a}	2.90±0.74^{b}	1.60±0.84^{c}	0^{a}	2.60±0.97^{b,c}	1.70±0.95^{c}
	Meningitis	0^{a}	2.60±0.69^{b}	0^{a}	0^{a}	2.20±1.14^{b}	0^{a}
	Overall	0.03±0.16^{a}	2.48±0.99^{b}	1.10±1.13^{c}	0.06±0.23^{a}	2.33±1.10^{b}	1.025±0.98^{c}
Liver	Congestion	0.30±0.67^{a}	1.90±0.99^{b}	1.86±0.94^{b}	0.11±0.33^{a}	2.10±0.74^{b}	1.40±0.70^{b}
	Hemorrhage	0^{a}	2.1±1.29^{b}	1.50±1.08^{b}	0.33±0.71^{a}	2.00±1.05^{b}	1.50±0.85^{b}
	Hepatitis	0^{a}	1.90±0.57^{b}	2.1±0.57^{b}	0^{a}	1.80±1.23^{b}	2.30±0.95^{b}
	Necrosis	0.10±0.32^{a}	1.60±0.84^{b}	1.80±0.92^{b}	0.10±0.32^{a}	1.00±1.05^{b}	1.80±1.03^{b}
	Overall	0.10±0.38^{a}	1.88±0.94^{b}	1.83±0.90^{b}	0.06±0.24^{a}	1.73±1.09^{b}	1.75±0.93^{b}
Spleen	Congestion	0.10±0.32^{a}	2.00±0.94^{b}	1.60±0.70^{b}	0^{a}	2.00±0.94^{b}	1.80±0.92^{b}
	Hemorrhage	0^{a}	0.90±0.99^{b}	1.1±0.88^{b}	0^{a}	1.40±1.08^{b}	0.60±0.70^{b}
	Infarction	0^{a}	1.60±1.27^{b}	2.10±1.42^{b}	0^{a}	1.30±1.42^{b}	2.30±0.74^{b}
	Overall	0.03±0.18^{a}	1.50±1.14^{b}	1.60±0.86^{b}	0^{a}	1.57±1.17^{b}	1.57±1.04^{b}

Note. Different superscripts (^{a,b}) within the same row indicate significant differences (P<0.05) between groups; SEM = Standard error of the mean

were previously conducted to understand this disease. However, to date, there is a very limited number of studies on the various species of *Streptococcus* that cause streptococcosis. The findings from this study are believed to significantly contribute to understanding the disease. In this study, the clinical signs observed following *S. agalactiae* or *S. iniae* infections were generally similar, making it impossible to distinguish between the two bacterial infections based solely on these criteria. In streptococcosis, it is postulated that damage to the brain and nervous system leads to behavioral changes such as abnormal swimming and grouping at the bottom of the tank (Abuseliana et al., 2011), while death is largely incriminated to septicemia (Song et al., 2017). It is consistent with the findings in this study, where histopathological changes were noted in the brain samples of tilapia exposed to *S. agalactiae* and *S. iniae*.

The behavioral changes mentioned earlier can be easily overlooked in real-world scenarios. Nonetheless, analyzing mortality patterns over time could offer significant clues to support the cause of infections. As observed in this study, earlier and higher mortality rates and higher percentages of isolation of pathogens in surviving fish suggested that streptococcosis caused by *S. agalactiae* develops rapidly. Furthermore, the transmission rate is faster for *S. agalactiae* than for *S. iniae*. Theoretically, in a septicaemic disease, rapid disease development and transmission are incriminated to rapid multiplication of the pathogens in the host tissues and circulation. It was previously observed that *S. agalactiae* usually results in severe septicaemic disease where it could be isolated from various organs, while *S. agalactiae* infection tends to be more localized, especially in the brain and the eye socket (Yuasa et al., 2008). Unfortunately, this study did not determine the concentrations of *S. agalactiae* and *S. iniae*. The mortality rate patterns between the four infected groups, as well as previous pathogenicity studies to determine the 50% lethal dose (LD_{50}) of these *S. agalactiae* and *S. iniae* isolated (Nur-Nazifah et al., 2011; Rahmatullah et al., 2017), proved that *S. agalactiae* is significantly more pathogenic compared to *S. iniae* in the tilapia. The findings from this and previous studies explain the previous field observation that *S. agalactiae* causes higher acute mortality in fish than *S. iniae* (Jantrakajorn et al., 2014; Yuasa et al., 2008). The field observations revealed that *S. agalactiae* results in cumulative mortalities that range from 40–60%. In contrast, mortality caused by *S. agalactiae* was low but consistent, accounting for only 0.1–0.2% per day (Yuasa et al., 2008).

Even though the gross lesions caused by both bacteria were similar, the histopathology evaluation showed some differences. These differences were primarily observed in the brain lesions, where *S. agalactiae* caused meningoencephalitis, while *S. iniae* caused only encephalitis. While the meningeal involvement in *S. agalactiae* infection in tilapia was recently explained (Eto et al., 2020), this comparative discovery has not been reported before. Furthermore,

these findings are actually in contrast with previous observations that both organisms cause similar histopathology findings (C.-Y. Chen et al., 2007). The different clinical and pathological features resulting from infection by *S. agalactiae* and *S. iniae* suggest different pathogenesis between these two pathogens.

The general pathogenesis of encephalitis or meningoencephalitis involves the early entry of the agent into the central nervous system (Quagliarello & Scheld, 1992). If the agent is equipped with certain virulence factors or due to the high concentration of the agent in the blood (Tunkel & Scheld, 1993), it could lead to meningeal invasion and breakdown of the blood-brain barrier causing the development of meningitis and cerebral edema (Quagliarello & Scheld, 1992). A recent study emphasized that a high concentration of pathogens in the bloodstream is crucial in invading the meninges and tropism of bacterial pathogens, such as Group B *Streptococcus* (Coureuil et al., 2017). On the other hand, without the meningeal invasion, inflammation occurs only in the brain. Studies to quantify the level of *S. agalactiae* and *S. iniae* in the circulation of infected tilapia, and their interactions with the blood-brain barrier should be conducted to elucidate the exact pathogenesis of infections of these two pathogens.

The variation in mortality rate and histopathological alterations observed in the two pathogens may be linked to the response of inflammatory cells to these pathogens. Previous research has demonstrated that the phagocytes of tilapia exhibit distinct reactions to *S. agalactiae* and *S. iniae*. When exposed to *S. agalactiae*, macrophages exhibit higher levels of chemotactic and chemokinetic activity than those exposed to *S. iniae*, as demonstrated by Klesius et al. (2007). Furthermore, macrophages exposed to *S. iniae* failed to show any chemokinetic activity. It is speculated that tilapia innate immunity could efficiently control *S. iniae* infection compared to *S. agalactiae* infection (C.-Y. Chen et al., 2007; Poyart et al., 2001), which possibly explains the meningeal invasion in the latter. Further investigations on the response and modulation of macrophages may offer valuable insights for the future prevention and treatment of streptococcosis.

CONCLUSION

This study showed that *S. agalactiae* is more pathogenic than *S. iniae*, leading to higher mortality rates and more severe clinical signs in red hybrid tilapia. Furthermore, the study also revealed that *S. agalactiae* caused meningoencephalitis, while *S. iniae* caused encephalitis in infected fish. These findings highlight the differences in pathogenicity and histopathological lesions between the two pathogens, possibly suggesting two different pathogenesis of infections of these two pathogens.

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Comparative Study of Thermal Pre-treatment on the Extraction, Antioxidant, Fatty Acid Profile, and Physicochemical Properties of Inca Inchi Seed Oil

Jeffi Christopher[1], Chin Ping Tan[2], Helmi Wasoh[3] and Oi Ming Lai*[1,3]

[1]Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[3]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Inca Inchi oil, an edible oil with high amounts of polyunsaturated fatty acids such as omega 3 and omega 6 fatty acids, has a wide range of applications in therapeutic, food, and pharmaceutical industries. Increasing its oil yield during oil extraction is important due to its high value. However, conventional techniques such as screw press extraction pose a limitation in terms of oil yield. Thus, in this study, the seeds were pre-treated in a microwave and hot air oven prior to oil extraction. The effects of this pre-treatment on the oil yield, fatty acid profile, antioxidant profile, and physicochemical properties were compared. Microwave treatment (4 min) was found to have the highest oil yield (43.39%) compared to control (37.76%). The proximate analysis revealed that the protein content in the oil meal was high (51–60%) compared to oil seed (24.2%), indicating that it has potential application to be developed into plant-based protein foods. The fatty acid profile indicates that the oil had high omega 3 (49%) and omega 6 (37%) fatty acids. The free fatty acids and peroxide values of the pre-treated oil samples were less than 1% and 10 meq O_2/kg oil, respectively, compared to the control (1%), while the iodine value was high due to double bonds. The 2,2-diphenyl-1-1picrylhydrazyl and 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid study shows that the oil has good radical scavenging activity (70 and 90%), which shows the oil's potential in functional food applications.

Keywords: Fatty acid profile, Inca Inchi oil, physicochemical properties, pre-treatment, oil extraction

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E-mail addresses:
jeffichris@gmail.com (Jeffi Christopher)
tancp@upm.edu.my (Chin Ping Tan)
helmi_wmi@upm.edu.my (Helmi Wasoh)
omlai@upm.edu.my (Oi Ming Lai)
* Corresponding author

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INTRODUCTION

Inca Inchi (*Plukenetia volubilis* Linneo), an oleaginous, perennial plant from the Euphorbiaceae family, is found naturally in the tropical rainforest of the Amazon region in South America. They are most abundant in Peru and grow at an altitude level below 900 m (Gillespie, 1993). The plants have been cultivated for centuries by the Peruvians (Guillén et al., 2003). The seed is commonly known as "forest peanut", or "sacha inchi" (Bussmann et al., 2009). The indigenous plant is also cultivated in Southeast Asia due to its high economic value (Medina-Mendoza et al., 2021).

Inca Inchi seeds are made up of seed layers (33–35%), which include the husk, shell, and oleaginous seed kernel (65–67%), which is commercially turned into oil (Chirinos et al., 2016). The oil content in Inca Inchi seeds is high (35–60%) and comparable to sunflower seeds (48%) and peanuts (45%) (Gutiérrez et al., 2011; Hamaker et al., 1992). Commercially, the oil is recognized for its health attributes and distinct sensory characteristics (taste and flavor) (Garmendia et al., 2011).

The major fraction of the oils is made up of polyunsaturated fatty acids (PUFAs) (77.5–84.4%). The remaining lipid fraction is composed of monounsaturated fatty acids (MUFAs) (8.4–13%) and saturated fatty acids (SFAs) (6.8-9.1%) (Chirinos et al., 2013; Follegatti-Romero et al., 2009; Gutiérrez et al., 2011; Kodahl et al., 2022; Maurer et al., 2012). A few vegetable oils with an equivalent high percentage of PUFA are linseed (*Linum usitatissimum* L.) (74%) and chia (*Salvia hispanica* L.) (80%) (Ciftci et al., 2012). The PUFAs are composed of two essential fatty acids, α-linolenic acid, an omega 3 (ω-3) (35–50%) and linoleic acid, an omega 6 (ω-6) (33–41%), respectively (Chirinos et al., 2013; Cisneros et al., 2014). These essential fatty acids are required for biological processes. However, they cannot be synthesized in the human body due to the lack of Δ-12 and Δ-15 desaturases and hence must be obtained through diet (Sinclair et al., 2002).

The primary mechanism by which α-linolenic acid is transformed to eicosapentaenoic acid (EPA) and ultimately docosahexaenoic acid (DHA) in the human body is by β-oxidation, which uses the same enzyme cascade that transforms linoleic acid to docosapentaenoic acid (DPA) (Glick & Fischer, 2013). The composition of the fatty acids varies depending on the factors such as climate, time of harvest, quality of seed, and storage condition (Torres Sánchez et al., 2021). The PUFAs aid in managing the cardiometabolic syndrome, specifically the prevention of coronary heart disease, hypertension, and exhibits hypocholesterolemic effect when used as a dietary supplement (Follegatti-Romero et al., 2009). Furthermore, topical products containing Inca Inchi oil have promising anti-inflammatory, anti-aging, antibacterial, and moisturizing properties (Gonzalez-Aspajo et al., 2015). Additionally, phytosterols, phenolic compounds, tocopherols, and carotenoids also improve health (Lagarda et al., 2006; Moreau et al., 2002). With the growing recognition of Inca Inchi

in international markets in recent years, dietary products such as gourmet oil, protein powder, and encapsulated oil are available. Other seeds, such as roasted, salted, or candied, are also available (Kodahl & Sørensen, 2021).

The oil from the Inca Inchi seeds is generally obtained through various methods such as solvent extraction, supercritical carbon dioxide (CO_2) extraction, and screw press extraction (Follegatti-Romero et al., 2009; Sayyar et al., 2009). Among these, screw press extraction is preferred due to the absence of solvent in the oil product, simple equipment, convenience of use, inexpensive investment, and low running costs (Siregar et al., 2016). The screw-pressing method is gaining popularity as an alternative to solvent extraction.

Additionally, screw pressing also helps retain the beneficial components, such as antioxidants, hence improving the quality of the oil (Lutterodt et al., 2011; Maier et al., 2009). In addition to the oil extraction process, pre-treatment is important in producing high oil yield with great reproducibility (Mwaurah et al., 2020). The applied pre-treatment will ease and speed up the process of releasing oil as the chemical bonds of the seed have been broken down (Mwaurah, 2020). It is important to ensure that the cellular structure of the plant seed has been loosened up before the extraction process.

This study aimed to extract the Inca Inchi oil using thermal pre-treatment methods such as microwaves and hot air ovens. Although oil extraction has been done using thermal pre-treatment methods on various oil seeds, very limited work has been done on Inca Inchi oil and its effect on the properties of the oil. Hence, in this work, the oil seeds were thermally pre-treated using a microwave with different times of exposure (1, 2, 4, and 6 min) and a hot air oven with three different temperatures (60, 80, and 120ºC) to evaluate the effects on oil yield, fatty acid profile, antioxidant activity, and physicochemical properties. Subsequently, the results from the study will be used to produce various geriatric functional foods.

MATERIALS AND METHODS

Materials

Inca Inchi seeds were purchased from Myanmar (Wusang Group Sdn. Bhd., Malaysia). The chemicals and solvents used in the study were high-performance liquid chromatography (HPLC) or analytical grades and purchased from Fisher Scientific Sdn. Bhd. (Malaysia). Standards such as gallic acid and quercetin were purchased from Sigma-Aldrich (USA). Folins-Ciocâlteu phenol reagent, 2,2-diphenyl-1-1picrylhydrazyl (DPPH), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) salt were purchased from Merck (Germany).

Inca Inchi Seeds Preparation

Inca Inchi seeds from the supplier were cleaned to remove the foreign particles. Then, the seeds were dried in an oven (Binder, Germany) at 50–60ºC for a day.

After drying, the seeds were cooled and packaged in re-sealable storage bags and stored in a dehumidifier room until further processing.

Pre-treatment Techniques

Pre-treatment of oilseeds is essential in increasing the quality and yield of oil. The major pre-treatment step goes to preheating to reduce the moisture content and weaken the cell structure of the oilseed. Applying heat prior to extraction enables the oil to flow more easily. Besides this, crushing plays an important role by destroying the cell wall of the oil-bearing material, which further aids the process of releasing the oil (Saurabh et al., 2011). Conventionally, hot air oven is commonly applied as pre-treatment before being replaced with modern techniques such as microwave radiation, ultrasonication, supercritical fluid, and enzyme treatment due to their excellent benefits (Danlami et al., 2014).

In this study, the seeds were pre-treated using a microwave and hot air oven prior to oil extraction to improve the oil yield. A control was performed without any pre-treatment.

Microwave Pre-treatment. The seeds were placed in the Pyrex Petri dish and inside the oven (Panasonic 800 W model, Japan). The sample rotates inside the oven during the process. This configuration allows the samples to pass through the electromagnetic field pattern, allowing unified energy absorption within the seeds. The samples were pre-treated for 1, 2, 4, and 6 min. The selection of time was based on a preliminary study in which the exposure to microwave pre-treatment beyond 6 min had burnt the Inca Inchi seeds (Azadmard-Damirchi et al., 2010). After pre-treatment, the seeds were loaded into an oil expeller for extraction.

Hot Air Oven Pre-treatment. Thermal treatment using hot air ovens inactivates antinutrients and improves the antioxidant activity of seed samples (Bueno-Borges et al., 2018). Dry roasting improves the general aroma of the seeds. In this method, 200 g of Inca Inchi seeds were roasted in an oven at 60 (T60), 80 (T80), and 120ºC (T120) for 15 min, respectively. After pre-treatment, the seeds were loaded into an oil expeller for extraction.

Oil Extraction

Oil extraction from Inca Inchi seeds was done using lab scale oil expeller (model: Household Oil Presser, Best Day, China). Two hundred (200) g of Inca Inchi seeds were manually dehulled for the study. Prior to the extraction of oil, the oil presser was pre-heated for a few minutes. After pre-heating, 200 g of dehulled seeds were loaded slowly into the hopper to obtain a steady flow of oil extraction. The crude oil obtained was kept in the chiller for the particles to sediment. The sedimented particles were removed by filtration to obtain the actual oil yield. The oil meal from the extraction was vacuum sealed and stored at room temperature to determine their proximate composition. The oil extraction process has been summarized in Figure 1.

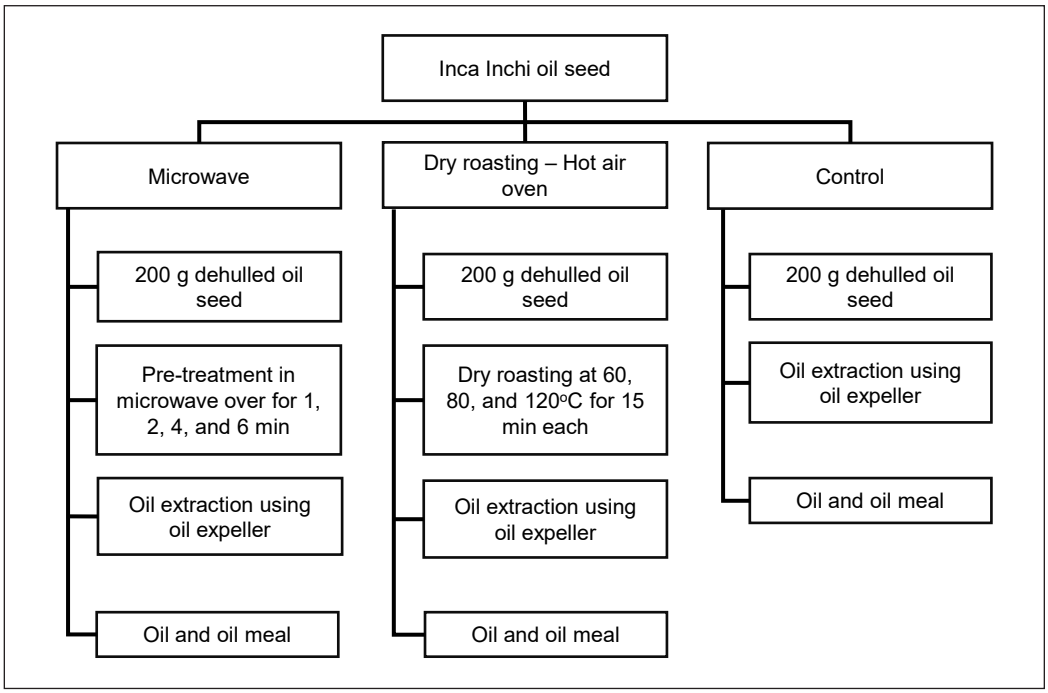

Figure 1. Summarized methods for the extraction of Inca Inchi oil

Proximate Analysis

The proximate analysis of the Inca Inchi oil seed and oil meal was analyzed in terms of moisture, ash, crude protein, crude fat, and crude fiber using the standard method of the Association of Official Analytical Chemists (AOAC) (Horwitz & Latimer, 2005). The protein content in the sample was analyzed by the micro Kjeldahl method by determining the total nitrogen in the sample (factor 6.25 was used to convert it to total protein). The moisture content in the sample was determined by drying the sample in a hot air oven at 105°C till constant weight was obtained. The ash content in the sample was analyzed by incinerating the sample in a muffle furnace at 550°C, while Soxhlet was used to analyze the crude fat. The crude fiber was analyzed by the acid-alkali digestion method. The difference in the above analysis calculated the carbohydrate content in the sample. All analyses were carried out in triplicate.

Thermal Profile Study

The melting profile of the oil samples was studied using differential scanning calorimetry (DSC). PerkinElmer DSC 8000 (USA) was used to determine the melting thermograms of the oil samples. Nitrogen gas was used to purge the system during the analysis. An empty and hermetically sealed aluminum pan was used as a reference. Seven (7.0) mg of sample was accurately weighed and hermetically sealed in an aluminum pan. The following temperature program was applied to the sample. The sample was initially heated to 80°C and held

for 5 min. The sample was then heated from 50 to 80°C at 5 min/s and held at 80°C for 10 min. Duplicate analysis was performed for each sample (Y.-Y. Lee et al., 2015).

Fatty Acid Composition

The fatty acid methyl esters (FAMEs) of the oil samples were derived by transesterification, based on O'Fallon et al. (2007). The analyses were done on Agilent Technologies (USA) gas chromatograph 7890A equipped with flame ionization detector (FID), a split/split less injector, and a capillary column (25 m × 0.32 mm × 0.25 µm, type BPX70, SGE™ Analytical Science, Australia). The injector and detector temperatures were set at 250 and 280°C. The temperature program for the analysis was as follows: Holding at 100°C for 0.5 min, increasing up to 180°C at the rate of 10°C/min, increasing from 180 to 220°C at the rate of 1.5°C/min, increasing from 220 to 250°C at the rate of 30°C/min and holding at 250°C for 5 min. Nitrogen was used as carrier gas. Triplicate analysis was done on each sample. Peaks were integrated using Agilent Chem Station software (version: B.04.01). The area under each fatty acid peak relative to the total area of all fatty acid peaks was used to quantify the fatty acids. Results are reported as a percentage of the total fatty acids.

Physicochemical Properties

The physicochemical properties of the oil studied were free fatty acid (FFA), peroxide value (PV), and iodine value (IV). The properties were analyzed using American Oil Chemist Society (AOCS) standards (Firestone, 2009). Free fatty acid (the method by Ca 5a-40), iodine value by cyclohexane-acetic acid method (the method by Cd 1d-92), and peroxide value using acetic acid-isooctane method (the method by Cd 8b-90), respectively. All the analyses were carried out in triplicate. FFA in oils was expressed as % oleic acid, while PV of the oil samples was expressed as meq (milli equivalent) O_2/kg of oil, and IV of the oil samples was expressed as grams of iodine absorbed by 100 g of sample (g I_2/100 g).

Antioxidant Study

Extraction of Bioactive Compounds. The oil's bioactive compounds, such as phenolics and flavonoids, were extracted using a solvent. A known amount of the oil was first dissolved in an equal proportion of *n*-hexane (Fisher Scientific Sdn. Bhd., Malaysia) and was vortexed vigorously. Then an equal volume of aqueous methanol (methanol: water, 80:20 v/v) (Fisher Scientific Sdn. Bhd., Malaysia) was added to the solution and vortexed vigorously. The methanolic phase was carefully collected to estimate the oil's total phenolic content and flavonoids. The analysis was conducted in triplicate (Muangrat et al., 2018).

Total Phenolic Content. The total phenolics in the oil extracts were analyzed using Folin's phenol reagent with slight modification. Twenty (20) µl of the sample extracts were diluted to 500 µl with distilled water. The solution was vigorously vortexed. One hundred fifty (150) µl of Folin's phenol

reagent (Merck, Germany) was added to the solution. The solution was incubated at room temperature for 10 min. Five hundred (500) µl of 7.5% (w/v) sodium carbonate solution (Fisher Scientific Sdn. Bhd., Malaysia) was added to this solution. The solution was incubated at room temperature in the dark for 60 min, and the absorbance was taken at 650 nm using a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, USA). Gallic acid (Sigma-Aldrich, USA) was used as the standard. The results were expressed as milligrams of gallic acid equivalents per gram of the oil sample (mg GAE/g oil sample) (Baba & Malik, 2015).

Estimation of Total Flavonoids. The total flavonoids in the oil extracts were analyzed using the method proposed by Djeridane et al. (2006) with slight modifications. This study mixed 0.5 ml of the oil extracts with 0.5 ml of 2% aluminum chloride methanolic solution (Fisher Scientific Sdn. Bhd., Malaysia). The samples were vigorously agitated and incubated at room temperature in the dark for 15 min. The absorbance was measured at 430 nm using a spectrophotometer (Cary UV-Vis, Agilent Technologies, USA). Methanol (Fisher Scientific Sdn. Bhd., Malaysia) was used as blank, and quercetin (Sigma-Aldrich, USA) was used as standard. The flavonoid content was expressed in mg quercetin equivalent (QE) /g oil extract (OE).

Radical Scavenging Activity. The radical scavenging activity of the oil sample was assessed by DPPH and ABTS scavenging assays. The method proposed by L. Liu et al. (2009) was followed with slight modifications to assess the oil's overall scavenging capacity by DPPH. The sample (20 µl) was taken in an Eppendorf tube and diluted to 1 ml using 95% ethanol (Fisher Scientific Sdn. Bhd., Malaysia). The sample was vigorously agitated. To this solution, 3 ml of 0.004% DPPH solution (4 mg of DPPH in ethanol, Merck, Germany) was added. The solution was vigorously agitated for a minute and incubated at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, USA). Ethanol (Fisher Scientific Sdn. Bhd., Malaysia) was used as a blank. The study was conducted in the dark as the DPPH chemical is light-sensitive. The % scavenging activity was calculated using Equation 1.

$$\text{Scavenging activity (\%)} = [A_{control} - A_{sample}/A_{control}] \times 100 \quad \text{(Equation 1)}$$

where, $A_{control}$ = Absorbance of control and A_{sample} = Absorbance of sample

The ABTS method is based on reducing the ABTS radical cation (Cheong et al., 2018; Marfil et al., 2011). In this assay, a stock solution consisting of 7 mM ABTS (Merck, Germany) and 2.45 mM potassium persulfate (Sigma-Aldrich, USA) were combined in the ratio of 1:1 and incubated at room temperature in the dark for 16 hr to create ABTS radical cation stock solution. The resulting solution was diluted with ethanol to adjust the absorbance to 0.700 ±

0.020 at 734 nm prior to sample analysis. To determine the radical scavenging activity of the sample, 100 µl of the oil sample was added to 2.0 ml of diluted ABTS solution and vortexed vigorously. The solution was incubated at room temperature in the dark for 3 min, and the absorbance was measured at 734 nm using a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, USA). Ethanol (Fisher Scientific Sdn. Bhd., Malaysia) was used as a blank. The percentage of scavenging activity was calculated using Equation 1.

Statistical Analysis

The experimental analysis data were presented as mean ± standard error (SE) for at least three analyses. Data were analyzed by one-way analysis of variance (ANOVA) to determine the significant difference, and Tukey pairwise comparisons were used to compare the significant difference among the thermal pre-treatment conditions. The statistical analysis was performed using Minitab version 17 (USA).

RESULTS AND DISCUSSION

Oil Extraction

The study results show that microwave and hot air oven thermal pre-treatment can improve oil yield. Among the thermal pre-treatment methods, microwave treatment for 4 min gave the highest oil yield of 43.39% compared to control (37.76%). The results of the study are shown in Table 1. The oil yield in the hot air oven and pre-treated method was between 41.44–42.68%, and in the microwave, the method was between 40.31–43.39%, indicating that pre-treating the oil seeds before oil extraction is necessary to increase the oil yield. Untreated Inca Inchi seeds had low oil yield as the intact cell wall significantly hindered oil extraction. Preliminary lab studies on microwave exposure duration revealed that Inca Inchi seeds thermally pre-treated longer than 4 min were slightly burnt and had reduced the oil yield. However, the Inca Inchi seeds were not affected in the hot air oven, so the oil yield increased with the roasting temperature.

Table 1
Effect of pre-treatment process on the yield of oil

Treatment	Sample weight (g)	Oil obtained (g)	Oil meal (g)	% yield of oil	% increase in oil yield
M1	200	80.63±0.09^{d}	90.90±0.63^{a}	40.31±0.05^{d}	6.7
M2	200	83.61±0.54^{c}	78.08±0.59^{f}	41.30±0.27^{c}	9.37
M4	200	86.77±0.59^{a}	81.49±0.58^{e}	43.39±0.29^{a}	14.9
M6	200	84.27±0.08^{bc}	77.11±0.47^{f}	42.14±0.04^{bc}	11.59
T60	200	74.88±0.54^{e}	82.80±0.57^{d}	41.44±0.27^{e}	9.74
T80	200	83.73±0.76^{bc}	87.92±0.07^{b}	41.87±0.38^{bc}	10.86
T120	200	85.10±0.43^{b}	85.39±0.17^{c}	42.68±0.22^{b}	13.0
Control	200	75.52±0.61^{e}	85.22±0.11^{c}	37.76±0.31^{e}	

Note. M1 = Microwave 1 min; M2= Microwave 2 min; M4 = Microwave 4 min; M6 = Microwave 6 min; T60 = Oven roasting 60ºC; T80 = Oven roasting at 80ºC; *T120* = Oven roasting at 120ºC; Different small letters indicate significant differences ($p < 0.05$)

The % increase in oil yield for microwave pre-treatment was between 6.7–14.9% for microwave and 9.74–13.0% for oven roasting. During heating, the microstructure of the seed changes due to water evaporation. It creates internal pressure and breaks the cell wall and the membrane, forming pores within. Generally, the oil droplets in the seed are in the form of an emulsion with protein. During roasting, the protein gets denatured, coagulates which causes the emulsion to break, and the oil droplets combine to form a bigger droplet. Moreover, the hot temperature reduces the surface tension and increases the fluidity. Thereby, the oil is easily released from the seed. Thus, the oil yield is better after thermal pre-treatment (Shahidi, 2005). The oil yield is better with microwaves as compared to oven roasting due to the efficient transfer of heat in the microwave. In the microwave, the energy is transmitted directly to the sample via molecular interaction with the electromagnetic field (Rękas et al., 2017).

Uquiche et al. (2008) conducted a study on Chilean hazel nuts seeds to determine the effect of pre-treatment on oil extraction using two different microwave power (400 and 600 W) for 3, 3.5, and 4 min. The highest oil yield of 45.3% was recorded in seeds exposed at 400 W for 4 min, followed by 43.5% at 600 W for 4 min, while control seeds recorded only 6.1%. Likewise, studies by Fathi-Achachlouei et al. (2019) on milk thistle seeds using microwave power (800 W) for 2 and 4 min revealed that the highest oil yield of 35.41% was obtained when the seeds were exposed to 4 min as compared to 32.33% when exposed to 2 min.

Studies on cold press extraction of oil from whole and dehulled seeds of Inca Inchi showed that the highest oil yield extraction (40.15%) was obtained in de-hulled seeds, as compared to whole seeds, which had only 27.20%, indicating that it is necessary to dehull the seeds prior to oil extraction (Gutiérrez et al., 2019).

Proximate Analysis

The proximate analysis of the Inca Inchi oil meal (with and without pre-treatment) and the oil seed were studied. The results are shown in Table 2.

Among the pre-treatments, the maximum and minimum moisture content obtained were in M1 (5.14%) and M6 (1.26%). The moisture content in the Inca Inchi oil seed was 3.58%, and a similar value (3.3%) was reported by (L. F. Gutiérrez et al., 2011). Moisture content is an important determinant of the shelf life of seeds. These values are within the safe range for storage and processing (0-13%) of Inca Inchi seeds without microbial deterioration of the triglyceride. Microbial growth can be prevented with low water activity (Alemu et al., 2022). The protein content of the Inca Inchi oil seed in this study was slightly lower (24.2%) than those reported by other studies (29.2 and 27%) (Bueno-Borges et al., 2018; Hamaker et al., 1992).

The protein content of the Inca Inchi oil seeds is similar to other oil seeds such as rape seeds (22%), sunflower seeds

Table 2
Proximate analysis of the Inca Inchi oil meal (with and without pre-treatment) and oil seed

Treatment	Moisture content (%)	Ash content (%)	Crude fat (%)	Crude protein (%)	Crude fiber (%)	Carbohydrate (%)
Microwave-oil meal						
M1	5.14±0.11^{a}	3.01±0.88^{a}	6.09±0.07^{d}	60.18±1.00^{ab}	11.47±0.20^{c}	14.11±1.96^{d}
M2	3.75±0.10^{b}	3.04±0.35^{a}	5.70±0.09^{d}	58.41±1.20^{abc}	12.07±0.40^{b}	17.03±1.11^{d}
M4	2.19±0.05^{e}	3.06±0.46^{a}	3.88±0.04^{f}	59.37±1.30^{abc}	10.87±0.15^{d}	20.63±1.85^{bc}
M6	1.26±0.08^{f}	3.08±0.02^{a}	4.21±0.14^{ef}	57.01±0.80^{c}	11.40±0.10^{c}	23.04±0.80^{b}
Hot air oven-oil meal						
T60	3.62±0.02^{b}	3.01±0.54^{a}	7.54±0.31^{c}	57.65±1.50^{bc}	12.70±0.10^{a}	15.48±0.71^{d}
T80	3.19±0.08^{c}	3.01±0.84^{a}	7.46±0.06^{c}	51.74±1.30^{d}	12.13±0.10^{b}	22.47±1.93^{b}
T120	2.75±0.03^{d}	3.00±0.49^{a}	4.70±0.06^{e}	60.97±0.90^{a}	11.13±0.30^{cd}	17.45±0.86^{cd}
Oilseed	3.58±0.01^{b}	2.49±0.14^{a}	40.95±0.44^{a}	24.20±0.70^{f}	12.47±0.20^{ab}	16.31±0.87^{d}
Control-oil meal	5.19±0.20^{a}	3.04±0.30^{a}	8.26±0.04^{b}	41.15±0.20^{e}	11.33±0.20^{cd}	31.03±0.18^{a}

Note. M1 = Microwave 1 min; M2= Microwave 2 min; M4 = Microwave 4 min; M6 = Microwave 6 min; T60 = Oven roasting 60ºC; T80 = Oven roasting at 80ºC; T120 = Oven roasting at 120ºC; Different small letters indicate significant differences ($p < 0.05$)

(18.72%), ground nut seeds (26.5%), and melon seeds (25.4%) (McKevith, 2005; Muhammad Anjum et al., 2012; Onyeike & Acheru, 2002). A plant product with more than 12% protein is regarded as an excellent source of proteins and is referred to as a protein-rich food (Pearson & Carr, 1976).

The crude fat content of the Inca Inchi oil seed in this study was 41%, and the value was within the range reported in earlier studies (35–60%) (Follegatti-Romero et al., 2009; Guillén et al., 2003). This variation in the oil content could be due to differences in geographical conditions and seed maturity. The fat content in the oil meal was between 3.88–8.26%. M4 had the lowest value, and oil meal (control) had the highest value.

Thermal Profile Study

The melting profile of Inca Inchi oil was studied using DSC. Figure 2 shows the DSC melting thermogram of the Inca Inchi oil obtained under different extraction conditions. At the given temperature program, when the oil was heated to 80°C, the melting curve showed a single prominent peak at about -20°C with a transition starting at -12°C and ending at -25°C. A small shoulder peak was visible at 25°C. This type of shoulder can be seen during the melting of more than one group of triglycerides with melting points that are too close to be distinguished by DSC. The melting of a triglyceride is determined by the extent of branching in the carbon chain, the degree of unsaturation of its fatty acids, and their stereo-specific arrangement along the glycerol molecules (Ferrari et al., 2007). Vegetable oils, in general, have a combination of triglycerides, and hence the melting occurs over a wide range of temperatures rather than at a single

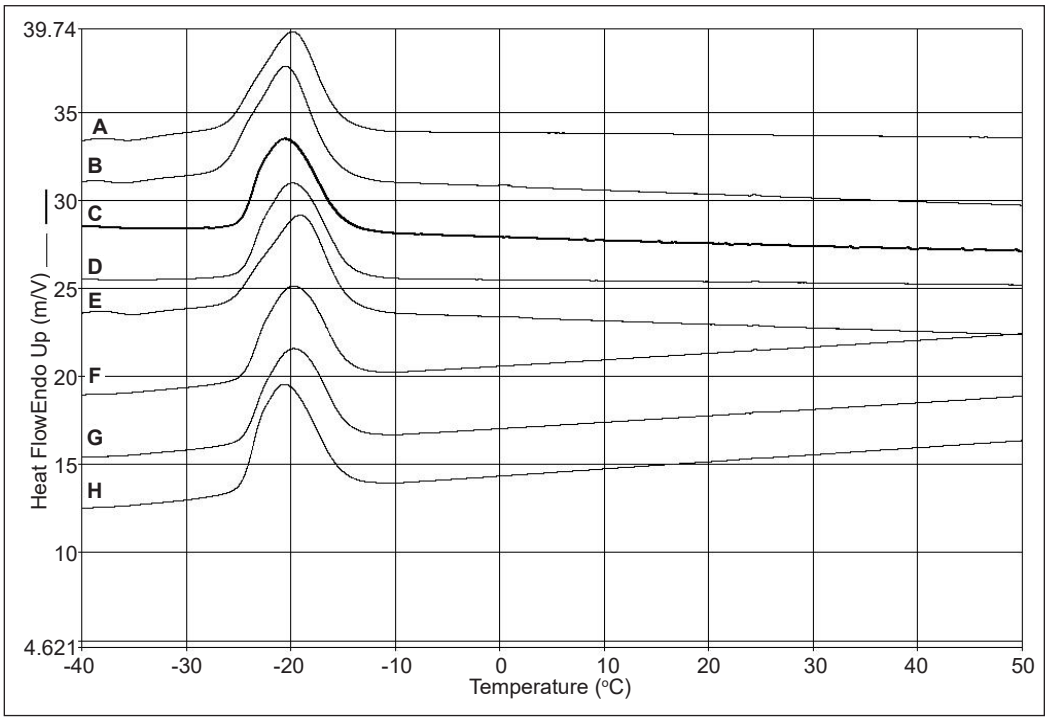

Figure 2. DSC melting curves of Inca Inchi oil samples with and without pre-treatment
Note. A = Control; B = M1 (microwave 1 min); C = M2 (microwave 2 min); D = M4 (microwave 4 min); E = M6 (microwave 6 min); F = T60 (oven roasting at 60ºC); G = T80 (oven roasting at 80ºC); H = T120 (oven roasting at 120ºC)

temperature (Tan & Che Man, 2002). Thus, due to the combination of triacylglycerols in most vegetable oils, the shoulder-like feature in a DSC curve is generally common in these oils. The melting curve of Inca Inchi oil also confirms that the oils rich in unsaturated fatty acids often melt at a lower temperature. The melting profile of Inca Inchi oil obtained through solvent extraction was at -18.5°C (Gutiérrez et al., 2011).

Fatty Acid Composition

Inca Inchi oil is an excellent source of the beneficial linolenic and linoleic acids that aid in preventing coronary heart disease, rheumatoid arthritis, diabetes, hypertension, and microbial infection (Alayón et al., 2018, 2019; da Silva Soares et al., 2019). Table 3 shows the fatty acid composition in Inca Inchi oil obtained through various pre-treatments. From the gas chromatography (GC) study, the major fatty acids in the oil were linolenic acid and linoleic acid, which were about 48 and 37%, respectively. The MUFA found in the oil was oleic acid, which was about 8%. The saturated fatty acids present in the oil were palmitic acid and stearic acid, which were about 4 and 2%, respectively. The oleic acid present in the oil helps to reduce the risk of coronary heart disease (Capurso et al., 2014; Lopez-Huertas, 2010). The PUFAs and MUFA

Table 3
Fatty acid composition of Inca Inchi oil with and without pre-treatment (%)

Treatment	ω-3	ω-6	ω-9	Palmitic acid	Stearic acid
M1	48.8±0.0^a	37.5±0.0^a	7.7±0.1^a	3.9±0.0	2.1±0.0^a
M2	48.7±0.0^a	37.6±0.0^a	7.7±0.0^a	4.0±0.0^a	2.0±0.0^a
M4	49.1±0.0^a	37.2±0.1^a	7.7±0.0^a	3.8±0.1^a	2.2±0.1^a
M6	48.5±0.0^a	37.6±0.0^a	7.8±0.1^a	4.0±0.0^a	2.1±0.0^a
T60	48.4±0.1^a	37.7±0.0^a	7.8±0.0^a	4.0±0.1^a	2.1±0.1^a
T80	48.7±0.0^a	37.6±0.0^a	7.7±0.0^a	3.8±0.0^a	2.2±0.0^a
T120	48.4±0.1	37.8±0.1^a	7.8±0.0^a	3.9±0.1^a	2.1±0.0^a
Control	48.3±0.0^a	37.3±0.0^a	7.7±0.0^a	4.3±0.1^a	2.4±0.0^a

Note. M1 = Microwave 1 min; M2= Microwave 2 min; M4 = Microwave 4 min; M6 = Microwave 6 min; T60 = Oven roasting 60ºC; T80 = Oven roasting at 80ºC; T120 = Oven roasting at 120ºC; Different small letters indicate significant differences ($p < 0.05$)

values in the Inca Inchi oil range between 77.5–84.4% and 8.0–13.2%, respectively.

However, the total SFA in the Inca Inchi oil is lower than other oil seeds such as sunflower, flaxseed, and canola (Follegatti-Romero et al., 2009; Maurer et al., 2012). Studies on the fatty acid composition of cold press extraction of oil from Inca Inchi seeds using whole seeds and dehulled seeds reveal that the fatty acid composition was not affected in both the samples, and there was no significant difference ($p > 0.05$) between the samples (Gutiérrez et al., 2019). Likewise, Gutiérrez et al. (2017) reported that γ-irradiation treatments of Inca Inchi shell at doses 1, 5, and 8 kGy did not affect the fatty acid composition of the oil. The omega 3 and omega 6 fatty acids were not affected at the high dose of 8 kGy, thereby maintaining the nutritional value of the oil.

Physicochemical Properties

The physicochemical properties of the Inca Inchi seed oil with and without pre-treatment are shown in Table 4. Peroxide value and free fatty acid are the most crucial parameters for assessing the oil quality. The hydrolysis of oils and fats results in the production of free fatty acids. Oils and fats are exposed to various conditions such as processing, storage heating, and frying, and due to this, the level of FFA varies with time, temperature, and moisture content. FFA are more susceptible to oxidation, which becomes rancid as they are less stable than neutral oil. Therefore, FFA is a crucial component associated with oils and fats' quality and commercial viability (Mahesar et al., 2014). To be acceptable for oral consumption, the free fatty acid in the oil must not be higher than 5% (Esuoso & Odetokun,1995). Results show that the % free fatty acid for Inca Inchi oil for pre-treated oil samples was lower (0.3–0.4%) than for the control (1.1%). A similar value (1.2%) was reported by Gutiérrez et al. (2011). The low free fatty acid in the pre-treated samples was due to moisture loss during roasting. The moisture content of the oil is one of the important factors that cause

Table 4
Physicochemical properties of Inca Inchi oil with and without pre-treatment

Treatment	Free fatty acid (%)	Peroxide value (meq O_2/kg oil)	Iodine value (g I_2/100 g oil)
Control	1.1±0.04^{a}	8.32±0.07^{a}	194.35±0.96^{a}
M1	0.4±0.03^{b}	3.82±0.02^{b}	192.42 ±0.26^{a}
M2	0.4±0.02^{b}	3.67±0.04^{cdc}	192.56±0.96 ^{a}
M4	0.3±0.01^{b}	3.54±0.03^{dc}	193.90±1.00 ^{a}
M6	0.3±0.03^{b}	3.50±0.29^{c}	191.30±1.12^{a}
T60	0.3±0.01^{b}	3.78±0.02^{bc}	192.66±0.34^{a}
T80	0.3±0.02^{c}	3.69±0.03^{cd}	192.55±0.80^{a}
T120	0.3±0.02^{b}	3.58±0.03^{dc}	191.48±1.58^{a}

Note. M1 = Microwave 1 min; M2= Microwave 2 min; M4 = Microwave 4 min; M6 = Microwave 6 min; T60 = Oven roasting 60ºC; T80 = Oven roasting at 80ºC; T120 = Oven roasting at 120ºC; Different small letters indicate significant differences ($p < 0.05$)

rancidity (Roger et al., 2010). Hence, Inca Inchi oil is an edible oil.

PV determines the amount of peroxides (primary oxidation products) in the oil. Peroxides are important intermediate products of oxidative reactions because they decompose to produce free radicals when exposed to transition metal irradiation and high temperatures. The organoleptic properties of the oil are also correlated with PV, which denotes the oil's freshness (Uquiche et al., 2008). PV greater than 10 meq O_2/kg oil is unacceptable (Shahidi, 2005). Results of the study exhibit that the PV for the oil samples was less than 10 meq O_2/kg. The PV of control is 8.32 meq O_2/kg, which is closer (7.36 meq O_2/kg) to commercial Inca Inchi oil (Vicente et al., 2015). Oils with peroxide levels of more than 10 meq O_2/kg are less stable and have a shorter shelf life. Lower the peroxide values better the resistance towards oxidation and lipolytic hydrolysis (Akanni et al., 2005). Studies by Fathi-Achachlouei et al. (2019) on the effect of microwave pre-treatment of milk thistle seeds reveal that the PV was low (2.09 meq O_2/kg) against control (5.11 meq O_2/kg).

However, studies by Mazaheri et al. (2019) on *Nigella sativa* seeds reveal that the PV increased with microwave and oven roasting compared to unroasted seeds. A similar result was seen in cottonseed oil due to the presence of reactive radicals that might be formed by exposure to microwaves (Farag et al., 1992). IV quantifies the average degree of unsaturation in oils and fats. The lower the IV, the lesser the number of unsaturated bonds. Thus, the oil is stable to rancidity (Haile et al., 2019).In this study, the IV was between 191–194 g I_2/100 g oil, and there was no significant difference ($p > 0.05$) between the extraction conditions. A similar value (193.1 g I_2/100 g) on non-treated Inca Inchi oil was reported by (Gutiérrez et al., 2011). The slight reduction in the iodine value in the pre-treated samples could be due to the breakage of long-chain

fatty acids, oxidation, or polymerization (Anjum et al., 2006). Generally, the iodine value decreases with roasting.

A study was done to determine the effect of IV on sunflowers seeds using microwave power (500 W) at 5, 10, and 15 min prior to solvent extraction. The study exhibits the highest IV of 140 g I_2/100 g of oil for control against 113.5 g I_2/100 g at 15 min. Likewise, studies by Fathi-Achachlouei et al. (2019) on milk thistle seeds using microwave power (800 W) for 2 and 4 min showed that pre-treatment by microwave had a significant effect on the IV. Studies on oven roasting (6, 9, 10, and 12 min) at 210°C on red pepper seed oils did not affect the iodine value (Jung et al.,1999). A similar result was reported by (Arab et al., 2022) on oven-roasted white sesame seeds.

Antioxidant Study

An antioxidant is a substance that can delay or block the oxidation of lipids or other molecules by reducing the beginning or propagation of oxidative chain reactions, thereby preventing or repairing damage caused by oxygen to the body's cells. Various functions of antioxidants are free radical scavenging, reducing activity, quenching singlet oxygen, and potential complexing of pro-oxidant metals (Tachakittirungrod et al., 2007). The presence of naturally existing or newly formed antioxidant compounds during the seed pre-treatment process is represented by the antioxidant activity of seed oil. Enhancing the oil's antioxidant properties through pre-treatment is important in food, cosmetic and pharmaceutical applications. Antioxidant compounds such as flavonoids, tannins, and phenolics are present in various parts of the plant, such as seed and oil leaves (Jeong et al., 2004). Bioactive compounds such as flavonoids and phenolic acids have health-promoting properties, especially for oxidative-stress-related diseases such as cancer, diabetes, neurodegenerative, and cardiovascular (Gutfinger, 1981; Li et al., 2014).

The antioxidant activities of the Inca Inchi oil were studied in terms of DPPH, ABTS, flavonoids, and total phenolic content. The results are presented in Table 5.

Total Phenolic Content and Flavonoid Content. Phenolic compounds are important plant constituents with antioxidant activity due to their redox properties. The phenolic content is analyzed using Folin's reagent. Folin's reagent is a mixture of phosphomolybdic acid and phosphotungstic acid. Under alkaline conditions (due to the presence of sodium carbonate) during the oxidation process, these acids are reduced to blue oxides of molybdene and tungstene, respectively. The intensity of the blue color represents the concentration of phenolic compounds present, which is measured using a spectrophotometer (Conforti et al., 2006). In this study, the phenolic content was (6.3-6.4 mg GAE/g oil), and the value reported was similar to that reported (6.2 mg GAE/g of oil extract) by (Q. Liu et al., 2014). In another study, the seeds were subjected to three different roasting conditions (lightly roasted, medium roasted, and highly roasted), and the phenolic content

Table 5
Antioxidant activities of Inca Inchi oil with and without pre-treatment

Treatment	Total phenolic content (mg GAE/g oil extract)	Total flavonoids (mg QE/g oil extract)	DPPH (%) scavenging activity	% increase in activity	ABTS (%) scavenging activity	% increase in activity
M1	6.4±0.0^{a}	0.6±0.01^{a}	71.23±0.1^{b}	3.2	90.49±0.48^{a}	11.71
M2	6.4±0.0^{a}	0.6±0.01^{a}	72.45±0.0^{ab}	4.96	90.75±0.23^{a}	12.03
M4	6.4±0.1^{a}	0.7±0.0^{a}	74.43±0.1^{a}	7.83	91.55±0.48^{a}	13.02
M6	6.3±0.0^{a}	0.6±0.1^{a}	73.44±0.0^{ab}	6.40	90.44±0.18^{a}	11.65
T60	6.4±0.0^{a}	0.6±0.0^{a}	73.44±0.0^{ab}	6.40	90.44±0.17^{a}	11.65
T80	6.3±0.01^{a}	0.6±0.0^{a}	73.46±0.1^{ab}	6.43	90.46±0.12^{a}	11.67
T120	6.3±0.0^{a}	0.7±0.0^{a}	73.43±0.0^{b}	6.38	90.54±0.11^{a}	11.77
Control	6.4±0.0^{a}	0.6±0.01^{a}	69.02±0.2^{c}		81.00±1.00^{b}	

Note. M1 = Microwave 1 min; M2= Microwave 2 min; M4 = Microwave 4 min; M6 = Microwave 6 min; T60 = Oven roasting 60ºC; T80 = Oven roasting at 80ºC; T120 = Oven roasting at 120ºC; Different small letters indicate significant differences ($p < 0.05$)

was compared to unroasted seeds. The study shows that the lowest phenolic content was in unroasted seeds (2.32 mg GAE/g of oil extract). However, it was noted that with an increase in roasting condition, there was an increase in phenolic content (3.72, 9.40, and 12.32 mg GAE/g of oil), respectively (Cisneros et al., 2014).

However, this concentration is lower than that reported by other commercial oils such as flax seed oil (39.16 mg GAE/g of oil extract), perillartine (20.38 mg GAE/g of oil extract), rice bran oil (19.59 mg GAE/g of oil extract), grape seed oil (15.56 mg GAE/g of oil extract), Inca Inchi oil (13.29 mg GAE/g of oil extract), respectively (Xuan et al., 2018). Commercial Inca Inchi oil in Japan had phenolic content of 13.29 mg GAE/g of oil extract). However, the value is still lower than the above commercial oils. These differences in the values of total phenolic content can be attributed to the processing condition or the environmental conditions where the oil seed was cultivated.

From the study, the flavonoid content in the oil extract was low. The flavonoid content in the oil extract is between 0.6–0.7 mg QE/g oil extract, and there was no significant difference ($p > 0.05$) among the processing condition. Incidentally, the flavonoid content reported by commercial Inca Inchi oil is 0.34 mg QE/g oil extract (Xuan et al., 2018). Thus, the oil had low phenolic and flavonoid content in our study, irrespective of the processing condition.

Radical Scavenging Activity. The radical scavenging activity of Inca Inchi seed oil with and without pre-treatment was investigated using DPPH and ABTS. DPPH indicates an antioxidant's ability to donate electrons throughout an assay since it is a persistent free radical (purple color) that transforms into a non-radical form (yellow color) by removing a single electron. Low absorbance at 530 nm measures the

reduction in the color transition from purple to yellow (Brand-Williams et al., 1995). In this study, the radical scavenging activity was 71–74% for the pre-treated oil, and the control had a scavenging activity of 69%. The percentage increase in DPPH scavenging activity was between 3.2 to 7.83. The higher the antioxidant activity, the higher the stability towards oxidation. Moreover, the slight reduction within the microwave pre-treatment conditions could be because antioxidants deteriorate at higher temperatures.

The ABTS method also studied the free radical scavenging activity of the oil. The percentage increase in scavenging activity was between 11 to 13. The radical scavenging activity for Inca Inchi oil was higher in the ABTS method when compared to the DPPH method.

Most plant compounds appear to have higher antioxidant activity against ABTS radicals than against DPPH radicals. It is due to the ABTS assay's increased sensitivity in detecting antioxidant activity, which causes the kinetic reaction to be faster, resulting in higher antioxidant activity (Floegel et al., 2011; K. J. Lee et al., 2015). The scavenging activity positively correlates to pre-thermal conditions (Cisneros et al., 2014).

In a nutshell, the variation between the DPPH and ABTS studies can be attributed to differences in the mechanism of action for each method. Since an extract is made up of several molecules with varied biological properties, its synergism could result in strong antioxidant activity; hence antioxidant activities cannot be attributed to only one or two components (Viuda-Martos et al., 2010).

CONCLUSION

Common thermal pre-treatment methods such as oven roasting, microwave, infrared radiation, boiling, and steaming impact oil yield and, to a considerable extent, the concentration and types of extracted minor lipid components in oil. In this work, microwave, and oven roasting of the Inca Inchi oil seed prior to oil extraction improved oil yield (40.31–43.39%). Among the pre-treatment conditions, M4 (microwave 4 min) had the highest oil yield (43.39%), and the pre-treated oil samples had good radical scavenging activity (about 70 and 90%) as studied through DPPH and ABTS assays. The thermal pre-treatment condition did not affect the fatty acid composition. The quality of the oil in terms of free fatty acid and peroxide values was lower in pre-treated oil, which indicates that pre-treating helps to improve the oil quality.

Further studies can be done to determine the effect of microwave and oven roasting on the anti-nutritional factors, as the Inca Inchi seeds are edible. Thus, to summarize, microwave and oven roasting are suitable methods to produce Inca Inchi oil since there are no undesirable changes in the fatty acid composition or the quality of the oil.

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CONFLICT OF 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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Optimization of Process Conditions for the Production of High-yield and High-quality Edible Bird's Nest (EBN) Hydrolysate

Bee-Hui Yeo[1], Shew-Fung Wong[3], Chin-Ping Tan[4], Yaya Rukayadi[1] and Oi-Ming Lai[1,2]*

[1]Natural Medicines and Products Research Laboratory (NaturMeds), Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Malaysia
[2]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Malaysia
[3]School of Medicine, International Medical University, 57000 Kuala Lumpur, Malaysia
[4]International Joint Laboratory on Plant Oils Processing and Safety (POPS) JNU-UPM, Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 Serdang, Malaysia

ABSTRACT

Edible bird's nest (EBN) hydrolysate is widely used in EBN downstream products. This study aimed to optimize the process conditions (combination of heat treatment and enzymatic hydrolysis) to produce high-yield and high-quality EBN hydrolysate. The effects of four factors in the process were studied by response surface methodology. The experimental factors are EBN temperature during double boiling (DB), DB duration, enzymatic hydrolysis duration, and the ratio of EBN to water. The recovery (yield) and quality (sialic acid [SA], 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid [ABTS], and 2,2-diphenyl-1-picrylhydrazyl [DPPH]) of the final product were used as response variables. The Pearson correlation coefficient showed that: EBN temperature during DB affected product recovery ($p < 0.01$) and ABTS ($p < 0.01$), DB Duration affected DPPH ($p < 0.01$), and the ratio of EBN to water affected product recovery ($p < 0.01$). The duration of enzymatic hydrolysis was not significantly correlated with any of the responses and least significant factors in the model. Two optimal conditions for the processes obtained from this study were yield (product recovery) and quality. This study also showed that EBN hydrolysate produced from EBN by-products could be used as a nutraceutical because of the antioxidant activity and high SA content.

Keywords: Edible bird's nest, enzymatic hydrolysis, heat treatment, sialic acid

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E-mail addresses:
y_beehui@yahoo.com (Bee-Hui Yeo)
shewfung_wong@imu.edu.my (Shew-Fung Wong)
tancp@upm.edu.my (Chin-Ping Tan)
yaya_rukayadi@upm.edu.my (Yaya Rukayadi)
omlai@upm.edu.my (Oi-Ming Lai)
*Corresponding author

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INTRODUCTION

For centuries, edible bird's nest (EBN) has been a tonic recognized by the Chinese community. The efficacy of EBN is documented in Traditional Chinese Medicine (TCM) records. Over the past 10 years, EBN has been known for its nutritional and medicinal properties, and its beneficial properties have also been confirmed by modern scientific research. EBN contains carbohydrates, protein, glycoprotein, moisture, fat, and ash. Compositional analysis of the purified EBN glycoprotein from a previous study showed that the EBN glycoprotein contained approximately 14% SA, 63% protein, and 21% total saccharide (Xu et al., 2019). SA is the signature component of EBN. The basic molecular structure of SA is an acidic amino sugar with a pyranose structure containing nine carbon atoms; it is a group of derivatives of neuraminic acid. EBN was reported to have the highest SA content in the natural world (Dai et al., 2022). Consumers always refer to the presence and percentage of SA to determine the purity of EBN. SA accounts for about 10% of EBN (Dai et al., 2021).

Before consumption, EBN must undergo a series of treatments. Different EBN products require different treatments and, therefore, different processing techniques to remove harmful substances, retain nutrients, control content, and improve taste. Processing technology is divided into three categories according to technological progress: primary processing, deep processing, and biotechnology processing. Primary processing (raw material: raw uncleaned (RUC) EBN, product: raw cleaned (RC) EBN) is a necessary process for most EBN products. RUC EBN sorting, primary washing, softening, picking, molding, drying, quality control, sterilization, packaging, and other primary processing processes. Primary processing involves laborious cleaning procedures resulting in high processing costs (Ng et al., 2020). Picking is the most tedious step, which determines the cleanliness of the EBN and the reliance on labor, so product cleanliness and recovery rates from RUC EBN to RC EBN are inconsistent. The determinants of the price of EBN products are the shape, cleanliness, and color of EBN products (Dai et al., 2021). Deep processing has opened more markets for EBN products by expanding a variety of ready-to-eat products while reducing prices. The product lineup of ready-to-eat options comprises various items such as candy, jelly, oral liquid, beverages, effervescent tablets, and nano EBN particles (Dai et al., 2021; Fan et al., 2020; Li et al., 2020; Yao, 2017). Biotechnology processing mainly obtains specific nutrients in EBN through extraction, enzymatic hydrolysis, separation, and other methods.

In general, the use of EBN in downstream products (e.g., nutraceutical and skin care products) involves only the extract and not EBN as a whole material (RC EBN) due to limitations of certain physical and chemical properties such as insolubility. The enzymatic hydrolysis technology product (EBN hydrolysate) has broad application prospects and is widely

used in EBN downstream products. After DB (heat treatment), the EBN enzymes were used to break down EBN sialylated mucin (SiaMuc) glycoproteins into simpler SiaMuc glycopeptides and free peptides. This enzymatic process improves EBN solubility, digestibility, and bioavailability (Amiza et al., 2019; Yan et al., 2021). Enzymatic hydrolysis is an alternative to the EBN cleaning process, using enzymes to improve cleaning efficiency and EBN product value (Noor et al., 2018).

The benefits of EBN extract have also been confirmed by scientific research, uncovering its nutritional value and pharmacological activity.

EBN extracts can be divided into three groups: (1) aqueous/water extract, (2) pancreatic digest extract, and (3) enzyme hydrolysis extract. The pharmacological activities have been shown with EBN water extract, which include eye care effect (Abidin et al., 2011), chondro-protective agent for human chondrocytes (Chua et al., 2013), anti-aging, anti-inflammatory, and wound-healing activities of edible bird's nest in human skin keratinocytes and fibroblasts (Hwang et al., 2020), reno-protective (Lim et al., 2021), and enhances fertility and embryo implantation rate (Albishtue et al., 2019). Previous studies have shown that using pancreatic enzymes, the pharmacological activities of EBN extract include enhancement of bone strength (Matsukawa et al., 2011), improving learning ability and memory (Careena et al., 2018), neuroprotection in Alzheimer's or Parkinson's Disease (PD) (Yew et al., 2018), and health-enhancing and antiviral activities against influenza A virus (Haghani et al., 2016).

Previous studies on EBN extract (using enzymes alcalase/papain/neutrase) and EBN hydrolysate have shown its nutritional value and pharmacological activity, which include increased angiotensin I-converting enzyme (ACE) inhibitor (Nurfatin et al., 2016) and antioxidant (Babji et al., 2018; Cao et al., 2022). EBN hydrolysate also showed higher free radical scavenging activities (antioxidant) and SA content (Cao et al., 2022; Chong et al., 2022; Yan et al., 2021, 2022).

Aqueous extracts are EBN extracts obtained by heating/boiling raw materials (RUC EBN/RC EBN/EBN by-products). Previous studies reported that protein solubility, degree of hydrolysis (DH), antioxidant activity, SA, and peptide content of EBN were positively correlated with heating time and temperature (Dai et al., 2022; Hun et al., 2015; Nasir et al., 2021; Ramachandran et al., 2017). Therefore, heating time and temperature will affect the extraction efficiency of EBN. Enzymatic hydrolysis (without heat treatment as pre-treatment) process to obtain EBN hydrolysate was reported to have: (1) influence of five factors on SA EBN extraction: pH > enzyme dosage > enzymatic hydrolysis temperature > ratio of liquid to material > enzymatic hydrolysis duration (Dai et al., 2022), and (2) influence of 4 factors on DH of EBN: pH > enzymatic hydrolysis temperature > enzymatic hydrolysis duration > enzyme concentration

(Bang et al., 2017). Factors affecting the efficacy of enzymatic hydrolysis with heat treatment as pre-treatment (double boiled at 90/100°C, 30 min) are also similar to the enzymatic hydrolysis without heat treatment, which includes enzymatic time, temperature, and enzyme dosage (Bang et al., 2017; Cao et al., 2022; Yan et al., 2022).

Optimization implies enhancing the functioning of a process, a system, or a product to get the most out of it. Optimization has become common in analytical chemistry to discover the conditions under which a procedure that produces the best response is applied (Bezerra et al., 2008). The response surface method (RSM) is an effective tool for building and parameterizing optimization models (Brightman, 1978). EBN proteolysis can be optimized to meet certain targets, such as obtaining high DH, high bioactivity, and desirable properties by using RSM (Amin et al., 2019). EBN produces a certain amount of by-products of EBN in the cleaning process, which is hard to use effectively, resulting in a certain amount of waste after primary processing (Babji et al., 2018). Adding waste or by-products of EBN to production, in other words, reduces the overall recovery of the product and ultimately increases the cost of the product. A few proteolytic enzymes that have been used in several previous studies include alcalase, pancreatin, protamex, proteases, and papain. Bromelain has been less studied, but it is also an efficient protein digesting enzyme for protein in milk and collagen (Nanda et al., 2020) and stable over a broad pH range (pH 4-8) (Ee et al., 2019).

This study aimed to optimize the process conditions (combination of heat treatment and enzymatic hydrolysis) to produce high-yield and high-quality edible bird's nest (EBN) hydrolysate. Previous studies only used heat treatment as their pretreatment for a limited period (30 min). This study includes optimizing heat treatment and enzymatic hydrolysis conditions. Bromelain's stability within a broad pH range was an advantage if the enzymatic process was applied in an industrial setting. Thus, in this study, bromelain was used in enzymatic hydrolysis process. Four factors were investigated in this study: (1) temperature of EBN during DB, (2) duration of DB, (3) duration of enzymatic hydrolysis, and (4) ratio of EBN to water. Product cost is very important in the EBN processing industry, so the raw materials used in this study are EBN by-products. One of the goals is to produce high yields of EBN hydrolysate. In addition to high yields, the quality of the product is also important. SA and antioxidant activity are important components that represent the quality of EBN products. RSM was used in this study to obtain the optimal conditions to produce high yield (product recovery) and high quality (SA, DPPH, and ABTS) EBN hydrolysate.

MATERIALS AND METHODS

Materials

The raw material here refers to the EBN by-product, which was provided by Think Birdnest (Segamat, Malaysia) Sdn. Bhd. EBN by-products are fragments of EBN

with tiny feathers. In the primary processing quality control step, EBN fragments with tiny feathers attached were picked from RC EBN to increase the cleanliness of RC EBN before sterilization and packaging. This EBN fragment is an EBN by-product here.

Chemicals used in all laboratory analysis were analytical grade, which was purchased from Sigma-Aldrich (USA) (phenol, N-acetylneuraminic acid, resorcinol, and DPPH) and Thermo Fisher Scientific (USA) (ABTS, potassium persulfate, and methanol). Other reagents and solvents were of analytical grade.

The Process to Produce EBN Hydrolysate from the By-product of EBN

The EBN hydrolysate process involves heat treatment (here, DB was used) and enzymatic hydrolysis (bromelain was used as an enzyme). The process conditions were optimized computationally by the Box-Behnken design of the RSM. The software Design Expert 13.0.0 was used. The software generated 29 runs based on 4 influencing factors, and their ranges were provided in Table 1. The range for each factor was determined through preliminary studies. Influencing factors include (A) temperature of EBN during DB, (B) duration of DB, (C) duration of enzymatic hydrolysis, and (D) ratio of EBN to water. The final product recovery rate is the response of the RSM. Most previous studies were not based on the internal DB temperature and duration of EBN itself. Thus, in this study, the internal temperature of the EBN (not the external temperature) during DB was considered, while the duration of DB was calculated after the initial temperature reached the target temperature. The samples were soaked in different proportions of water for 10 min.

Table 1
Range for each factor

Factors	Lower range	Upper range
A: Temperature of EBN during DB (°C)	85	95
B: Duration of DB (min)	20	60
C: Duration of enzymatic hydrolysis (min)	30	120
D: Ratio of EBN to water	20	100

Note. DB = Double boiling; EBN = Edible bird's nest

The samples were double-cooked at different temperatures and durations and cooled to 48°C before enzymatic hydrolysis. Bromelain (2,400 GDU/g) was added to the samples at 0.8% by EBN dry weight. Enzymatic hydrolysis was performed for different durations (give the different durations) at 48°C. The hydrolysate is then filtered through a sieve (80 mesh) to remove larger size impurities and then

sieved through smaller size WYPALL® X70 Wipers (Kimberly-Clark Professional, USA) to remove tiny impurities. After enzymatic hydrolysis, the samples were double boiled at 80°C for 20 min to denature the bromelain. Subsequently, the liquid hydrolysate and retentate (on a sieve) were fan dried in an air-conditioned room (18-20°C) for 16-24 hr. The air-dried hydrolysate was later ground to a powder and stored in a sealed plastic bag for future use.

Product and Waste Recovery

Raw material (EBN by-product) was weighed before DB. The product (hydrolysate) and the waste (retentate) were weighed after drying. The product recovery and waste recovery were calculated by using the equations below:

$$\text{Product recovery (\%)} = (\text{Product/Raw material}) * 100\% \quad (1)$$

$$\text{Waste Recovery (\%)} = (\text{Waste/Raw material}) * 100\% \quad (2)$$

A total of 9 samples out of the 29 runs were selected for quality analyses. The nine samples were the first 3 with the highest product recovery, the middle recovery, and the last 3 with the lowest recovery.

Total Polysaccharide

The phenol-sulfuric acid method (Dubois et al., 1956; Yan et al., 2022) was used to assay the total polysaccharide content in the sample. One ml of sample (EBN weight: 2.0 mg/ml) was mixed with 0.50 ml of 5% phenol solution (w/w) (Sigma-Aldrich, USA). Then, 1.5 ml of concentrated sulfuric acid (H_2SO_4) (Merck Millipore, USA) was added to the mixture. The samples were gently shaken and incubated at room temperature for 10 min. Absorbance was measured at 490 nm (Shimadzu UV-VIS Spectrophotometer mini-1240, Japan). Glucose monohydrate was used as standard.

Total SA Content

The periodate-resorcinol assay (Jourdian et al., 1971; Yan et al., 2022) was used to analyze the total SA content in the sample. A 0.5 ml (2 mg/ml) sample was mixed with 0.5 ml resorcinol reagent in a test tube. The tube was covered with chilled marble, and the sample was incubated in boiling water for 15 min. After incubation, the samples were cooled (10 min) to room temperature. Then, 2.0 ml of 1-butanol (Merck Millipore, USA) was added to the sample. The sample was vortexed vigorously for at least 10 s to form a single-phase solution. The samples were then incubated in a 37°C water bath for 3 min to stabilize the color, and the absorbance was read after cooling to room temperature. An amount of 0.22 g resorcinol (Sigma-Aldrich, USA) was mixed with 10 ml of distilled water, 80 ml of concentrated hydrochloric acid (HCl) (Merck Millipore, USA), and 0.25 ml of 0.1 M copper sulfate ($CuSO_4$, Merck Millipore, USA) solution and then top up with distilled water to make 100 ml of resorcinol reagent. Absorbance was measured at 580 nm (Shimadzu UV-VIS Spectrophotometer mini-1240,

Japan). N-acetylneuraminic acid (analytical standard) (Sigma-Aldrich, USA) was standard.

Antioxidant Activity

DPPH Assay. One ml sample (2 mg/ml) was mixed with 14 ml DPPH reagent. The DPPH reagent (0.036 mM) was prepared by dissolving 14.07 mg of DDPH powder (Sigma-Aldrich, USA) in 1 L of methanol (Thermo Fisher Scientific, USA). In the control sample, distilled water was used instead of EBN. The mixture was incubated in the dark for 30 min. The sample/control was then filtered with a polytetrafluoroethylene (PTFE) syringe filter (0.45 µm) and measured at 517 nm (Shimadzu UV-VIS Spectrophotometer mini-1240, Japan). Distilled water was used as blank. The free radical scavenging activity (%) was calculated using the equation below:

[(DPPH control absorbance - DPPH sample absorbance)/ DPPH control absorbance] * 100% (3)

ABTS Assay. A 0.2 ml sample (2 mg/ml) was mixed with 1.8 ml of ABTS reagent. The ABTS reagent was prepared by mixing 7 mM ABTS (Thermo Fisher Scientific, USA) and 2.45 mM of potassium persulfate (Thermo Fisher Scientific, USA) with a ratio of 1:1. This stock ABTS reagent was incubated for 14–16 hr at room temperature in the dark. Then ABTS reagent with absorbance 0.7 ± 0.2 was done by diluting the stock ABTS reagent with methanol (Thermo Fisher Scientific, USA). In the control sample, distilled water was used instead of EBN. The mixture was incubated in the dark for 10 min. The sample/control was then filtered with a PTFE syringe filter (0.45 µm) and measured at 734 nm (Shimadzu UV-VIS Spectrophotometer mini-1240, Japan). Distilled water was used as blank. The free radical scavenging activity (%) was calculated using the equation below:

[(ABTS control absorbance - ABTS sample absorbance)/ABTS control absorbance] * 100% (4)

Model Validation

The optimized samples were prepared according to the section 'Process to Produce EBN hydrolysate from By-product of EBN'. Samples were collected after DB/heat treatment, before enzymatic hydrolysis, and after the enzymatic hydrolysis process was completed.

Statistical Analysis

The EBN quality data were presented as mean ± standard error (SE) for at least three analyses. The IBM SPSS Statistics 28.0.0.1 calculated the Pearson correlation coefficient and the statistical relationship between two continuous variables. The software Design Expert 13.0.0 was used to calculate the analysis of variance (ANOVA) for the model.

RESULTS AND DISCUSSION

Product and Waste Recovery

Table 2 shows the conditions (factors) and product and waste recovery results for the 29 runs. The product recovery was between 54.88 and 96.22%, the waste recovery was between 0.19 and 23.31%, and the total recovery (product recovery plus waste recovery) was between 64.26 and 99.03%. Most of the runs reported a total recovery of above 90%. The overall total recovery obtained was less than 100% and may be due to (1) liquid hydrolysate being absorbed by wipers (WYPALL® X70 Wipers) during the second filtration, and (2) the remaining samples were found stuck to the glass bottle, especially sample with a ratio of EBN to water is 20. It is supported by the overall recovery results, where the samples with a ratio of 20 had the lowest recovery among the 5 samples.

Table 2

The simulated design parameters by Design Expert 13.0.0 and product and waste recovery results

Run	Condition				Recovery (%)		
	A: Temperature of EBN during DB (°C)	B: Duration of DB (min)	C: Duration of enzymatic hydrolysis (min)	D: Ratio of EBN to water	Product	Waste	Total
1	90	40	75	60	79.96	10.71	90.67
2	90	40	120	100	91.82	2.40	94.21
3	90	40	75	60	82.60	8.20	90.80
4	95	40	120	60	96.06	1.57	97.64
5	95	60	75	60	94.08	0.19	94.27
6	95	40	75	20	87.70	0.39	88.09
7	85	40	120	60	81.51	7.95	89.46
8	95	20	75	60	80.48	9.16	89.64
9	95	40	30	60	83.13	5.36	88.49
10	85	40	75	100	84.24	14.79	99.03
11	90	40	75	60	85.86	7.16	93.01
12	90	20	30	60	81.51	9.54	91.05
13	85	20	75	60	60.36	23.31	83.67
14	90	20	75	100	82.09	13.78	95.87
15	90	60	75	100	90.87	0.19	91.06
16	85	40	30	60	74.55	14.51	89.07
17	90	60	120	60	86.96	4.55	91.50

Table 2 *(Continue)*

Run	Condition				Recovery (%)		
	A: Temperature of EBN during DB (°C)	B: Duration of DB (min)	C: Duration of enzymatic hydrolysis (min)	D: Ratio of EBN to water	Product	Waste	Total
18	90	60	75	20	73.37	7.10	80.47
19	90	40	120	20	63.24	3.95	67.19
20	90	40	30	20	54.88	9.38	64.26
21	90	20	120	60	89.48	1.98	91.47
22	85	60	75	60	82.63	9.98	92.61
23	95	40	75	100	96.22	1.99	98.21
24	90	20	75	20	55.29	11.18	66.47
25	90	40	75	60	80.08	11.72	91.80
26	90	40	30	100	86.03	8.98	95.01
27	90	60	30	60	77.09	15.34	92.43
28	85	40	75	20	62.55	10.59	73.14
29	90	40	75	60	83.50	13.52	97.02

Total Polysaccharide, Total SA, and Antioxidant Activity of Selected Samples

A total of 9 samples out of the 29 runs were selected for quality analyses. The 9 samples included the first 3 samples with the highest product recoveries (runs 23, 4, and 5), middle recovery rates (runs 9, 3, and 14), and the last 3 samples with the lowest recoveries (runs 13, 24, and 20). Four response tests were performed on EBN hydrolysates, including DPPH free radical scavenging activity, ABTS free radical scavenging activity, total SA content, and total polysaccharide content.

Table 3 shows the responses of the 9 EBN hydrolysate samples. DPPH free radical scavenging activity was between 5.97 and 18.15%. Sample H3 shows a significant ($p < 0.05$) higher DPPH value, while L3 shows a significant ($p < 0.05$) lower DPPH value. ABTS free radical scavenging activity was between 67.65 and 78.35%. Among the sample, samples H1 and H2 show a significant ($p < 0.05$) higher ABTS value, while L2 shows a significant ($p < 0.05$) lower ABTS value. The total SA content in the EBN hydrolysate was between 16.03 and 20.49%. Sample M1 shows ($p < 0.05$) lower SA significantly compared to other samples. Sample H1 had the highest SA but was not significantly different ($p > 0.05$) from the H2, H2, L1, L2, and L3 samples. Thus, sample M1 was not denoted as a significantly higher sample in Table 2. The total polysaccharide content in the EBN hydrolysate was between 8.44 and 14.29%.

Among the samples, samples H2 and L1 showed significantly ($p < 0.05$) higher total polysaccharide content, while H1, H3, and M2 showed significantly ($p < 0.05$) lower total polysaccharide content. A *t*-test was performed between the two samples (rows), and there was no significant difference between the samples ($p > 0.05$).

Table 3

The analyst results of total polysaccharide content, total SA content, and antioxidant activity of nine selected samples

Run	Sample ID	Product recovery (%)	DPPH free radical scavenging activity (%)	ABTS free radical scavenging activity (%)	Total SA content (%)	Total polysaccharide content (%)
23	H1	96.22	12.60 ± 0.39	78.35 ± 0.68[a]	20.49 ± 0.73	8.88 ± 0.17[b]
4	H2	96.06	10.26 ± 0.40	77.97 ± 0.26[a]	19.21 ± 0.77	14.07 ± 0.04[a]
5	H3	94.08	18.15 ± 0.66[a]	73.99 ± 0.50	19.28 ± 0.27	8.68 ± 0.08[b]
9	M1	83.13	10.49 ± 0.62	75.13 ± 0.69	16.03 ± 0.15[b]	10.55 ± 0.00
3	M2	82.60	10.39 ± 0.36	72.25 ± 0.46	18.01 ± 0.28	8.44 ± 0.01[b]
14	M3	82.09	7.72 ± 0.82	72.94 ± 0.83	16.68 ± 0.45	13.44 ± 0.11
13	L1	60.36	7.34 ± 0.48	70.00 ± 0.65	18.61 ± 0.87	14.29 ± 0.34[a]
24	L2	55.29	10.11 ± 0.20	67.65 ± 0.60[b]	19.27 ± 0.51	10.99 ± 0.03
20	L3	54.89	5.97 ± 0.30[b]	72.78 ± 0.69	19.69 ± 0.53	12.97 ± 0.04

Note. Superscript a = Significantly higher in the same column ($p < 0.05$); Superscript b = Significantly lower in the same column ($p < 0.05$)

The IBM SPSS Statistics 28.0.0.1 calculated the Pearson correlation coefficient (Table 4) and the statistical relationship between two continuous variables. The variables included the responses and factors. Some correlations were observed from Table 4: (1) product recovery was positively correlated with ABTS ($p < 0.05$) and negatively correlated with waste recovery ($p < 0.01$), (2) DPPH was negatively correlated with total polysaccharide and waste recovery ($p < 0.01$), (3) waste recovery was negatively correlated with product recovery and antioxidant activity ($p < 0.01$), (4) EBN temperature during DB was positively correlated with product recovery rate and ABTS ($p < 0.01$), and negatively correlated with waste recovery rate ($p < 0.05$), (5) DB duration was positively correlated with DPPH ($p < 0.01$) and negatively correlated with waste recovery ($p < 0.05$), (6) ratio of EBN to water was positively correlated with product recovery ($p < 0.01$), and (7) total SA and enzyme duration were not correlated with any other variables ($p > 0.05$).

Results from Box-Behnken Design of the Response Surface Methodology (RSM)

Table 5 shows the response surface regression model analysis of variance results for the linear model of product recovery. The model *F*-value of 17.12 suggests that it is a significant model. The probability of such a large *F*-value due to noise is only 0.01%. *P*-values less than 0.0500 reveals that the model terms are significant. In this study, A, B, C, and D are significant model terms. In other words, the effects of all factors on the product recovery were significant ($p < 0.05$). In accordance with the *F*-value, the order of impact of the four factors on product recovery was the ratio of EBN to water (D) > temperature of EBN during DB (A) > duration of DB (B) > duration of enzymatic hydrolysis (C). Based on the results of simple linear regression analysis, the function of product recovery (Y, %) with temperature of EBN during DB (°C, A), duration of DB (min, B), hydrolysis time (min, C), and ratio of EBN to water (D) was established. The formula was as follows:

$$Y = 80.19 + 7.56*A + 4.50*B + 4.43*C + 11.12*D \quad (5)$$

No significant linear model can be developed for another 3 responses (SA, DPPH, and ABTS).

Enzymatic hydrolysis duration shows no significant correlation with any response (Table 4) and is the least significant factor in the model (Table 5). Cao et al. (2022) used RSM to study the optimal enzymatic hydrolysis and reported that the sequence of the impact of three factors on DH was hydrolysis temperature > enzyme concentration > hydrolysis time. EBN is affected by the degree of heat treatment, which promotes the accessibility of enzymes to the cleavage site, and the denatured protein after heat treatment is more easily hydrolyzed (Amiza et al., 2019). Duration of enzymatic hydrolysis was the least significant factor in the model ($p < 0.05$). A possible reason could be that heat-treated denatured proteins are easily cleaved by enzymes when other factors are at their optimum, so the duration of enzymatic hydrolysis is not as important. The duration tested was between 30 and 120 min.

Figures 1, 2, and 3 are the optimal conditions the software gave after inputting the data for the responses. The criteria for Figure 1 show the optimal conditions (1 out of 100 solutions) selected by RSM for response product recovery only. This solution includes 29 run conditions, as shown in Table 2. The product recovery rate was set at 100 with a 95–100% range. The optimal condition was suggested as follows: (1) EBN temperature during DB = 93.6°C, (2) DB duration = 57.5 min, (3) Enzymatic hydrolysis time = 76.4 min, and (4) ratio of EBN to water = 1:96.6. The desirability of this solution is 1.000.

Figure 2 shows the optimal conditions selected by RSM for the 4 responses. In addition to product recovery, the hallmark beneficial parameters of EBN, total SA, and antioxidant activity (DPPH and ABTS) were included as responses. This solution included 9 selected run (3, 4, 5, 9, 13, 14,

Table 4

Pearson correlation coefficient between variables

	Product recovery (%)	DPPH free radical scavenging (%)	ABTS free radical scavenging (%)	Total SA (%)	Total polysaccharide (%)	Waste recovery (%)	Temperature of EBN during DB (°C)	Duration of DB (min)	Duration of enzymatic hydrolysis (min)	Ratio of EBN to water
Product recovery (%)	1	0.647	0.823**	-0.040	-0.395	-0.718*	0.767*	0.580	0.442	0.704*
DPPH free radical scavenging (%)	0.647	1	0.317	0.224	-0.711*	-.687*	0.654	0.720*	0.242	0.211
ABTS free radical scavenging (%)	0.823**	0.317	1	0.128	-0.141	-0.709*	0.773*	0.560	0.212	0.556
Total SA (%)	-0.040	0.224	0.128	1	-0.127	-0.252	0.079	0.246	0.299	-0.219
Total polysaccharide (%)	-0.395	-.711*	-0.141	-0.127	1	0.560	-0.461	-0.574	0.167	-0.122
Waste recovery (%)	-0.718*	-.687*	-.709*	-0.252	0.560	1	-.951**	-0.809**	-0.138	-0.116
Temperature of EBN during DB (°C)	0.767*	0.654	0.773*	0.079	-0.461	-0.951**	1	0.707*	0.098	0.250
Duration of DB (min)	0.580	0.720*	0.560	0.246	-0.574	-0.809**	0.707*	1	-0.069	0.000
Duration of enzymatic hydrolysis (min)	0.442	0.242	0.212	0.299	0.167	-0.138	0.098	-0.069	1	0.294
Ratio of EBN to water	0.704*	0.211	0.556	-0.219	-0.122	-0.116	0.250	0.000	0.294	1

Note. ** = Correlation is significant at the 0.01 level (2-tailed); * = Correlation is significant at the 0.05 level (2-tailed); Number of samples for each variable = 9

20, 23, and 24) conditions. The responses were set at (1) target 100% for product recovery and a range of 95–100%, and (2) maximized total SA and antioxidant activity. The suggested optimal conditions are: (1) EBN temperature during DB = 95.0°C, (2) DB duration = 60.0 min, (3) enzymatic hydrolysis time = 117.1 min, and (4) ratio of EBN to water = 72.9. The desirability of this solution is 0.798.

Some combinations of these factors have been made to obtain higher desirability and did not significantly affect the response. (1) The setting target factor for EBN temperature during DB was 95°C, and the range was 85–95°C (same as above), and (2) the ratio of EBN to water was targeted at 80, and the range was 20–100 (same as above). The response settings are the same as in Figure 2. Figure 3 shows the optimal conditions after factor and response settings. The suggested optimal conditions were: (1) EBN temperature during DB = 95.0°C, (2) DB duration = 60.0 min, (3) enzymatic digestion time = 97.3 min, and (4) EBN to water ratio = 79.9. The desirability of this setting (0.858) is higher than the above solution (Figure 2—0.798). The response for this setup is like the solution above, with a slight decrease in DPPH from 15.18 to 14.92%. Therefore, these conditions are recommended as the optimal ones to obtain EBN hydrolysate with high yield and quality.

Table 5

Regression model variance analysis results of a response surface for product recovery linear model

Source	Sum of squares	df	Mean square	*F*-value	*p*-value
Model	2648.46	4	662.11	17.12	< 0.0001**
A: EBN temp during DB	686.67	1	686.67	17.75	0.0003**
B: DB duration	243.54	1	243.54	6.30	0.0193 *
C: Enzymatic duration	235.59	1	235.59	6.09	0.0211 *
D: Ratio EBN to water	1482.66	1	1482.66	38.33	< 0.0001**
Residual	928.43	24	38.68		
Lack of fit	903.89	20	45.19	7.37	0.0328*
Pure error	24.54	4	6.13		
Cor total	3576.88	28			

Note. ** = Correlation is significant at the 0.01 level (2-tailed); * = Correlation is significant at the 0.05 level (2-tailed)

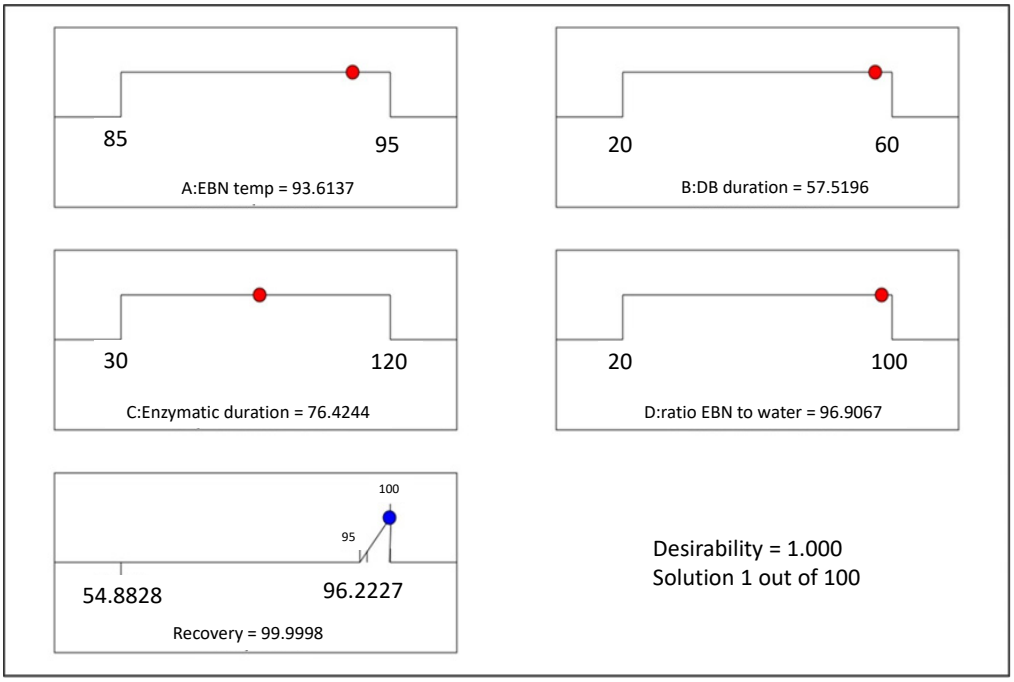

Figure 1. Optimization solution to get 100% product recovery

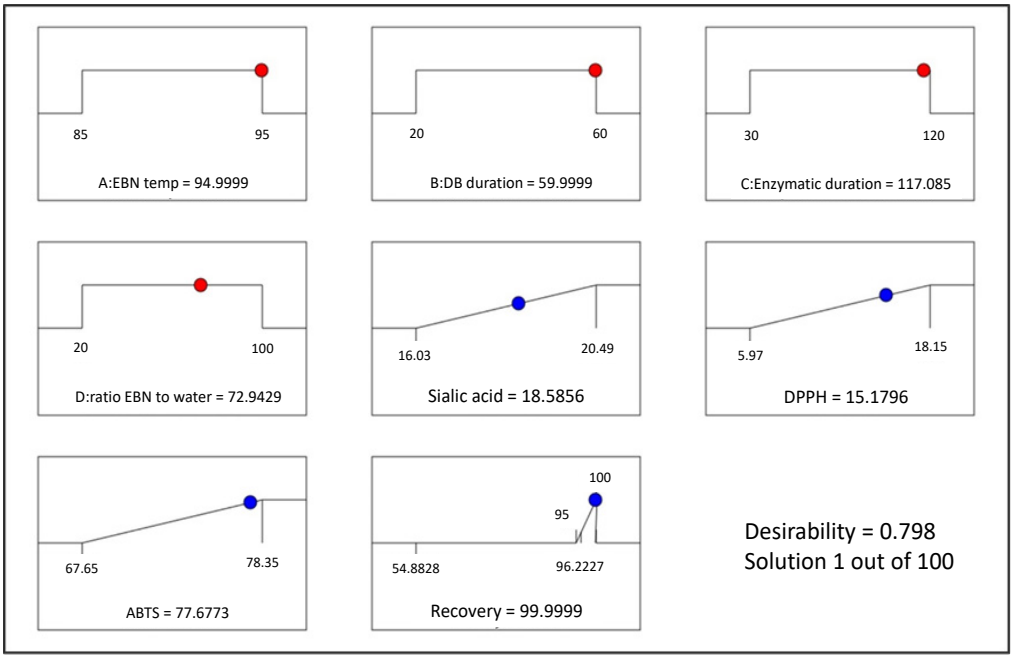

Figure 2. Optimization solution to get 100% product recovery and maximum total SA, DPPH, and ABTS

Note. SA = Sialic acid; DPPH =2,2-diphenyl-1-picrylhydrazyl; ABTS = 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid

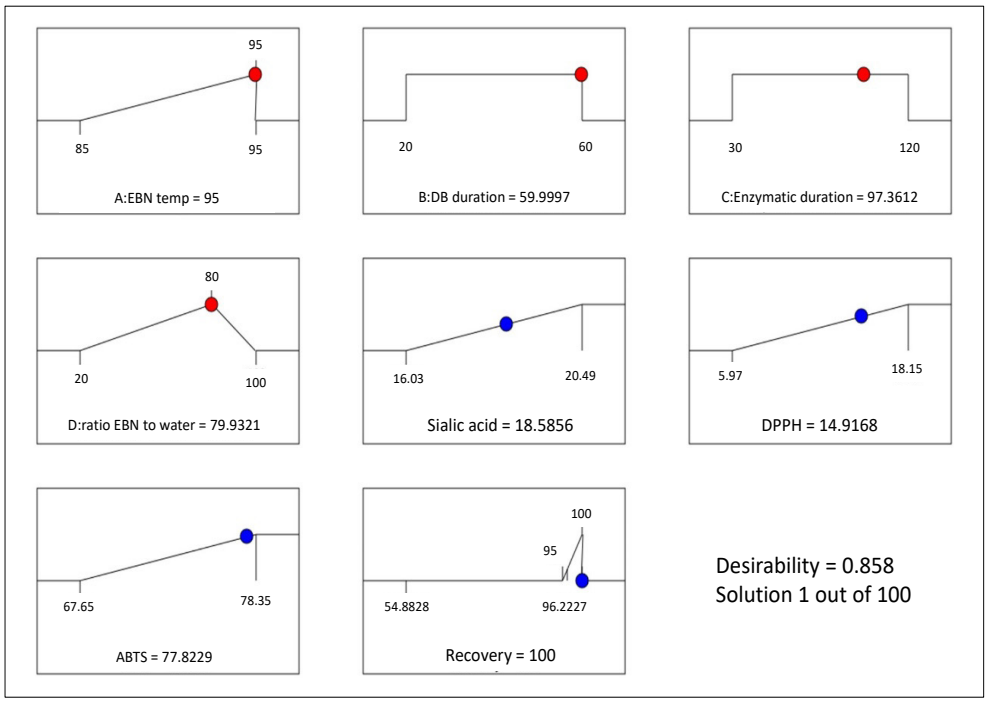

Figure 3. Optimization solution to get 100% product recovery and maximum total SA, DPPH, and ABTS. Factors EBN temperature during DB was set at 95°C, and the ratio of EBN to water was set at 80
Note. SA = Sialic acid; DPPH =2,2-diphenyl-1-picrylhydrazyl; ABTS = 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid

Model Validation

Figure 4 shows the raw material (EBN by-product) and EBN hydrolysate. Table 6 shows the results of the samples before (after heat treatment/DB) and after enzymatic hydrolysis using the optimal conditions. The optimal process parameters were: (1) temperature of EBN during DB = 95°C, (2) DB duration = 60 min, (3) duration of enzymatic hydrolysis = 97 min, and (4) ratio of EBN to water is 1:80. DPPH and ABTS assays are performed directly from liquid samples after collection without any dilution. Therefore, the antioxidant activity results shown here are for samples with 12.5 mg/ml EBN (ratio 1:80). Sample concentrations for total SA and total polysaccharide determinations were the same as above (2 mg/ml).

The antioxidant activities (DPPH and ABTS) of the samples before (after heat treatment) and after complete enzymatic hydrolysis showed similar results. Total SA and polysaccharides significantly increased after hydrolysis ($p < 0.05$). Previous studies have shown that low molecular weight EBN fractions do not affect DPPH free radical scavenging activity (Chong et al., 2022) but have positive effects on SA and total polysaccharides (Chong et al., 2022; Ng et al., 2020). It was suggested that, after heat treatment, the protein may have been

hydrolyzed to polypeptide or dipeptide, which play a role in the free radical scavenging activity. These polypeptides/ dipeptides did not increase after enzymatic hydrolysis.

The product recovery rate (96.44%) was lower than the result of the predicted model (99.99%), while the total SA concentration in the hydrolysate (19.86%) was higher than the result of the predicted model (18.59%).

Figure 4. Left = EBN by-product; Right = EBN hydrolysate after the enzymatic process
Note. EBN = Edible bird's nest

Compared to the previous study by Ling et al. (2020), which showed an 89.09% product recovery, this study demonstrated a higher product recovery (96.44%). The total SA content (a signature component of EBN) was also within the range of other studies (15–22.4%) (Chong et al., 2022; Ng et al., 2020; Yan et al., 2022). The advantage of using bromelain is that bromelain is less sensitive to pH, so no pH adjustment is required. It is especially important for the EBN industry because no additional chemicals were needed to adjust the pH, reducing processing steps. The conventional primary process for the EBN

Table 6
Product recovery, waste recovery, and quality of EBN before and after enzymatic hydrolysis

	Recovery (%)		Quality parameters			
	Product	Waste	DPPH free radical scavenging activity (%)	ABTS free radical scavenging activity (%)	Total SA content (%)	Total polysaccharide content (%)
Before hydrolysis	-	-	28.73 ± 1.08	98.59 ± 0.27	11.32 ± 0.11	6.21 ± 0.45
After hydrolysis	96.44 ± 2.94	2.77± 0.60	28.03 ± 0.78	98.68 ± 0.27	19.86 ± 0.36	9.73 ± 0.35

cleaning method was claimed to incur high processing costs caused by the laborious cleaning procedure (Ng et al., 2020). Moreover, the traditional EBN cleaning methods report losses of up to 35–40% of EBN after cleaning (Noor et al., 2018). If the raw material is an EBN by-product with many fine feathers (Figure 4), the cleaning loss may be higher than the reported value using the traditional cleaning method. This research shows that the enzymatic process has higher product recoveries (96.44%), less wastage (2.77%), and simpler cleaning procedures. This processing method saves time and does not require skilled labor, as the feathers can be removed through filtration, eliminating the need for picking. The enzymatic process demonstrated here could be a promising alternative to EBN cleaning with less reliance on skilled labor and lower product cost.

CONCLUSION

This research led to two optimal conditions, one focusing on yield (product recovery) and the other on yield and quality. Suggestions for optimal conditions for maximum yield (target 100%) are: (1) EBN temperature during DB = 93.6°C, (2) DB duration = 57.5 min, (3) duration of enzymatic hydrolysis = 76.4 min, and (4) ratio of EBN to water = 1:96.6. The optimal conditions suggested for maximum yield and quality are: (1) EBN temperature during DB = 95°C, (2) DB duration = 60 min, (3) duration of enzymatic hydrolysis = 97 min, and (4) ratio of EBN to water = 1:80. In addition, under this optimal process conditions, a 96% product recovery can be obtained, which is believed to be higher than the normal traditional cleaning process of EBN by-products (35-40%).

The factors that affect the yield and quality of EBN hydrolysate include (1) the influence of EBN temperature on product recovery and ABTS during the DB process, (2) DPPH is affected by DB Duration, and (3) the ratio of EBN to water affects product recovery rate. In this study, the duration of enzymatic hydrolysis had the least significant effect on the yield and quality of EBN hydrolysate.

This study suggests that RSM may be a good tool for determining the optimal conditions for the enzymatic process and tailoring the yield and physicochemical properties of EBN hydrolysates. This study also shows that the conversion of EBN by-products into EBN hydrolysate can be used as a nutraceutical because EBN hydrolysate was found to have high antioxidant activity and high SA content. As an extension to the current study, future studies on the degree of hydrolysis and evaluating the peptide bond cleaved after enzymatic hydrolysis will enhance knowledge of the bromelain hydrolysis process.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Review Article

The Use of Nest Boxes in Malaysia: Design and the Potential for Research and *In-situ* Conservation of Birds

Amera Natasha Mah Muhammad Adam Mah[1], Chong Leong Puan[1,2]* and Mohamed Zakaria[1]

[1]*Faculty of Forestry and Environment, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2]*Institute of Tropical Forestry and Forest Products, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

Nest boxes have been used as artificial cavities for decades to attract cavity-nesting birds that rely on such structures to roost and breed. Nest boxes aid research efforts to understand the biology and behaviour of birds and may increase breeding success, thereby contributing to the conservation of a species. The type and dimension of nest boxes and the survey design used in the tropics vary, depending on the targeted species. This paper reviewed 30 published *in-situ* works using nest boxes and their survey design in Malaysia. Most studies were done on Barn Owls (*Tyto alba javanica*) (25), followed by hornbills (5), where they were conducted in oil palm plantations (50%), rice fields (20%), forest habitats (16.7%), urban landscapes (10%), and different habitat matrices (3.3%). Attempts to enhance/manage the local populations were the main research purpose. Unlike studies from other tropical countries, studies involving nest boxes in Malaysia are still in their infancy. This review suggests future research focus on forest species, especially those requiring immediate conservation attention, and delineate parameters highlighted in published works to counter literature inconsistency. Such research involving nest boxes may also be further expanded to the study and/or conserve other interconnected fauna species.

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E-mail addresses:
ameranatashamah@gmail.com (Amera Natasha Mah Muhammad Adam Mah)
chongleong@upm.edu.my (Chong Leong Puan)
mzakaria@upm.edu.my (Mohamed Zakaria)
* Corresponding author

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INTRODUCTION

Secondary cavity-nesting birds often rely on tree cavities that are naturally available or are excavated by primary cavity-nesting birds (e.g., woodpeckers) and mammals (e.g., sun

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bears *Helarctos malayanus*) to roost and breed. As tree cavities are often competed for, especially those of good quality as nest cavities, such structures may serve as a limiting factor for the breeding population of a species (Newton, 1994) and researchers' efforts in understanding the birds' biology and behaviour. To either achieve the goal of population expansion or improve the research of these birds, nest boxes have been used to act as artificial nest cavities to attract these birds (Lambrechts et al., 2012). These secondary cavity-nesting birds may range from certain passerines (Cooper & Bonter, 2008; Gelter & Tegelström, 1992; Hanmer et al., 2017; Hudin et al., 2017; Miller, 2002; Riyahi et al., 2022; Zhang et al., 2021) and non-passerines especially raptors (Calabrese et al., 2020; Geduhn et al., 2016; Liébana & Sarasola, 2013; Raid, 2012; Rejt, 2001; Richards et al., 2004; Zárybnická et al., 2016), parrots (Olah et al., 2014; Ortiz-Catedral & Brunton, 2009; Wimberger et al., 2017), hornbills (Cremades et al., 2011; Pasuwan et al., 2011), and waterfowls (Davis et al., 2007; Gong et al., 2018).

The use of nest boxes has contributed to a wide range of research topics varying from breeding ecology (Arct et al., 2022; Beasley & Parrish, 2009; Davis et al., 2007; Hanmer et al., 2017; Mainwaring et al., 2015; Palko et al., 2011), feeding ecology (Balčiauskienė et al., 2005; Hudin et al., 2017; Rejt, 2001), nest site preference (Cooper & Bonter, 2008; Hanmer et al., 2017; Miller, 2002; Olah et al., 2014; Ortiz-Catedral & Brunton, 2009), conspecific and heterospecific interactions (Gong et al., 2018; Zárybnická et al., 2016), as well as occupancy rate (Liébana & Sarasola, 2013; Sudarmaji et al., 2021; Zhang et al., 2021). Additionally, some studies retrieved nestlings, breeding pairs, or even nest materials from nest boxes to assess the presence of ectoparasites (Hanmer et al., 2017; Proudfoot et al., 2006; Soltész et al., 2018) and the effect of rodenticide exposure (Richards et al., 2004; Geduhn et al., 2016), as well as for population genetic studies (Gelter & Tegelström, 1992; Riyahi et al., 2022). Otherwise, nest boxes may be solely deployed for conservation purposes to increase local bird populations (Calabrese et al., 2020; Cremades & Ng, 2012; Pasuwan et al., 2011).

The type and dimension of nest boxes and the method in nest box provisioning are variable depending on the targeted species. Nest boxes are often carefully designed to facilitate access to the nest cavities and their contents (Lambrechts et al., 2012; Olah et al., 2014). For a particular species, those designs and methods may differ across habitats and countries as well (Lambrechts et al., 2010) with attempts to control the biotic (Davis et al., 2007) and abiotic factors (Arct et al., 2022; Pasuwan et al., 2011) that serve as explanatory variables of species ecology. A few studies have supplemented details on nest box dimensions (Arct et al., 2022; Cooper & Bonter, 2008; Maziarz et al., 2017; Zhang et al., 2021) and illustrations (Olah et al., 2014; Pasuwan et al., 2011; Stanback, 2020; Wimberger et al., 2017) as reference for future studies of species. While most nest boxes were built with a single chamber, some studies may

include additional chambers apart from the nest chamber to place monitoring equipment [e.g., camera surveillance system or camera traps; see Surmacki and Podkowa (2022) and Zárybnická et al. (2016)]. Most nest boxes were constructed from wood material (Arct et al., 2022; Miller, 2002; Palko et al., 2011; Zhang et al., 2021), while others were from other non-wood materials (Beasley & Parrish, 2009; Olah et al., 2014).

Depending on the study sites, the nest boxes' distribution may be placed randomly (Davis et al., 2007) or with a specific distance between the nest boxes (Arct et al., 2022; Palko et al., 2011; Zhang et al., 2021). Some studies may consider surveying targeted species' breeding or home range before nest box provisioning (Calabrese et al., 2020; Mainwaring et al., 2015; Richards et al., 2004). These nest boxes were either nailed or tied to trees (Arct et al., 2022; Maziarz et al., 2017; Olah et al., 2014; Ortiz-Catedral & Brunton, 2009; Palko et al., 2011) or else placed on man-made structures (Beasley & Parrish, 2009; Hudin et al., 2017). While many studies may not report the orientation of the nest boxes, some studies from the temperate and subtropical regions have highlighted the preferred position of the entrance facing the east to the southeast direction (Miller, 2002; Zhang et al., 2021). The height at which the nest boxes were placed is different across species from those that can easily be accessed (Arct et al., 2022; Lambrechts et al., 2010; Miller, 2002; Ortiz-Catedral & Brunton, 2009; Palko et al., 2011) otherwise higher into the canopy (Pasuwan et al., 2011).

For decades, studies using nest boxes were frequently done in the temperate region (Arct et al., 2022; Calabrese et al., 2020; Cooper & Bonter, 2008; Gelter & Tegelström, 1992; Gong et al., 2018; Hudin et al., 2017; Liébana & Sarasola, 2013; Ortiz-Catedral & Brunton, 2009; Raid, 2012; Rejt, 2001; Riyahi et al., 2022; Soltész et al., 2018; Zhang et al., 2021), with fewer studies conducted in the subtropic (Miller, 2002; Davis et al., 2007; Wimberger et al., 2017) and tropical regions (Cremades et al., 2011; Palko et al., 2011; Pasuwan et al., 2011; Olah et al., 2014; Sudarmaji et al., 2021). The implementation of nest boxes in the Southeast Asian region is still relatively new, apart from provisioning nest boxes for barn owls (*Tyto alba javanica*) to encourage the local population to breed and control the rodents in agricultural areas (Adidharma, 2002; Sipayung, 1992).

APPLICATION OF NEST BOXES IN MALAYSIA

Unlike studies from other tropical countries, *in-situ* studies involving nest boxes in Malaysia are still in their early stages, although they have been implemented since the late 1970s (Lenton, 1983). From a total of 30 published works, it is found that most studies involving nest boxes were done on barn owls (25), followed by hornbills (5), of which the works were conducted in oil palm plantations (50%), rice fields (20%), forest habitat (16.7%), urban landscapes (10%), and different habitat matrices (3.3%). The main research topics covered by these studies comprised methods in enhancing/

managing local populations (43.3%), prey selection (16.7%), breeding behaviour (13%), home range (10%), growth performance (6.7%), sex identification (6.7%), and egg measurement (3.3%). A summary of the studies using nest boxes, nest box dimensions, and respective survey designs is presented in Table 1.

Barn Owls

Barn owls, formerly a vagrant in Peninsular Malaysia, became a common resident species when they were introduced in agricultural areas to provide biological control of pests (i.e., rodents) as a complementary or alternative approach to rodenticide treatment (Puan et al., 2020). As the availability of nest sites was the limiting factor of barn owl numbers, nest boxes were provisioned to increase their population in oil palm plantations (Duckett, 1991; Lenton, 1983). The success in elevating the population had helped control the rat populations in some cases, lessening the dependency on anticoagulant rodenticide since the late 1970s (Duckett, 1991; FFTC, 2002; Lenton, 1983). Additionally, the cost of maintaining nest boxes was relatively cheaper than the costs of rodenticide and labour, which had been shown to reduce the overall cost spent by the plantation owners (Abidin et al., 2021). Other than in plantations with immature (Naim et al., 2010) or mature oil palms (Salim et al., 2014, 2015, 2016), nest boxes had also been provided in rice fields [(Amzah et al., 2014; FFTC, 2002; Hafidzi et al., 2003, 2007; Hafidzi & Na'im, 2003a, 2003b), urban landscapes, e.g., urban garden; see Saufi et al. (2020a, 2020b), and different habitat matrices, e.g., cocoa-coconut farmland; see Lee (1997)].

Before the 2000s, studies that involved provisioning nest boxes focused mainly on the methods to enhance/manage the local population (Duckett, 1991; Lee, 1997; Lenton, 1983), with few studies investigating their ecology (Lim et al., 1993). However, recent studies not only focused on upgrading previous methods (Abidin et al., 2021; Amzah et al., 2014; FFTC, 2002; Hafidzi et al., 2007; Hafidzi & Na'im, 2003b; Saufi et al., 2020b) but had expanded into their biology and ecology including breeding behaviour (Abidin et al., 2022; Naim et al., 2011; Salim et al., 2014), growth performance (Naim et al., 2010; Salim et al., 2016), egg measurement (Salim et al., 2015), home range (Hafidzi et al., 2003; Naim et al., 2012; Saufi et al., 2019), prey selection (Hafidzi & Na'im, 2003a; Puan et al., 2011, 2012; Saufi et al., 2020a), and sex identification (Ravindran et al., 2018, 2019). In addition, some studies retrieved eggs, nestlings, and even adults from nest boxes to assess the level of anticoagulant rodenticide exposure and the associated effects towards their growth and behaviour (Naim et al., 2012; Salim et al., 2014, 2015, 2016).

In the past, nest boxes were constructed from wood with zinc sheet roofs placed on top of telegraph or hardwood poles (Duckett, 1991; FFTC, 2002; Lenton, 1983; Naim et al., 2010, 2011). Studies from the past two decades may also use wood material (Amzah et al., 2014) as well as fibreglass

Table 1
Summary of the nest box dimension and survey designs in Malaysian bird studies

Species	Reference	Main study focus	Study site	Number of nest boxes provisioned	Material	Dimension (width × length × height; cm)	Distribution	Provisioned height and structure	Other methods
Barn owl	Lenton (1983)	A review of biology and study design to introduce population	Oil palm plantation	-	Wood	50 × 50 × 100	-	Placed on top of telegraph poles	-
Barn owl	Duckett (1991)	Joint barn owl Project from stakeholders to introduce population	Oil palm plantation in Negeri Sembilan	200	Weatherproof plywood (6mm thickness) with zinc sheet roof (28 gauge)	Follows Lenton (1980) with adjustments [see Lenton (1983) instead], i.e., 46 × 122 × 51 with the entrance of 15 cm width by 20 cm height; 19 cm width by 20 cm height door placed at the long side of the box secured by rubber tyre as hinges; partition placed half of nest box width on the same side as the entrance door	1 box per 5 ha	At least 4.5 m above ground using a minimum of 10 cm × 10 cm hardwood pole and buried at least 1.2 m deep; hole prepared with tractor-mounted auger (0.5 to 0.6 m diameter); pole placed centrally and filled with rubber then cement mix	Nest boxes were provisioned under the shade of a canopy tree; for exposed nest boxes, roof insulation and ventilation were made; a metal cowl was placed on a pole; two mild steel bars were placed as struts; ventilation holes of 2.5 cm diameter on the nest box ceiling
Barn owl	Lim et al. (1993)	Prey selection	Oil palm plantation in Johor	182 (pre-established by plantation owner)	-	-	1 nest box per 10.9 ha	-	-

Table 1 *(continue)*

Species	Reference	Main study focus	Study site	Number of nest boxes provisioned	Material	Dimension (width × length × height; cm)	Distribution	Provisioned height and structure	Other methods
Barn owl	Lee (1997)	Study design to introduce population	Oil palm plantation and cocoa-coconut farmland in Perak	24 (12 nest boxes in each site)	-	-	Set between third and fourth oil palm rows at 500 m apart for oil palm plantation, in 2 rows within an old coconut field	-	-
Barn owl	Food and Fertilizer Technology Center (FFTC) (2002)	Study design to enhance population	Rice field	20	Plywood painted with white paint	Detailed dimensions were provided in the figure but were visually unclear	1 nest box per 40 ha	Using a 55 m length pole buried 10 m deep	Place broken bark, pine needles, and leaves as nesting materials; deposits or pellets do not need to be cleaned out
Barn owl	Hafidzi and Na'im (2003a)	Prey selection	Rice field in Selangor	-	-	-	-	-	-
Barn owl	Hafidzi and Na'im (2003b)	Evaluate rice damage by rice field rats concerning barn owl provisioning	Rice field in Selangor	15	-	-	5 nest boxes each in 5, 10, and 20 ha	-	-
Barn owl	Hafidzi et al. (2003)	Home range	Rice field in Selangor	79	-	-	-	-	-

Table 1 *(continue)*

Species	Reference	Main study focus	Study site	Number of nest boxes provisioned	Material	Dimension (width × length × height; cm)	Distribution	Provisioned height and structure	Other methods
Barn owl	Hafidzi et al. (2007)	Influence of wet and dry seasons on breeding individuals and relation to rat damage	Rice field in Selangor	33 (18 and 15 nest boxes in the first and second sites, respectively)	-	Follows Duckett (1976)	1 nest box per 45 ha in the first site; 5 nest boxes each in 5, 10, and 20 ha in the second site	-	-
Barn owl	Naim et al. (2010)	Growth performance of nestling in rat baiting area	Immature oil palm plantation in Perak	22	Wood	-	1 nest box per 25 ha	-	-
Barn owl	Naim et al. (2011)	Breeding performance	Oil palm plantation in Perak	22	Wood	-	1 nest box per 25 ha	-	-
Barn owl	Puan et al. (2011)	Prey selection	Oil palm plantation in Negeri Sembilan	Pre-established by plantation owner	-	-	1 nest box per 10 ha	-	-
Barn owl	Naim et al. (2012)	Home range concerning rodenticide	Oil palm plantation in Perak	24	-	-	Average of 1 nest box per 25 ha	-	-
Barn owl	Puan et al. (2012)	Prey selection and predatory behaviour	Oil palm plantation in Negeri Sembilan	Pre-established by plantation owner	-	-	1 nest box per 10 ha	-	-

Table 1 *(continue)*

Species	Reference	Main study focus	Study site	Number of nest boxes provisioned	Material	Dimension (width × length × height; cm)	Distribution	Provisioned height and structure	Other methods
Barn owl	Amzah et al. (2014)	Study design to enhance population	Rice field in Penang	20	Plywood painted with white paint	37.5 × 67.5 × 42.5	-	4.5–6.0 m above ground, attached to a random coconut tree	-
Barn owl	Salim et al. (2014)	Breeding performance concerning rodenticide	Mature oil palm plantation in Pahang	42 (pre-establish by plantation owner)	High durable fibreglass	50 × 115 × 61	1 box per 15 ha	7 m above the ground	-
Barn owl	Salim et al. (2015)	Effect of rodenticide deposited in eggs	Mature oil palm plantation in Pahang	-	-	-	-	-	-
Barn owl	Salim et al. (2016)	Nestling growth concerning rodenticide	Mature oil palm plantation in Pahang	42	High durable fibreglass	50 × 115 × 61	1 box per 15 ha	7 m above the ground	-
Barn owl	Ravindran et al. (2018)	Sex identification based on morphology and molecular-based methods	Oil palm plantation in Pahang	-	-	-	-	-	-
Barn owl	Ravindran et al. (2019)	Molecular sexing using blood and feather	Oil palm plantation in Pahang	-	-	-	-	-	-

Table 1 *(continue)*

Species	Reference	Main study focus	Study site	Number of nest boxes provisioned	Material	Dimension (width × length × height; cm)	Distribution	Provisioned height and structure	Other methods
Barn owl	Saufi et al. (2019)	Homing instinct of female individual	University campus in Penang	1	-	-	-	-	-
Barn owl	Saufi et al. (2020a)	Prey selection	Urban garden in Penang	14	Wooden and fibreglass	-	-	-	-
Barn owl	Saufi et al. (2020b)	Release method	Urban garden in Penang	14	Fibreglass	63 × 72 × 28	1 box per 15 ha	5 m above ground on metal poles buried one meter deep and secured with concrete	Entrance faced North to Northeast
Barn owl	Abidin et al. (2021)	Effectiveness of introducing population to control the rat population	Oil palm plantation in Sabah	16	-	-	-	-	-
Barn owl	Abidin et al. (2022)	Breeding of translocated individuals	Oil palm plantation in Sabah	16	-	-	16 nest boxes per 25 ha and at least 36 m from the nearest road	5.3 m above ground on smooth poles	Placed two weeks prior to the study, the entrance faced the East or North
Hornbill	Kaur and Ancrenaz (2016)	Study design to enhance population	Forest in Sabah	5 [including 4 plastic drums from HUTAN (2018) and a	Recycled plastic for rectangular nest box; plastic drum	64 × 67 × 120; diamond-shaped entrance hole about 10 cm wide and 29 cm length	-	-	Camera traps were placed near the nest box, while a data logger

Table 1 *(continue)*

Species	Reference	Main study focus	Study site	Number of nest boxes provisioned	Material	Dimension (width × length × height; cm)	Distribution	Provisioned height and structure	Other methods
				rectangular nest box]	nest box wrapped with styrofoam, a wire mesh, and a layer of cement and sand for cylindrical	at 30 cm from the bottom			was placed inside the nest box
Hornbill	HUTAN (2018)	Study design to enhance population	Forest in Sabah	4	Plastic drum insulated with a layer of polystyrene covered with another plastic drum and a coating of cement	210 x 90 (diameter × height); entrance hole at 30 cm from the bottom	-	15–25 m above ground attached to trees with adequate cover using rot-proof webbing	A perch was constructed at the lower part of the entrance hole; the base was covered with soil and sawdust
Hornbill	Gaia (2019)	Study design to enhance population	Forest in Sabah	20 (including from Kaur and Ancrenaz (2016))	-	-	-	Installed 30 m high on 50 m tall tree next to Helmeted Hornbill (*Rhinoplax vigil*) dying nest tree	-
Hornbill	Kaur (2019)	Study design to enhance population	Forest in Sabah	-	Follows Kemp (1995)	-	-	-	-
Hornbill	Vercoe et al. (2021)	Study design to enhance population	Forest in Sabah	25 [including from Gaia (2019)]	Wood, fibreglass, and plastic barrel	-	-	-	Lichens were allowed to grow on the nest box surface

(Salim et al., 2014, 2016; Saufi et al., 2020b) or both (Saufi et al., 2020a) for box construction. However, only several studies have reported the nest box dimension in brief or detail (Amzah et al., 2014; Duckett, 1991; FFTC, 2002; Lenton, 1983; Salim et al., 2014, 2016; Saufi et al., 2020b). From these studies, dimensions differed from the relatively bigger base (Duckett, 1991; Salim et al., 2014, 2016) to vertically longer nest boxes (Lenton, 1983). In addition, a partition was placed with the width of half the nest box to separate the nest and entrance chamber. It was to prevent the chicks from falling out and darkening the interior nest box (Duckett, 1991). In some cases, white paint may be applied to wooden nest boxes to increase shelf life (Amzah et al., 2014; FFTC, 2002).

Depending on the study's purpose, the nest boxes' distribution may differ across studies, while certain studies used pre-established nest boxes by landowners (Lim et al., 1993; Puan et al., 2011, 2012; Salim et al., 2014). For example, in oil palm plantations, the density of the nest boxes ranged from one box per 5 ha (Duckett, 1991), 10 ha (Puan et al., 2011, 2012), 10.9 ha (Lim et al., 1993), 15 ha (Salim et al., 2014, 2016) to 25 ha (Naim et al., 2010, 2011, 2012). Other studies may provision 16 nest boxes per 25 ha (Abidin et al., 2022) or between two adjacent oil palm rows 500 m apart (Lee, 1997). In rice fields, nest boxes were installed at a density of one box per 40 ha (FFTC, 2002) and 45 ha (Hafidzi et al., 2007); otherwise, 5 nest boxes per 5, 10, and 20 ha (Hafidzi et al., 2007; Hafidzi & Na'im, 2003b). In other landscapes, such as the cocoa-coconut farmland, nest boxes were placed in two rows within the old coconut field (Lee, 1997), and nest boxes in urban gardens were provisioned at a nest box per 15 ha (Saufi et al., 2020b).

The nest boxes were often placed on poles ranging from 4–7 m above ground to deploy nest boxes in designated study sites (Abidin et al., 2022; Duckett, 1991; FFTC, 2002; Salim et al., 2014, 2016; Saufi et al., 2020b) but may also be placed onto trees in landscapes lacking suitable trees [e.g., rice fields; see Amzah et al. (2014)]. Cement mix may be applied into the pole's hole for more support (Duckett, 1991; Saufi et al., 2020b). At the same time, most studies did not specify the time allocated before observation, a study considered provisioning two weeks before a study commenced (Abidin et al., 2022). Certain studies have highlighted specific areas or orientations to which nest boxes should be installed to increase their occupancy rate. For example, nest boxes may either be placed under the shade of a canopy tree (Duckett, 1991) or have their entrance facing towards the north to the northeast direction (Abidin et al., 2022; Saufi et al., 2020b). For nest boxes installed in open habitats, roof insulation and ventilation were built to maintain a relatively cooler nest box interior (Duckett, 1991).

Hornbills

In Malaysia, the early efforts in provisioning nest boxes for hornbill conservation were initiated by a French Non-Government

Organization, HUTAN Kinabatangan Orang-utan Conservation Program (HUTAN/KOCP) and Gaia, a local social enterprise in Sabah (Kaur, 2019) as well as Piasau Camp Miri Nature Park Society (PCMNPS) in Sarawak (Lai, 2021). Complementary to the efforts to survey natural hornbill nests or tree cavities and restore them, nest boxes were also constructed based on recommendations by Kemp (1995) and installed in Lower Kinabatangan Wildlife Sanctuary (LKWS), Sabah (Kaur, 2019). As a result, 25 nest boxes with materials varying from wood, fibreglass, and plastic barrel were provisioned (Vercoe et al., 2021). In addition, a rectangular nest box was built from recycled plastic material, while four cylindrical nest boxes were built from a plastic drum wrapped with styrofoam, a wire mesh, and a layer of cement and sand (Kaur & Ancrenaz, 2016).

A perch was placed at the lower part of the diamond-shaped entrance hole, allowing the hornbills to perch close to the nest box (HUTAN, 2018; Kaur & Ancrenaz, 2016). The inner base of the nest box was covered with soil and sawdust (HUTAN, 2018). Some of the nest boxes were attached using rot-proof webbing to trees with adequate cover about 15-25 m high, while others were about 30 m high on a 50 m tall tree next to the hornbill's dying nest tree (Gaia, 2019; HUTAN, 2018). Camera traps were placed facing the nest box entrance, whereas data loggers were placed inside the nest box to monitor the occupants and microclimate within the nest box (Kaur & Ancrenaz, 2016). Lichens were allowed to grow on the nest box surface to create a more natural appearance and provide light energy absorption (i.e., to reduce nest box temperature). Some difficulties in provisioning the nest boxes for birds were addressed, where wooden nest boxes tend to decay rapidly and often take over by stingless bees, civets, ants, and flying squirrels (Vercoe et al., 2021).

CONSIDERATIONS IN IMPLEMENTING NEST BOXES INTO *IN-SITU* STUDIES

As seen in studies conducted throughout the world, provisioning nest boxes may help in understanding the behaviour and ecology of bird species, especially those that are considered vulnerably threatened (Soltész et al., 2018) or rather elusive (Balčiauskienė et al., 2005; Zárybnická et al., 2016). Provisioning nest boxes may also help elevate local bird populations with adequate management planning (Calabrese et al., 2020; Katzner et al., 2005; Mänd et al., 2005). When successfully implemented, this could benefit about 32 obligate secondary cavity-nesting resident species out of the 74 cavity-nesting bird species of 18 families recorded in Malaysia (Puan et al., 2020; van der Hoek et al., 2017). Likewise, about seven vulnerable secondary cavity-nesting species (Black Hornbill *Anthracoceros malayanus*, Great Hornbill *Buceros bicornis*, Plain-pouched Hornbill *Rhyticeros subruficollis*, Rhinoceros Hornbill *Buceros rhinoceros*, Wreathed Hornbill *Rhyticeros undulatus*, White-fronted Scops-owl *Otus sagittatus*, and

Long-tailed Parakeet *Psittacula longicauda*) along with an endangered (Wrinkled Hornbill *Rhabdotorrhinus corrugatus*) and a critically endangered species (Helmeted Hornbill) (Puan et al., 2020; van der Hoek et al., 2017) may also benefit from such implementation. Additionally, nest boxes may also be provisioned for *in-situ* studies involving other fauna, such as mammals (e.g., bats, civets, squirrels), feral bees, and even herpetofaunas that refuge in tree cavities (Goldingay et al., 2020; Griffiths et al., 2020; Vercoe et al., 2021). However, it should be noted that provisioning nest boxes is rather supplementary and not a complete solution to species conservation as some, if not all, rare and threatened species may be unable to use and adapt to the presence of nest boxes. Natural tree cavities are highly encouraged to be maintained and restored (Le Roux et al., 2016).

CONCLUSION

While studies using nest boxes in Malaysia are still in their early stages, this paper suggests that future research or publications should provide as much information as possible concerning parameters highlighted in Lambrechts et al. (2010) and Wesołowski (2011) to counter literature inconsistency. In some cases, a species utilising two different habitats or different nest box sizes may show different outcomes leading to inconclusive results on a species' nest requirements (Lambrechts et al., 2010; Le Roux et al., 2016; Mänd et al., 2005). Detailed information on the monitoring efforts is crucial to provide guidelines for other studies, although often overlooked by many researchers. This review highlighted the lack of studies involving nest boxes and the bias towards species of special interest. For many cavity-nesters in the tropics, locating and reaching their nests in the field may be challenging. Despite being man-made, installing nest boxes is a better alternative to increase research into the feeding and breeding ecology of many little-known tropical birds that use such structures. Therefore, this paper recommends the continued use of nest boxes in this part of the world due to their potential for autecology research and *in-situ* conservation in natural or agroecosystems.

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Review Article

Avian Metapneumovirus Infection in Poultry Flocks: A Review of Current Knowledge

Wafaa A. Abd El-Ghany

Poultry Diseases Department, Faculty of Veterinary Medicine, Cairo University, 12211 Giza, Egypt

ABSTRACT

Avian metapneumovirus (aMPV) is one of the respiratory viruses that cause global economic losses in poultry production systems. Therefore, it was important to design a comprehensive review article that gives more information about aMPV infection regarding the distribution, susceptibility, transmission, pathogenesis, pathology, diagnosis, and prevention. The aMPV infection is characterized by respiratory and reproductive disorders in turkeys and chickens. The disease condition is turkey rhinotracheitis in turkeys and swollen head syndrome in chickens. Infection with aMPV is associated with worldwide economic losses, especially in complications with other infections or poor environmental conditions. The genus Metapneumovirus is a single-stranded enveloped RNA virus and contains A, B, C, and D subtypes. Meat and egg-type birds are susceptible to aMPV infection. The virus can transmit through aerosol, direct contact, mechanical, and vertical routes. The disease condition is characterized by respiratory manifestations, a decrease in egg production, growth retardation, increasing morbidity rate, and sometimes nervous signs and a high mortality rate, particularly in concurrent infections. Definitive diagnosis of aMPV is based mainly on isolation and identification methods, detection of the viral DNA, as well as seroconversion. Prevention of aMPV infection depends on adopting biosecurity measures and vaccination using inactivated, live attenuated, and recombinant or DNA vaccines.

Keywords: Distribution, pathology, swollen head syndrome, turkey rhinotracheitis, vaccine

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E-mail address:
wafaa.soliman@cu.edu.eg (Wafaa Abd El-Ghany)

INTRODUCTION

Respiratory viral diseases are the primary causes of severe economic losses in poultry production systems. Avian metapneumovirus (aMPV) infection is regarded as a significant and highly contagious respiratory viral

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disease (Tucciarone et al., 2018). Infection with aMPV is associated with poor feed conversion ratio, a drop in egg production with alterations of egg quality, and mortalities, especially in complications.

In 1978 in South Africa, aMPV induced a disease condition in turkey named 'turkey rhinotracheitis' (TRT) (Buys et al., 1989). However, in 1984 in France and the United Kingdom, the same virus was first termed in chickens and described as 'swollen head syndrome' (SHS) (Morley & Thomson, 1984). In the 1980s, aMPV was detected in turkeys and commercial fowl in some European countries and elsewhere in the 1990s, such as the United States of America (USA). The previous infections aMPV induced were avian infectious pneumoniae, avian pneumovirus, and avian rhinotracheitis (Alkahalaf et al., 2002).

The virus mainly affects turkeys, chickens, and other avian species with variable mortality rates (Brown et al., 2019; Tucciarone et al., 2022). Air is the main method of aMPV transmission. However, direct contact with wild birds is possible (Alkahalaf et al., 2002). Concurrent secondary infections dramatically exacerbate the virus infection. *Escherichia coli*, *Bordetella avium*, *Ornithobacterium rhinotracheale* (ORT), *Riemerella anatipestifer*, *Mycoplasma gallisepticum*, *Avibacterium paragallinarum*, *Chlamydophila psittaci*, Newcastle disease virus (NDV), infectious bronchitis virus (IBV), avian orthoavulavirus-1, infectious laryngotracheitis virus, or *Aspergillus fumigatus* can considerably enhance the incidence and severity of aMPV infection (Croville et al., 2018). The aMPV infections are globally distributed in poultry-producing areas with adverse economic losses. Based on reactivity against monoclonal antibodies, serological tests, and nucleotide sequence analysis, subtypes A, B, C, and D of aMPV are found to be antigenically distinctive (Cook et al., 1993). Recent sequence analysis of the virus showed the presence of additional subtypes in wild and game birds of North America. Based on L gene sequences, the phylogenetic analysis of aMPV revealed that the new subtypes are close to the subgroup C (Retallack et al., 2019; World Organisation for Animal Health [WOAH], 2022). Vaccination against aMPV has been adopted to protect flocks from infection with variable immune responses. Several attempts have been carried out to develop inactivated, living attenuated, and recombinant vaccines against aMPV infection in commercial turkey and chicken flocks (Śmiałek et al., 2021).

This review article was designed to give more information about aMPV infection regarding distribution, susceptibility, transmission, pathogenesis, pathology, diagnosis, and prevention.

THE VIRUS

Avian metapneumovirus is a member of *Mononagavirales*, the family *Paramixoviridae*, the sub-family *Pneumoviridae*, and the genus *Metapneumovirus* (Pringle, 1998). There are two genera in the family of *Pneumoviridae*: the *Orthopneumovirus* and

the *Metapneumovirus*. The virus is a single-stranded, negative-sense RNA, enveloped, linear, non-segmented, and pleomorphic (80 to 200 nm) or spherical. The viral genome in a nucleocapsid is about 14 kilo-bases with a helical symmetry (M. Yu et al., 2019). The viral gene order is 3′-leader-N-P-M-F-M2-SH-G-L-trailer-5′ (Ling & Pringle, 1988). Moreover, the aMPV characteristics differ from mammalian pneumoviruses at the molecular level (Kuhn et al., 2020). As aMPV has no hemagglutination or neuraminidase activity in G attachment protein, it could be distinguished from other paramyxoviruses.

Avian metapneumovirus has 4 subtypes: A, B, C, and D, according to the nucleotide and amino acids sequences of the G attachment gene as well as the serological properties (Chacón et al., 2007), and they are circulating in many countries all over the world. Subtypes A and B of aMPV were first recognized in the 1980s (McDougall & Cook, 1986), and they are prevalent, especially in Europe (Giraud et al., 1986; Naylor, Shaw, et al., 1997). However, the field reports of aMPV pointed to the higher incidence of subtype B over subtype A in some countries for unclear reasons (Chacón et al., 2011). Moreover, sub-populations of subtype B may be found due to escaping of vaccine mutants with inadequate protection. Subtype C of the virus was demonstrated in turkey flocks of the USA (Seal, 1998) and wild avian populations (Turpin et al., 2008). Moreover, subtype C was detected in breeder flocks of ducks in France and China (Wei et al., 2013). In Europe, a second distinctive lineage of subtype C has been found (Toquin et al., 2006). In the USA, though strains of aMPV are classified as subgroup C, a novel subgroup showed a higher genetic similarity to human MPV than avian types (Govindarajan & Samal, 2004). Later, subtype C was distributed in France (Toquin et al., 2006) and South Korea in avian species. Also, in France (Bäyon-Auboyer et al., 2000) and other countries (Cook et al., 2000), subtype D of aMPV was isolated from turkeys. Subtypes A, B, and D of the virus are nearly similar.

The presence of an envelope in aMPV increases the sensitivity of the virus to the lipid solvents and some physical and chemical agents (Townsend et al., 2000). The viability of aMPV can be decreased after exposure to some disinfectants including ethanol, quaternary ammonium compounds, iodophor, phenol, and sodium hypochlorite (L. Zhang et al., 2002). The virus may remain active for up to 26 weeks at -20°C and 60 days at 12°C (Velayudhan et al., 2003).

DISTRIBUTION

Since aMPV was isolated in the late 1970s, the virus has been distributed worldwide. The virus infection is characterized by a rapid spread and distribution within the flock and between flocks such as those of European countries (Franzo et al., 2020; Mescolini et al., 2021). The distribution of aMPV depends on hygienic measures, shedding rate, seasonal variation, and stocking density (Jardine et al., 2018). Despite the development of management and hygiene measures and vaccination programs, the incidence of aMPV is still

high in many countries and continents worldwide, including Japan (Mase et al., 2003), Israel (Banet-Noach et al., 2005), Korea (Kwon et al., 2010), China (S. Sun et al., 2014), Romania (Franzo et al., 2017), Greece (Tucciarone et al., 2017), Bangladesh (Ali et al., 2019), Brazil (Rizotto et al., 2019), Pakistan (Umar et al., 2019), and Italy (Graziosi et al., 2022; Legnardi et al., 2021).

There are limited data about the incidence of aMPV in the Middle East region, possibly due to other more significantly important respiratory viruses. However, the virus was detected in some of the Middle East countries such as Egypt, Iraq, Iran, Jordan, Morocco, Algeria, and Turkey (Table 1).

Table 1

The incidence of avian metapneumovirus in some countries of the Middle East region

Country	History	Findings	Method of detection	Reference
Egypt	Serum samples were collected from 30-38-week-old broiler breeder chickens	Six of 38 serum samples were serologically positive for aMPV	ELISA	Youssef and Ahmed (1996)
	Seventy-five serum samples were taken from 20 and 52-day-old broiler chickens representing 15 farms in different provinces. Ten farms had respiratory signs, and the other 5 farms were apparent healthy	High seroprevalences to aMPV were found in the 14 flocks. Antibodies were detected in broilers from apparent healthy farms	ELISA	Aly et al. (1997)
	Ten oropharyngeal swabs were collected from 3-week-old turkey poults with upper respiratory disease in Fayoum province	Subtype A of aMPV was identified	RT-PCR	Abdel-Azeem et al. (2014)
	Oropharyngeal and nasal sinus swabs were taken from 100 broiler turkeys representing 10 farms and showed respiratory signs in Giza, Beni-Suif, and Cairo provinces	Subtype B of aMPV strains was detected in 8 samples, which were very close to the VCO3 vaccine strain	Embryonated chicken eggs, Vero cells, RT-PCR, and genetic analysis	Arafa et al. (2015)

Table 1 *(Continue)*

Country	History	Findings	Method of detection	Reference
	Serum samples were taken from 40 chicken flocks (23 broilers and 17 layers), and 8 duck flocks (6 Pekin and 2 Muscovy)	Five out of 23 broilers, 6 out of 17 layers, 1 out of 6 Pekin ducks, and 1 out of 2 Muscovy ducks were serologically positive for aMPV	ELISA	Nagy et al. (2018)
Egypt	Trachea, lung, and choanal cleft were collected from broiler chicken flocks in different provinces	Subtype B of aMPV was detected. In addition to co-infection with *E. coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*	RT-PCR and real-time polymerase chain reaction (qPCR) for viral detection. Besides conventional bacteriological examination and PCR	Abdelmoez et al. (2019)
	Swabs from the trachea, sinuses, lungs, and air sacs were collected from 67 3-6-week-old broiler chickens with SHS in Baghdad, Wasit, Karbal, Al Muthanna, Al-Najaf, and Al-Qadisiyyah provinces	Detection of ORT as one of the etiological factors that cause SHS in poultry	Conventional bacteriological examination and RT-PCR	Al-Hasan et al. (2021)
Iraq	Sixty-seven swabs from the trachea, sinuses, lungs, and air sacs were taken from 3-6-week-old broiler farms in Baghdad, Wasit, Karbal, Al Muthanna, Al-Najaf, and Al-Qadisiyyah provinces	Subtype B of aMPV was found in 16 (23.8%) samples; 51 (76.11%) were negative from typical SHS-infected flocks, and no positive samples for other subtypes were found	RT-PCR	Al-Hasan et al. (2022)

Table 1 *(Continue)*

Country	History	Findings	Method of detection	Reference
Iran	A total of 540 serum samples were collected from 27 broiler breeder flocks in 11 provinces	Serologically, 92.59% were positive, while 2 were suspected of aMPV infection. Besides, 92.77% were positive, 3.70% were suspected, and the others were negative	ELISA	Sheikhi and Masoudian (2011)
	Oropharyngeal and turbinate swabs were collected from un-vaccinated 4 broiler chickens with swollen heads, and 10% mortality in Alborz province	Subtype B of aMPV was isolated	RT-PCR	Hosseini and Ghalyanchi-Langeroudi (2012)
	A total of 525 blood samples and trachea/ nasal turbinates swabs were taken from 35 non-vaccinated broiler flocks with respiratory signs in northern Iran	Ten (28.5%) of flocks had positive antibodies to aMPV. Of the 35 flocks, 8 (23%) were positive aMPV	ELISA and RT-PCR	Seifi et al. (2015)
	Tissue samples of broiler chickens were taken	Subtype B of aMPV was identified based on the fusion (F) gene	RT-PCR	Hosseini et al. (2017)
	Sixty-three meat-type unvaccinated turkey flocks from several provinces were sampled in major turkey abattoirs	Twenty-six samples from three flocks (4.10%) were positive for the virus RNA, while all viruses were detected as aMPV subtype B	RT-PCR	Mayahi et al. (2017)

Table 1 *(Continue)*

Country	History	Findings	Method of detection	Reference
Iran	Tracheal swabs were collected from 20 broiler chicken farms that had respiratory disease complex in Qazvin province	Thirteen out of 20 flocks were infected with aMPV, which accounted for 65 infection rates of the flocks	RT-PCR	Zahirabadi et al. (2017)
	Four hundred and fifty oropharyngeal samples were taken from 8 migratory and local species of birds from live bird markets in Gilan province	The aMPV subtype B was detected in 30.60%, including chickens (37%), turkeys (33%), Eurasian teal (25%), common blackbirds (33%), and Eurasian woodcock (25%)	RT-PCR	Chaboki et al. (2018)
	Samples from the trachea, choana, and sinuses of more than 3-week-old broiler chickens were collected from 85 broiler flocks in Semnan province	Thirty out of 85 (35.3%) flocks were positive for aMPV. Besides, 28 positive samples were subtype B, and the other 2 were non-subtype B, possibly A, C, or D	RT-PCR	Darebaghi et al. (2021)
Jordan	In northern and central Jordan, 38 chicken flocks (23 broilers, 8 layers, and 7 broiler breeders) were tested for serology. However, 150 chicken flocks (133 broiler flocks, 7-layer flocks, and 10 broiler breeder flocks) were tested for molecular virus detection	Antibodies against aMPV were detected in 5 of 23 broiler chickens (21.7%), 6 of 8-layer chickens (75%), and 7 of 7 (100%) broiler breeder chickens. Molecularly, aMPV was detected in 17 broiler flocks (12.8%) and 3-layer flocks (42.9%). All the broiler breeder chickens were negative. All aMPV isolates were subtype B	ELISA and RT-PCR	Gharaibeh and Algharaibeh (2007)

Table 1 *(Continue)*

Country	History	Findings	Method of detection	Reference
Jordan	Trachea swabs from 115 diseased broiler chicken flocks were tested for avian influenza virus (AIV) subtype H9N2, IBV, NDV, and aMPV	Thirteen and 14.8% were infected with NDV and IBV, respectively, whereas 5.2, 6.0, 9.6, 10.4, 11.3, and 15.7% were infected with *Mycoplasma gallisepticum (*MG) and NDV; aMPV and MG; NDV and IBV; MG and IBV; AIV and NDV; as well as AIV and IBV, in a respective manner. Moreover, 2.6% showed aMPV, NDV, and IBV infections. However, 11.3% were negative for all these respiratory viruses	PCR and RT-PCR	Roussan et al. (2008)
Morocco	Serum samples were taken from 48, 5-week-old non-vaccinated broiler chicken flocks in different bioclimatic zones	From 1,142 sera, 912 (79.86%) samples were positive serologically to aMPV. The arid zone had the highest seroprevalences. Moreover, 94.16% during winter and 84.82% during spring were positive	ELISA	Mernizi et al. (2022)
Algeria	Tissues were collected from the respiratory tract of broiler chickens	Subtype B of aMPV was detected with IBV, AIV, and MG	RT-PCR	Sid et al. (2015)

Table 1 *(Continue)*

Country	History	Findings	Method of detection	Reference
Turkey	A total of 624 tracheal samples were taken from a local turkey abattoir. Besides, 20 tracheal swabs were collected from turkeys with respiratory problems. In addition, 23 vaccinated healthy turkey flock Vaccinated turkey flocks with a subtype A of aMPV vaccine were healthy, while the others that were immunized with a subtype B virus vaccine showed respiratory signs	Out of 62,418 (2.9%) of 624 tracheal samples and 18 of 20 tracheal swabs were aMPV positive. Moreover, 1 of 23 healthy and 4 flocks with signs were aMPV positive. Thirty-six samples were positive for subtype B of aMPV	Vero cells, chicken embryo fibroblast cells, and RT-PCR	Ongor et al. (2010)
	Trachea tissues and swabs were taken from 110 non-vaccinated broiler flocks distributed in different geographical regions	Eight out of 110 (7.2%) broiler farms were positive for subtype B of aMPV. Three aMPV isolates were clustered closely with Israel isolate, and the remaining 5 ones were closely related to a vaccine strain from nearby vaccinated turkey farms	RT-PCR	Bayraktar et al. (2018)

Note. AIV = avian influenza virus; aMPV = avian metapneumovirus; IBV = infectious bronchitis virus; MG = *Mycoplasma gallisepticum*; NDV = Newcastle disease virus; ORT = *Ornithobacterium rhinotracheale;* SHS = swollen head syndrome

SUSCEPTIBILITY

Species

Turkeys and chickens are the natural hosts of aMPV subtypes A and B (M. Yu et al., 2019). However, all aMPV subtypes are adapted to Galliformes, particularly turkeys. Subtype C of duck origin is well adapted to ducks; however, chickens and turkeys showed seroconversion and positive virus isolation. It has been investigated that aMPV strains of chicken origin are antigenically closely related to those of turkey origin (Cook et al., 1993). Chickens showed less frequency of aMPV infection, which may be due to

the low shedding capability of this species, as the virus tends to cluster at the temporal and geographical levels (Brown et al., 2019). Chicken flocks are also susceptible to subtype B of aMPV, and they showed seroconversion without signs or shedding of subtypes A and C of turkey and duck lineages. Despite the tropism of subtype C for ducks, chickens could be infected by this subtype (Wei et al., 2013). Turkeys are susceptible and transmit all aMPV subtypes except for subtype C of the duck lineage (S. Sun et al., 2014). They showed clinical signs following an experimental challenge of ducks with subtype C of aMPV (Brown et al., 2019). Antibodies to aMPV have been detected in non-vaccinated, apparently healthy Pekin and Muscovy duck flocks for the first time in Egypt (Nagy et al., 2018). Jardine et al. (2018) isolated subtype C from wild waterfowl (37-44%) in Canada. Recently, Tucciarone et al. (2022) in Italy molecularly detected subtype C of aMPV in a mallard duck and concluded that the short period of the virus infection and transmission reduces the possibility of its detection and increases its significance. In Northern Italy, a mallard duck flock was found to be seropositive for subtype C at slaughter (Legnardi et al., 2021). The virus also was demonstrated in wild geese (Bennett et al., 2005).

In the USA, aMPV originated from wild bird populations and was detected in turkeys. Moreover, pheasants (Gough et al., 2001) and Guinea fowl (Cecchinato et al., 2018) are susceptible to infection and development of swollen head signs. Ostriches were found to be seropositive for aMPV (Cadman et al., 1994). In Brazil, a subtype A of aMPV has been reported in some wild populations of ducks (wood ducks, mandarin ducks, and white-faced whistling ducks), American kestrels, and white-eyed parakeets (Rizotto et al., 2019), but subtype B has been found in rock pigeons, white-faced whistling, and white-cheeked pintails (Felippe et al., 2011). The RNA of subtype C of the virus was detected in black, mallards, wood ducks, geese, blue-winged teals, shovelers, wigeons, sparrows, barn swallows, and starlings in the USA (Bennett et al., 2004; Shin, Njenga, et al., 2000; Shin, Rajashekara, et al., 2000), as well as in mallards, greylag geese, and common gulls in Europe (van Boheemen et al., 2012). Additionally, the RNA of aMPV was found in snow geese, house sparrows, blue-winged teal, earns, and round-billed gulls in Canada (Jardine et al., 2018). Seroconversions against aMPV have been detected in American crows, American coots, Canadian geese, rock pigeons, and cattle egrets in the USA (Turpin et al., 2008). The recent results of aMPV sequencing revealed the presence of 2 new subtypes in a monk parakeet and a great black-backed gull (Canuti et al., 2019). These subtypes may be intermediate between the avian clusters of A, B, C, and D and the human clusters of A and B (Retallack et al., 2019).

Age

All age groups can get infected with aMPV. Broilers are more susceptible to the virus infection than layers and breeders (Tamam et al., 2015). Turkeys are susceptible

for TRT infection at 3 to 12 weeks old. However, 4 to 9 weeks old turkey poults are severely affected. The aMPV tends to affect birds older than 26 days of age (Franzo et al., 2020).

MODE OF INFECTION AND TRANSMISSION

Infection with aMPV is most likely horizontal (airborne) via aerosol or dust particles (Alkahalaf et al., 2002). The virus is highly infectious and characterized by a rapid spread. Direct contact between susceptible birds and infected, contaminated objects is the main route of aMPV transmission (Alkahalaf et al., 2002; Cha et al., 2013). However, the short releasing period of the virus from the infected birds allows for a quick absence of it from the surrounding environment (J. Sun et al., 2014).

Wild and migratory free-living birds are another important means of aMPV transmission (Shin et al., 2002). Bennett et al. (2004) found a 95% nucleotide sequence identity between the strains of aMPV isolated from wild birds and those isolated from turkeys. Wild birds can spread the virus from one region to another. For instance, migratory birds have been incriminated in the early immigration of aMPV from South Africa to European countries (M. Yu et al., 2019; S. Sun et al., 2014). Therefore, free-living birds may act as virus reservoirs for commercial poultry flocks (Jardine et al., 2018). Since migratory birds may have repeated contact with domestic birds in farming regions, a particular correlation has been found between aMPV isolated from wild and domestic birds.

Vertical transmission of subtype C of aMPV has been documented in specific pathogen-free laying turkey hens, as the virus has been isolated following experimental contamination of eggs up to 7 days post-infection (PI) (Cook et al., 2000; Jones et al., 1988). However, this pathway of virus transmission may be short-lived with slight importance (Ganapathy et al., 2007).

Mechanical transmission of aMPV via vectors, including contaminated feeders, drinkers, litter, bedding materials, people, and vehicles, is considered an indirect transmission (Alkahalaf et al., 2002).

PATHOGENESIS

The pathogenesis of aMPV is affected by several factors, including the degrees of macroscopic and microscopic damage, immunity, virus shedding, and clinical outcomes (Cha et al., 2013).

After the respiratory affection of the bird with aMPV, the target tissue of the virus is mainly the upper respiratory tract epithelial layer. Moreover, the virus will likely spread in layers and breeders from the respiratory tract to the reproductive organs.

The attachment of aMPV to the epithelial cells is mediated by the G-protein, while the envelope of the virus fuses with the cell membrane of the host through the F protein. Following that, the virus's genome is released in the host cell cytoplasm with a rapid and multiple propagation or multiplication of the virus (Q. Yu et al., 2013). An attenuated or virulent strain of aPMV replicates in the upper respiratory

tract, followed by a short stage of viremia and persistence of the virus for almost 10 days PI (Van de Zande et al., 1998). In this stage, the virus shows fast dissemination and shedding in the surrounding environment. The clinical signs usually appear to concur with the viral shedding and may be first detected during 2 to 10 days PI, while the severity of the signs is the greatest between 5 to 7 days PI (Choi et al., 2010). Cook (2000) reported on the limited replication of aMPV in the trachea, lung, or other tissues following natural infection.

For routine isolation of aMPV, the samples should be taken from day 1 till 10 PI. However, the viral genomes could be present in the nasal cleft swabs for up to 28 days PI. When layering turkeys with aMPV, the virus can be detected in the respiratory and genital tracts for up to 9 days after infection. It poses a significant challenge in terms of replication and detection. The presence of virus carriers is limited as the viral shedding is very short. Gharaibeh and Shamoun (2012) correlated the presence or absence of aMPV in the respiratory mucosa with the existence or recovery of microscopic lesions, respectively. The histopathological and immunohistochemical results showed that on the second day PI, aMPV could replicate in the turbinates tissues causing serious rhinitis and increased glandular activity. Besides, the virus could induce epithelial damage and decilliation, congestion, and infiltration of mononuclear inflammatory cells and intracytoplasmic eosinophilic inclusions in the ciliated epithelial cells and submucosa. However, on the third and fourth day, PI, catarrhal rhinitis, mucopurulent exudate, exfoliation of the epithelium, and moderate to severe submucosal mononuclear inflammatory cell infiltration could be observed. Temporary lesions were seen in the trachea, with few or no lesions in the conjunctiva and the other organs (Majó et al., 1995).

It was detected that transient immunosuppression of birds may occur during the acute stage of aMPV infection (Timms et al., 1986). Multiplication of the virus in the cilia of the upper respiratory cells reduces the amount of mucoid secretion and consequently enhances bacterial replication (Jirjis et al., 2004). Moreover, the virus could reduce the thymus weight in turkey poults (Timms et al., 1986). The early stage of aMPV infection may inhibit the later efficacy of the turkey hemorrhagic enteritis virus vaccine and induces immune suppression of vaccinated birds (Chary, Rautenschlein, et al., 2002).

PATHOLOGY

The severity of clinical signs, mortality rate, and aMPV lesions is affected by other infectious and non-infectious complications (Figure 1).

The incubation period of aMPV usually ranges from 3-7 days (Jones et al., 1987). Some infected cases show a sudden onset of signs and rapid virus transmission. An acute, highly contagious upper respiratory disease is usually associated with aMPV infection. Affected birds show unilateral and/or bilateral swelling of the infraorbital sinuses, facial edema, and accumulation

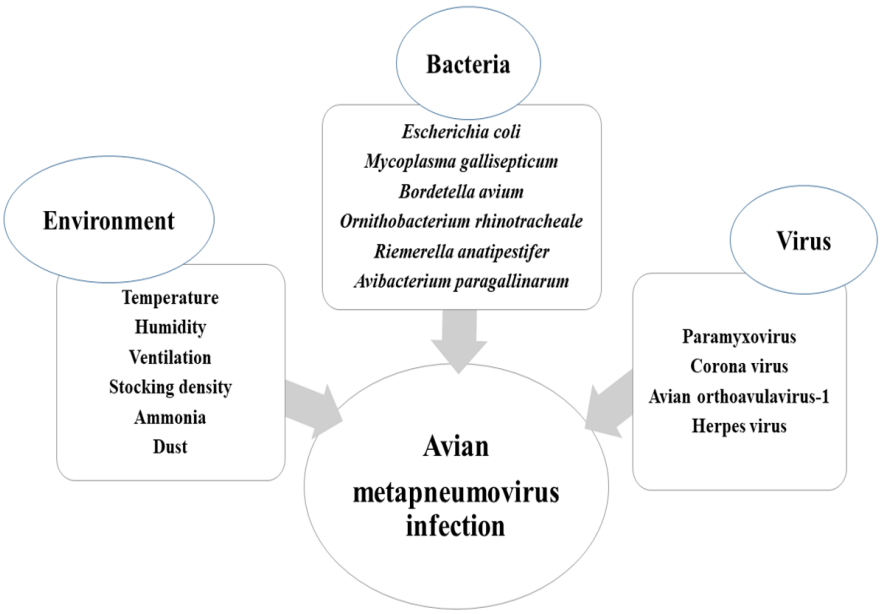

Figure 1. Factors affecting the severity of avian metapneumovirus infection in poultry flocks

of discharge in nostrils, sinuses, and eyes (Jirjis et al., 2002). Snicking, sneezing, periorbital and submandibular edema, and watery frothy to mucopurulent nasal and ocular discharges are the upper respiratory signs that are mostly observed, especially in young birds (Pringle, 1998). Coughing, gasping, dyspnea, and rales can also be detected in the later stages, especially with complications (Jones et al., 1988). General signs, including depression, anorexia, ruffled feathers, and reduced growth and egg performance parameters, are usually associated with respiratory manifestations (Seifi & Boroomand, 2015). Besides the mild respiratory manifestations, aMPV-affected adult layers may exhibit a 10–40% decrease in egg quantity and changes in eggshell quality (Sugiyama et al., 2006). It has been documented that chickens with SHS may show upper respiratory manifestations, cerebral disorientation, torticollis, and opisthotonos (Morley & Thomson, 1984). Concurrent infection with aMPV and IBV may be associated with reduced fertility and orchitis in roosters (Villarreal et al., 2007).

Infection with aMPV is generally characterized by high morbidity (40–100%) and low mortality (1–5%) rates (Sun et al., 2014b) depending on the presence of many factors, including the age of birds, secondary infections, and constitution of the affected flock (Umar et al., 2019). For instance, the mortality rate ranges from 0.5% in adults to 80% in young turkeys (Van de Zande et al., 1998). In cases with concomitant secondary bacterial infection, the mortality rate reached 90% (Awad et al., 2014).

Sudden onset and rapid recovery of aMPV could be observed within 7–10 days

PI, especially in experimentally infected birds with a good constitution and absence of complications (Jones et al., 1988). However, a prolonged disease course for several weeks may be seen in cases reared under poor hygienic conditions and other infections (Zuo et al., 2018). It is assumed that there is no latent or carrier status, and the shedding time of aMPV virus is limited. Without complications, aMPV could be re-isolated from infected birds only for a few days under natural and experimental conditions (Bäyon-Auboyer et al., 1999; Choi et al., 2010). However, in complication, the virus could be detected for up to 7 days following infection.

Differences in replication ability and virulence of aMPV subtypes have been noticed following experimental infections of ducklings, turkeys, and chickens (Aung et al., 2008; dos Santos et al., 2012). For instance, the challenge of domestic chickens with aMPV revealed less severe clinical signs than those in turkeys. Chickens seem vulnerable to experimental but not natural infection with the USA "Colorado" strain or subtype C of aMPV (Cook et al., 1999). Moreover, this subtype in ducks may induce respiratory manifestations and a drop in egg production (Toquin et al., 2006). It is important to note that some aMPV infected flocks did not show any respiratory manifestations, but serological evidence of specific neutralizing antibodies indicates infections in the infected birds (Owoade et al., 2006).

The post-mortem lesions of aMPV revealed the presence of excessive exudates in the respiratory tract, which represented rhinitis, conjunctivitis, sinusitis, laryngitis, and tracheitis. Lower respiratory infections, including airsacculitis, pneumonia, pericarditis, and perihepatitis, could be observed in the presence of concomitant bacterial infection (Zuo et al., 2018). Furthermore, osteomyelitis and subcutaneous head edema may be noticed (Cha et al., 2007; Jirjis et al., 2002).

The histopathological examination of aMPV-affected tissues revealed the presence of necrosis and damage of upper respiratory mucosa, loss of cilia, severe infiltration with inflammatory or lymphoid cells in the submucosa, and substantial tracheitis (Cha et al., 2007; Chary, Rautenschlein, et al., 2002). The virus or the intracytoplasmic inclusion bodies could be seen in the epithelial cells of the respiratory tract following the second or third day of PI.

LABORATORY DIAGNOSIS

The observation of signs and post-mortem lesions of aMPV infection can be considered a preliminary diagnostic step for the diagnosis**.** However, many similar bacterial, viral, and fungal diseases may be confusing. Therefore, the laboratory diagnosis is very important to confirm the aMPV infection.

The aMPV diagnosis relies on virus detection using molecular techniques and serology. Sometimes, isolation of aMPV from chicken tissues is harder than from turkey tissues, possibly due to secondary *E. coli* infection concurrent with SHS. Moreover, other viruses in samples may overcome AMPV propagation during the isolation process on tissue cultures.

Isolation and Identification

The sampling time is crucial, as aMPV is only detected in the upper respiratory organs in the early first week of infection. Sample collections for virus isolations should be done during the early phase of the infection because the virus is present in the sinuses, choanal clefts, and turbinates for a very short time. It is why early sampling in acute infection is commended (Jones et al., 1988). The isolation process of aMPV, a few days of PI, may be difficult because the secondary bacterial infections with the short shedding period of the virus create difficulties in the isolation process (Cook et al., 2001). Swabs from the secretion and tissues of the upper respiratory organs and trachea in the acute phase of aMPV infection are the samples of choice (Cook & Cavanagh, 2002). The virus could be isolated from the trachea, lungs, and sometimes visceral tissues of the infected young turkeys. Successful isolation of the virus from birds in chronic stages of infection is rare. Samples should be kept on ice and immediately transported or frozen for further processing.

Samples could be processed for the conventional isolation techniques. Primary isolation of aMPV could be applied through yolk sac inoculation of 6–8-day-old specific pathogen-free embryonated chicken or turkey eggs (Buys et al., 1989). Positive AMPV samples show stunting and few deaths of the embryos within 7–10 days. However, the virus may need 2 to 9 embryo passages to induce embryonic lesions and deaths.

Moreover, the yolk sac membrane homogenate could be inoculated on a specific tissue culture to detect the viral cytopathic effect (Coswig et al., 2010). However, this method for isolation is laborious, slow, expensive, and needs successive repeated passages to detect the virus. The tracheal organ culture from turkey embryos or turkey poults (Cook & Cavanagh, 2002), chicken embryo fibroblast cells (Coswig et al., 2010), Vero cells (Shin et al., 2002), chick embryo rough cells (Dani et al., 1999), and QT35 quail cells (Coswig et al., 2010) have been used for conventional isolation of aMPV. The virus may take 7 days on the primary passage (Jones et al., 1991), but mostly, cultures should be kept under observation for 11 days with 7 to 8 blind passages (Coswig et al., 2010) to produce ciliostasis and a characteristic cytopathic syncytia formation. Immunofluorescence and staining could be done for confirmation of cryostasis (Bhattacharjee et al., 1994).

Because of the fastidious nature of aMPV, molecular techniques are now applied as alternatives to the traditional isolation methods for rapid virus detection (Mo et al., 2022). Reverse transcription-polymerase chain reaction (RT-PCR) can be used as a highly specific, sensitive, and rapid technique for the detection and subtyping of aMPV from mouth, nose, and tracheal swabs (Marianna et al., 2019; Lemaitre et al., 2018). The primer sequences for the RT-PCR have been designed using targeted M, F, N, and G genes (Bäyon-Auboyer et al., 1999). Isolates of aMPV are molecularly heterogenic. Therefore,

most RT-PCR techniques are subgroup-specific and cannot identify all subgroups (Pedersen et al., 2000). Primers should be directed to the conserved regions of N and G genes to obtain broader specificity and to detect all subtypes (Lwamba et al., 2005). Sequencing techniques can confirm the PCR product identity. Quantitative RT-PCR has also been applied to detect the viral load in a sample (Guionie et al., 2007). Virus sequencing using RT-nested PCR or restriction endonuclease digestion is essential for differentiating between the vaccine and field virus strains (Listorti et al., 2014) and detecting subtypes A and B viruses (Cook & Cavanagh, 2002). The subtype C of aMPV was detected by isolation and RT-PCR up to 6 days PI of 3-weeks-old turkeys (Jirjis et al., 2000).

Other techniques, such as immunohistochemistry staining (Shin et al., 2002), immunofluorescent assay (Cook & Cavanagh, 2002), and *in situ* hybridization (Velayudhan et al., 2005) have also been applied for the detection of aMPV. Although monoclonal antibodies are used to characterize and detect subtypes A and B (Cook et al., 1993), they are laborious, time-consuming, and costly (Cook & Cavanagh, 2002).

Serological Tests

The success of aMPV isolation from turkeys displaying severe chronic symptoms is rare (Naylor & Jones, 1993). It is important to note that the serological response to aMPV infection in chickens is weaker than in turkeys. However, serology is the most common diagnostic tool for the virus, especially in non-vaccinated flocks where seroconversion is a clear indicator of field virus contact (Cook, 2000). Moreover, serology is a common detection method due to difficulties isolating and identifying aMPV.

Some serological tests such as enzyme-linked immunosorbent assay (ELISA) (Ali et al., 2019; Nagy et al., 2018; Zuo et al., 2018) and serum neutralization (SN) (Kapczynski et al., 2008) test is used for serological monitoring and screening of antibodies against aMPV. Commercial ELISA kits containing aMPV-specific monoclonal antibodies have broad specificity and sensitivity for every subtype in various avian species (Aly et al., 1997). Some commercial ELISA kits can detect subtypes A and B of aMPV without differentiation, as both belong to a common serotype (Toquin et al., 1996). However, ELISA kits cannot detect antibodies to aMPV subtype C with subtypes A and B antigens. Choi et al. (2010) suggested that detecting antibodies to aMPV infection in layer chickens using yolk ELISA may be an appropriate substitute for serum. It has been found that there is a close relation between ELISA and other serological tests but subtypes A and B of the virus showed a cross-reaction in the SN test (Cook & Cavanagh, 2002; Jones et al., 1988). The commercial ELISA kit detected aMPV during the first 10 days of PI, while the competitive ELISA started to detect antibodies as early as 5 days of PI (Choi et al., 2010). The competitive ELISA showed specificity and sensitivity of 100 and 98.0%,

respectively, compared to the SN test (Choi et al., 2010).

Additionally, indirect immunofluorescence, virus neutralization, and immunodiffusion tests may be applied to tissue sections to detect specific aMPV antibodies. It is well known that aMPV does not cause hemagglutination of red blood cells. Thus, the hemagglutination-inhibition test cannot be used to detect specific antibodies.

THE IMMUNE RESPONSE AGAINST AMPV INFECTION

Both the humoral and cell-mediated immune response plays a role in aMPV infection. The immune reaction against aMPV in broiler chickens differs from that in turkeys. Experimental infection of broiler chickens with subtypes A and B of aMPV of turkey origin provoked a mild respiratory sign, correlated with the induction of local and systemic virus-neutralizing specific antibodies (Rautenschlein et al., 2011). The neutralizing antibodies began to rise at the peak of signs (6 days PI), while IgG-ELISA titers were high between 24 and 28 days PI (Rautenschlein et al., 2011). The levels of specific IgA-ELISA and neutralizing antibodies in tracheal washes decreased by 10 and 14-days PI, respectively, which may clarify the re-infection with the field AMPV (Rautenschlein et al., 2011). Both subtypes A and B induced up-regulation of the nasal interferon-γ mRNA expression but only subtype A enhanced this expression in the Harderian gland of the eye. Moreover, aMPV-infected broiler chickens showed CD4 and T cells in the Harderian gland, while turkeys showed increased CD8alpha and cells at 6-day PI (Rautenschlein et al., 2011). In the study of Liman and Rautenschlein (2007), the local and systemic humoral and cell-mediated immune reactions following infection of turkeys with an attenuated vaccine strain of a subtype B of aMPV and virulent strains of subtypes A and B were investigated. The results indicated that the neutralizing antibodies were seen in the tracheal washes and the serum 5–7 days PI and then declined, while the level of ELISA antibody appeared from 14–28 days PI. Moreover, subtypes A and B of aMPV infection induced humoral and cell-mediated immunity in comparison with subtype C infection.

PREVENTION AND CONTROL

Biosecurity measures and vaccination programs play an important role in preventing deaths, drops in egg production, and changes in egg quality resulting from aMPV infection. Vaccines succeeded in providing cellular and humoral protective immune responses against the virus.

Biosecurity

Good biosecurity measures should be adopted in the farms to prevent and control aMPV. Prohibit multiage sites as they are always at risk. It is important to decrease stress on the respiratory tract that may predispose birds to secondary bacterial infections. For example, controlling air

quality, ventilation, heating, and misting of dry litter is very important. Control measures should be carried out after aMPV infection, including depopulation, hygienic disposal of carcasses, washing, a reasonable period between batches, marketing restrictions, and good hygienic measures.

Vaccination

Inactivated Vaccines. Oil emulsion or water adjuvant inactivated aMPV vaccines containing subtypes A and B have been used. These vaccines provoke long-term protection of aMPV infection, replication, excretion, reduction of clinical picture severity, and prevention of egg quantity and quality changes (Hess et al., 2004; Tamam et al., 2015). They are given before laying in breeder and layer turkeys (Van de Zande et al., 2000). Intramuscular vaccination of breeder turkeys with aMPV inactivated vaccine at 30 weeks old after priming with a live attenuated vaccine at 7 days of age prevented the drop in egg production at 38 weeks of age. Also, broiler breeders and layers may be given a live aMPV vaccine at 10 to 12 weeks, followed by a killed vaccine at 16 to 20 weeks. Turkey breeders could be primed with a coarse spray of a live aMPV vaccine at 2 weeks old, followed by inoculation of the inactivated vaccine at 22 weeks old (Cook et al., 1996). However, the usual program in turkey's vaccination includes the administration of an inactivated aMPV vaccine 4–6 weeks of age following the last vaccination with a live vaccine up to 28 weeks of age but avoid the last 4 weeks before laying. Turkey poults could be vaccinated with an inactivated aMPV vaccine with promising results. In the Egyptian study of Tamam et al. (2015), the findings indicated that the locally prepared aMPV inactivated vaccine provoked significant high humoral and cell-mediated immune responses as well as protection rates up to 100% in vaccinated and challenged 3-week-old-turkey poults.

Live Attenuated Vaccines. Living attenuated vaccines against TRT infection were first described in Europe in the early 1990s. These vaccines are given for growing turkey poults to prevent the development of clinical respiratory signs in young and to prime inactivated vaccines in future breeders. Live vaccines could successfully control TRT infection (Cook, 2000). Live attenuated TRT vaccines have been derived from aMPV subgroups A and B in Europe and from a subgroup C in the USA. According to the dominant subtype of aMPV in the region, subtype B and A live vaccines may maximize protection. A single dose of live vaccine can enhance the poor immune response when compared with infection with a virulent field aMPV. These vaccines have been applied to provide excellent local and cell-mediated immune responses in the absence of maternal-derived antibodies. Accordingly, turkeys that received live attenuated aMPV vaccines showed activation of CD8+ and CD4+ T lymphocytes in both the Harderian glands of eyes and tracheal mucosa as well as production of immunoglobulin (Ig) A. Whereas, there is a minor role of humoral immune response in the protection of turkey

poults against TRT infection (Jones et al., 1992; Naylor, Worthington, et al., 1997). The development of humoral immune response and, consequently, the efficacy of the vaccination process against TRT might be affected by the maternal-delivered antibodies (Rubbenstroth & Rautenschlein, 2009; Śmiałek et al., 2016, 2021). It has been documented that the titers of serum antibodies rapidly increased during the first 7 days after immunization of turkeys having low maternal antibodies (Śmiałek et al., 2015). Some interference between maternal-derived antibodies and living TRT vaccines may occur in young turkeys with higher maternal immunity. Turkeys with maternally derived antibodies displayed low virus-specific interferon (IFN)-gamma-secreting cells after vaccination with the TRT vaccine (Śmiałek et al., 2020).

Different vaccination protocols have been applied for the live TRT vaccine. The first one is the vaccination of turkeys one day till 7 days old, while the second protocol may be applied around 3 to 6 weeks old or after 6 weeks old. Repeated vaccinations may be important to induce a prolonged immune response and to prevent interference with maternal immunity to the hemorrhagic enteritis virus vaccine that is given concurrently with the TRT vaccine.

Birds can receive live vaccines against aMPV in the form of a spray, drinking water, or eye drops. Spray or drinking water routes gave satisfactory protection against the development of the clinical picture compared with the ocular-oral route (Ganapathy et al., 2010).

Subtypes A and B of aMPV could be detected in immunized and non-immunized birds. Moreover, a vaccine containing a subtype A strain may become virulent and distributed in turkey flocks inducing typical TRT signs (Lupini et al., 2011). Some reports showed that live vaccines against TRT may return to their virulence after repetitive passages in susceptible hosts (Catelli et al., 2006; Lupini et al., 2011). Besides, mutation of the field aMPV in response to sustained vaccination is evident (Catelli et al., 2010). Some variant strains of aMPV showed an increased capability to resist the immunity provoked by the used vaccines (Catelli et al., 2010). Formulating new vaccines that do not revert to virulence has gained a great effort. Accordingly, new methyltransferase-defective live attenuated aMPV vaccines have been developed to reduce the viral return to virulence and stop the emergence of field strains that may disturb the vaccine immunity (Y. Zhang et al., 2016).

The cross-protection between the live aMPV vaccine and the subgroups A or B of the challenging virus has been detected (Velayudhan et al., 2005). Vaccines of aMPV containing subtypes A and B gave some protection against the challenge with subtype C but not vice versa (Jones, 2010). Though the presence of good cross-protection between subtypes A and B in the live aMPV vaccines (Cook et al., 1995; Toquin et al., 1996), vaccinated birds still show the disease in different countries with high stocking density (Catelli et al., 2010; Chacón et al., 2011). Unfortunately, some studies in Brazil and Italy revealed that the

current vaccines are not completely efficient against the novel isolates of aMPV (Banet-Noach et al., 2009). In Brazil, dos Santos et al. (2012) found full heterologous protection induced by a live vaccine containing subtypes A and B of aMPV, and they expected that subtype A replicated less efficiently than the subtype B isolates. Moreover, the field viruses may multiply in the immunized birds and shed into the environment.

Infections with aMPV are caused in different ways, including infection by a field subtype that is not involved in the used vaccine (Banet-Noach et al., 2005, 2009), genetic variations between the vaccine and the field strains causing immune evasion (Banet-Noach et al., 2009; Catelli et al., 2010), or insufficient vaccine dose that leads to returning the virus to its virulence (Catelli et al., 2006). Some reports showed a genetic deviation between the field aMPV of subtype B origin and the vaccine strain, which resulted in the absence of homologous protection (Banet-Noach et al., 2009; Catelli et al., 2010). The field virus could overcome the immune response provoked by the vaccine-virus strain owing to the variation in the amino acids between both viruses in the G gene products and SH, respectively. Although a live aMPV vaccine containing subtype B gave 100% protection against clinical disease, one of 10 challenged chicks was positive by virus isolation or RT-PCR after challenge at 21 or 49 days of age (Ganapathy & Jones, 2007).

Some live vaccines may contain aMPV and other viruses such as IBV or NDV (bi or tri-valent vaccine). The interaction between these viruses in one vaccine has been studied. Cook et al. (2001) found that the IBV vaccine interfered with the replication of aMPV vaccine due to the competition of both viruses for the respiratory cell receptors and consequently causing the damage of cells and the reduction of aMPV replication. To avoid the problem of vaccine interference in chickens, live aMPV vaccines should be given at different intervals from IBV vaccines. Besides, dual vaccination against aMPV and NDV induced temporary suppression of aMPV replication, but the virus was found for up to 24 days post-immunization of chickens (Ganapathy et al., 2005). Therefore, immunization against NDV and aMPV did not affect their efficacies (Jones, 2010). Despite other reports showing that the produced protection by live vaccines of IBV, NDV, and aMPV did not cooperate when the vaccines were given concurrently in dual or triple mixtures, the humoral immune response to aMPV may be insufficient (Awad et al., 2015). In addition, giving live vaccines containing subtype B of aMPV, NDV, alongside the classical and variant strains of IBV vaccines, to maternal antibodies-positive day-old broiler chicks did not restrict any protection produced against these viruses (Ball et al., 2019).

Recombinant and DNA Vaccines. Recombinant and DNA vaccine, which encodes fusion glycoprotein (F) of aMPV and a fowlpox virus, has been produced and successfully investigated in turkeys

(Qingzhong et al., 1994). Reduction of clinical manifestations, post-mortem lesions, and the virus multiplication in the respiratory system was reported after vaccination and challenge with the virulent aMPV. The same results were also observed after the vaccination of turkeys with DNA vaccines containing N protein (Kapczynski & Sellers, 2003). Hu et al. (2011) demonstrated that a single dose vaccination of turkeys with a recombinant NDV, encoding glycoprotein G of subtype C of aMPV, provoked moderate immune response and partial protection against aMPV but satisfactory immune response to NDV. The vector vaccine containing the F and G genes of subtype C of aMPV and NDV has been recently produced in turkeys, and the vaccinated birds showed specific antibodies against both viruses and resisted the challenges with both virulent viruses (Hu et al., 2017). Furthermore, the DNA vaccine with bacterial vectors encoding the F gene revealed significant prevention from subtype B of aMPV infection in chickens (Madbouly et al., 2014) and in turkeys (Kapczynski & Sellers, 2003). A recombinant vaccine containing subtype C of aMPV and expressing M and N glycoproteins proteins (Chary et al., 2005) and virosome (Kapczynski, 2004) showed a good immune response in vaccinated turkeys.

In addition, *in ovo*, vaccination with DNA or subunit aMPV vaccines has been shown to be an effective and practical method for protecting chicks and turkey poults against early infection (Jones, 2010).

CONCLUSION

The aMPV is an important infection that affects a wide range of domestic and wild poultry species with different ages. Such infections are widely distributed and recorded in many continents and countries worldwide. Adverse effects on the growth and egg-laying performance parameters have usually been recorded in different aMPV infections, particularly environmental and infectious complications. Despite the development of inactivated, live, and recombinant aMPV vaccine, the different subtypes of the virus are still circulating in the commercial broiler and turkey production system. Therefore, regular surveillance and monitoring of the circulating aMPV and adotping strict biosecurity measures are essential for preventing such infection. Besides, preventing predisposing or concurrent infections is a must to avoid increasing the incidence and severity of aMPV infection.

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Molecular Characterisation of Partial Structural Genes of Fowl Adenovirus Serotype 8b UPMT1901 Field Strain Isolate Associated with the Inclusion Body Hepatitis in Malaysia's Commercial Broiler Chickens

Bahiyah Azli[1], Nur Farhana Salim[1], Abdul Rahman Omar[3], Mohd Hair-Bejo[3], Norfitriah Mohamed Sohaimi[4] and Nurulfiza Mat Isa[1,2]*

[1]Laboratory of Vaccine and Biomolecules, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Pura Malaysia, 43400 Serdang, Selangor, Malaysia
[3]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[4]Department of Veterinary Medicine, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Fowl adenovirus (FAdV) is reported to pose a severe risk to the poultry industry, affecting food and nutrient security nationally and globally. FAdV is identified as the primary pathogen for inclusion body hepatitis (IBH) disease in avians during outbreaks in farms. Numerous interventions have been employed to reduce the chicken's mortality rate in future outbreaks, such as local autogenous vaccine production that has yet to be successfully commercialised. Fibre and hexon protein are two out of the three major components of the adenoviral capsid, identified to contribute towards FAdV virulence. Hence, this study aims to determine the fibre and hexon gene molecular changes of a local isolate, FAdV UPMT1901, in Malaysia's recent IBH outbreak and identify the evolutionary relationship with known FAdV strains. Propagation of FAdV UPMT1901 was performed in specific pathogen-free embryonated chicken prior to genomic extraction. The genes were amplified, and the retrieved nucleotide sequences were aligned with the published FAdV sequences. The phylogenetic tree analysis showed that UPMT1901 partial fibre and hexon genes are 99% similar to other known FAdV serotype 8b (FAdV-8b) species, especially the published Malaysian FAdV-8b isolates. Interestingly, the amino

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E-mail addresses:
bahiyahazli@gmail.com (Bahiyah Azli)
nur.farhanasalim97@gmail.com (Nur Farhana Salim)
aro@upm.edu.my (Abdul Rahman Omar)
mdhair@upm.edu.my (Mohd Hair-Bejo)
fitriahsohaimi@upm.edu.my (Norfitriah Mohamed Sohaimi)
nurulfiza@upm.edu.my (Nurulfiza Mat Isa)
*Corresponding author

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acid residue analysis further supported consistent residues amongst Malaysian isolates at fibre positions T176A, Q195H, D213E, S243T, A258V, F335Y, and F353V and hexon position T422M. These findings elucidate the structural proteins' functional capacity and molecular diversity, specifically amongst Malaysian FAdV isolates and FAdV-8b, while contributing to global initiatives to establish biosecurity, such as a vaccine or antiviral production against future outbreaks.

Keywords: Adenovirus, FAdV, fibre, fowl adenovirus, hexon, inclusion body hepatitis, phylogenetic analysis, serotype

INTRODUCTION

Malaysia is among the top global consumers of poultry meats and eggs as 63 kg of poultry per capita consumption (PPC) volume was reported in the year 2019 (Kinsley, 2020), followed by a record of 66.9 kg (PPC) in the year 2021 (Department of Statistics Malaysia [DOSM], 2022b). Besides that, DOSM (2022a) has documented an annual increment of 5.6% in chicken production in 2021 compared to 2022, deeming the poultry industry significant and robust compared to other livestock such as red meat and pork. Constant outbreaks in farms are considered a major contributing factor that jeopardises the poultry industry and causes losses to the nation's revenue. For several years, pathogens such as bacteria, viruses, and parasites have been commonly isolated in commercialised farms hit with uncontrolled and sudden outbreaks. One of the viral pathogens being identified is fowl adenovirus (FAdV).

FAdV is a double-stranded DNA virus with a non-enveloped structure adenovirus. This avian-infecting pathogen is subgrouped into five species (A-E) and subtyped into 12 (CELO 1, 2, 3, 4, 5, 6, 7, 8a, 8b, 9, 10, 11) serotypes. The virus is reported to be a primary pathogen of a few common avian diseases called inclusion body hepatitis (IBH), hepatitis hydropericardium syndrome (HHS), and avian gizzard erosion (AGE) in chickens. However, interestingly, different diseases and clinical symptoms were reported with different FAdV serotypes, further indicating the serotypes' virulence capacity variability. The highly lethal HHS symptoms were commonly manifest among FAdV subgroup C–infected outbreaks (Zhang et al., 2018), while IBH was routinely seen in FAdV subgroups B, D, and E (Islam et al., 2023). In contrast, FAdV species A was generally reported as non-pathogenic (Gupta et al., 2018).

The major constituents of FAdV are fibre, hexon, and penton proteins. These three proteins are elucidated to play a massive role in the virulence of FAdV as the aetiological agent of IBH, HHS, and AGE. Additionally, the antigenic specificity among FAdV isolates was commonly determined and identified in the fibre and hexon gene regions. These major antigenic determinants located in fibre and hexon genes will be translated into the nucleus of host cells to manifest the infection's clinical signs, such as ruffled feathers, diarrhoea, huddling, and reduction in body weight.

The fibre protein is also speculated to aid the viral attachment and internalisation of the virus into host cells (Wang & Zhao, 2019). Meanwhile, the penton protein characteristics and functionality in infection are still understudied but mainly reported to enhance the interaction of cellular components and the virus-neutralising antibodies (Wang & Zhao, 2019).

Fibre protein is responsible for the antigenicity of the virus and poses type-specific epitopes. It is divided into three domains, positioned explicitly in the tail, shaft, and head domains, which specific domains carry specific features (Grgić et al., 2013). The three-dimensional structure of fibre protein is reported to be bound non-covalently and projected to the penton base, involved in viral entry during infection and compromised in the virulence variation among FAdV groups. The head (knob) domain is characterised as the receptor-binding domain (RBD) of FAdV, hence acting as the critical factor associated with adenoviral infection's virulence and tissue tropisms properties. While the shaft region constitutes the largest portion of fibre protein, its specific role towards FAdV virulence is not fully established. However, the "VYPF" residues at position 55-58 amino acid of fibre protein have been elucidated to be involved in the penton base adenoviral interaction (Grgić et al., 2014). The previous report has established that base substitution mutational changes in fibre gene sequence would result in different pathogenicity of strains (Pallister et al., 1996).

Among the three main structural proteins, hexon makes up the largest structure of the virus protein and is further divided into two major parts: variable and conserved loops. The conserved regions, divided into P1 and P2, located at the inner side of the capsid, were elucidated to provide stability for the hexon protein structure. Meanwhile, L1, L2, and L4 loops are found to be a part of the outer side of the capsid and observed to be non-conservative and vary between serotypes, exposing different surfaces to produce type-specific epitopes. Interestingly, compared to other loops, only the L4 loop is conserved and found internally in the capsid to stabilise the interface between P1 and P2 regions as hexon protein structures the global three-dimensional stability of the overall virus. Previously, the antigenic specificity region (epitope) was highlighted in the hypervariable region (HVR) of L1 and L2 of hexon, as it was reported to be the predominant target for serotype-specific neutralising antibodies during immune infection (Rux et al., 2003). Interestingly, 7 HVRs were identified within the region of three loops; four HVRs in L1, two in L2, and one in L4 (Crawford-Miksza & Schnurr, 1996). The type-specific antigenic determinants responsible for triggering an immune reaction in the host were elucidated in HVR between L1 and L2 (Niczyporuk, 2018). A previous study has also elucidated a cell-culture-adapted FAdV strain into a complete attenuation due to amino acid substitution in the variable region of the L1 loop (Majdi & Bejo, 2015).

The first FAdV in Malaysia was isolated in 2005 from an IBH outbreak and identified as the FAdV-8b strain (Hair-Bejo, 2005). To date, the mortality of IBH ranges from 10 to 30% per outbreak in Malaysian farms and continues to increase (Sohaimi, Omar, et al., 2018). To this date, FAdV pathogens isolates identified worldwide exhibit predominant nature to the reporting countries, such as FAdV-4 being the predominant serotypes in China and Japan, and FAdV-8b in Turkey, Indonesia, Malaysia, and Australia (Cizmecigil et al., 2020; Juliana et al., 2014; Majdi & Bejo, 2015; Steer et al., 2009; Wang & Zhao, 2019; Wei et al., 2019). However, suitable and efficient autogenous vaccines to combat all IBH-caused FAdV strains, especially against Malaysian local FAdV isolates, are unavailable in the market (Juliana et al., 2014). Despite being profiled with low mortality and morbidity rate, the presence of FAdV in the environment or the avian host system still poses horizontal or vertical transmission potential, respectively, which could eventually lead to continuity of problems within the avian industry (Sohaimi & Clifford, 2021). Hence, there is an urgent need for national-specific initiatives to propose a control and measure protocol for preventing future IBH outbreaks.

FAdV was established by a few researchers as an opportunistic pathogen as different strains have different pathogenicity and mortality rate against susceptible flocks (Jørgensen et al., 1995). Due to the previously discussed variations of FAdV activities, it is essential to fully understand essential gene sequences, amino acid residues, and their functional capacity. Hence, this study aims to identify the DNA sequence and amino acid changes of fibre and hexon of the newly isolated FAdV strain UPMT1901 isolate with other previous isolates, as well as establish the evolutionary relationship with other FAdV isolates reported from other countries globally.

MATERIALS AND METHODS

Isolation of the Field Strain

The liver of infected broiler chickens in the 2019 outbreak in Johor, Malaysia, were harvested and stored in Acson® International -40ºC freezer (Acson®, Malaysia) for future virus isolation in the lab. The virus isolated from the harvested liver is FAdV strain UPMT1901 isolate. The infected broiler chicken flocks were crouching and exhibiting IBH clinical symptoms such as ruffled feathers, low performance, and severe depression. These chickens were also pathologically reported to have friable, swollen, pale, and haemorrhagic livers gross lesions upon necropsy.

The collected liver tissue samples were homogenised thoroughly in phosphate buffer saline (PBS, 0.1 M, pH 7.4, Life Technologies™, USA) to produce 50% (w/v) suspension to isolate the virus. The homogenate liver suspension was centrifuged with a 32R centrifuge (Hettich Universal, Germany) at 350 x*g* for 5 min at 4ºC, post-three sets of freeze-thaw cycles. The retrieved supernatants were filtered using 0.45 and 0.22 µm polyethersulfone (PES) membrane syringe filters (Microlab Scientific Inc., China) prior to viral propagation and viral DNA extraction.

Viral Propagation in Specific Pathogen-free Chicken Embryonated Eggs

Specific pathogen-free (SPF) chicken embryonated eggs were purchased from Malaysian Vaccine Pharmaceutical® Sdn. Bhd. (Malaysia). The Committee of IACUC Universiti Putra Malaysia approved the animal experiment under the UPM/IACUC/AUP-R065/2020.

Eight-day-old SPF eggs were disinfected with 70% Systerm® ethyl alcohol solution (Classic Chemicals Sdn. Bhd., Malaysia) and proceeded with candling for viability, followed by marking the desired position for a new artificial route in the chorioallantoic membrane (CAM). The new artificial route marking was 90º from the original air sac position at the region, with fewer capillaries observed. Both original and newly marked routes were poked using a sterilised thumbtack, followed by making an artificial air sac via the suction of a sterilised rubber. Then, 0.1 ml virus supernatant from the virus isolation step was inoculated via the CAM route using a 1 cc/ml syringe (Terumo, China). The holes were sealed with thick fast-drying glue and positioned on the egg rack horizontally. An egg was inoculated with 0.1 ml of PBS during each passage, while one was non-inoculated to act as the negative control per batch. The inoculated eggs were then incubated in a sterile incubator with a 37ºC setting, and observations were made daily. The propagations were performed until a passage with 100% mortality was observed and tabulated. The total observation window frame is between 9 to 20 days, approximately until the control egg was observed with a physical sign of hatching. Dead eggs' CAM and liver organs were harvested within two days maximum to prevent significant loss of viral titre concentration.

The harvested CAM and liver organs proceeded with viral inoculum preparation using the method mentioned in the viral isolation step. The prepared 50% (w/v) suspension inoculum of CAM and liver for all passages were labelled and stored in Innova® U725 ultra-low temperature freezer -80ºC (Eppendorf, Germany) for future use such as deposition into institutes microbiology catalogue or subjected onto viral DNA extraction.

Extraction of Viral DNA

The total viral DNA extraction was performed on both viral supernatants retrieved from the field and subsequent passages from the viral propagation stage. InnuPREP® Viral DNA/RNA Extraction kit (Analytik Jena, Germany) was used according to the manufacturer guide with few optimisations. Two hundred (200) µl of freshly prepared viral inoculum (supernatant) were subjected to extraction. 200 µl of CBV/carrier mix mixture, 200 µl of viral inoculum and 20 µl of proteinase K were mixed vigorously and incubated for one hour in a shaking heat block Thermomixer® R Mixer (Eppendorf, Germany) with 400 rpm continuous shaking and 70°C setting. The remaining extraction and purification processes were followed per the manufacturer's guidelines. The final purified viral DNA was eluted into 50 µl and subjected to quantification via an

Eppendorf BioSpectrometer® (Eppendorf, Germany). Only extracted viral DNA with high concentration and good purity is further used in the amplification process.

Amplification via Polymerase Chain Reaction

The extracted viral DNA was amplified via the Polymerase Chain Reaction (PCR) method to obtain the partial gene sequence of fibre and hexon. The primers were designed with the available nucleotide sequence of FAdV-8b strain 764 (accession no.: KT862811) as the reference sequence. Only primer sets with suitable GC content, target region and optimum amplicon length were chosen. The primer sets were: FibreF/FibreR: 5'-AGCCACTACCGAGGGAATA-3'/5'-GCTCCAACCCAGAAGGTAAA-3' and HexonF/HexonR: 5'-CAGCTCGAACGCTACATCTT-3'/5'-ACGTAGTCCTCCTTGTCTCTAC-3'. The PCR protocol was performed using the MyTaq™ Mix kit (Meridian Bioscience, USA) on a PCR thermocycler C1000 Touch™ Thermal Cycler (Bio-RAD, USA). The PCR reaction mixture of 50 µl contained 1 µl of both 20 µmol of forward and reverse primers, 150–200 ng of DNA template, 25 µl of 2x MyTaq Mix and deionised distilled water. The thermal condition protocol was as follows: 95ºC for 1 min (initial denaturation), followed by 35 cycles of 95ºC for 15 s (denaturation), 55ºC for 15 s (annealing), 72ºC for 30 s (elongation), and final elongation at 72ºC for 1 min. Each gene was run with three technical replicates. The final product of the amplicons was then further subjected to gel electrophoresis for validation. A known FAdV-8b UPM11134CELP10 isolate was selected as the positive control of the concurrent PCR protocol.

The gel electrophoresis was done onto partial fibre and partial hexon gene amplicon products by running 1% gel agarose under 70 V, 400 A settings for 45 min. The 1% gel agarose was prepared using 1 g of HyAgarose™ LE agarose powder (Hydragene, China) in 100 ml 1x Tris-acetate-EDTA (TAE) (pH 8.0, 1st BASE™, Singapore). One microliter RedSafe nucleic acid staining solution (Chembio, UK) was mixed with the prepared gel agarose solution when the room temperature was reached, followed by gel casting. During electrophoresis, 1 µl 6x blue/orange loading dye (Promega®, USA) was mixed with 5 µl of the PCR products before loading into the well alongside 5 µl of 1 kb DNA ladder (1st BASE™, Singapore). Gel Doc™ Transillumination UV XR+ machine (Bio-RAD, USA) was used to visualise the conducted gel electrophoresis.

Sequencing and Molecular Analysis

Only amplicons samples with bright and linear bands from the gel visualisation process were pooled between technical replicates of each gene and further sent for a sequencing service (1st BASE™, Singapore). These amplicons were sequenced using the Sanger sequencing method. The retrieved raw sequences were

cleaned via Sequence Scanner Software v2.0 (Thermo Fisher Scientific, USA) to ensure that only high-quality sequences with nil noise were subjected to further molecular analysis. All clean nucleotide sequences were assembled and analysed using BioEdit v7.2.5 (Alzohairy, 2011), followed by a translation into amino acid residue sequences via ExPASY online tool (Gasteiger et al., 2003). Consensus nucleotide and amino acid sequences were functionally annotated using the Basic Local Alignment Search Tools (BLAST), a bioinformatics tool available from the National Centre for Biotechnology (NCBI) (Conesa et al., 2005).

Upon functional annotation, the high-quality sequences were aligned with homologous difference FAdV species available in the GenBank database of NCBI via MEGA-X v10.2.6 software (Kumar et al., 2018). All sequences were performed multiple sequence alignment (MSA) using the ClustalW algorithm (Thompson et al., 1994) for nucleotide and amino acid residues. Additionally, phylogenetic trees for fibre and hexon were generated to analyse the relationship between isolate and other known FAdVs (Tables 1 and 2), employing the maximum-likelihood (ML) analysis with 1,000 bootstrap replicates.

RESULTS

Mortality and Gross Lesion

The inoculation of UPMT1901 viral inoculum into viable SPF chicken embryonated eggs was observed within 12 days post-infection (dpi). In every passage, the inoculated eggs were candled in a 24-hr interval. Interestingly, inoculated eggs from Passage 0 (P0) and 1 (P1) were observed with 4/4 and 15/15 death of inoculated eggs, respectively. It suggests that UPMT1901 isolate virus propagated in P0 and P1 could reach 100% mortality within the expected observation window frame of a FAdV infection in chicken embryonated eggs, further implying UPMT1901 to be pathogenic. It is also worth noting that P0 achieved 100% mortality at 10 dpi, whereas P1 reached a full-mortality profile at 7 dpi (Table 3).

Subsequently, according to Table 3, the highest mortality rate throughout the infection window time frame for both passages was observed at 4 dpi. It suggests that 4-dpi of UPMT1901 isolate in SPF embryonated eggs have the highest virulence activity compared to another dpi. Meanwhile, the mock-inoculated eggs with PBS and non-inoculated eggs in both passages are still viable at 12 dpi, ensuring the propagation passages are in a controlled environment.

Upon harvesting, all embryos in the control group were normal without lesions throughout both passages, displaying a transparent and thin CAM (Figure 1). Meanwhile, gross lesions were mainly observed in all mortalities of FAdV-inoculated eggs, especially at the CAM and liver tissues. CAM of inoculated eggs was cloudy and thickening, while the embryos' liver organs were seen as swollen and friable, with pale yellow discolouration.

Table 1

Fibre gene sequences of fowl adenovirus from all species retrieved from NCBI for phylogenetic analysis

No.	Strain	Accession number		Group	Year	Country	Reference
		Nucleotide	Amino acid				
1	Strain 8b 764	KT862811.1	ANJ02574.1	E	2016	Austria	Marek et al. (2016)
2	**UPMT1901	OQ162224	GenBank: WIF20012.1	E	2022	Malaysia	This study
3	UPM04217	KU517714.1	ANA50324.1	E	2017	Malaysia	Juliana et al. (2014)
4	UPM1137E15	KY911371.1	ATI09499.1	E	2017	Malaysia	Sohaimi, Bejo, et al. (2018)
5	UPM1137E10	KY911370.1	ATI09498.1	E	2017	Malaysia	Sohaimi, Bejo, et al. (2018)
6	UPM1137E5	KY911369.1	ATI09497.1	E	2017	Malaysia	Sohaimi, Bejo, et al. (2018)
7	UPM1137CEL35	KY305957.1	AQZ26947.1	E	2017	Malaysia	Sohaimi, Bejo, et al. (2018)
8	UPM1137CEL25	KY305955.1	AQZ26945.1	E	2017	Malaysia	Sohaimi, Bejo, et al. (2018)
9	UPM1137CEL10	KY305952.1	AQZ26942.1	E	2017	Malaysia	Sohaimi, Bejo, et al. (2018)
10	Strain HG	GU734104.1	ADE58406.1	E	2011	Canada	Grgić et al. (2011)
11	SD1356	MG712775.1	AYC35477.1	E	2018	China	Qinghua et al. (2019)
12	ON NP2	KP231537.1	AKR76204.1	D	2016	Canada	Slaine et al. (2016)
13	HBQ12	KM096545.1	AIS19830.1	D	2015	China	Zhao et al. (2015)
14	MX95-S11	KU746335.1	AOS51019.1	D	2016	Mexico	Absalón et al. (2017)
15	CH/HNJZ/2015	KU558760.1	ANV21404.1	C	2016	China	Liu et al. (2016)
16	AG234-CORR	MK572849.1	QGQ62347.1	C	2019	Japan	Schachner et al. (2019)
17	HLJFAd15	KU991797.1	APA19531.1	C	2016	China	Pan et al. (2017)
18	Strain-340	NC_021221.1	YP_007985662.1	B	2020	Austria	Marek et al. (2013)
19	40440-M/2015	MG953201.1	QCC26484.1	B	2019	Hungary	Kaján et al. (2019)
20	Isolate-340	FR872928.1	CCB84856.1	B	2012	Austria	Marek et al. (2012)

Table 1 *(Continue)*

No	Strain	Accession number		Group	Year	Country	Reference
		Nucleotide	Amino acid				
21	CELO-CORR	MK572875.1	QGQ63308.1	A	2019	Japan	Schachner et al. (2019)
22	Punjab 1	DQ864435.1	ABI20702.1	A	2016	India	Unpublished
23	Duck FJ12025	KF286430.1	-	-	2013	China	Fu et al. (2013)
24	Turkey A	AC_000016.1	-	-	2018	USA	Davison et al. (2003)

Note. - = Unrelated; ** = The sequence identified in this study

Table 2

Hexon gene sequences of fowl adenovirus from all species retrieved from NCBI for phylogenetic analysis

No.	Strain	Accession number		Group	Year	Country	Reference
		Nucleotide	Amino acid				
1	Strain 8b 764	KT862811.1	ANJ02569.1	E	2016	Austria	Marek et al. (2016)
2	**UPMT1901	OQ162223	GenBank: WIF20011.1	E	2022	Malaysia	This study
3	SD1356	MG712775.1	AYC35472.1	E	2018	China	Qinghua et al. (2019)
4	SD15-24	KY426992.1	AUB29588.1	D	2017	China	Unpublished
5	ON NP2	KP231537.1	AKR76197.1	D	2016	Canada	Slaine et al. (2016)
6	JL/1407	KY012057.1	ATG30790.1	D	2017	China	Li et al. (2022)
7	ZZ	MN337322.1	QGS70289.1	C	2019	China	Unpublished
8	SDSX1	KY636400.1	AUO29782.1	C	2018	China	Unpublished
9	SD1601/FAdV-4	MH006602.1	AZJ17899.1	C	2018	China	Qinghua et al. (2019)
10	AG234-CORR	MK572849.1	QGQ62336.1	C	2019	Japan	Schachner et al. (2019)
11	CH/GDYF/201706	MK387062.1	QDY98296.1	C	2019	China	Unpublished

Table 2 *(Continue)*

No	Strain	Accession number		Group	Year	Country	Reference
		Nucleotide	Amino acid				
12	CH/HNJZ/2015	KU558760.1	ANV21395.1	C	2016	China	Unpublished
13	Strain-340	NC_021221	YP_007985654.1	B	2020	USA	Marek et al. (2013)
14	CELO-CORR	MK572875.1	QGQ63303.1	A	2019	Japan	Schachner et al. (2019)
15	CELO	AF339914.1	AAL13217.1	A	2001	Belgium	Meulemans et al. (2001)
16	Duck FJ12025	KF286430.1	-	-	2013	China	Fu et al. (2013)
17	Turkey A	AC_000016.1	-	-	2018	USA	Davison et al. (2003)

Note. - = Unrelated; ** = The sequence identified in this study

Table 3

Tabulated cumulative mortality frequency of specific pathogen-free (SPF) chickens embryonated eggs infected with FAdV strain UPMT1901 isolate in two subsequent passages

Passage	Cumulative mortality (Days post-inoculation, [dpi])													Total death	Mortality (%)
	0	1	2	3	4	5	6	7	8	9	10	11	12		
P0	$0^a/4^b$	0/4	1/4	2/4	2/4	2/4	3/4	3/4	3/4	3/4	4/4	-	-	4/4	100
P1	0/15	0/15	0/15	3/15	11/15	11/15	14/15	15/15	-	-	-	-	-	15/15	100

Note. The data are presented in a/b representation: a = The number of infected embryos that died in the passage; b = The total number of eggs inoculated in the passage

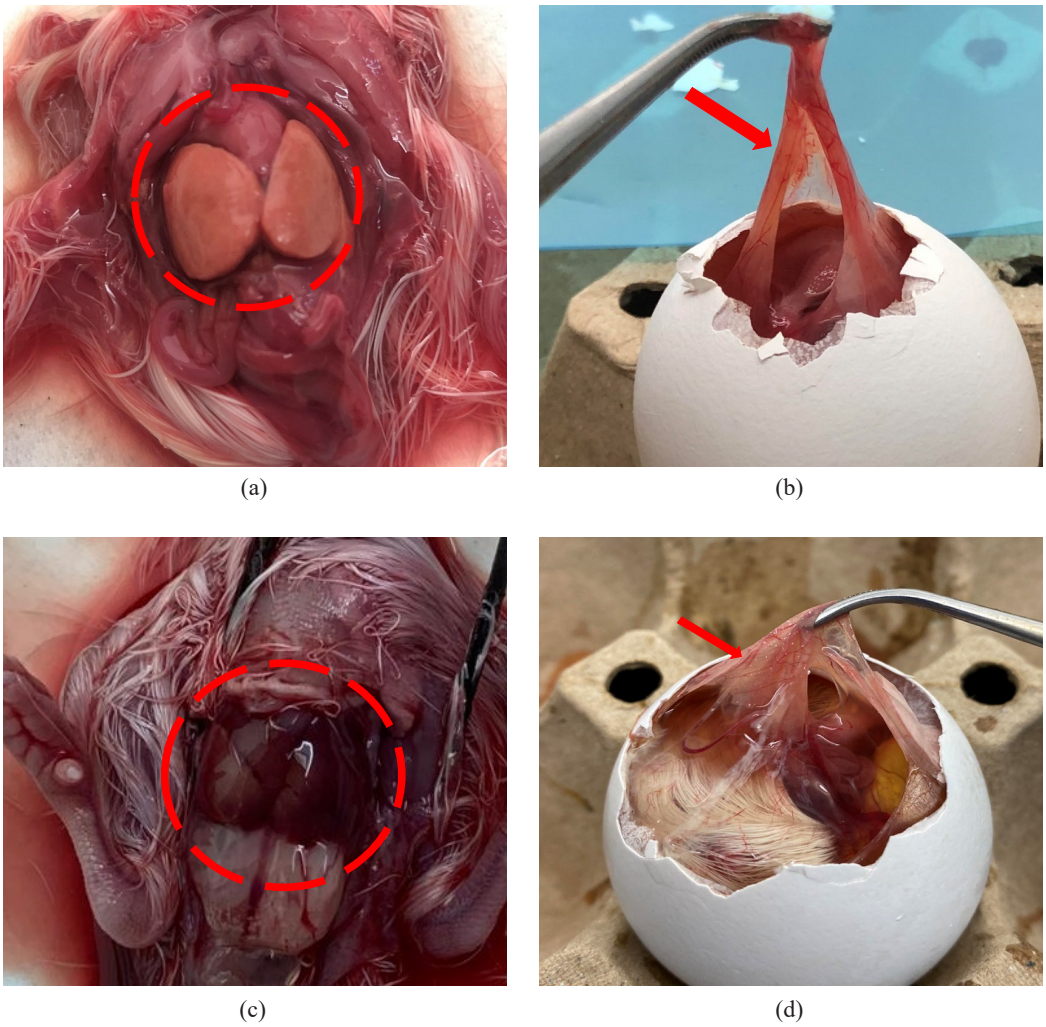


Figure 1. Gross lesion of the (a) liver and (b) chorioallantoic membrane (CAM) of FAdV strain UPMT1901-inoculated Specific pathogen-free (SPF) eggs, as well as the (c) liver and (d) CAM of phosphate buffer saline mock-inoculated SPF eggs

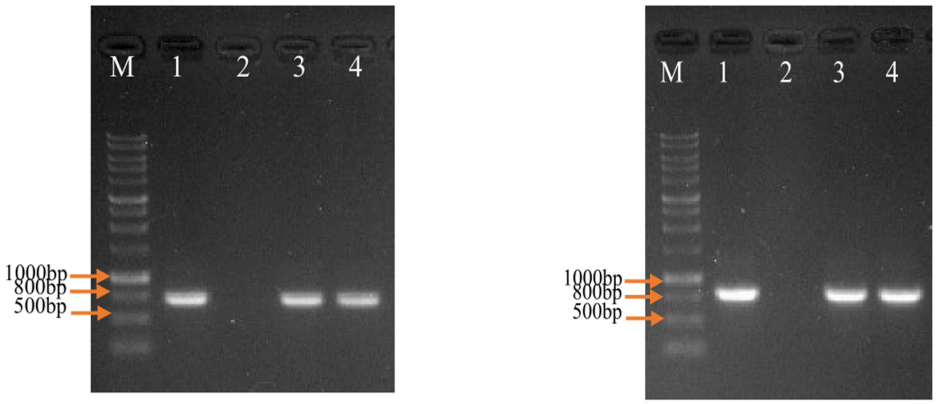
Note: FAdV = fowl adenovirus; PBS = phosphate buffer saline; SPF = specific pathogen-free

Partial Gene Prediction, Amino Acid Annotation, and Phylogenetic Analysis

DNA fragments of the expected amplicon lengths of 747 bp of partial fibre and 821 bp of hexon were successfully amplified, as observed in the agarose gel (Figure 2).

Next, only 723 bp of clean partial fibre and 789 bp of clean partial hexon nucleotide sequences proceeded to functional annotation via BLAST. According to BLAST hits of both partial genes, the nucleotide sequence revealed that the FAdV strain UPMT1901 isolate of this study has high nucleotide identity of other reported fibre genes [accession no.: KY911369.1 (99.86%), KY305954.1 (99.86%), KY305950.1

(99.86%), KY911370.1 (99.72%), KY305955.1 (99.72%), KY305952.1 (99.72%), KU517714.1 (99.72%), and MW885275.1 (99.72%)], and previous hexon gene sequences [accession no.: KU517714.1 (99.48%), KT2862809.1 (98.97%), KX258422.1 (98.71%), and MK572858.1 (98.58%)] of other Malaysian FAdV-8b isolates.



(a) (b)

Figure 2. Amplification of (a) partial fibre (747 bp) and (b) partial hexon gene (821 bp), using primer set Fibre F/R and Hexon F/R of fowl adenovirus serotype 8b (FAdV-8b) strain UPMT1901 isolate and other isolates

Note. M = 1 kbp DNA ladder; Lane 1 = FAdV-8b positive control FAdV UPMT11134CELP10F isolate (accession no.: MT525011.1); Lane 2 = No template control; Lanes 3 and 4 = FAdV-8b UPMT1901

Moreover, the phylogenetic tree analysis of both partial fibre (Figure 3) and partial hexon (Figure 4) nucleotide sequences also displayed that FAdV strain UPMT1901 isolate has a close relationship with other FAdV-8b Malaysian isolates, further supporting previous BLAST hits results. The constructed tree also reported UPMT1901 to share the same FAdV serotypes 8b subgroup ancestor into FAdV Group E.

Meanwhile, the multiple sequence alignment (MSA) performed via ClustalW of the partial fibre gene (Figure 5) presented similar and identical nucleotide sequence concurrently observed in FAdV-8b Malaysian isolates (KY911369.1, KY911370.1, KY305955.1, KY305952.1, and KU517714.1). However, a nucleotide base of UPMT1901 at 33,217 nt, which is sequenced as 'Y' (coded for any pyrimidine base), was identified, whereas the thymine (T) base was consistently reported at the same position in other Malaysian FAdV-8b isolates. Accordingly, X365F was exhibited in the UPMT1901 strain in the MSA analysis of the partial fibre amino acid residues analysis (Figure 6). Interestingly, 'X' was often discussed as a possibility of translating into more than one amino acid probability. Nevertheless, consistent amino acid substitution observed in other FAdV-8b Malaysian isolates are still present in UPMT1901, such as T176A, Q195H, D213E, S243T, A258V, F335Y, and F353V.

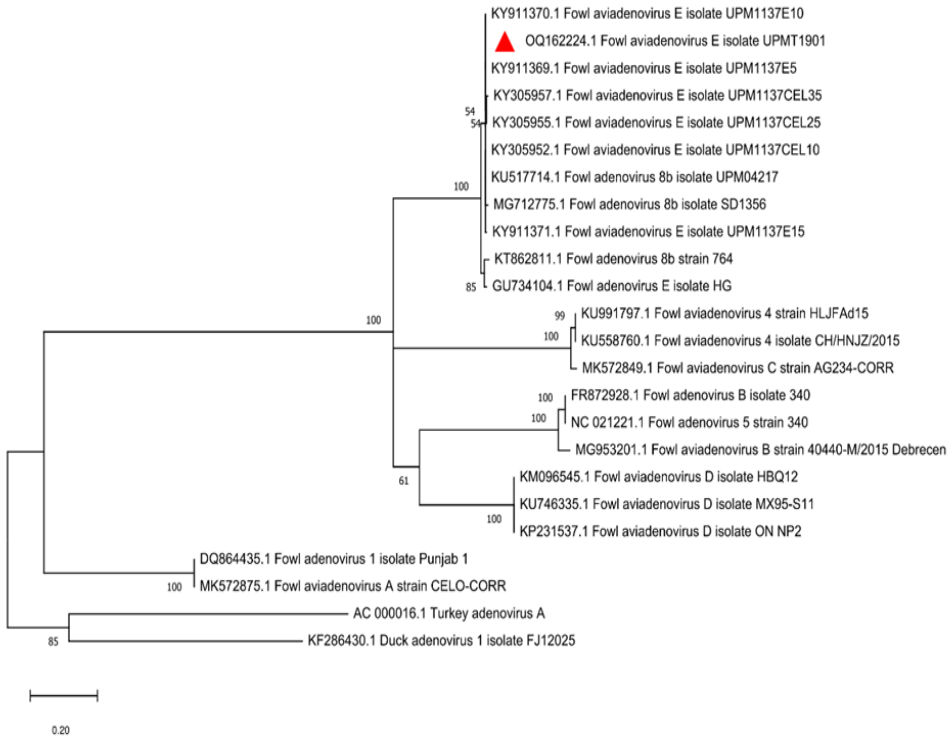


Figure 3. Phylogenetic analysis and sequence identity of partial fibre gene based on the 723 bp nucleotide in the shaft region, with selected Fowl adenovirus (FAdV) groups, performed via MEGA-X v10.2.6 software with maximum-likelihood algorithm applied with 1,000 bootstraps. The red triangle indicates FAdV strain UPMT1901 partial fibre gene

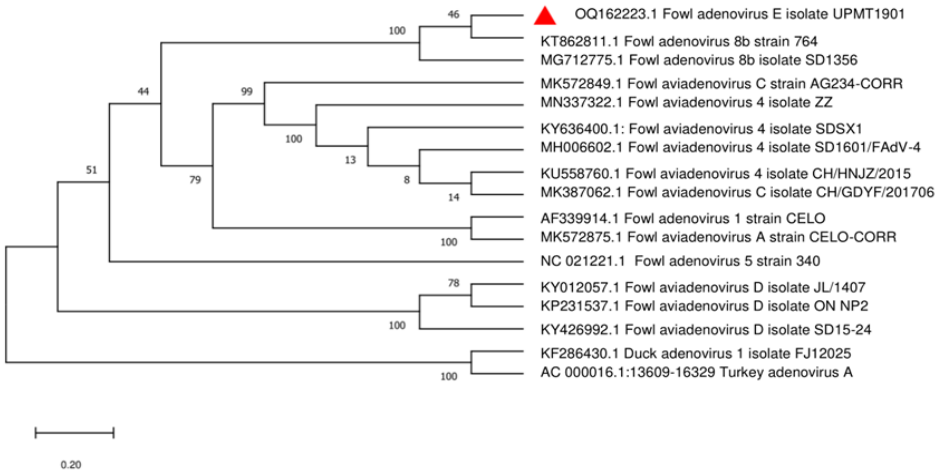


Figure 4. Phylogenetic analysis and sequence identity of partial hexon gene based on the 789 bp nucleotide in the L1 loop region, with selected Fowl adenovirus (FAdV) groups, performed via MEGA-X v10.2.6 software with maximum-likelihood algorithm applied with 1,000 bootstraps. The red triangle indicates FAdV strain UPMT1901 partial hexon gene

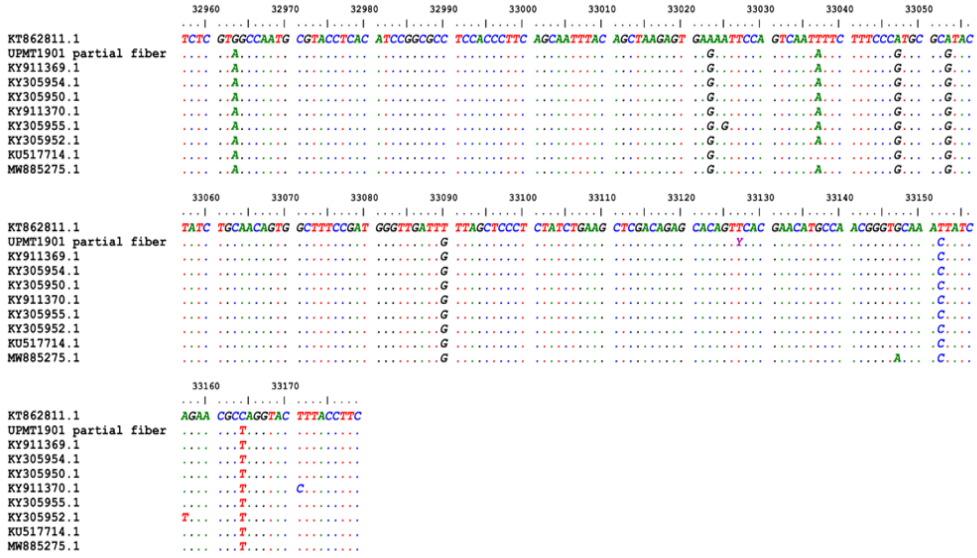


Figure 5. Multiple sequence alignment of partial fibre gene nucleotide sequence based on the 723 bp of nucleotide positioned within the shaft and head domain regions between Fowl adenovirus (FAdV) strain UPMT1901 isolate and other FAdV isolates with BLAST hits similarity of >99%, performed using ClustalW algorithm in the BioEdit software. Only nucleotide positions 32,957-33,180 nt are shown

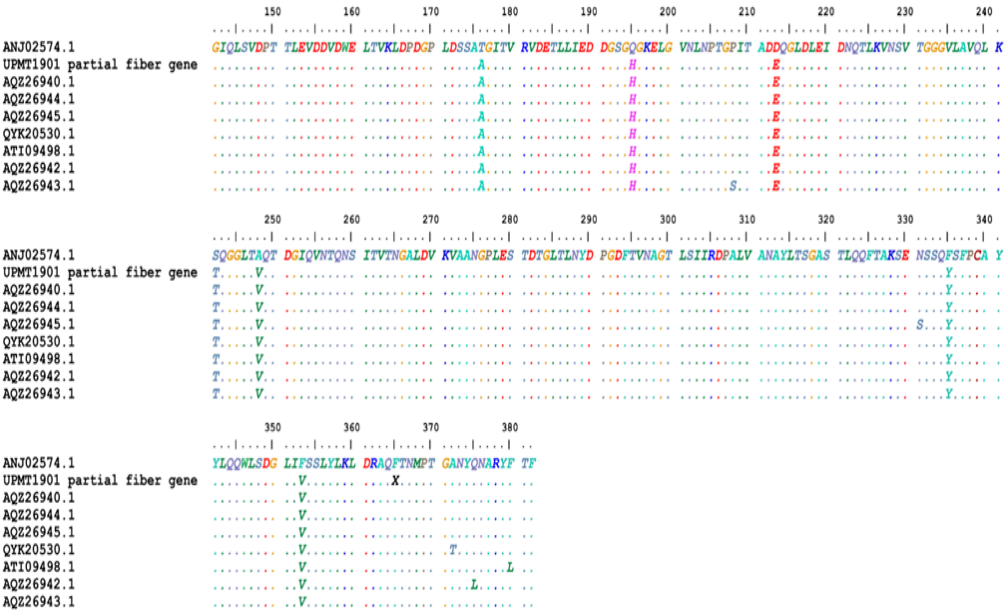


Figure 6. Multiple sequence alignment of partial fibre amino acid residues sequence based on the 241 amino acids positioned within the shaft and head domain region Open reading frame between Fowl adenovirus (FAdV) strain UPMT1901 isolate and other FAdV isolates with BLAST hits similarity of >99%, performed using ClustalW algorithm in the BioEdit software

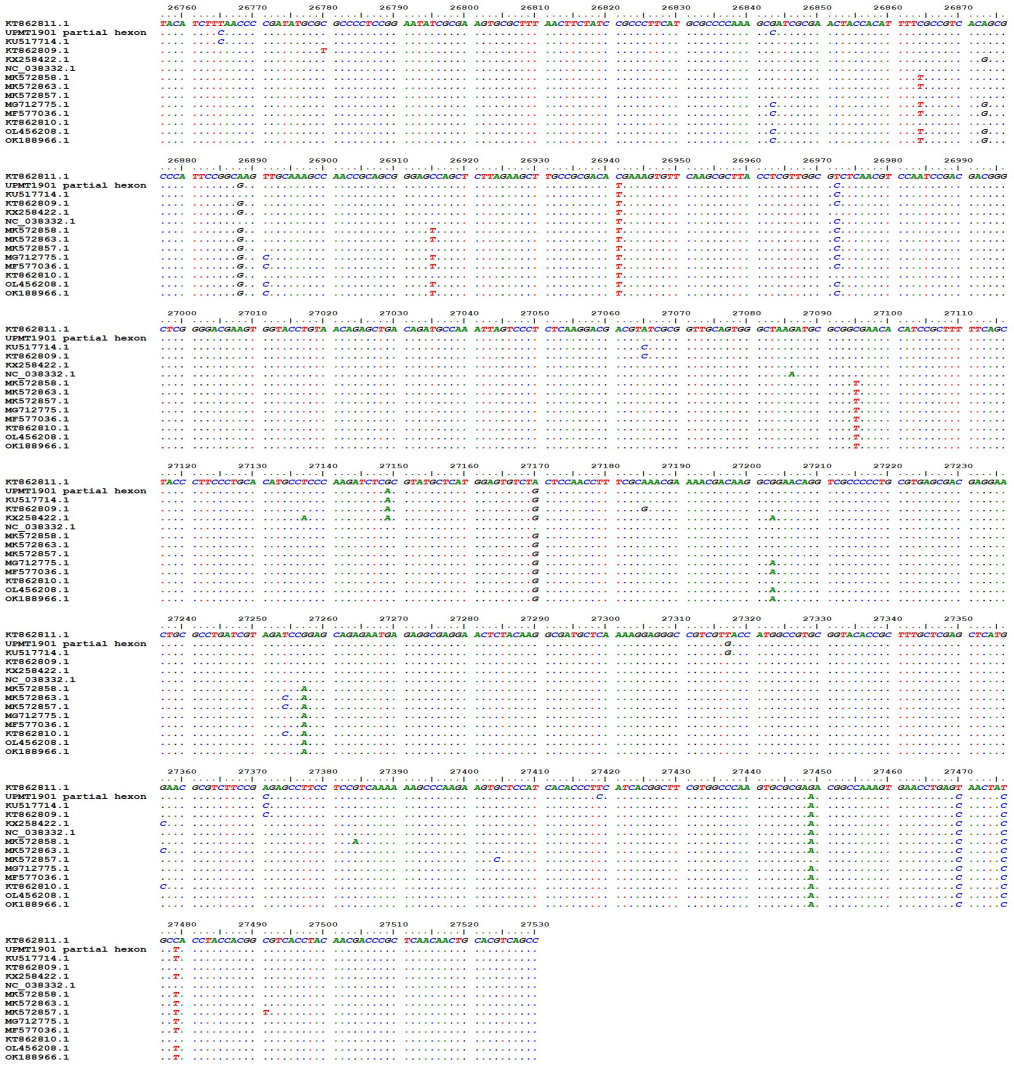


Figure 7. Multiple sequence alignment of partial hexon gene nucleotide sequence based on the 789 bp nucleotide positioned within the L1 loop region between Fowl adenovirus (FAdV) strain UPMT1901 isolate and other FAdV isolates with BLAST hits similarity of >99%, performed using ClustalW algorithm in the BioEdit software

On the contrary, the MSA analysis of both the partial hexon gene and protein of the UPMT1901 strain displayed high similarity with consistent nucleotide sequence (Figure 7) and amino acid residues (Figure 8) substitutions as observed in other Malaysian FAdV-8b isolates, specifically T422M.

DISCUSSION

The mortality pattern of FAdV-infected diseases such as HHS, IBH, and AGE significantly affects the poultry industries, nationally and globally. The loss of chicken as one of the many sources of proteins will lead to food and nutrition security, as well

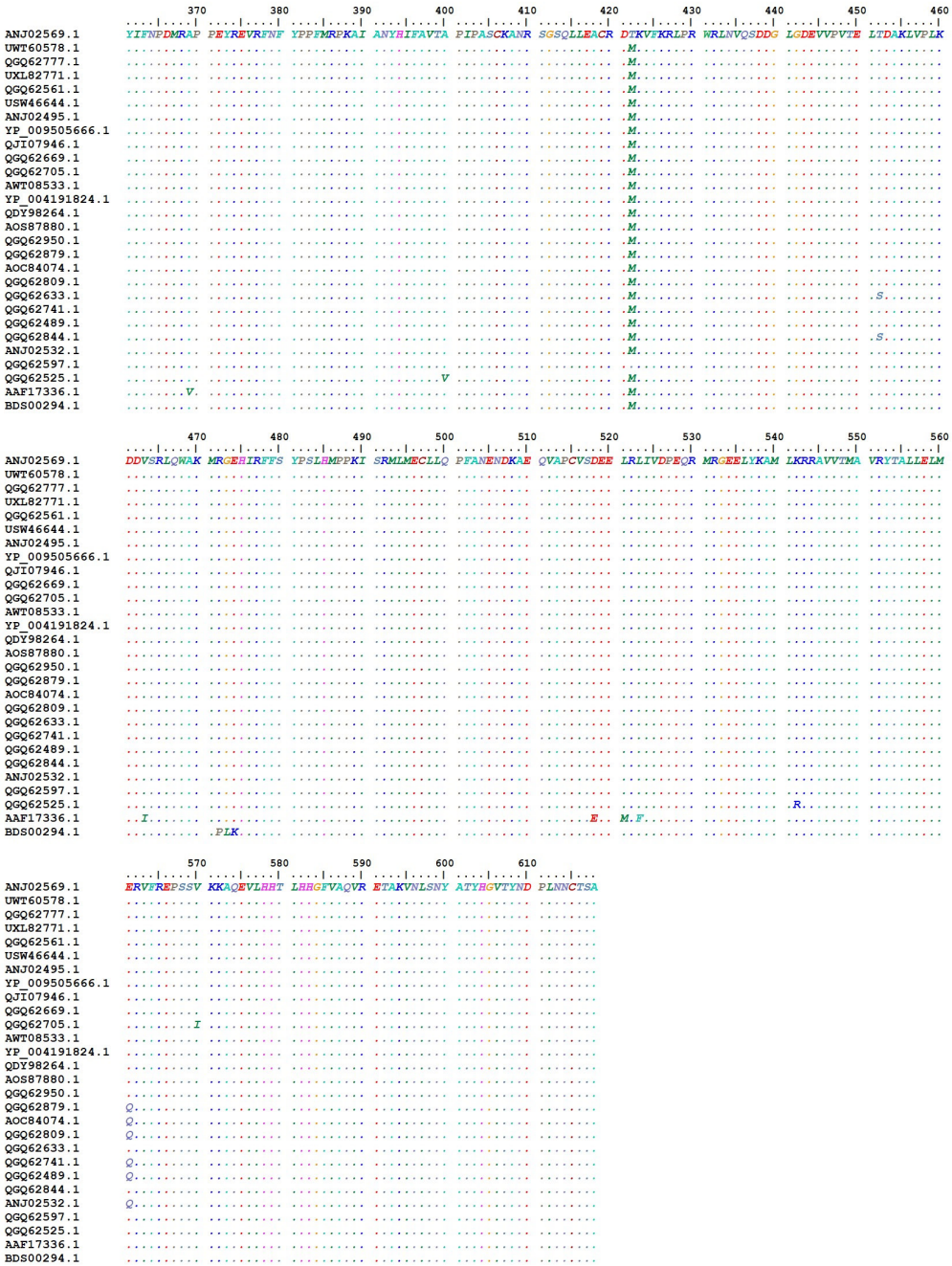


Figure 8. Multiple sequence alignment of partial hexon amino acid residues sequence based on the 263 amino acids positioned within the L1 loop region open reading frame between Fowl adenovirus (FAdV) strain UPMT1901 isolate and other FAdV isolates with BLAST hits similarity of >99%, performed using ClustalW algorithm in the BioEdit software

as food scarcity issues. Multiple records of FAdV-associated infection signs have been documented from every continent, with an increasing number of FAdV outbreaks reported for the past 15 years (Schachner et al., 2021). As tabulated in Table 3, UPMT1901 displayed 100% mortality occurrence in SPF chicken embryonated eggs in early passages as early in P0 and replicated in the subsequent passage of P1. Previous FAdV-8b UPM1137 Malaysian isolate propagation in a series of passages trial was reported to achieve 14.3% mortality (7-dpi; 1/7) in the first passage and only achieved 100% in the second passage (9-dpi; 5/5) (Sohaimi, Bejo, et al., 2018). The full-mortality occurrence observed in inoculated eggs between P0 and P1 suggests that UPMT1901 has a high virulence capacity.

Next, the designated Fibre F/R and Hexon F/R primers about FAdV-8b strain 764 isolate had successfully amplified partial regions of both fibre and hexon genes of UPMT1901, approximately 747 bp and 821 bp expected length of DNA fragments respective to each gene. However, only 723 bp of the sequenced partial fibre and 789 bp of sequenced partial hexon genes were produced after cleaning bases with high noise, further ensuring the significance and confidence in downline analyses performed in this study. As mentioned in the result, BLAST alignment outputs of both partial gene sequences also resulted in hits by FAdV serotypes 8b organisms (e-value = 0, percentage identity = 99%). This result indicates that UPMT1901 isolates retrieved from the farm outbreak with observed IBH signs are due to FAdV serotype 8b-infection, similar to previously reported IBH outbreaks cases in Malaysia (Ahmed et al., 2021; Majdi & Bejo, 2015; Mat Isa et al., 2019; Sabarudin et al., 2021; Sohaimi, Omar, et al., 2018). To date, most FAdV isolates identified in Malaysia are predominant by the FAdV-8b strain; it facilitates the veterinarians and researchers in prioritising potential target genomic composition to act as a template in autogenous vaccines or antiviral production as biosecurity to fight against local FAdV infection. Several numbers of countries with FAdV outbreaks were constantly reporting similar patterns of a predominant serotype as their nation's primary aetiological agent, such as FAdV-4 in China (Niu et al., 2022), FAdV-11 in Australia (Steer et al. 2011) and FAdV-2 in Japan (Nakamura et al., 2011), a study from the Caribbean has reported the identification of multiple serotypes within an outbreak in the same farm, with FAdV-8a, -8b, -9, and -11 isolates circulating among the low-performed chickens in the farm (Jordan et al., 2019). It suggests the probability of other FAdV serotypes emerging in Malaysian farms or currently residing in the environment is low and yet to cause an alarming outbreak.

Next, upon performing the MSA of the genes, both partial fibre and hexon exhibit similar nucleotide base substitutions with other previously published Malaysian FAdV isolates such as UPM04217 (Mat Isa et al., 2019) and UPM1137 (Sohaimi, 2018). The 723 bp of fibre and 789 bp of hexon genes phylogenetic analysis also

displayed that both partial gene sequences are closely related to known FAdV-8b isolates, especially the published Malaysian FAdV-8b isolates. This finding highlights that UPMT1901 shared common ancestors with FAdV species E serotype 8b. Although FAdV outbreak cases in Malaysia have yet to be reported with the identification of various serotypes, lack of biosecurity, such as vaccine intervention in the nation, could lead to a critical infection in the future due to uncontrolled low management husbandry ethics, such as movement of contaminated workers between poultry houses, sharing of contaminated water sources within farm perimeters and more (Butterworth & Weeks, 2010).

Next, the 789 bp of partial fibre sequence was observed to reside within the coding region of the shaft and head domain of the fibre protein. Albeit identified as FAdV-8b, there are still chances of having variety between isolates, which could lead to the rise of alterations in amino acid residues sequence upon translation as laid in central dogma protocol. As shown in Figure 6, base 'Y' is located at 33,217 nt (in reference to KT862811.1), whereas previously known Malaysian FAdV isolates were only reported with base T (thymine pyrimidine). The presence of the 'Y' base in the sequence suggested an equal chance of any pyrimidine bases, either C (cytosine) or T (thymine), to be recruited during viral replication. Hence, the expected amino acids translated from a codon TCC and TTC are serine (S) and phenylalanine (F). These amino acids differ in structures and chemical characteristics as S is a polar, uncharged amino acid (Group II), while F is identified as a non-polar, aromatic amino acid (Group I). A single base substitution mutation within genes of FAdV has the potential to alter the functional capacity of the protein performance, resulting in a variety of FAdV virulence as a primary pathogen of IBH, specifically during viral attachment and entry into the host cells (Pallister et al., 1996; Shah et al., 2017). Previous studies supported this notion as amino acid differences observed in the fibre knob domain region and hexon L1 loop domain result in a degree of virulence and tissue tropism variety for canine and human adenovirus (Zhang et al., 2018).

As two of the main major constituents of the structural capsid of adenovirus, alterations of the fibre and hexon genes would result in a better viral adaptation, depending on the various degree of pathogenicity of the FAdV isolates. Different FAdV serotypes are elucidated to have serotype-specific gene characteristics such as numbers of fibre proteins (long and short fibre proteins in FAdV group C) and epitope-region gene sequences, which suggested the difference in pathogenicity and FAdV-infections clinical symptoms observed in chickens. However, observing slight difference base substitution mutations between isolates of similar serotypes may also suggest an individualistic and opportunistic profile of each adenoviral isolate for viral adaptation. Interestingly, it was observed that changes in fibre genes among isolates of FAdV-8b lead to a different pathogenicity profile

(Ojkic & Nagy, 2000). It promotes the notion that investigating structural protein such as penton, fibre, and hexon, which play a huge role in viral survival, are essential to evaluate and determine the pathogenicity of groups, serotypes, and isolates as individuals. However, it is only possible to observe patterns between gene and amino acid residues sequence prior to concluding a definite indicator of FAdV isolates' pathogenicity by deciphering the overall virulence determinants of FAdV. The age of the chicken during inoculation, viral inoculum titre, route of administration, chicken susceptibility, chicken breed, and other factors contribute towards the chicken's mortality and the virus's pathogenicity.

Equally important, FAdV is also known to be resistant to disinfectants and high temperatures, increasing the threat posed by this pathogen when it is widespread globally (Hafez, 2011). Developing a vaccine against FAdV infection has not been a priority in research because of the absence of important diseases with severe mortality rates and morbidity profiles caused by an adenovirus (McFerran & Smyth, 2000). However, frequently reported outbreaks of HHS and IBH worldwide have sped up the development of an autogenous vaccine, either live attenuated, subunit, virus-like particles, or genetically-modified vaccine (Ahmed, 2020; De Luca et al., 2020; Gupta et al., 2018; Schachner et al., 2014; Schonewille et al., 2010; Sohaimi, 2018; Yin et al., 2021). In Malaysia, a commercialised vaccine developed from the country's local strain is yet to be produced to protect the flocks against FAdV infections (Juliana et al., 2014). However, the effort in establishing vaccines with reports of varying success are efforts worth acknowledging (Ahmed, 2020; Sohaimi, 2018). Indeed, any autogenous vaccine production as a potential biosecurity in controlling infectious diseases requires a thorough understanding and established molecular characteristics of its known isolates.

With the advance in technology in the research field, whole genome sequencing (WGS) could be performed on FAdV strain UPMT1901 isolate to evaluate further the isolate's evolutionary relationship with other known FAdV isolates molecular profiles, as well as establish the factors that lead to virulence of the virus.

CONCLUSION

The UPMT1901 isolate is a FAdV serotype 8b under the group E strain. This isolate was reported to have evolutionary relationships with other FAdV-8b, with precisely 99% identity with other known Malaysian FAdV isolates. The presence of a nucleotide base substitution in the partial fibre gene sequence of UPMT1901 indicates the possibility of having a variety of pathogenicity between and within the serotype, indicating a potential opportunistic nature as an individual isolate. The control and preventive measure protocols, such as vaccine production, are ongoing to curb avian FAdV diseases, especially in future farm outbreaks that would jeopardise the nations' food scarcity and economic growth.

The study of FAdV isolates will be a stepping point to elucidate the epidemiological factor of FAdV in Malaysia and provide essential information in building a detailed understanding at the molecular level to produce an effective vaccine against FAdV infections in the future.

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Tropical Forests Stand Recovery 30-year After Selectively Logged in Peninsular Malaysia

Aldrich Richard, Mohamad Roslan Mohamad Kassim, Kamziah Abd. Kudus and Mohd. Nazre Saleh*

Department of Forest Science and Biodiversity, Faculty of Forestry and Environment, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

This article analysed the 25–30 years of growth of dipterocarps forests that were logged under the Selective Management System (SMS) at three sites in Peninsular Malaysia to understand how management regimes affected forest stem density and basal area. The management regimes were (1) unlogged, (2) moderately logged forests that logged all dipterocarps ≥ 65 cm diameter at breast height (dbh) and all non-dipterocarps ≥ 60 cm dbh, and (3) intensely logged forests that logged all dipterocarps ≥ 50 cm dbh and non-dipterocarps ≥ 45 cm dbh. The intensely logged regime is similar to the SMS practices in Peninsular Malaysia. This result showed that one-year post-logging, there was no difference in the total stem density and basal area between forests logged according to the two management regimes. Forest stem density decreased over time in all management regimes, significantly greater in unlogged forests (-15.1 stems/ha/yr, confidence interval (CI): -16.9 to -13.3). This decline in stem density reflected that mortality exceeded recruitment in all management regimes. Despite the consistent decline of forest stem density, the basal area increased over time, and the rate of increase in the intensely logged forest (0.22 m^2 /ha/yr, CI: 0.19 to 0.25) was significantly greater than the other management regimes. Our study showed that 30 years post-logging, the effect of selective logging remained evident. Both logged forest stem density and the basal area did not recover to that of unlogged forests, indicating the importance of enrichment planting and extending the cutting cycles beyond 30 years for the sustainability of dipterocarps forests.

Keywords: Dipterocarps forests, management regime, natural recovery, selective logging

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E-mail addresses:
aldrich@forestry.gov.my (Aldrich Richard)
mohdroslan@upm.edu.my (Mohamad Roslan Mohamad Kassim)
kamziah@upm.edu.my (Kamziah Abd. Kudus)
nazre@upm.edu.my (Mohd. Nazre Saleh)
* Corresponding author

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INTRODUCTION

Removing large commercial trees is the main driver of forest degradation during logging (Sist et al., 2014). Selective logging, widely practised in southeast Asia, greatly impacts stand structure, species composition and regeneration dynamics of tropical forests (Hayward et al., 2021, Okuda et al., 2003; Yamada et al., 2013). However, these logged-over forests still possess significant elements of their original biodiversity and will recover over time, especially with silvicultural treatments such as enrichment planting using indigenous tree species (Berry et al., 2010; Philipson et al., 2020). Currently, 2.92 million ha or 59.1% of the total forest in Peninsular Malaysia are classified as production forests and will be selectively logged in the future (Forestry Department Peninsular Malaysia [FDPM], 2020).

The Selective Management System (SMS) is a selective logging system formulated in 1978 to manage Hill Dipterocarp Forests in Peninsular Malaysia (FDPM, 2003). The system prescribes a cutting cycle of 25-30 years, a cutting limit of 45 cm diameter at breast height for non-dipterocarps and 50 cm dbh for dipterocarp species and a minimum availability of 32 sound residual trees of 30–45 cm dbh/ha post-logging. This regime was defined on diameter growth of 0.77 cm/yr, annual recruitment of 0.60%, and mortality rate of 0.90%/yr for all trees above 30 cm observed over three years in 200 ha of permanent sample plots established in virgin and logged-over hill forests subjected to silvicultural treatments. It is expected that after 30 years, these logged forests will regenerate and be available for the next harvest (Thang, 1987).

Previous studies have shown that logged-over forests recovered more slowly in terms of the diameter growth above the threshold size for logging than was assumed by the SMS (Ismail et al., 2010; Rosli & Gang, 2013; Yong, 1996). However, these studies were limited by the small areas available for study (Rosli & Gang, 2013), the short duration of the census data (Yong, 1996), or possible biases associated with the timing of the study, such as when a census was carried out after extreme drought events that may have affected the growth and mortality of trees (Ismail et al., 2010). There is also limited information on the forest stand density and basal area recovery. The SMS is in the second cutting cycle since 2015 across most production forests in Peninsular Malaysia. Understanding the forest stand recovery of these logged forests will assist forest managers in planning for the third cutting cycle.

The Dipterocarpaceae is the dominant family in lowland and hill forests, compromising 30% of the total basal area or over 40% of the emergent, making them the main structure and support for other species (Saw & Sam, 1999). The Dipterocarpaceae dominates the canopy and sub-canopy forests in Southeast Asia (Ashton & Kettle, 2012). This dominance may be associated with their possession of root-inhabiting ectomycorrhizas that promote faster growth than other tree families, especially in a close canopy environment (Ashton, 1988; Banin et al., 2014; Brearley et al., 2016). This family is the main timber produced in

Peninsular Malaysia, and current practice in the SMS prescribes that the percentage of dipterocarps among the residual trees should not be less than in the pre-logging stand (Yong, 1996). The total timber production derived from dipterocarps amounted to 1.82 million m^3 or 46.6% for 2019 in Peninsular Malaysia (FDPM, 2020). The implication of the logged-over forest growth by removing mature Dipterocarpaceae and leaving small or intermediate-sized trees remains poorly understood (Ashton & Kettle, 2012).

The data presented the impact of the SMS on the stand structure and basal area of the logged-over forest through a time series of data collected over 25–30 years on permanent sample plots established in Peninsular Malaysia. This study aims to understand:

i. the impact of management regimes on the recruitment and mortality rate,
ii. the impact of management regimes and the recovery rate of forest stem density, and
iii. the impact of logging and the recovery rate of the total basal area, especially for Dipterocarpaceae tree species.

These insights provide a complete understanding of logged-over forest recovery status and the implication for sustainable forest management practices in Peninsular Malaysia.

METHODS

Study Area

The study was conducted in Lesong Forest Reserve, Sungai Lalang Forest Reserve, and Ulu Muda Forest Reserve, widely distributed across three different states in Peninsular Malaysia (Figure 1). The Forestry Department of Peninsular Malaysia established permanent sample plots in all three sites to evaluate the recovery of forests to logging in response to different management regimes and, in comparison, to an unlogged control. These sites were chosen to represent the lowland (Lesong) and hill (Sungai Lalang and Ulu Muda) Dipterocarp forests of Peninsular Malaysia. Over the past three decades (1990–2019) mean annual rainfall averaged across the three sites was 2,549.5 mm, and the mean annual temperature was 26.3°C. The climate of Peninsular Malaysia is characterised by two monsoon seasons: north-eastern winds bring rains from November to March during the boreal winter season,

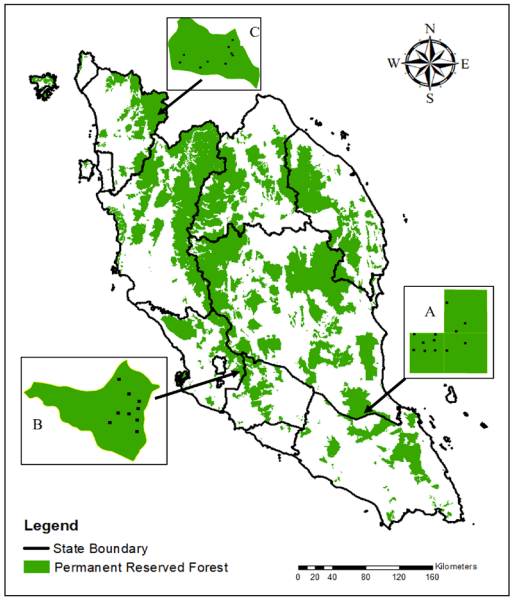


Figure 1. The study sites (black dots) in Peninsular Malaysia: (A) Lesong Forest Reserve, Pahang; (B) Sungai Lalang Forest Reserve, Selangor; and (C) Ulu Muda Forest Reserve, Kedah

and south-western winds bring strong winds from May to September during the boreal summer (Ministry of Environment and Water [MEWA], 2020). A detailed description of each site is shown in Table 1.

Thirty management blocks, each of 10.0 ha (316 m × 316 m), were established across the three sites: 12 in Lesong, 9 in Sungai Lalang, and 9 in Ulu Muda. Three management treatments were then implemented equally among management blocks within each site, as follows: (1) unlogged, (2) moderate logging, involving commercial extraction of all dipterocarp trees ≥ 65 cm dbh and all non-dipterocarp trees ≥ 60 cm dbh, and (3) intensive logging, involving commercial extraction of all dipterocarp trees ≥ 50 cm dbh and all non-dipterocarp trees ≥ 45 cm dbh. The intensive logging management regime replicated the SMS in terms of the diameter-cutting limits. The average volume logged was 131.8 m^3/ha

Table 1
Particular of each study site

Site	Lesong Forest Reserve	Sungai Lalang Forest Reserve	Ulu Muda Forest Reserve
Compartment no.	351, 371, and 372	50	10
Size of area (ha)	445.99	420	302.176
Forest type	Lowland dipterocarp forest	Hill dipterocarp forest	Hill dipterocarp forest
Altitude (meter above sea level)	100	400-800	300-400
Forest status	Virgin forest	Virgin forest	Virgin forest
Year logged	1989	1991	1993
Year of study plot established	1990	1992	1994
Year census	1990, 1991, 1992, 1993, 1994, 1996, 1998, 2000, 2002, 2009, 2014, 2019	1992, 1993, 1994, 1995, 1996, 1998, 2000, 2002, 2006, 2011, 2018	1994, 1995, 1996, 1997, 1998, 2000, 2002, 2004, 2006, 2008, 2013, 2018
Management regime	A, B, C	A, B, C	A, B, C
Size per plot (ha)	1.0	1.0	1.0
No. of a plot per regime	4	3	3
Total plot size (ha)	12	9	9
Plot number	A: 1, 2, 3, and 4 B: 5, 6, 7, and 8 C: 9, 10, 11, and 12	A: 13, 14, and 15 B: 16, 17, and 18 C: 19, 20, and 21	A: 22, 23, and 24 B: 25, 26, and 27 C: 28,29, and 30
Latitude Longitude	102° 65' 50" 2° 40' 54"	101° 57' 35" 3° 7' 35"	100° 56' 53" 5° 51' 15"
Mean annual temperature (°C) (1990-2019)	26.4	25.9	26.7
Mean annual rainfall (1990-2019) (mm)	2,629.6	2,481.4	2,537.7

Note. Management regime:
A: Control/Unlogged
B: Moderately logged (all dipterocarps ≥ 65 cm and non-dipterocarps ≥ 60 cm dbh logged)
C: Intensely logged (all dipterocarps ≥ 50 cm and non-dipterocarps ≥ 45 cm dbh logged

for moderately logged, and intensely logged, was 157.0 m^3/ha. The logged areas were left to regenerate naturally without any post-logging silvicultural treatments.

A 1.0 ha permanent sample plot was established in the centre of each management block's one-year post-logging, yielding four replicates of each management treatment in Lesong and three replicates in the other two sites. All stems ≥ 5 cm dbh were numbered, tagged, measured, and identified to genus or family on all plots at the first census and on 10 (Sungai Lalang) or 11 (Lesong and Ulu Muda) subsequent censuses, which were conducted at intervals of one to seven years for 25 (Ulu Muda), 27 (Sungai Lalang), or 30 (Lesong) years. The mean census interval length was 2.5 years, and the mode of interval durations was one year. None of the sites was exposed to anthropogenic or natural disturbances, such as burning, encroachment and landslides, over the study period. Further details of the design and layout of the plots are presented in Figure 2 and Table 2.

Data Processing

The diameter growth data were screened for errors based on the methods adopted by Qie et al. (2017) and Talbot et al. (2014). These protocols were modified to accept growth rates up to 6 cm/yr for individuals of fast-growing *Macaranga* spp. and *Mallotus* spp. (Manokaran & Kochummen, 1987). The data set comprised 147,687 diameter measurements on 18,247 individual stems over 82 years of censuses. The total data corrected were 3,659 or 2.48% of the total data set.

For analysis, stems were partitioned into 2 diameter size classes labelled poles (5.0-29.9 cm dbh) and trees (30.0 and above dbh). Mortality, recruitment, and growth rates are important demographic variables

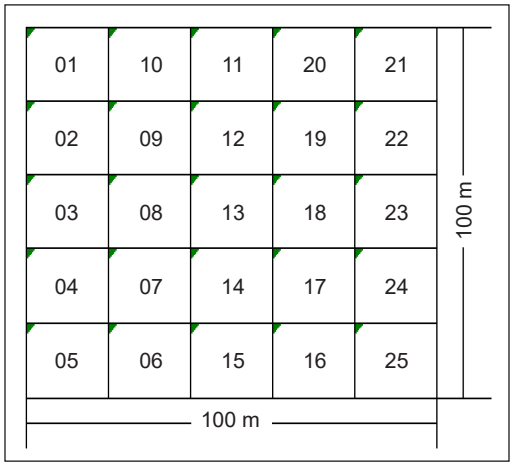


Figure 2. Layout design for the study plot. Each plot was divided into 25 subplots with a size of 0.04 ha (20 m × 20 m)

Table 2
Enumeration of stem for the study plot

Subplot sizes (m)	Subplot number	Tree dbh sizes (cm)	Stem class
20 × 20	1, 2, 3, 4, 5, 6, 10, 11, 15, 16, 20,21, 22, 23, 24, 25	15.0-29.9 30.0-44.9 > 45	Big pole, Small tree Big tree
20 × 20	7, 8, 9, 12, 13, 14, 17, 18, 19	5-14.9 15.0-29.9 30.0-44.9 > 45	Small pole, Big pole, Small tree Big tree

that may help managers make decisions to manage and conserve production forests (Dionisio et al., 2018). The rates of mean annual mortality (m) and recruitment (r) were calculated based on Sheil et al. (1995, 2000) as follows:

$$m = 1 - (nm / N_0)^{(1/t)}$$

$$r = 1 - [1 - (nr/N_1)^{(1/t)}]$$

where N_0 and N_1 are population sizes at the beginning and end of the census interval, nm is the number of dead stems, nr is the number of recruits, and t is the census interval length.

Mortality and recruitment rates were adjusted to account for the variation in census interval length (Lewis et al., 2004) as follows:

$$\lambda_{corr} = \lambda \times t^{0.08}$$

where λ_{corr} is the corrected mortality or recruitment rate in %/yr, λ is the mortality or recruitment rate, and t is the census interval.

Basal area is the cross-sectional area of all trees at diameter breast height (dbh) in a unit area. It is an informative measure of stand density used in prescribing silvicultural options in managed forest stands (Zhao et al., 2020). Basal area is used to compute the growth rate in reflecting the growth of trees over time. The basal area will provide information on the growth of trees in a unit area which will fluctuate over time due to mortality and recruitment of trees. The formula for basal area is as follows:

$$BA = \frac{\pi \times dbh^2}{4 \times 10{,}000}$$

where BA is the basal area in m^2/ha, and dbh is the diameter at breast height in cm.

The census enumerates stems of 5–14.9 cm in only 9 subplots. For estimation of stem density and basal area at the 1-ha plot scale, the mean values computed for stems of 5–14.9 cm dbh sampled on 9 subplots were multiplied by 25.

Statistical Analysis

Repeated measurements of sample trees determine forest growth over time. Separate values in the time series are not independent because they are based on the same sample of subject trees (Shek & Ma, 2011; Zuur et al., 2007). Analysing forest growth data with a linear mixed effects model addresses the issue of non-independence inherent to longitudinal time series data sets by specifying the plot as a random effect nested within spatial covariates as required by the sampling design (Qie et al., 2017). In this paper linear mixed effects model was fitted to values of mortality, recruitment, stem density, poles density, trees density, basal area, Dipterocarpaceae basal area, and non-Dipterocarpaceae basal area with fixed effects of management regime and time since logging. Plots and sites were fixed as random effects. The analysis was carried out using the *lmer* function in the R package *lme4* (Bates et al., 2015) using the syntax shown in Equation 1 below. Recruitment and mortality rates were calculated per year, and the equation applied to understand the changes is shown in Equation 2.

Response_variable ~ Year * regime + (1|Plot) + (1|Site)

(Equation 1)

Response_variable ~ regime + (1|Plot) + (1|Site)

(Equation 2)

The linear mixed effect model assumed equal variances and a normal distribution of residuals, validated by examining the residual of fitted variance value against plots and sites (Philipson et al., 2020). The 95% confidence intervals (CI) of parameter estimates were obtained using restricted maximum likelihood estimation and bootstrapping. The *LmerTest* package was used to obtain the *p*-value (Kuznetsova et al., 2017). The model for recruitment rate was $\log_{10}$ transformed as the distribution of variances was not homogenous without transformation. The results were back-transformed before being presented in this paper.

RESULTS

Effect of Management Regime on Mortality and Recruitment Rate

There was a significant difference in mortality rate between unlogged forests with the other two management regimes (Figure 3). The unlogged forest has the lowest mortality rate at 1.8%/yr (CI: 1.0 to 2.5). There was no significant difference between moderately (4.0%/ yr, CI: 3.3 to 4.8) and intensely logged (3.9%/yr, CI: 3.40 to 4.50) forests. As for the annual recruitment rate (stems ≥ 5 cm dbh), the lowest was in unlogged forests at 0.5%/yr (CI: 0.40 to 0.70), which was significantly different from the other two management regimes (Figure 4). Moderately and intensely logged forests had the same recruitment rate at 2.1%/yr (CI: 1.4 to 2.9) and 2.1%/yr (CI: 1.6 to 2.6), respectively.

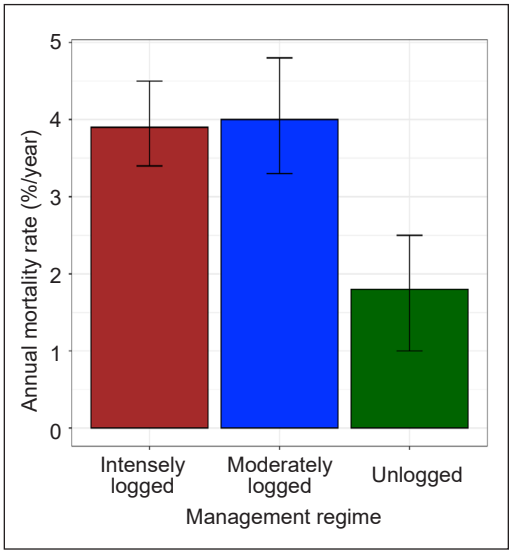


Figure 3. Annual mortality rate for all management regimes. The interval bar showed the confidence interval for each management regime

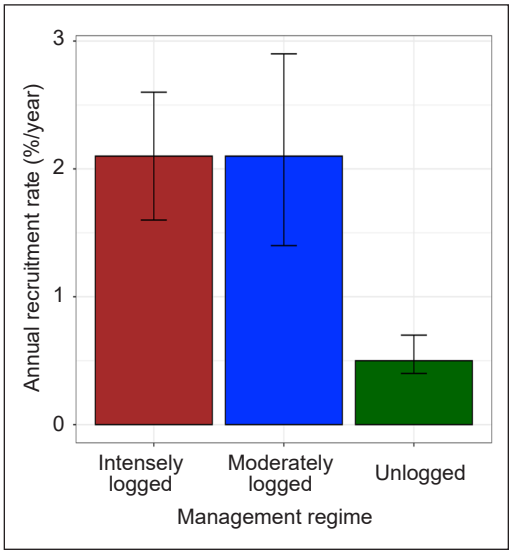


Figure 4. Annual recruitment rate for all management regimes. The interval bar showed the confidence interval for each management regime

Effect of Management Regime on Forest Stem Density

There was a significant difference in the forest stem density between unlogged and the two logging regimes one-year post logging for all diameter classes (Table 3, Figure 5). There were no differences in the stem density between intensely and moderately logged forests. The only diameter class that had a significant difference between all management regimes are the trees class (30 cm dbh and above). The stem density of trees was highest in the unlogged forest (108.0 stems/ha, 95% CI 95.2 to 120.3) and lowest in the intensely logged forests (37.1 stems/ha, 95% CI 24.4 to 49.1).

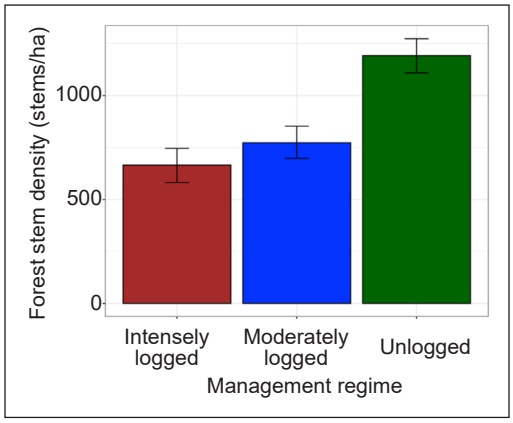
Stem Density Changes Over Time

The forest stand for all management regimes showed decreased stem density over time

Table 3
Diameter class, mean, and the confidence interval for all management regimes one-year post logging

Class diameter / Management regime	Stem/ha		
	Mean	2.5%	97.5%
Forest stem (5 cm and above)			
Unlogged*	1,192.2	1,111.6	1,269.6
Moderately logged	772.9	694.6	846.8
Intensely logged	665.2	588.9	750.4
Poles (5–29.9 cm dbh):			
Unlogged*	1083.1	1,003.5	1,162.8
Moderately logged	719.0	636.4	807.3
Intensely logged	627.0	537.7	715.2
Trees (30 cm and above dbh):			
Unlogged*	108.0	95.2	120.3
Moderately logged*	52.8	39.9	64.3
Intensely logged*	37.1	24.4	49.2

Note. * indicates management regime is significantly different



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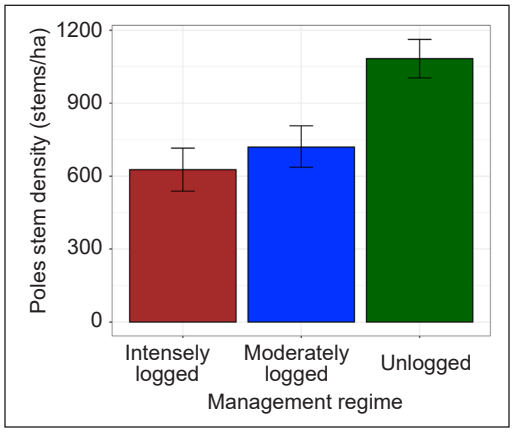

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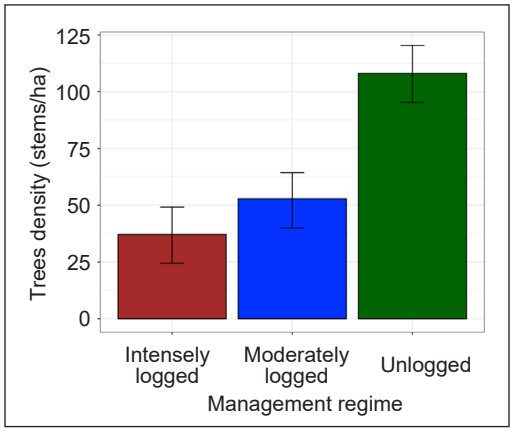


(c)

Figure 5. Total stem density one year post logging for all management regimes: (a) Forests (5 cm and above dbh); (b) Poles (5–29.9 cm dbh); and (c) Trees (30 cm and above dbh). The interval bar showed the confidence interval

(Figure 6a). The unlogged forest had the highest rate of decrease at -15.1 stems/ha/yr (CI: -16.9 to -13.3) and the second highest at -12.90 stems/ha/yr (CI: -14.7 to -11.1) was in moderately logged forest. The lowest rate of decrease was in the intensely logged forest at -9.50 stems/ha/yr (CI: -11.5 to -7.7). The only significant difference in the rate of stem density changes over time of forest stand was between unlogged forests and intensely logged forests. The unlogged forests had the highest decrease in stem density at 453 stems/ha in 30 years. The decrease in moderately and intensely logged forests was 387 stems/ha and 285 stems/ha, respectively, over the 30 years since logging. Although the unlogged forests had the highest decrease in stem number, the total stem density/ha was still the highest compared to the other two management regimes (Table 4).

The changes in pole stem density over time significantly differed between all management regimes (Figure 6b). The unlogged forests have a greater decrease (-15.3 stems/ha/yr, CI: -17.1 to -13.6) followed by moderately logged forests (-13.9 stems/ha/yr, CI: -15.5 to -12.1) and lowest in intensely logged forests (-11.0 stems/ha/yr, CI: -12.9 to -9.3). The highest decrease of poles was in the unlogged logged forest with 459 stems/ha and the lowest in the intensely logged forest at 330 stems/ha 30 years post-logging.

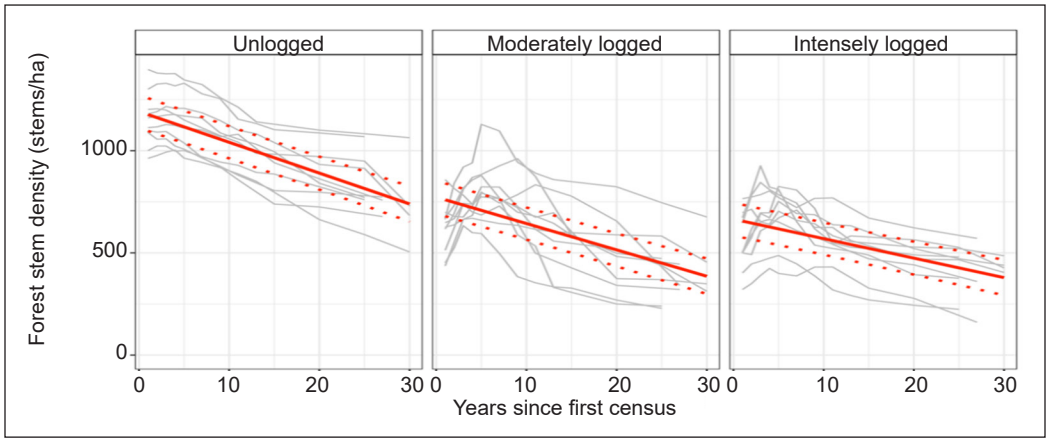
The trees class stem showed an increasing stem density between all management regimes compared to the poles class stem (Figure 6c). The changes over time in trees class stems were significant among all management regimes, with the highest rate in intensely logged forests (1.5 per ha per yr, CI: 1.4 to 1.6 stems). The second highest rate was in intensely logged forests (1.0 stems/ha/yr, CI: 0.9 to 1.1) and lowest in unlogged forests (0.2, CI: 0.1 to 0.3). The highest increase was in intensely logged forests at 45.0 stems/ha and the lowest in unlogged forests at 6.0 stems/ha 30 years post-logging. For the intensely and moderately logged forests to recover to the pre-logging tree stem density of unlogged forests, it will take 47 and 55 years, respectively.

Effect of Management Regime on Basal Area

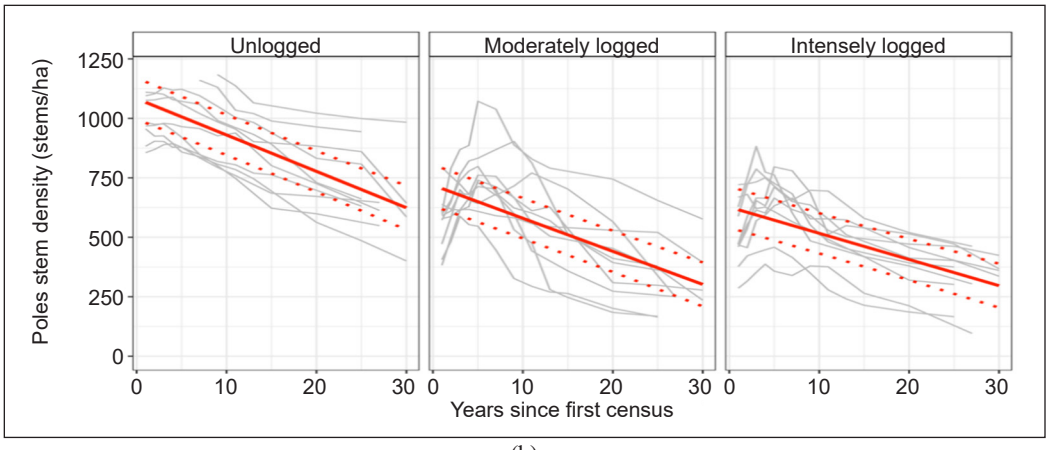
As expected, the total basal area was significantly greater one year after logging in the unlogged forest than the other two management regimes at 36.7 (CI: 33.2

Table 4
Total stem density changes in 30 years for all management regimes

Management regime	Total stem density during the first census (stem/ha)	Total stem density changes in 30 years (stem/ha)	Total stem density in 30 years (stem/ha)
Unlogged	1,192.2	- 453	739.2
Moderately logged	772.9	- 387	385.9
Intensely logged	665.2	- 285	380.2



(a)



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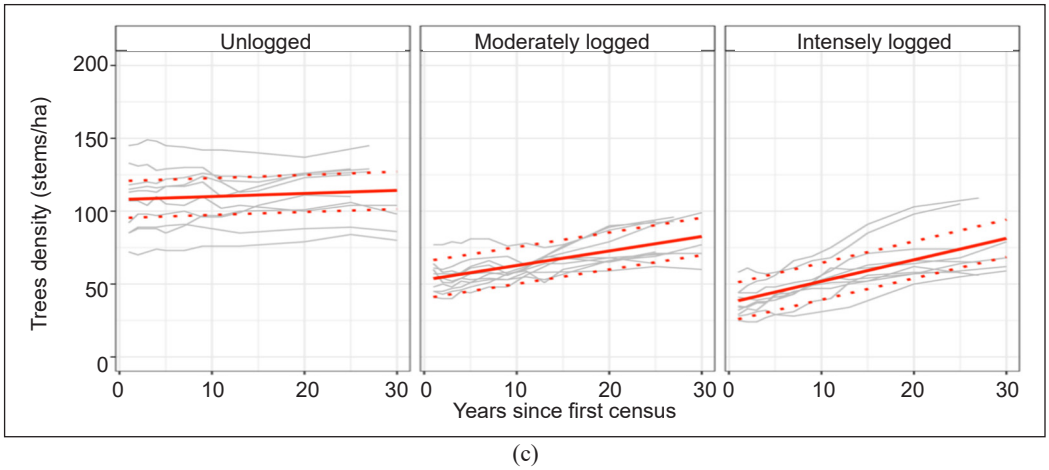


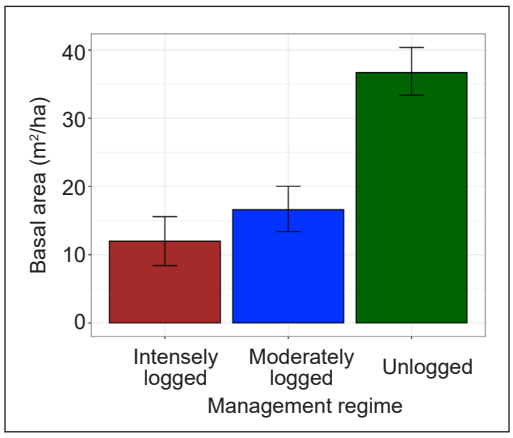
(c)

Figure 6. Total stem density as a function of time: (a) Forests stem; (b) Poles; and (c) Trees. The red solid line represents the mean timber volume growth, while the red dotted line represents the 95% confidence interval. The grey line represents each plot no. of stem growth in each census interval

to 39.8) m²/ha (Figure 7a). There was no difference in the total basal area between moderately logged (16.6 m²/ha, CI 13.1 to 19.9) forests and intensely logged (12.0 m²/ha, CI 8.4 to 15.5) forests at this stage.

The unlogged forests had the highest total basal area for Dipterocarpaceae at 7.7 m²/ha (CI: 5.6 to 9.7), significantly different from moderately and intensely logged forests one year after logging (Figure 7b). The basal area of dipterocarps in moderately (0.7 m²/ha, CI -1.3 to 2.7) and intensely logged (0.7 m²/ha, CI -1.3 to 2.8) forests did not vary one year after logging. Moderately and intensely logged forests lost an average of 7.0 m²/ha basal area compared to values of unlogged forests one-year post-logging.

All management regimes significantly differed in the total basal area for non-Dipterocarpaceae one-year post-logging (Figure 7c). The unlogged forest had the highest total basal area for non-Dipterocarpaceae species at 29.1 m²/ha (CI: 26.3 to 31.8 m²/ha). The total basal area for non-Dipterocarpaceae in the moderately logged forest was 16.0 m²/ha (CI: 13.3 to 18.7 m²/ha), higher than the value of 11.3 m²/ha (CI: 8.6 to 13.8) in intensely logged forest. Intensely logged forests lost the greatest amount of basal area of non-dipterocarps with an average of 17.8 m²/ha compared to the value of 13.1 m²/ha in moderately logged forests one-year post logging.



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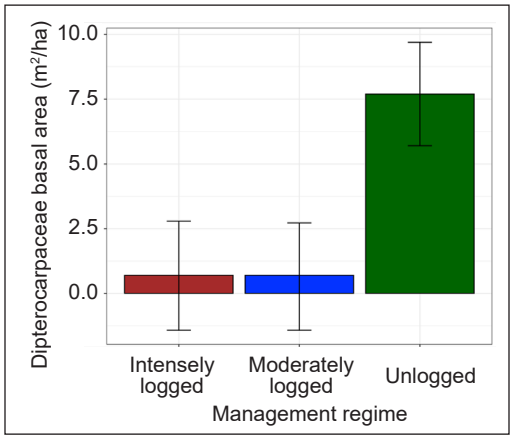

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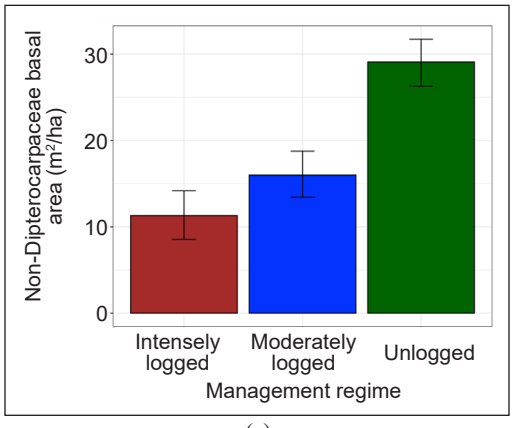


(c)

Figure 7. Total basal area one year post logging for all management regimes: (a) All species; (b) Dipterocarpaceae; and (c) Non-Dipterocarpaceae. The interval bar indicates the confidence interval

Basal Area Changes Over Time

The rate of change in basal area over time was significantly different among the three

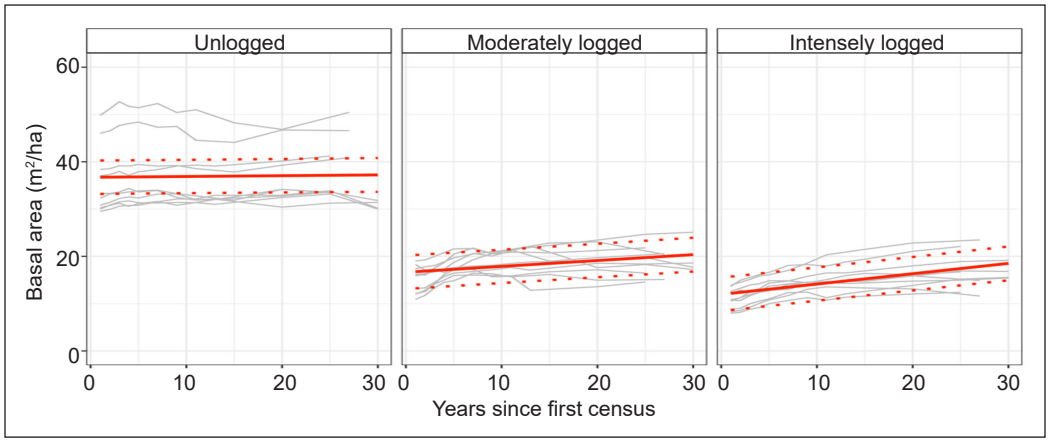
management regimes but showed positive values in all cases (Figure 8). The rate of change in the intensely logged forest was highest at 0.22 m²/ha/yr (CI: 0.19 to 0.25), followed by 0.12 m²/ha/yr (CI: 0.10 to 0.15) in moderately logged forest. The unlogged forests had the lowest rate at 0.02 m^2/ha/yr (CI: -0.01 to 0.05). Thirty years post-logging, intensely logged, and moderately logged forests had recovered 6.60 m^2/ha and 3.6 m^2/ha (Table 5). The recovery of the basal area to pre-logging status would require 112 years for intensely logged forests and 167 years for moderately logged forests. There were no changes in the basal area for unlogged regimes in the past thirty years, as the gain is just 0.6 m²/ha.

There was no significant difference between the three management regimes in the rate of change in basal area over time for Dipterocarpaceae basal area, which showed an increasing trend (Figure 8b). The highest changes over time rate occurred in the unlogged forests at 0.05 m^2/ha/yr (CI: 0.03 to 0.06). The intensely logged forest rate of 0.04 m^2/ha/yr (CI: 0.02 to 0.05) was higher than the value of 0.03 m^2/ha/yr (CI: 0.01 to 0.04), which occurred in moderately logged forests. The biggest total change over time of Dipterocarpaceae basal area after 30 years since the first census was in the unlogged forests, which saw a 1.5 m^2/ha growth. The second highest increase was in the intensely logged forest at 1.2 m^2/ha, and the lowest at 0.9 m^2/ha occurred in moderately logged forest. The recovery of the Dipterocarpaceae basal area to the pre-logging status would require 175 and 234 years in intensely and moderately logged forests, respectively.

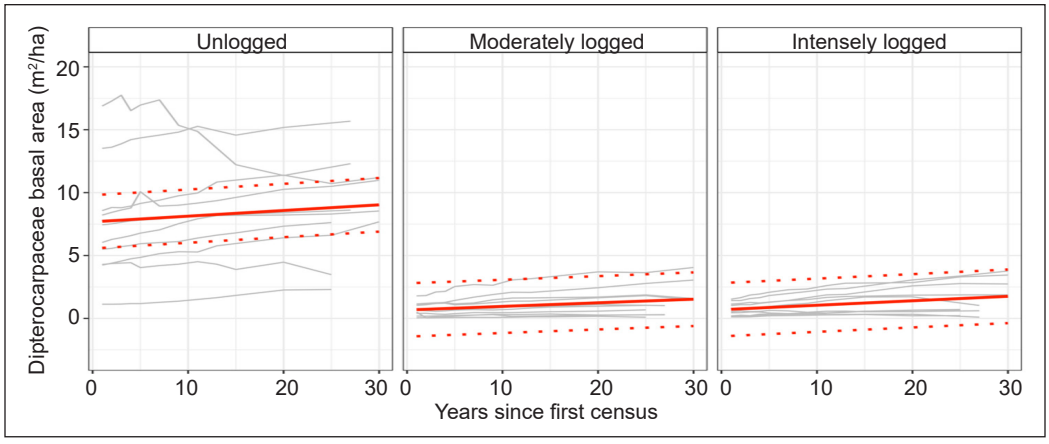
The basal area of non-Dipterocarpaceae increased through time in moderately and intensely logged forests but decreased in the unlogged forest (Figure 8c). The changes over time in the non-Dipterocarpaceae basal area were significantly greater in the intensely logged (0.18 m^2/ha/yr, CI: 0.15 to 0.21) than in the moderately logged forest (0.10 m^2/ha/yr, CI: 0.07 to 0.13). The unlogged forest displayed a decrease in non-Dipterocarpaceae basal area of -0.03 m^2/ha/yr (CI: -0.06 to 0.00). The unlogged forests lost 0.9 m^2/ha of non-Dipterocarpaceae basal area over the 30 years since the first census, but this forest retained a higher basal area than the two logged forest types, which gained 3.0 m^2/ha (moderately logged forest) and 5.4 m^2/ha (intensely logged forests), respectively. It is estimated that intensely logged forests would require 98.4 years to recover to the pre-logging basal area, while moderately logged forests would require 130.8 years.

Table 5
Total basal area changes in 30 years for all management regimes

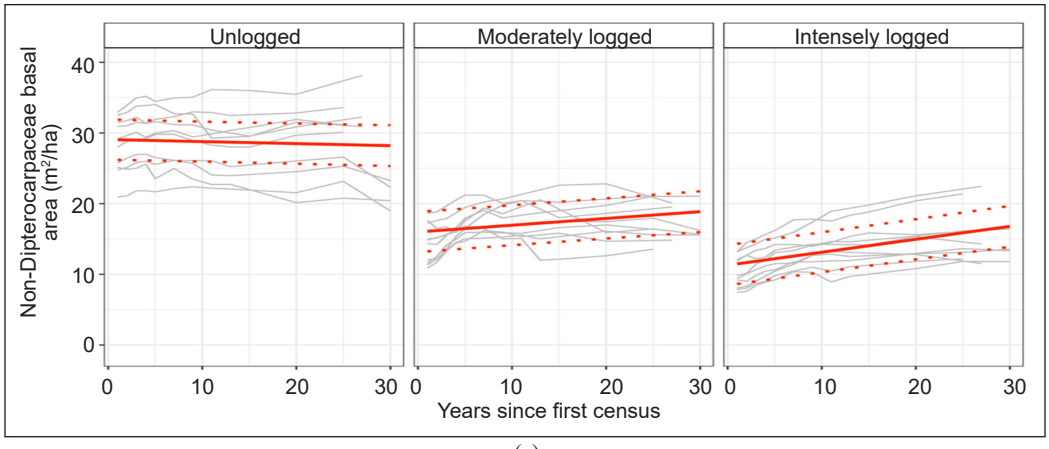
Management regime	Total basal area during the first census (m^2/ha)	Total basal area changes in 30 years (m^2/ha)	Total basal area in 30 years (m^2/ha)
Unlogged	36.7	0.6	37.3
Moderately logged	16.6	3.6	20.2
Intensely logged	12.0	6.6	18.6



(a)



(b)



(c)

Figure 8. Total basal area growth as a function of time; (a) All Species; (b) Dipterocarpaceae; and (c) Non-Dipterocarpaceae. The red solid line represents the mean timber volume growth, while the red dotted line represents the 95% confidence interval. The grey line represents each plot's basal area growth in each census interval

DISCUSSION

Annual Mortality and Recruitment Rate

The mortality rate in unlogged forests did not vary from previous studies of below 2.0%/yr (Ismail et al., 2010; Yong, 1996). In the logged forest, the rate is higher than 0.9%/yr in the SMS for 30 cm and above trees (FDPM, 2003); 2%/yr in Ismail et al. (2010); and 2.50%/yr from Yong's (1996) result. The recruitment rate in the logged forest plots was higher than the value of 0.6%/yr reported in studies of the SMS (FDPM, 2003). It was lower compared to the study by Yong (1996) at 3.35% but did not vary from the value of 2.0%/yr reported by Ismail et al. (2010). Our mortality and recruitment rate results differed because SMS was based on a 30 cm and above dbh tree class. Yong's study (1996) was based on 5 cm and above dbh trees but from only one site and a shorter period of only 14 years, which may explain the greater recruitment rate.

The annual mortality in unlogged and logged forests was higher than the recruitment rate in previous studies, as shown (Ismail et al., 2010; Manokaran & Kochummen, 1987; Yong, 1996). It indicates that the tropical forests lost stem density over time as recruitments could not offset the high mortality rate. This trend is especially prevalent in logged forests, as the mortality is twice the recruitment rate. The mortality and recruitment rate also did not vary between logging intensity.

Effect of Logging on Forest Stem Density

The intensely logged regime lost more stem/ha compared to moderately logged regimes. One-year post-logging, the intensely logged forests lost 44% of the original stem density, while 35% in moderately logged forests. Poles stem density (5–29.9 cm dbh) was reduced by 34% in moderately logged and by 51% in the intensely logged forest compared to the unlogged forest. The percentage of stem loss will be higher for the trees class (30 cm and above dbh), which may go up to 51 and 65% for moderately and intensely logged regimes, respectively. The result showed no difference in the effect of logging intensity on the overall forests except for tree class stems. Even though the logging operation only involved the tree above 45 cm dbh class, the effect was also evident in smaller classes stem. The loss of stem density after logging operation can also be seen in other selective logged-over forests with different management regimes (Dionisio et al., 2018; Hayward et al., 2021; Okuda et al., 2003). The losses of stem density post-logging were not solely due to the removal of commercial tree species but also to mortality caused by logging activities (Shenkin et al., 2015). Mortality in logged-over forests is usually high immediately post-logging, and the effect may last up to three years (Ismail et al., 2010; Sist et al., 2014).

The unlogged forests' stem density of 5.0 cm and above of 1,192.2 stems/ha (CI: 1,111.6 to 1,269.6) was lower than the Amazon basin of 1,561 stems/ha (Myster, 2016) but higher than Africa at 425.6 stems/ha (95%, CI: +11.1) for 10 cm an above tree (Lewis et al., 2013). There was variation in stem density/ha between the three blocks of tropical forests, with the lowest in Africa and

the highest in the Amazon. The unlogged forest stem density was also lower than Yong's (1996) 1,376.57 stems/ha results.

The increasing stem density trend of trees above 30 cm dbh showed that the logged-over forest is still recovering from logging operations. Selective logging eliminates the competition of big trees for growth resources and allows small trees to grow into the next diameter class. Bigger trees (30 cm in dbh and above) are not as greatly affected by this competition as smaller trees (below 30 cm in dbh), which resulted in an increasing trend over time (Rozendaal et al., 2020). The decreasing trend for unlogged forests, especially for poles, is contributed by the close canopy characteristic of mature forests that limits light availability to the understory. The limited availability of light will decrease the photosynthesis rate and halt growth, which eventually causes tree mortality due to carbon starvation (Gora & Esquivel-Muelbert, 2021). Changing climate conditions will also contribute to the decreasing trend of poles as for the past four decades, Malaysia has been experiencing an increased mean temperature of 0.13 to 0.24°C with Peninsular Malaysia having the highest changes (Fung et al., 2020; Ministry of Energy, Science, Technology, Environment and Climate Change [MESTECC], 2018). The first signal of response by trees to climate change is through changes in phenology (Deb et al., 2018). Southeast Asian tropical forest trees need a combination of drought and low temperatures during the day and night for general flowering to be triggered. The ideal flowering conditions, especially for Dipterocarpaceae, are a 54–90-day period of low temperature between 25.5–27.8°C and 93-186 mm precipitation per month (Chen et al., 2018; Yeoh et al., 2017). An increase in temperature or extreme drought events will disrupt the flowering of Dipterocarpaceae, decreasing the seed availability for forest natural regeneration.

The SMS emphasises that a minimum of 32 residual trees of 30–45 cm dbh should be retained post-logging as these trees are used to determine that the logged-over forest is fully stocked and are scheduled to become the next crop trees without the requirement of silvicultural treatments such as enrichment planting. However, the logged-over forests showed that the number of poles stem densities one \year post-logging will decrease by 35–45%. The low stem density with high turnover will affect the ability of small poles to grow into the next size class and eventually drive the recovery process of logged-over forests. Silvicultural treatment such as enrichment planting needs to be carried out to ensure that enough poles are present, even though the number of residual trees is sufficient.

Effect of Logging on Basal Area

The moderate and intense logging regimes greatly impacted the basal area as the losses were 54.8 and 67.3%, respectively, one year post-logging compared to the unlogged regime. The logged-over forests had a faster recovery rate of basal area, but even 30 years post-logging, these forests

are still recovering. However, the recovery rates are lower than Yong's (1996) result of 0.25 m^2/ha/yr. The lower recovery rate for the logged-over forests is due to the higher number of big trees, especially Dipterocarpaceae, being logged and a higher proportion of the non-Dipterocarpaceae family in the residual stands (Ismail et al., 2010). Dipterocarpaceae has higher growth than non-Dipterocarpaceae trees (Banin et al., 2014).

The mean basal area of 36.7 m^2/ha (CI: 33.25 to 39.84) in unlogged forests does not vary from Borneo of 37.5 + 2.62 m^2/ha but is higher than Amazon of 31.5 and 30.3 + 0.77 m^2/ha of Africa (Lewis et al., 2013; Myster, 2016). The high basal area in unlogged forests indicates that the trees are bigger in Peninsular Malaysia compared to Amazon and Africa. The increase in basal area of 0.6 m^2/ha in unlogged forests over 30 years may indicate that these old-growth forests are not in equilibrium with changes to the total basal area over time. Thirty years after the first census, the basal area of the unlogged forest showed an increase in the proportion of Dipterocarpaceae and a decrease in non-Dipterocarpaceae. It suggests that species of the Dipterocarpaceae family are increasing in dominance over time.

The basal area for Dipterocarpaceae accounted for about 20.9% of the total basal area in the unlogged regime. This figure is lower than Pasoh Forest Reserve, which recorded 27.3% of the total basal area for trees > 1 cm (Okuda et al., 2003). One-year post logging, the basal area for Dipterocarpaceae was reduced to 4.2 and 5.8% in moderately and intensely logged regimes, respectively. The remaining small and intermediate-sized trees of Dipterocarpaceae stems contributed a small percentage of the total basal area. The logging of big Dipterocarpaceae trees also affected the basal area recovery rate, removing the family's competitive advantage and forest ecosystem dominance (Brearley et al., 2016). Our study highlighted the importance of maintaining big Dipterocarpaceae trees to improve the recovery rate of logged-over forests.

CONCLUSION

The effect of selective logging on the forest stand structure and the basal area remained evident even after decades post-logging. The high mortality and low recruitment rates showed that the natural regeneration of logged-over forests would be challenging. The stand density and total basal area, especially the Dipterocarpaceae composition of the logged-over forests, have not recovered to the pre-logging status of unlogged forests even 30 years post-logging. It shows that a logging regime that is only based on cutting limits without volume control will require a cutting cycle that is substantially longer than 30 years to deliver a sustainable yield. The SMS prescription of maintaining the Dipterocarpaceae stem percentage during pre- and post-logging may need to be reviewed as the recovery period of logged-over forests depends on the availability of big Dipterocarpaceae trees rather than small intermediate trees. Residual trees that will form the next crop

trees need to combine small and big trees to improve the recovery rate of logged-over forests. Carrying out enrichment treatments with indigenous timber trees will increase the stem density of poles and assist in the regeneration of logged-over forests.

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Effect of Straw Compost (*Oryza sativa* L.) on Crop Production

Nana Danapriatna[1]*, Ismarani[1], Ridwan Lutfiadi[1] and Moh. Dede[2,3,4,5]

[1]*Faculty of Agriculture, Universitas Islam 45 Bekasi, 17113 Bekasi, Indonesia*
[2]*Postgraduate School, Universitas Padjadjaran, 40132 Bandung, Indonesia*
[3]*Cakrabuana Institute for Geoinformation, Environment and Social Studies, 45188 Cirebon, Indonesia*
[4]*National Research and Innovation Agency of Indonesia, 10340 Jakarta, Indonesia*
[5]*Faculty of Social Sciences Education, Universitas Pendidikan Indonesia, 40154 Bandung, Indonesia*

ABSTRACT

Agricultural productivity depends mainly on soil fertility, particularly in intensified-paddy fields. Heavily relying on synthetic agrochemicals in intensified agriculture could be changed to regenerative agriculture utilizing cultivation wastes to achieve sustainable food production. Therefore, this study aims to analyze the effectiveness of rice (*Oryza sativa* L.) straw compost for intensified-rice cultivation. Rice straw compost from the previous planting season was composted on the field (*in situ*). The composting used "Effective Microorganisms version 4" (EM-4), which contains *Lactobacillus* sp., *Rhodopseudomonas* sp., *Actinomycetes* sp., *Streptomyces* sp., yeast, and cellulose-decomposing fungus. The test field used 4 tons of straw compost and treatments adopted from the local farmers' planting style named Legowo 4:1. Observations on these treatments include the plant nutrients, plant contents, rice components, and yield production. The differences in the results were analyzed using the paired *t*-test. The results show that the application of straw compost provides a significant increase in dry grain weight, panicle length, and the number of grains per rice plant. However, the treatment did not give significant results on the clumps number and rice grain weight. Besides improving rice production, straw compost improved the C-organic, total N, and K levels in the soil. Based on this study, rice straw compost brings benefits for paddy cultivation as well as the reuse of agricultural waste in a simple way, especially in tropical lowland areas of Indonesia.

Keywords: Agricultural waste, crop productivity, paddy, rice cultivation, sustainability

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E-mail addresses:
danapriatna.nana@gmail.com | nana.danapriatna@unismabekasi.ac.id (Nana Danapriatna)
raniisma@gmail.com (Ismarani)
ridwanlutfiadi@gmail.com (Ridwan Luthfiadi)
m.dede.geo@gmail.com (Moh. Dede)
*Corresponding author

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INTRODUCTION

The degradation of farmland poses a significant threat to both biodiversity and soil fertility. Consequently, a shift towards regenerative agricultural practices is needed to counteract this trend. Regenerative agriculture can address global challenges such as limited arable land, greenhouse gas emissions, and biodiversity loss (Lal, 2020; Meybeck & Redfern, 2016). The main objective of regenerative agriculture is to prevent soil degradation and agroecosystem threats through high-quality as well as non-hazardous soil management practices (El-Ramady et al., 2014; LaCanne & Lundgren, 2018). These methods for improving soil quality can positively affect crop yield and resource management efficiency (Ogunwole et al., 2014). De Moura et al. (2016) showed that continuous cultivation depends on soil quality, including nutrient content and organic matter.

Intensive rice cultivation is a growing threat to biodiversity, especially in tropical and sub-tropical regions such as Southeast and East Asia. In Indonesia, lowland rice production relies heavily on soil nutrients threatened by erosion, pollution, land conversion, and climate change (Dede, Asdak et al., 2022; Wahyunto & Dariah, 2014). Consequently, farmers use excessive inorganic fertilizers to maintain soil fertility, leading to new problems such as soil acidity, nutrient imbalances, and low crop yields (Médiène et al., 2011). Unsustainable cultivation practices also result in greenhouse gas emissions and increased production costs (Thiyageshwari et al., 2018). However, sustainable agriculture should prioritize productivity, quality yield, and preservation of resources (Bilali et al., 2018). Rice straw can help maintain soil fertility, but burning it damages soil nutrients and contributes to environmental pollution, also causing the loss of important nutrients, especially N (up to 80%), P (25%), K (21%), Si (4-60%), and soil organic matter (Mandal et al., 2004).

About 73% of paddy fields in Indonesia have low organic content (2%) due to past volcanic eruptions and sedimentation processes (Adviany & Maulana, 2019; Dede, Wibowo et al., 2022; Rahayu et al., 2014). Straw is an organic material that contains carbon and major nutrients such as N, P, K, Ca, and Mg (Simarmata et al., 2016), but this requires an ideal treatment. Rice straw increases nitrogen fixation when converted into compost containing *Azotobacter* and cellulolysis microorganisms, making the soil healthier and more fertile for plant growth (Galsim et al., 2021). Research has shown that combining rice straw with organic fertilizers is more effective than rice ash in improving nutrient levels as well as soil quality for plant development (Watanabe et al., 2017; W. Lin et al., 2019). Incorporating rice straw and organic fertilizers into intensive rice cultivation practices can be a regenerative solution for tropical and sub-tropical regions.

Unlike previous research that referred to the usage of rice straw in soil quality and health, this study aims to analyze the effectiveness of straw compost (*Oryza sativa* L.) on crop production. This research was conducted in Karawang, Indonesia, a well-

known place for national rice production. Currently, rice farmers in Karawang heavily rely on agrochemicals material, whose input reaches more than 100,000 tons per year for 98,000 ha of lands, such as urea, sulfur-phosphate "SP-36", ammonium sulfate, or *zwavelzure ammoniak* "ZA," and NPK fertilizers (Sundari & Halim, 2020). Also, large external input is necessary to produce more than 1 million tons of rice annually (Aenunnisa et al., 2022). The researchers hypothesized that applying straw compost can improve soil quality that affects paddy nutrients and productivity. This study addresses a field experiment of straw compost on lowland paddy varieties (IR-32) for tropical areas.

MATERIALS AND METHODS

Research Experiment

The research was conducted in Rengasdengklok, Karawang Regency, West Java, Indonesia, for two seasons, covering the cultivation period of dry and rainy. This area has a tropical monsoon climate with an annual rainfall of 1,000-1,500 mm yearly (Aruminingsih et al., 2022; Sukowati & Kusratmoko, 2019). According to Nugrahatama and Utami (2021), Rengasdenglok has an annual average temperature of 27^{o}C and 85% humidity, making it a fertile-alluvial region for intensive rice farming in Pantura, Northern Java. This region has a large expanse of Entisols, making it ideal for rice cultivation; the detailed characteristics are presented in Table 1. Materials for this study are rice straw, decomposer organisms named EM-4 (*Laktobacillus* sp., *Rhodopseudomonas* sp., *Actinomycetes* sp., *Streptomyces* sp., yeast, and cellulose-decomposing fungus), clean water, inorganic fertilizers, and rice seed (variety IR-32).

EM-4 is an "Effective Microorganisms" (EM) Indonesia product widely sold in agricultural stores. This product is an effective inoculum derived from tropical microorganisms (Syahid et al., 2020). The decomposer organism needs to be prepared before mixing with rice straw; this solution becomes an activator. Composting started by making a solution made from EM-4 (PT Songgolangit Persada, Indonesia), molasses (Tetes Murni, Indonesia), and water (ratio 1:1:1,000). Every 1 m^3 rice straw needs 100-200 liters of activator solution (PT Songgolangit Persada, Indonesia). The compost is ready for use when the rice straw is physically changed to brown-black color, soft texture, and crushed. Additionally, the pile's temperature should be near the initial composting conditions, and this process typically takes around one month. A trial was set up in the lowland rice cultivation area with a local cropping culture called "Legowo 4:1," adapted from the farmers in Karya Sari Village. Legowo 4:1 is the rice cultivation practice, where an empty row intersperses every four rows of crops, providing air circulation and sunlight for paddy (Abdulrachman et al., 2012). The paddy field for this research was two plots, with 1,000 m^2 each.

Table 1

Characteristics of Entisols at the study site

Parameter	Value	Information	Acquisition
Soil texture		Silt loam	
Sand	3		Hydrometer (Faé et al., 2019)
Silt	70.7		
Clay	26.3		
pH H_2O	7.04	Neutral	Potentiometer (Singh et al., 2021)
pH KCI	6.2	Neutral	
C-organic (%)	2.22	Medium	Walkey and Black (Munawaroh et al., 2022)
Total N (%)	0.162	Low	Kjeldahl (Todorova et al., 2011)
K_2O (mg/100 g)	12.4	Low	$HClO_4$ + HNO_3 (Sulaeman et al., 2005)
P_2O_5 (mg/kg)	10.2	Medium	Olsen (Steinfurth et al., 2021)
Cation (cmol/kg):			
K	0.1	Low	Conductometric (Makarychev & Motuzova, 2013)
Na	0.1	Low	
Ca	9.5	Medium	
Mg	3.6	High	
CEC (cmol/kg)	36.1	High	Colorimeter (Matula, 2011)
Alkaline saturation (%)	37.1	Medium	Spectrophotometer (X. Lin et al., 2021)
Exchangeable aluminum (cmol/kg)	0.01	Low	KCl (Antonangelo et al., 2022)
Exchangeable hydrogen (cmol/kg)	0.4	Low	
Azotobacter (× 10^6 cfu/g)	7.4	Not analyzable	Plate-count (Aasfar et al., 2021)
Azospirillum (× 10^6 cfu/g)	8.3	Not analyzable	

Note. KCI = Potassium chloride; K_2O = Potassium oxide; P_2O_5 = Phosphorus pentoxide; CEC = Cation exchange coefficient; $HClO_4$ = Perchloric acid; HNO_3 = Nitric acid

The effect of combining straw with compost was determined by referring to an experimental approach that compares a test field (A) and the control field (B) (Hansson, 2019). This research is based on an experimental approach that intends to analyze the effect of treatments on certain samples with the same characteristics (de Janvry et al., 2017). In the first field A, cultivation of lowland rice applied 4 tons of straw per hectare (ha) with compost from paddy stubble obtained through the

cultivation style adopted by the local farmers in Karawang (Legowo 4:1). However, field B, which is the control only applied the cultivation style of these local farmers. Field A was a regenerative rice farming model that is environmentally friendly and seeks to reuse the remaining resources, whereas field B was a conventional model highly dependent on external inputs.

Cultivation Stages and Data Analysis

This research started by composting rice straw and stubble from the previous harvest, lasting one month before planting. The composted materials are the major input for rice cultivation in field A. Characteristics of the treatment in field A include land flooding, application of fertilizer (urea 300 kg/ha and SP-36 200 kg/ha, Petrokimia Gresik, Indonesia), plant spacing of 25 x 25 cm^2, pests control method adopted from the local farmers, and application of compost the day before planting. However, field B with the control only applied to cultivate styles of the local farmers. Also, soil and plant sampling took place during the maximum vegetative phase (fifth-seventh leaf stage), which helps to know the effectiveness of the treatment (Moldenhauer et al., 2001). The rice IR-32 was sampled on the 55th day after planting, with six samples from each group. These samples were obtained through random selection and transects (crop plots) measuring 1 x 1 m^2 (Purnama et al., 2020; Riginos et al., 2011). Furthermore, the content analysis for soil and plants was carried out in collaboration with university laboratories and the government's agricultural research centers in West Java.

The response variables in soil and plants (N, P, K, C, Si), water content, and pH were all measured. In addition, the rice yield and plant components from the two fields were measured (Table 2). Data obtained were analyzed using the paired t-test, employed when analyzing the difference in means of the two correlated groups (Dede, Wibowo, et al., 2022). Paired t-test is commonly used to analyze the differences and significance in data involving the test and control groups, as shown in Equation 1 (Hugar & Savithramma, 2017). Significant differences are shown by the *t*-value, which is greater than the t-table. It is also referred to as the *p*-value with a 95% confidence level.

$$t = \frac{D}{SD / \sqrt{N}} \qquad (1)$$

where *t* is the t-count value, *D* is the average measured value for groups 1 and 2, SD indicates the standard deviation, and N represents the number of samples.

RESULTS AND DISCUSSION

Soil Characteristics and Paddy Nutrients

The results showed that the field with the compost treatment was beneficial to both the plants and the soil. The increase observed in the nutrient levels are as follows: C-organic (5.69%), total N (16.67%), P (7.53%), and K (42.34%). Statistically, it was shown that there was a significant increase in nutrient levels, although there was an exception for P (Table 3). Descriptively, the P level increase was higher than the C-organic in the soil. It

Table 2

Parameters of soil and rice observations

Parameter	Acquisition
Water	Gravimetry (Pasha et al., 2016)
pH	Potentiometer (Singh et al., 2021)
C-organic (%)	Walkey and Black (Munawaroh et al., 2022)
Total N (%)	Kjeldahl (Todorova et al., 2011)
C/N	Ratio (Zhang et al., 2016)
P_2O_5 (%) K_2O (%)	$HClO_4$ + HNO_3 (Sulaeman et al., 2005)
Ash (%) Si (%)	Wet incineration (Sulaeman et al., 2005)
Number of clumps	Count (Longland & Dimitri, 2016)
Straw weight (kg) Dry grain weight (g)	Scale (Kumar et al., 2021)
Panicle length (cm)	Tape measure (Bao et al., 2019)
Grains per panicle	Count (Wu et al., 2019)
Weight per 1,000 gram	Scale (Thakur et al., 2010)

Note. P_2O_5 = Phosphorus pentoxide; K_2O = Potassium oxide; $HClO_4$ = Perchloric acid; HNO_3 = Nitric acid

Table 3

Differences in the nutrient content of the soil

Parameter	Treatment	Mean	SD	*t*-value	*p*-value
C-organic (%)	A	2.97	0.11	-2.726	0.02
	B	2.81	0.09		
Total N (%)	A	0.28	0.02	-3.413	0.01
	B	0.24	0.02		
P (ppm)	A	2.57	0.15	-2.208	0.05
	B	2.39	0.15		
K (ppm)	A	104.25	17.44	-3.826	0.00
	B	73.24	9.49		

Note. Treatment A = Test field (with straw compost); Treatment B = Control field

is because the C-organic at the study site was at moderate levels, different from P, which was previously low. The P content was the lowest compared with other nutrients, as shown in Table 4. Almohammedi et al. (2014) and Rakotoson et al. (2021) argued that the insignificant P increase in the soil after using straw compost is an indication that the presence of this nutrient depends on inorganic fertilizers and naturally occurring phosphate compounds excreted as wastes by bats (guano). Also, significantly increasing C-organic, N, and K levels have previously been partially investigated by scientists. Based on the study of T. Li et al. (2019), increasing C-organic is caused by the ability of microorganisms to break down carbon compounds, which easily blend with the soil. However, Moe et al. (2019) showed that applying 50% fertilizer mixed with compost contained less than 4% of N, showing a similar effect to conventional cultivation, which is fully dependent on inorganic fertilizers. Consequently, using straw-based compost should be encouraged for optimal doses of this nutrient while reducing farmers' dependence on inorganic fertilizers.

Table 4

Content of rice straw compost

Parameter	Value	Parameter	Value
Water	60	P_2O_5 (%)	0.17
pH	7.25	K_2O (%)	0.6
C-organic (%)	15.24	CaO (%)	5.52
Total N (%)	0.75	MgO (%)	1.25
C/N	20.32	CEC (cmol/kg)	19.08

Note. P_2O_5 = Phosphorus pentoxide; K_2O = Potassium oxide; CaO = Calcium oxide; MgO = Magnesium oxide; CEC = Cation exchange coefficient

Furthermore, the increasing nutrients in the soil after applying straw compost are well-absorbed by rice plants. It is evident from the rice yield and the nutrient in the crops, such as C-organic, total N, K, and Si. According to Table 5, the weight of the dry straw in field A increased by 29.96% compared with the conventional method, and there was an increase in the nutrient content of the plants. However, this treatment did not increase the ash and P content of the rice, while the conventional cultivation in the control field resulted in higher values for both components, 4.84 and 4.35%, respectively. Statistically, the increase in nutrients for Si is not significant. This compost treatment showed increasing C-organic, total N, and K without affecting ash, P, and Si contents. Ash and Si are components associated with each other and useful for stemming pest attacks, plant resistance to diseases, and producing

agroecosystems restoring enzymes (Karam et al., 2022; Mahmad-Toher et al., 2022; Ratnayake et al., 2018). The ash and Si levels were not significant in plants due to the high pH of the soil, thereby preventing the well-absorption of these substances by the roots. Increasing C-organic, N, and K should be compatible with P in the crop (Figure 1). Zhao et al. (2016) found that organic matter and essential nutrients contribute positively to agricultural land productivity because plants grow optimally. Hence, it is observed that there is a need for the supply of P from inorganic fertilizers while reprocessing straw compost is needed for the compounds making up the plant nutrients.

Table 5
Differences in straw weight and rice nutrients

Parameter	Treatment	Mean	SD	*t*-value	*p*-value
Dry grain weight (g)	A	89.91	12.92	-2.84	0.02
	B	62.97	19.34		
C-organic (%)	A	51.63	9.79	-3.21	0.01
	B	38.78	0.71		
Ash (%)	A	16.12	0.8	1.35	0.21
	B	16.9	1.16		
Si (%)	A	11.08	0.93	-0.88	0.4
	B	10.63	0.83		
N (%)	A	1.33	0.14	-3.81	0
	B	1.08	0.08		
P (%)	A	0.23	0.01	1.61	0.14
	B	0.24	0.02		
K (%)	A	1.76	0.12	-2.39	0.04
	B	1.63	0.06		

Note. Treatment A = Test field (with straw compost); Treatment B = Control field

Paddy Yield and Its Components

Significantly applying straw compost increased the number of clumps and dry grain weight in rice. It also increased the grains per panicle, panicle size, and grain weight, as shown in Table 6. Also, a significant increase, with a *p*-value of less than 0.05, was seen in dry grain weight, panicle length, and grains per panicle. Furthermore, the treatment did not affect straw weight due to the same value obtained from the two fields. It shows the effectiveness of applying straw compost with the conventional cultivation style by the local farmers, as the dry grain

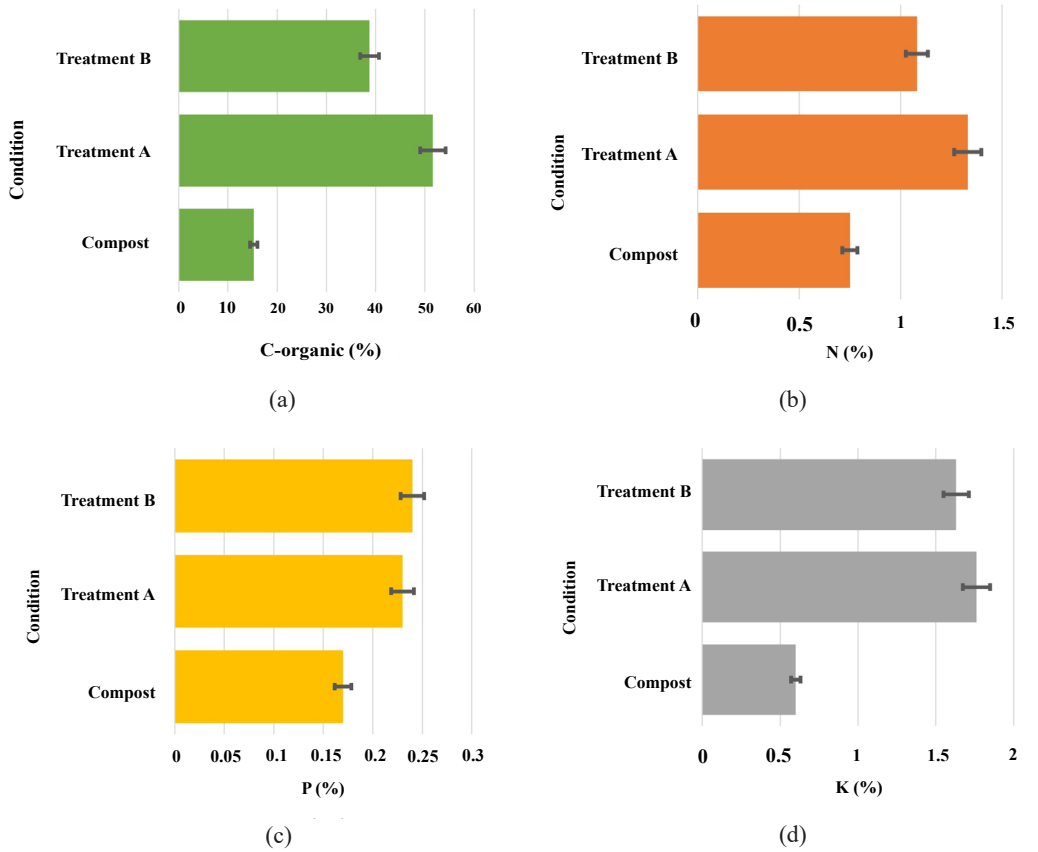

Figure 1. Comparison of various nutrient content in compost and its absorption in rice plant. The application of straw compost increased C-organic (a), nitrogen (b), and potassium (d) levels but not phosphorus (c)

Note. Treatment A = Test field (with straw compost); Treatment B = Control field

weight increased by 10.95%. These results align with research conducted by Xie et al. (2015), which showed the effectiveness of compost on soil organic matter content and rice production up to 7.1–12.1%. Additionally, the increase in dry grain weight indicates the abundance of grains per panicle. Applying straw compost raised the number of grains in each panicle by almost 10%, showing that the abundance of rice grains does not reduce its quality. Combining compost, proper treatment, and the right timing for harvest are the keys to improved rice production (Ho et al., 2022).

Straw compost triggered the proper growth of the rice components, such as the clumps, panicle length, and grain. This treatment benefits the farmers by reducing costs for agrochemical fertilizers since it has proven to produce higher quality rice plants compared with the conventional cultivation method. In tropical regions, rice straw compost is good for increasing the growth and productivity of crops. Its application

Table 6

Plant components and rice productivity after treatments

Parameter	Treatment	Mean	SD	*t*-value	*p*-value	Difference (%)
Number of clumps	A	32	3.56	-1.69	0.12	6.67
	B	28	4.59			
Straw weight (kg)	A	2.58	0.26	0.00	1.00	0.00
	B	2.58	0.45			
Dry grain weight (g)	A	1,274.17	122.56	-2.53	0.03	10.95
	B	1,022.67	210.47			
Panicle length (cm)	A	23.3	0.67	-2.59	0.03	3.88
	B	21.56	1.5			
Grains per panicle	A	163	13	-2.70	0.02	9.76
	B	134	23			
Weight per 1,000 gran	A	30	0	-1.58	0.14	2.86
	B	28.33	2.58			

Note. Treatment A = Test field (with straw compost); Treatment B = Control field

also improved the soil health for rice cultivation. Straw compost helps to solve the problem of low organic matter because the C-organic in the soils is low to moderate (80.1%) during rice cultivation in Karawang (Balai Penelitian Tanah [Balittanah], 2010). Also, treatment with straw compost during rice cultivation increased soil organic matter and reduced leaching of soil nutrients (Lenin et al., 2021). Straw compost improved the absorption of essential nutrients for rice plants, thereby increasing the cation exchange coefficient (CEC) in the soil (J. Li et al., 2014).

This positive result shows that the success of restoring paddy fields is vital to food security and self-sufficiency. There is a need for general coordination from the government, academics, researchers, and agricultural activists on properly using straw compost during rice cultivation, thus improving the farmers' welfare. In addition, using harvested straw as compost is a good means of eradicating the burning of agricultural wastes, which releases greenhouse gases into the atmosphere. Regenerative farming activities are easier to achieve because the life cycle of rice plants produces zero or less waste, as all its parts, except for rice grain and bran, are useful on the fields as input for the next cultivation process. Furthermore, rice is one of the major cereals humans depend on for carbohydrates; hence, the sustainability of

its cultivation needs serious attention from all stakeholders. Conclusively, using straw compost during rice cultivation is more environmentally friendly and aligns with sustainable principles.

CONCLUSION

Using straw compost during intensive rice cultivation has increased soil and plant nutrients. It also improved rice growth and productivity compared to conventional agrochemical cultivation practices. Using straw in the previous planting season is more profitable for farmers than burning it to ashes. Specifically, it increased the C-organic, N, and K contents of the soil and the rice plants. The rice plants had better growth, as seen from the number of clumps, panicle length, and weight per panicle. Additionally, this treatment during rice planting resulted in higher dry grain and grain weight compared with the conventional methods used by local farmers. Furthermore, it helps solve problems by tackling agricultural waste, is more environmentally friendly, prevents high emission greenhouse gases into the atmosphere, and is a major means of implementing regenerative agriculture for intensive rice planting. These results can be a reference for wider trials through the development of large-scale research in rice cultivation. However, it requires further studies to know the effects of straw compost when applied to a wider area and varying elevation, different soil, rice varieties, and climates in tropical to sub-tropical environments, especially in lowland areas. Research on carbon sequestration in soils in the future can support the impact of utilizing straw compost at a certain time.

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Review Article

Mosquito as West Nile Virus Vector: Global Timeline of Detection, Characteristic, and Biology

Jafar Ali Natasha[1], Abd Rahaman Yasmin[1,2]*, Reuben Sunil Kumar Sharma[1], Saulol Hamid Nur-Fazila[3], Md Isa Nur-Mahiza[3], Siti Suri Arshad[3], Hussni Omar Mohammed[4], Kiven Kumar[5], Shih Keng Loong[6] and Mohd Kharip Shah Ahmad Khusaini[7]

[1]Department of Veterinary Laboratory Diagnosis, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Laboratory of Vaccines and Biomolecules, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[3]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[4]Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca, 14853 New York, USA
[5]Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[6]Tropical Infectious Diseases Research and Education Centre, Universiti Malaya, 50603 Kuala Lumpur, Malaysia
[7]Department of Conservation of Biodiversity of Wildlife and National Park Malaysia, Jalan Cheras, 56100 Kuala Lumpur, Malaysia

ABSTRACT

Mosquitoes are extremely important vectors that transmit zoonotic West Nile virus (WNV) globally, resulting in significant outbreaks in birds, humans, and mammals. The abundance of mosquito vectors combined with the migratory flying behaviour of wild birds across the globe has exacerbated the dynamics of WNV infection. Depth understanding of the WNV infection requires a comprehensive understanding of the character of the vector in terms of their taxonomy, morphology, biology, behaviours, preferences, and factors that promote their breeding. Most susceptible animals and humans may experience serious neurological illnesses such as encephalitis. Little is known about the susceptibility of mosquitoes to WNV

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E-mail addresses:
natashali036@gmail.com (Jafar Ali Natasha)
noryasmin@upm.edu.my (Abd Rahaman Yasmin)
reuben@upm.edu.my (Reuben Sunil Kumar Sharma)
nurfazila@upm.edu.my (Saulol Hamid Nur-Fazila)
nurmahiza@upm.edu.my (Md Isa Nur-Mahiza)
suri@upm.edu.my (Siti Suri Arshad)
hussni.mohammed@cornell.edu (Hussni Omar Mohammed)
kivenkumar@yahoo.com (Kiven Kumar)
loongsk@um.edu.my (Shih Keng Loong)
khusaini@wildlife.gov.my (Mohd Kharip Shah Ahmad Khusaini)
*Corresponding author

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infection. This review provides insightful knowledge about the characteristics of mosquitoes that carry WNV and their susceptibility to WNV infection. The context of mosquito's involvement in WNV transmission is demonstrated through space and time from the 1950's until to date. The historical timeline of WNV transmission strength was significantly intensified via the complex interactions between vector, virus, and environment. Such knowledge will provide valuable insights into vector control intervention mitigation strategies, especially in tropical climate countries like Malaysia.

Keywords: Characteristics, mosquitoes, timeline, vectors, West Nile virus

INTRODUCTION

Approximately 3,500 mosquito species have been discovered globally, which can be categorised into 41 genera (Xia et al., 2018). Among them, some mosquitoes are associated with viruses, which have been further classified into two categories: mosquito-specific and mosquito-borne (Xia et al., 2018). Mosquito-specific viruses do not replicate in other species except mosquitoes, while mosquito-borne viruses replicate in mosquitoes and infect other animals (Bolling et al., 2015). The latter category of viruses replicates within mosquitoes and is regarded as the most significant cause of arboviral diseases, causing significant public health issues in medical and veterinary fields (Bolling et al., 2015).

West Nile virus (WNV) is an emerging, zoonotic arthropod-borne virus transmitted by mosquitoes, primarily *Culex* species, across the continent (Rizzoli et al., 2019). In 2020, a 63-year-old man was reported dead in the United States due to WNV complications (Lewis, 2020). Meanwhile, approximately 25 people were diagnosed with West Nile fever in the Seville district in Spain, while 39 and 66 cases were detected in Greece and European Union regions during the same year. About six deaths were recorded in Greece, yet the disease remained unnoticed and unresolved over time (Allen, 2020). Not only limited to temperate countries, but WNV has also been detected in tropical climate countries like Malaysia in the mosquito, humans, and animals (Ain-Najwa et al., 2020; Bowen et al., 1970; Marlina et al., 2014).

WNV is a positive-sense single-stranded RNA virion containing an icosahedral capsid with a spherical envelope grouped in the *Flaviviridae* family (Rossi et al., 2010). As the name implies, the virus was first isolated from a febrile woman in the West Nile district of Uganda in 1937 (Smithburn et al., 1940). It unexpectedly spread to the Western Hemisphere in 1999 and expanded to various regions globally (Chancey et al., 2015). WNV causes significant clinical outcomes in birds, humans, and other susceptible mammals. The infected incidental hosts are mostly dead-end hosts, resulting in various clinical manifestations ranging from predominantly asymptomatic infection to clinical syndromes such as fever and skin rashes. Severe cases involve

neurological infections such as encephalitis, meningitis, and death (Rossi et al., 2010).

WNV transmission occurs in the enzootic cycle between wild birds, the reservoir host, and mosquitoes as vectors (Ahlers & Goodman, 2018). Naïve mosquitoes become infected during the blood-feeding of infected birds and subsequently transmit WNV to a wide range of susceptible hosts, including humans and animals. The renowned role of mosquitoes as vectors of WNV infection has been widely discussed. Being prevalent across the globe, including in Malaysia making, mosquitoes are an interesting subject to dig into in-depth, especially in terms of their character and effects during WNV infection. As the characteristics and susceptibility of mosquitoes in WNV infections are poorly discussed, this review will provide the information in depth.

GLOBAL TIMELINE OF WNV DETECTION IN MOSQUITO

WNV infection has been dispersed globally across Africa, Europe, North America, South America, Asia, and Australia. Various species of *Culex* spp. have been reported based on species diversity and vector competence (Figure 1). The first evidence of the detection of WNV in mosquito communities was revealed in Egypt with the discovery of *Culex univittatus* and *Culex antennatus* (Taylor et al., 1953). During the 1960s, WNV was detected in *Culex modestus* in France, isolated in humans (Hannoun et al. as cited Balenghien et al., 2008, p. 592). In Malaysia, the first study was published in 1970, at which about three *Culex* spp. (*Culex tritaeniorchynchus*, *Culex vishnui*, and *Culex pseudovishnui*) was found positive for the Kunjin virus, a subtype of WNV (Bowen et al., 1970). Later, various mosquito species was recognised, notably from *Culex* spp., as potential WNV vector in every continent up to the year 2020 in this world except Antarctica (DiMenna et al., 2006; Eastwood et al., 2011; Farajollahi et al., 2011; Hamer et al., 2009; Lu et al., 2014; Maquart et al., 2016; Martínez-de la Puente et al., 2018; Mixão et al., 2016; Orshan et al., 2008; Reisen et al., 2014).

TAXONOMY AND MOSQUITO GENUS THAT HARBOUR WNV

Mosquitoes belong to the phylum Arthropoda, class Insecta, subclass Pterygota, order Diptera, and suborder Nematocera. The Culicidae family is divided into three subfamilies, Culicinae, Anophelinae, and Toxorhynchitinae (Table 1). Culicinae (*Aedemomyia*, *Aedes*, *Armigeres*, *Coquillettidia*, *Culex*, *Culiseta*, *Ficalbia*, *Haemagogus*, *Heizmannia*, *Mansonia*, *Orthopodomyia*, *Psorophora*, *Topomyia*, *Trichoprosopon*, *Uranotaenia*, and *Verralina*) and Anophelinae (*Anopheles*) are of medical and veterinary importance as they carry deadly pathogens to animals and humans (Becker et al., 2010). The female mosquitoes from both subfamilies feed on blood to spawn progenies, contributing extensively to the spread of mosquito-borne diseases (Becker et al., 2010; Tandina et al., 2018). In contrast, members of Toxorhynchitinae feed on nectar as the source of nourishment (Taylor et al., 1953)

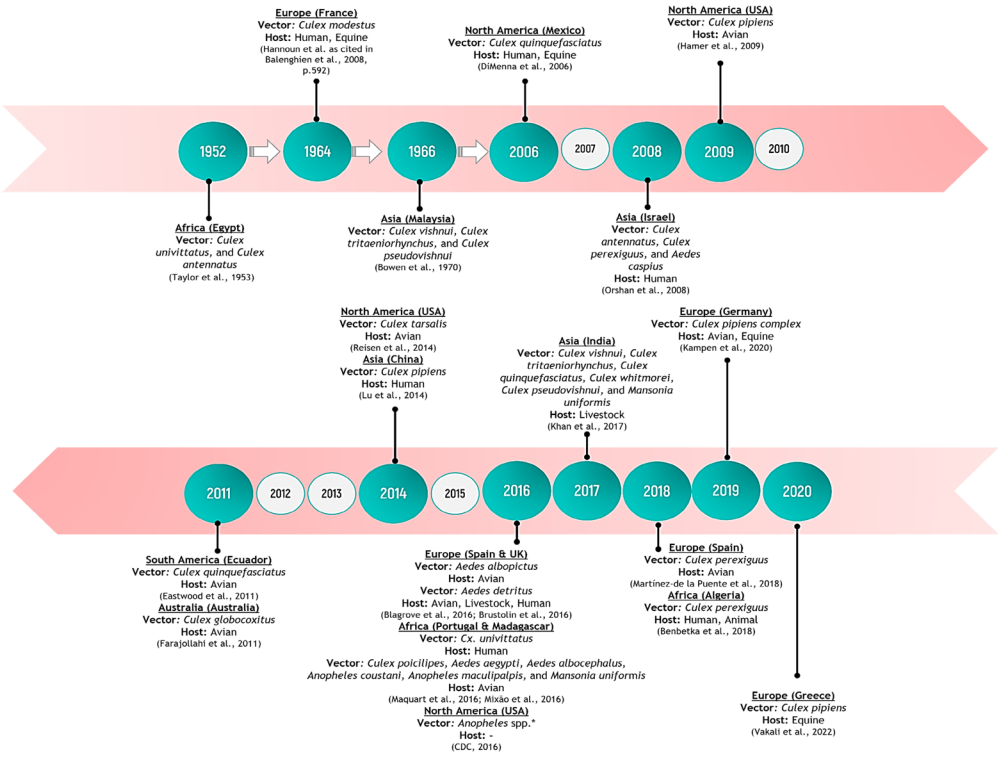

Figure 1. Global timeline of West Nile virus (WNV) vector according to species, continents, and host. This worldwide distribution was detected in different mosquito species from the year 1950's until 2020

Note. *WNV was detected in the United States from multiple countries from 1999 to 2016. *Anopheles* spp. are comprised of *Anopheles atropos*, *Anopheles barberi*, *Anopheles bradleyi*, *Anopheles franciscanus*, *Anopheles freeborni*, *Anopheles hermsi*, *Anopheles punctipennis*, *Anopheles quadrimaculatus*, and *Anopheles walkeri*

Table 1

Taxonomical classification of mosquito

Taxonomy	Classification		
Kingdom	Animalia		
Phylum	Arthropoda		
Class	Insecta		
Order	Diptera		
Suborder	Nematocera		
Family	*Culicidae*		
Subfamily	*Culicinae*	*Anophelinae*	*Toxorhynchitinae*
Genus	*Aedemomyia*, ***Aedes***, *Armigeres*, *Ayurakitia*, ***Coquillettidia***, ***Culex***, ***Culiseta***, ***Deinocerites***, *Eretmapodites*, *Ficalbia*, *Galindomyia*, *Haemagogus*,	***Anopheles***, *Bironella*, *Chagasia*	Toxorhynchites

Table 1 *(Continue)*

Taxonomy	Classification		
Genus	*Heizmannia*, *Hodgesia*, *Isostomyia*, *Johnbelkinia*, *Kimia*, *Limatus*, *Lutzia*, *Malaya*, ***Mansonia***, *Maorigoeldia*, *Mimomyia*, *Onirion*, *Opifex*, ***Orthopodomyia***, ***Psorophora***, *Runchomyia*, *Sabethes*, *Shannoniana*, *Topomyia*, *Trichoprosopon*, *Tripteroides*, ***Uranotaenia***, *Verralina*, *Wyeomyia*, *Zeugnomyia*	*Anopheles*, *Bironella*, *Chagasia*	Toxorhynchites

Note. The vectors are classified into seven taxonomic ranks. The ten genera of vectors involved in West Nile virus infection were bolded, and their distinctive features were discussed further in Table 2

and are poorly known due to the lack of medical significance (Hayes et al., 2005). Nevertheless, their larvae are used as a mosquito biological control as they prey on other mosquito larvae, gradually reducing the number of medically important mosquitoes (Service, 2012).

Mosquito species are mostly found in warm and humid climates due to their conducive environmental conditions (Paz, 2015). According to Chancey et al. (2015), although WNV has been documented throughout all continents except Antarctica, vectors of WNV among the mosquito populations are different in every continent, as presented in Figure 1. Currently, 62 mosquito species from 10 genera are involved in WNV infection, i.e., *Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Culiseta*, *Deinocerites*, *Mansonia*, *Orthopodomyia*, *Psorophora*, and *Uranotaenia* (Centers for Disease Control and Prevention [CDC], 2016). Every genus can be differentiated through morphological features of prespiracular setae, postspiracular setae, scutellum, wing vein scales, and sterna scales that are presented on the thorax and abdomen parts (Table 2).

Laboratory experiments have demonstrated that *Aedes albopictus* and *Culex pipiens* var. *pallen* can transmit WNV (Kitaoka et al., 1950; Philip & Smadel, 1943). Additionally, Egypt reported the first isolation of WNV in 1952 from *Cx. univittatus* and *Cx. antennatus* (Taylor et al., 1953). According to Hayes et al. (2005), the abundance of mosquito vectors and the prevalence of the infection among the mosquito population is directly correlated to the severity of WNV transmission. *Culex* spp. is more frequently infected with WNV than other genera and is considered a competent vector of WNV. It was evident in a major outbreak in New York City in 1999 (Asnis et al., 2006), where *Culex* spp. was the most prominent and abundant genus compared to other genera. In the outbreak, 81% of the *Culex* spp. mosquitoes were infected with WNV with a viraemia of $10^{7.2\pm0.4}$ PFU/ml. Conversely, *Aedes* spp.

mosquitoes are classified as moderately efficient WNV vectors even though WNV was isolated from the *Aedes* spp. population during the outbreak (Turell et al., 2000). In 2002, a strain of WNV (NY99) carrying a mutation in its envelope nucleotide sequence adapted to a new *Culex* species causing a widespread epidemic involving crows, horses, and humans in the USA (Moudy et al., 2007; Snapinn et al., 2007).

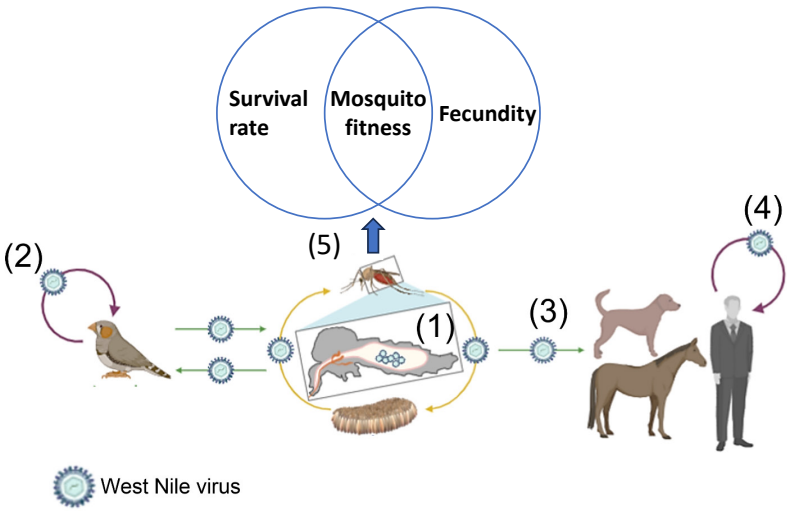

Figure 2. Overview of the West Nile virus (WNV) transmission cycle between reservoir hosts, i.e., diverse bird species and mosquitoes. Once the viraemic blood enters the body of the mosquitoes, the virus replicates in the midgut, followed by the dissemination to the salivary gland and ready to pass the virus to other hosts (1). When an infected mosquito bites the bird, the infected bird amplifies the virus and further spreads it to other birds during the roosting period (2). Concurrently, the infected mosquitoes will bite the dead-end host like humans, horses, and other mammals (3). Like in birds, the WNV transmission cycle can be circulated between humans through medical interventions such as blood transfusion, organ transplantation, and breastfeeding (4). Throughout the cycle, the mosquito population affects not only the hosts but also within the mosquito cycle via cytopathological effects in the body of mosquitoes, which are detrimental to mosquitoes with low survival rates and decreased fecundity effects (5)

Table 2

Distinctive features of mosquito genus involved in West Nile virus infection

Genus	Description	References
Aedes	Presence of postspiracular setae and dark scutum with the presence of white scales as well as broad scutellum	Becker et al. (2010); Jeffery et al. (2012)
Anopheles	Proboscis and maxillary palp have an equal length approximately with a round scutellum	Jeffery et al. (2012)
Coquillettidia	Absence on pulvilli, with yellow or purplish scutum with proboscis curved downward	Jeffery et al. (2012)

Table 2 *(Continue)*

Genus	Description	References
Culex	The absence of prespiracular and postspiracular setae on the thorax and pulvilli are present	Becker et al. (2010); Jeffery et al. (2012)
Culiseta	Presence of setae on the inferior part of mesepimeral and scale on remigium wing vein	Jeffery et al. (2012)
Deinocerites	Postspiracular setae and prespiracular setae absent Antenna longer than the proboscis	Becker et al. (2010)
Mansonia	Broad and intermixed with pale and dark on the wing vein in an asymmetrical pattern	Becker et al. (2010); Jeffery et al. (2012)
Orthopodomyia	Tarsomere leg part I is longer than the tarsomeres leg part from II-V	Jeffery et al. (2012)
Psorophora	Postspiracular setae and prespiracular setae are present. The apex of the abdomen pointed; the mesopostnotum bare	Becker et al., (2010)
Uranotaenia	Dark vertex with proboscis is swollen apically with no hair	Jeffery et al. (2012)

MORPHOLOGY OF MOSQUITOES THAT HARBOUR WNV

Mosquitoes are distinguished from other Nematocera such as Tipulidae, Bibionidae, Mycetophilidae, Sciaridae, Pschodidae, Chaoboridae, Simuliidae, Ceratopogonidae, and Chironomidae through the presence of a long-scaled proboscis feature, which is longer than the thorax and pointing forward with palps (Becker et al., 2010; Mullen & Durden, 2019). The body is divided into the head, thorax, and abdomen (Figure 3). The head consists of compound eyes, an antenna, a palp, and a proboscis (Service, 2012). The antenna and the palp are distinctive features of male and female mosquitoes and are used as sex determinants (Becker et al., 2010). Male mosquitoes have a plumose (bushy) antenna, with palp length as long as the proboscis. Female mosquitoes have a pilose (non-bushy) antenna with a shorter palp, as shown in Figure 4. A pair of pedicels is located on the head as touch and sound receptors. The proboscis is in the middle of the head in a cylindrical structure, which is responsible for injecting infected saliva and sucking the blood of hosts (Becker et al., 2010).

The thorax, which comprises three legs, each at the left and right sides of the body and a pair of wings, is placed between the head and abdomen of the mosquitoes. The vein of the wings is known as venation, which differs for some mosquito species. For instance, *Culex* spp. and *Coquillettidia* spp. wing scales are narrow and conspicuous with broad size (Becker et al., 2010). However, the wing veins are easily faded and similar in colour, causing these features to be not commonly used as key identification for species and sex determination. Eleven segments known

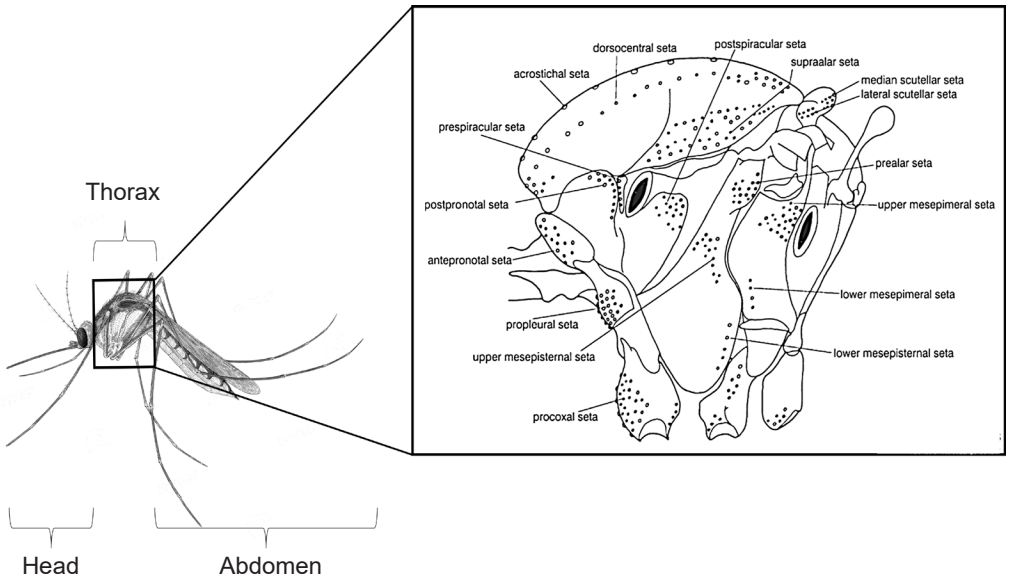

Figure 3. Morphology of mosquito. The lateral view of the thorax. The thorax is located between the head and abdomen, which comprises numerous setae in different sites to distinguish between genera and species of mosquitoes

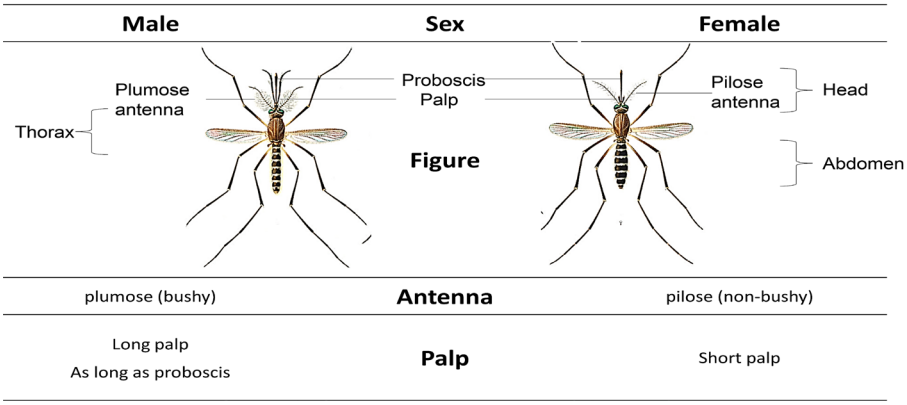

Figure 4. Male (Left) and Female (Right) *Culex* spp. mosquito based on distinctive features for sex identification, such as antenna and length of pulp (Becker et al., 2010)

as tarsals are in the abdomen and connected by an intersegmental membrane (Becker et al., 2010). The colour of the abdomen of an adult varies according to the species. For instance, *Culex* spp. mosquitoes generally appear yellowish to brown, while *Aedes* spp. normally appear black and white on the abdomen (Komp, 1923). The last tarsal comprises a pair of distinctive claspers and tiny finger-like cerci for males and females. The abdomen of an unfed female mosquito is thin and slender but expands, resembling an oval red balloon after a blood meal. The abdomen appears white when filled with eggs (Service, 2012).

BIOLOGY OF MOSQUITOES

All mosquitoes have four stages in their life cycle, i.e., the egg, larval, pupal, and adult stages, collectively known as complete metamorphosis (Becker et al., 2010). However, the development properties show great diversity between species as the mosquitoes emerge. The presence of water is necessary during the larval and pupal stages to allow them to grow (American Mosquito Control Association [AMCA], 2018). The lifespan of the mosquitoes is not more than two months on average. Typically, the life span of an adult male mosquito (≤ 10 days) is shorter than the female (Manimegalai & Sukanya, 2014). In contrast, adult female mosquitoes may live about 6 to 8 weeks in tropical regions and hibernate for 5 to 6 months in protected areas during winter (Service, 2012). These female mosquitoes come out during warm periods in the winter and lay successive batches of eggs (Lawler & Lanzaro, n.d.).

After copulation, the female mosquitoes hunt for a host to obtain blood meals for nourishment and egg maturation (Becker et al., 2010). The oviposition sites are influenced by water quality, local vegetation, light intensity, and food accessibility. The eggs are transparent initially and eventually darken to brown and black as they develop. The shape and features of the eggs are different depending on the mosquito family classification. For instance, Culicine mosquitoes lay elongated oval-shaped eggs. In contrast, Anopheline mosquitoes lay individual cigar-shaped eggs, which are laid in diverse forms, either individually or in a group adjacent to water bodies (Becker et al., 2010).

Eggs of most mosquito genera have a lengthy survival period but may easily die due to desiccation, except for *Aedes* spp., which can withstand a longer desiccation period (Becker et al., 2010). Greater chitin content and melanisation degree enhanced the egg resistance desiccation (ERD) level. Hence, *Aedes*' egg showed higher contents of chitin and melanin than other mosquito genera, contributing to its increased resilience towards desiccation under inhospitable environments (Farnesi et al., 2017). The species can withstand desiccation for up to 8 months (Kweka et al., 2018). Once the eggs are hatched, the larval stage develops and undergoes four instars before developing into the pupal stage. The larva inhabits aquatic conditions and continues to evolve in water bodies (Bashar et al., 2016). The size of the larva increases with every instar stage when they moult the outer skin. The level of each instar is determined by the head capsule size located anterior to the larvae. The tail, known as the siphon, is located on the posterior site and functions as a breathing apparatus via spiracles (AMCA, 2018). The breathing process occurs by having the larva hanging downward while exposing the siphon to the air. In water bodies, mosquitoes feed on algae, protozoa, and microorganisms as their source of nutrients (Becker et al., 2010; Diaz-Nieto et al., 2016). The development of the mosquito larval stage depends on environmental temperatures (Becker et al., 2010). For instance, the

floodwater mosquitoes, such as *Aedes vexans*, successfully develop at 30°C, while snow-melt mosquitoes like *Ochlerotatus cantans* prefer lower temperatures (~10°C) and cannot develop at temperatures over 25°C. In contrast, the larvae of *Cx. pipiens* thrive between 10 and 30°C (Becker et al., 2010).

The pupal stage continues after the fourth instar stage in the aquatic habitat. The pupa does not feed and can withstand a short period of desiccation. Tissue destruction of larval organs occurs prior to the development of the adult body within the pupal skin (Becker et al., 2010).

Once the adult emerges, the haemolymph pressure is raised for the legs and wings to stretch. The emerging males come out 1 to 2 days before the female completes sexual maturity concurrently with the emerging female. The mosquitoes take a short break on the water's surface before flying off to allow the body and wings to dry. After 24 to 48 hr of emergence, female and male adult mosquitoes feed on nectar before mating (Becker et al., 2010).

TRANSMISSION OF WNV

Horizontal transmission between the avian reservoirs, mostly from the family of passerine and charadriiform and the mosquitoes, primarily the *Culex* and *Aedes* genera, had contributed to the large outbreak of WNV in the USA (Komar et al., 2003). However, this transmission mode only partially explained the expansion of WNV infections. Other modes of transmission, such as vertical transmission, are important for maintaining WNV among mosquito populations, as shown in Figure 2 (J. F. Anderson & Main, 2006).

Vertical transmission depends on vector competence, which is affected by extrinsic and intrinsic factors. The extrinsic factors comprise virus dosage (Kramer et al., 1983) and extrinsic incubation temperature (Dohm et al., 2002). The extrinsic incubation period, which begins when the mosquito ingests the virus through an infected blood meal and ends when the host is infected (S. L. Anderson et al., 2010), is also crucial. Meanwhile, the intrinsic factors affecting vertical transmission are comprised of virus strains (Moudy et al., 2007), mosquito populations (Richards et al., 2007), and mosquito species (Goddard et al., 2003).

Consequently, mosquitoes are greatly affected by infection and resistance to WNV infection (Ciota et al., 2011). Studies reported mosquito-specific species influenced these conditions as measured parameters for determining the potential vector of WNV (Ciota et al., 2011). For instance, no significant differences in the survival and fecundity rates are observed, measured by the number of egg production and hatched eggs in WNV-infected female *Cx. pipiens* and unexposed female *Cx. pipiens* (Styer et al., 2007). Conversely, WNV-infected female *Culex tarsalis* experienced reduced fertility and increased blood-feeding rates (Ciota et al., 2011).

Nevertheless, WNV could replicate higher viral loads in *Cx. tarsalis* compared to *Cx. pipiens*, with up to $10^{7.7}$ PFU/ml, recovered under diverse experimental

conditions (Dohm et al., 2002; Styer et al., 2007). Distinctive viral loads were detected in four different body segments of the mosquito, including the head, thorax, abdomen, and leg segments, suggesting widespread dissemination to other body parts. The study estimated that the thorax, where the salivary gland is located, contained the highest viral load of WNV, ranging from $10^{5.5}$ to 10^{7} PFU/ml, followed by the head ($10^{4.8}$–10^{6} PFU/ml), abdomen ($10^{4.6}$–$10^{6.1}$ PFU/ml), and leg segment ($10^{3.5}$–10^{5} PFU/ml) (Styer et al., 2007).

The adaptation of the mosquito fitness towards WNV infection is shielded by the immune response of mosquito immunity against WNV, primarily on the salivary gland and midgut part (Ahlers & Goodman, 2018; Sim et al., 2014). The blood-feeding process begins with the insertion of the proboscis, a long thin needle-like structure found in the mouthpart of the mosquito. The proboscis comprises six stylets, including a hypopharynx, labrum, mandibles, and maxillae (Kong & Wu, 2010). It vertically pierces the host's skin (dermis) and injects saliva. The saliva contains heparin, acting as the anticoagulant (Ha et al., 2014), and sialokinin to increase endothelial permeability, which leads to vasodilation (Zeidner et al., 1999) and provides the anaesthetic effect (Ribeiro & Francischetti, 2003). During this process, the susceptible vector is infected with WNV from ingesting the viraemic blood meal of the amplifying host. A viral load at approximately 4.4 logs PFU WNV/ml is enough for the mosquitoes to establish WNV infection (Richards et al., 2007). Replication of the virus takes place in the midgut, followed by transfer to the salivary gland and dissemination throughout the mosquito body (Cheng et al., 2016). However, the midgut and salivary glands can act as barriers that impede the dissemination of the virus throughout the mosquito's body. As a result, not all mosquitoes serve as the vector of WNV (Vogels et al., 2017). The limitation of virus replication is species-specific, resulting in the distinct vector species being susceptible to the respective mosquito-borne viruses (Wanasen et al., 2004). The mosquito immune system and RNAi pathway in the innate immune response of the mosquito are protective, warding off death from WNV infection (Ahlers & Goodman, 2018). However, the maximum capacity for the different mosquito species to replicate WNV is unknown.

Unlike the high viral load in mosquitoes, dead-end hosts such as mice could only recover low amounts of WNV viral load (10^{2} PFU/ml of blood), impeding WNV transmission (Styer et al., 2007). Thus, other mammals, including humans, are specified as dead-end hosts for WNV, likely the endpoint in the WNV transmission cycle (Colpitts et al., 2012). Immunocompromised dead-end hosts could potentially develop higher levels of viraemia. For example, dogs treated with methylprednisolone (glucocorticoid) showed higher mean WNV titres than the control group, with up to 6.6×10^{8} PFU/mosquito values. Similarly, immunosuppressed hamsters treated with cyclophosphamide, one of the chemical

compounds used in chemotherapy, had increased WNV viraemic load, which continued to elevate for eight days post-infection of WNV, resulting in serious illness or death (Mateo et al., 2006). The exposure to the compound caused a decrease in the total leukocyte count, signifying that the animals were immunocompromised and developing clinical signs affecting several vital organs, i.e., the lungs, heart, kidney, and parts of the brain, involving the hippocampus, cerebral cortex, and cerebellum sections. Besides, high WNV titres (10^8 PFU/ml) were sustained for 8 days in the WNV-infected immunosuppressed hamster before death compared to the control hamster (Mateo et al., 2006).

BREEDING PREFERENCE OF MOSQUITOES THAT HARBOUR WNV

The ubiquity of adult mosquitoes in a habitat is dependent on diverse factors, including the host population and biotic and abiotic factors (Bashar et al., 2016). The lack of a hygienic environment contributes to various conducive breeding sites for mosquito species such as *Culex quinquefasciatus* and *Aedes aegypti* (Bashar et al., 2016). *Culex* mosquitoes are primarily abundant in peri-urban and rural areas due to continuous water sources from irrigation systems (Boyer et al., 2014). Water depth affects the suitability of the breeding habitat of different mosquito species. *Aedes aegypti* and *Ae. albopictus* are found at low water depth, whereas *Cx. quinquefasciatus* and *Culex tritaeniorhynchus* prefer high water depth for oviposition (Bashar et al., 2016). Additionally, Rueda et al. (2008) reported that freshwater and brackish water are preferable habitats for mosquitoes to thrive in, but they do not breed in marine habitats with high-salt concentrations. A study by Tandina et al. (2018) reported that, *Ae. aegypti* mosquitoes favour clean water with average organic content, which usually collects in tree holes and man-made containers. The abundance of artificial and natural containers has increased the habitats suitable for *Aedes* mosquitoes to grow (Tandina et al., 2018). Urbanisation and the abundance of vegetation contributed to increased habitats for *Aedes* spp. mosquitoes primarily, *Ae. aegypti* (Ebi & Nealon, 2016). It shows that every mosquito species has a distinct feeding habit. The *Culex* species are more active from dusk to dawn and are known as night-biting mosquitoes (Becker et al., 2010). According to Rohani et al. (2008), the *Culex* species favours polluted aquatic environments such as cesspools.

FACTORS THAT PROMOTE THE BREEDING OF MOSQUITOES AND ENHANCE WNV TRANSMISSION

Aquatic Site

According to Maquart et al. (2016), lakeshores with emergent vegetation promote the breeding of mosquitoes and present a higher number of positive WNV mosquito pools. However, there are WNV infections at sites far from lakeshores, even though the number of mosquito traps was lowered. Additionally, migratory birds are preferred to stay aloft in small lakes and

swamp areas for long or short rest where there are higher numbers of *Cx. pipiens* mosquito populations, the most WNV-competent vector (Gomes et al., 2013).

Migration of Wild Birds

WNV intensification and transmission by mosquito fully depends on introducing an immunologically naïve avian population (Reisen et al., 2014). Dispersion of birds into new geographic attributes to the prompt widespread of WNV transmission in North America in 1999 (Rappole, 2000). Various migratory and resident birds choose wetland habitats as resting and breeding sites, permitting the virus to be established through the migration route, which eventually allows rapid augmentation of WNV via enzootic transmission (Valiakos et al., 2014).

Ornithophilic Properties

The ornithophilic properties of some species of mosquitoes, such as *Cx. univittatus* that also feed on humans for their blood meal increases the potential threat of the species as WNV vectors (Mixão et al. 2016). Further, the blood preferences of *Cx. pipiens* are avian hosts, mainly from the Passeriformes family: Blue Jay, Common Grackle, House Finch, American Crow, House Sparrow, Cardinal, and American Robin. These bird species attribute to the reservoir host of the WNV transmission and viral expansion of WNV (Komar et al., 2003).

Climatic Condition

In terms of climate, the ambient temperature increases the transmission of WNV and vector abundance (Deichmeister & Telang, 2011). WNV was discovered throughout tropical and temperate regions of the world, which are conducive climates for *Cx. pipiens* and *Cx. quinquefasciatus* to breed and survive (Farajollahi et al., 2011). Environmental factors, mainly temperature and rainfall parameters are crucial in breeding mosquitoes and WNV transmission (Paz, 2015). Flooding and heavy rainfall are ideal environments for ardeid birds, which subsequently lead to the proliferation of the *Culex annulirostris* mosquito (van den Hurk et al., 2014). Further, higher temperatures increased the infectivity on the rate of virus replication (Kilpatrick et al., 2008).

Anthropogenic Activities

Anthropogenic activities involve the transformation in nature, which results from human activity such as deforestation, urbanisation, and global warming. Xia et al. (2018) revealed that the global warming and climate change phenomenon is prone to the indirect transfer of infected vectors, causing a higher number of mosquito viruses in China. Importantly in terms of vector ecology, *Culex* spp. mosquitoes prone to well adapted to the man-made habitat and rise in vector abundance, ultimately increasing the avian seroprevalence in urban sites (Epstein, 2001). Deforestation disrupts the natural habitat of vectors and influences the transmission of vector-borne diseases (Burkett-Cadena & Vittor, 2018). Besides, the emergence of puddles in deforested sites creates a favourable location for the development of larvae (Vora, 2008).

CONCLUSION

Successful WNV transmission from the amplifier host to the susceptible host has been largely contributed by the strong and impactful role of mosquitoes as vectors. By recognising the taxonomic and morphological characteristics of *Culex* spp. as the principal vector of WNV, its ornithophilic properties and factors that enhance their breeding provide a better understanding of WNV transmission and infection dynamics globally, including in Malaysia.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

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