



Pertanika Journal of

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***PERTANIKA* JOURNAL OF TROPICAL AGRICULTURAL SCIENCE**

About the Journal

Overview

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

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

Pertanika Journal of Tropical Agricultural Science currently publishes 6 issues per year (*January, February, May, June, August, and November*). It is considered for publication of original articles as per its scope. The journal publishes in **English** and it is open for submission by authors from all over the world.

The journal is available world-wide.

Aims and Scope

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

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Pertanika was founded in 1978. Currently, as an interdisciplinary journal of agriculture, the revamped journal, *Pertanika* Journal of Tropical Agricultural Science now focusses on tropical agricultural research and its related fields.

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To publish journals of international repute.

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

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

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

Welcome to the first issue of 2026 of the *Pertanika Journal of Tropical Agricultural Science (PJTAS)*!

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

This issue contains 25 articles: one short communication; two review articles; and the rest are regular articles. The authors of these articles come from different countries namely Brunei, China, Indonesia, Japan, Jordan, Malaysia, Nigeria, Pakistan, Thailand, United States of America, and Vietnam.

A selected article entitled “*Field Assessment of Plant Growth Performance and Residue Persistence of Saponin-Based Molluscicide Formulations*” evaluates the plant growth performance and environmental residue behaviour of natural saponin-based molluscicide formulations compared to commercial chemicals like niclosamide and fentin acetate. Using varying concentrations of rice crops and analysing residues via HPLC, the study found no significant negative impact on growth parameters such as plant height and biomass. Notably, saponin formulations demonstrated rapid biodegradability with half-lives under 10 days, presenting a safer and more environmentally friendly alternative to synthetic chemicals, which showed higher persistence and food safety risks. Further details of this study can be found on page 77.

The study entitled “*Improving Maize Yield, Biomass, and Selected Chemical Properties of an Acid Soil using Transformed Chicken Dung*” addresses the challenge of low agricultural productivity in Brunei’s acidic Ultisols and Oxisols. The researchers conducted a field trial using a Random Complete Block Design to assess the effectiveness of repurposed chicken dung as an organic amendment (OA). Findings revealed that integrating OA with chemical fertilisers significantly enhanced maize fresh cob yield and aboveground biomass by 89.42% and 50.95%, respectively, compared to using chemical fertilisers alone. This study provides a sustainable waste management strategy to restore degraded tropical soils. Full details of this study are available on page 145.

An article titled “*Optimising Pepper (Capsicum Annuum L.) Seedling Growth with the Synergistic Effects of Seaweed Waste Media and Beneficial Microbes Application*” explores the use of seaweed waste as a sustainable seedling medium to enhance plant productivity. Employing a Completely Randomised Factorial design, the study examined the synergistic effects of seaweed waste media combined with microbial consortia and mycorrhizal applications. Results indicated that these combinations significantly improved seedling growth, chlorophyll content, and nutrient levels, suggesting that seaweed waste can effectively substitute soil in nurseries to reduce transplanting stress and increase vigour. Further detailed information can be found on page 183.

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 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.

Editor-in-Chief
Kamal Md Uddin

Review Article

A Systematic Review of Seed Oils: From Extraction to Health-boosting Food Solutions

Norazlin Abdullah*, Nurulain Syuhada Mohamad Yazid, Laavanya Nadarajah, Nur Hafizah Malik, Norhayati Muhammad, and Fazleen Izzany Abu Bakar

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ABSTRACT

This systematic review presents recent research on seed oils, with an emphasis on extraction processes, health-promoting properties, and potential applications in food product development. As interest grows in sustainable and health-conscious food alternatives, seed oils have gained attention for their usefulness and nutritional value. However, there is a lack of up-to-date and systematic reviews that cover the entire scope of seed oil research, from extraction techniques to their nutritional and functional roles. To fill this gap, a systematic search was performed using the Scopus and ScienceDirect databases, with articles published between 2023 and 2025 being prioritised. The PRISMA guidelines guided this systematic review, with the resultant integration of relevant literature. The findings were categorised into three main themes: (1) extraction, characterisation, and functional properties of seed oils; (2) health benefits, nutritional value, and functional applications; and (3) seed oils in food product development and fat replacement. There has been significant improvement in extraction technologies, along with the identification of bioactive compounds present in seed oils,

which may provide beneficial health effects. This review emphasises the importance of seed oils in improving food quality and enhancing consumer health. These findings are an important resource for researchers and food industry professionals interested in exploring the broader applications of seed oils in food technology and health-oriented solutions.

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INTRODUCTION

The potential of seed oils to improve human health has been extensively studied, especially when they contain a considerable amount of unsaturated fatty acids. It has been found that substituting pumpkin seed oil for saturated fats in the diet can reduce the severity of atherosclerosis and non-alcoholic fatty liver disease. Another benefit to cardiovascular health is the phytochemical content of pumpkin seed oil, which enhances its anti-inflammatory properties (Morrison et al., 2015). Chia seed oil has been used to replace milk fat in ice milk because it is rich in omega-3 fatty acids. This makes the product more nutritious and gives it more antioxidant properties (Basuny et al., 2021). The fatty acid composition of seed oils makes them suitable replacements for industrial fats. Ficus sur seed oil contains stearic acid, which is considered a healthier option compared to industrial trans fats, while high stearic sunflower oil has saturated fats that can replace hydrogenated oils and palm oil in various applications (Nwanisobi et al., 2021; Salas et al., 2014).

Seed oils are also valued for their rich content of bioactive compounds. Tocopherols and polyphenols are among the compounds that contribute to the health-promoting properties of seed oils. This bioactive profile supports not only health benefits but also a variety of non-food applications. The diverse range of bioactive compounds found in the seed oils has led to their increasing use in the cosmetic industry and as sustainable alternatives to petroleum-based products (Pachau et al., 2019). In the cosmetic industry, oils derived from fruit seeds, including raspberry and grape seed oils, are increasingly used due to their beneficial effects on skin health. These oils help slow down the aging process and relieve stress-related skin conditions (Kaseke et al., 2020). Seed oils are known to improve skin conditions and are used in cosmetic formulations due to their moisturising and protective properties (Sumara et al., 2023). The oils extracted from sunflower, sesame, canola, and flaxseed seeds contain high amounts of essential fatty acids together with antioxidants and bioactive compounds. The high polyunsaturated fat content of seed oils, particularly omega-3 and omega-6 fatty acids, sustains cellular structure and promotes overall health (Quintero-Angel et al., 2023; Tiencheu et al., 2021).

Recent studies have highlighted the health-promoting properties of specific seed oils. Some examples include omega-3 rich flaxseed oil and its anti-inflammatory properties (Romanić et al., 2021) or pumpkin seed oil and its ability to enhance urinary functions and prostate health (Kang et al., 2021). Sesame and chia seed oils are rich in polyunsaturated fatty acids (PUFA), which have been shown to improve blood lipid profiles and lower cholesterol levels, thus reducing the risk of heart disease (Morrison et al., 2015; Parker et al., 2018; Sohoulou et al., 2022). The presence of bioactive compounds such as tocopherols and carotenoids contributes to the antioxidant and anti-inflammatory effects of seed oils, which help mitigate chronic inflammation and oxidative stress, both of which are commonly linked to various diseases (Šamec et al., 2022; Sumara et al., 2023). Pumpkin

seed oil has been shown to help regulate blood glucose, which is important for managing diabetes (Morrison et al., 2015; Sohoulı et al., 2022). Kiwifruit seed oil has also demonstrated potential in weight management by regulating inflammation, enhancing thermogenesis, and improving gut microbiota, which are essential factors in obesity management (Qu et al., 2019). Prickly pear seed oil is valued for their antioxidant and anti-inflammatory properties, which help protect against diseases related to oxidative stress and inflammation (Al-Naqeb et al., 2021).

As global awareness about the environmental impact of conventional fat sources rises, seed oils are emerging as sustainable options. For example, researchers have studied the rheology of fat blends containing rapeseed and sunflower oils to develop practical alternatives to palm oil in food applications (Kovács et al., 2024). This effort highlights the potential of seed oils to contribute to more sustainable and environmentally friendly practices in the food industry and beyond. Seed oils also show potential in the energy sector. Tomato seed oil has been identified as a good candidate for alternative fuel due to its favourable properties such as low volatility, low sulfur content, and high viscosity, making it suitable for use in diesel engines (Giannelos et al., 2005). The high oil yield and effective conversion of mandarin seed oil into biodiesel, which shows comparable properties to regular diesel fuels, have made it a promising alternative fuel (Azad, 2017).

This systematic review aims to provide a comprehensive overview of seed oils from their composition and extraction methods to the usage in health-boosting foods. The information will also provide insights on the health benefits offered by different seed oils ranging from disease prevention to overall wellness. It will highlight unresolved issues and propose future research directions to maximise the potential of seed oils in functional food development.

In this systematic review, the PICO framework was applied to formulate the research questions (Wurschi et al., 2025). This structured approach helps outline focussed research questions and ensures a systematic evaluation of existing literature. The four components of PICO are defined as follows:

1. Population (P): This is the population or context of interest, which in this study includes the use of seed oils in food systems and functional food development.
2. Intervention (I): This involves the application of seed oils, either through different extraction techniques or as fat substitutes in food products.
3. Comparison (C): This includes conventional fats or oils, such as animal fats and palm oil, used as the standard or baseline for comparison.
4. Outcome (O): This refers to the nutritional, functional, technological, or health-related effects of using seed oils.

Based on this framework, the following research questions were outlined:

1. How do different seed oil extraction methods affect the functional properties and quality of seed oils used in food systems?
2. What are the health benefits of incorporating seed oils in human diets compared to conventional fat sources?
3. How can seed oils replace animal fats or palm oil in food products while maintaining desirable nutritional and sensory qualities?

MATERIALS AND METHODS

An established framework that is frequently used for conducting systematic literature review (SLR) is the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) approach. It ensures transparency, completeness, and consistency throughout the entire process (Page et al., 2021). These guidelines provide clear instructions on how to systematically identify, screen, and choose studies to be included in a review. The method also highlights the significance of including randomised studies, which are essential in reducing bias and providing stronger evidence. Scopus and ScienceDirect were selected for this analysis because of their reliability and broad coverage.

The selection and screening process of the articles included in this review is summarised in Figure 1. The PRISMA approach was structured into four primary phases: identification, screening, eligibility, and data abstraction. In the identification stage, relevant studies were located through database searches. The screening part requires assessing these studies against predetermined criteria in order to exclude studies that were either inappropriate or of insufficient quality. In the eligibility step, the remaining studies were thoroughly evaluated to ensure they met the inclusion requirements. Lastly, the data abstraction phase includes extracting and synthesising information from the selected research, which is an important step for drawing relevant and valid conclusions. This systematic process guarantees that the review was performed with high accuracy, which results in reliable findings that can inform future studies and practice.

Identification

In the identification stage of the SLR, relevant records were obtained from Scopus and ScienceDirect by performing keyword-based searches using “seed oil” and “fat substitute” terms. These terms were employed to construct detailed search strings, which were then used to query both databases, as outlined in Table 1. Filters were applied to restrict the search to academic journal articles and conference proceedings published within recent years, ensuring the retrieved literature was both up-to-date and pertinent. This process resulted in a total of 960 records, with 921 records from Scopus and 39 from ScienceDirect.

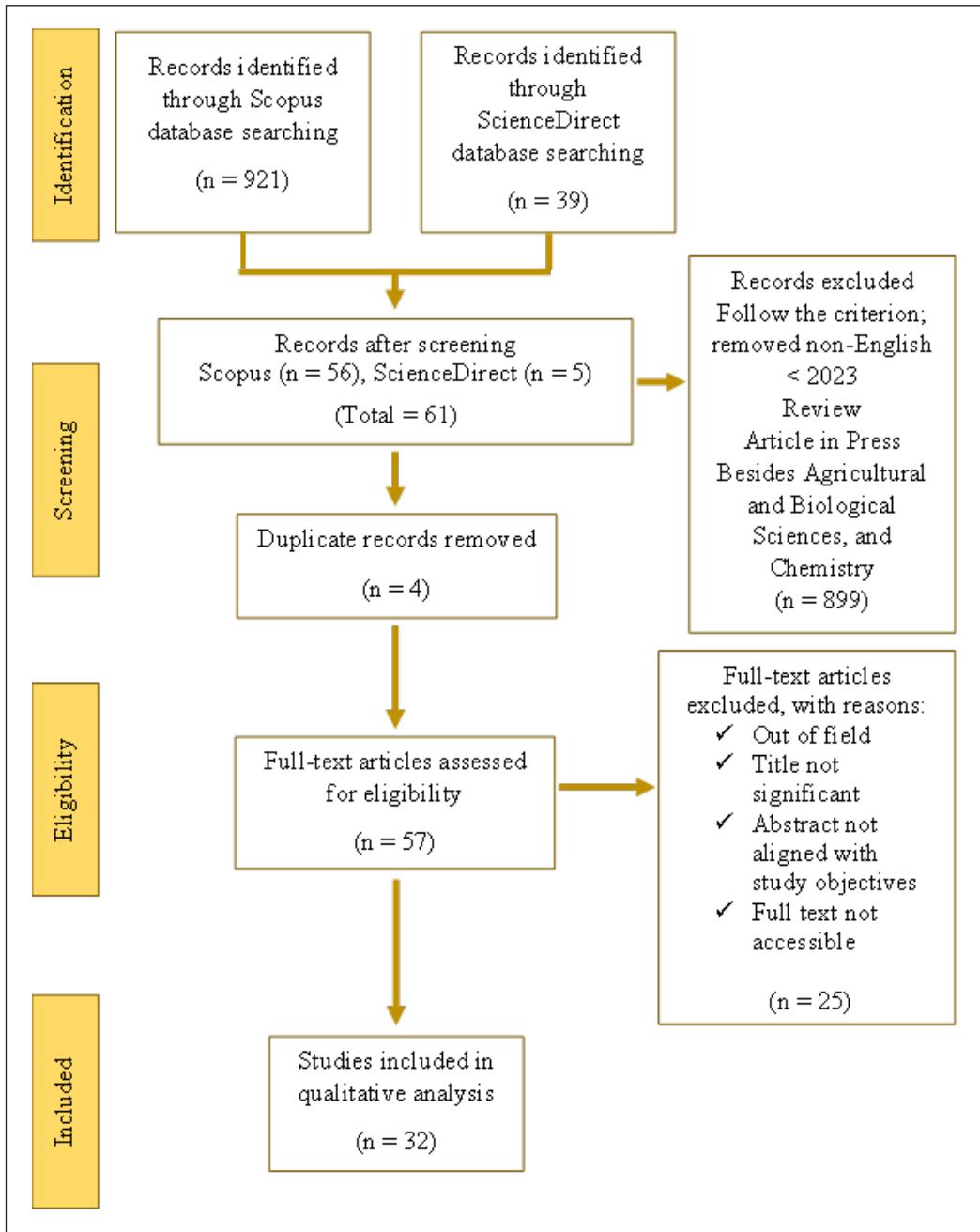


Figure 1. Flow diagramme of the proposed searching study (Moher et al., 2009)

Table 1
The search string

Scopus	ScienceDirect
TITLE-ABS-KEY ("seed oil" AND fat AND (substitute OR alternat* OR replace*)) AND (LIMIT-TO (SUBJAREA , "AGRI") OR LIMIT-TO (SUBJAREA , "CHEM")) AND (LIMIT-TO (DOCTYPE , "ar")) AND (LIMIT-TO (PUBYEAR , 2023) OR LIMIT-TO (PUBYEAR , 2024) OR LIMIT-TO (PUBYEAR , 2025)) AND (LIMIT-TO (PUBSTAGE , "final")) AND (LIMIT-TO (SRCTYPE , "j")) AND (LIMIT-TO (LANGUAGE , "English"))	"Seed oil" AND fat AND (substitute OR alternat OR replace) Date of Access: December 2024
Date of Access: December 2024	

Screening

In the screening stage, records retrieved during the identification phase were subjected to screening based on predefined inclusion and exclusion criteria (Table 2). Non-English articles, those published before 2023, review articles, and articles in press were excluded, along with records from journals outside the fields of Agricultural and Biological Sciences, and Chemistry. A total of 899 records were excluded following these criteria. Titles and abstracts of the remaining records were examined, and irrelevant articles were excluded. This process resulted in 56 records from Scopus and 5 from ScienceDirect, resulting in a total of 61 articles. Duplicate records across the two databases were removed, with 4 duplicates eliminated. The final 61 records were then prepared for further quality assessment and detailed analysis in subsequent stages of the SLR.

Table 2
The chosen criteria for searching

Criterion	Inclusion	Exclusion
Language	English	Non-English
Timeline	2023 – 2025	< 2023
Literature type	Journal (Article)	Review
Publication Stage	Final	Article in Press
Subject	Agricultural and Biological Sciences; Chemistry	Besides Agricultural and Biological Sciences, and Chemistry

Eligibility

In the third step of the SLR process, eligibility criteria were applied to 57 articles, resulting in the exclusion of 25 studies. These exclusions were based on various reasons such as irrelevance to the field, non-significant titles, abstracts not aligned with the study objectives, lack of full text access, or being out of scope. After applying these exclusion criteria, the remaining 32 studies were considered eligible and included in the qualitative analysis

(Table 3). This step ensures that only studies that closely align with the research objectives and meet the predefined quality standards are considered for in-depth analysis.

Table 3
Details of primary studies database

No.	Study	Journal	Scopus	ScienceDirect
1	Piasecka et al. (2023)	European Food Research and Technology	/	
2	Martínez et al. (2023a)	Foods	/	
3	Martínez et al. (2023b)	Foods	/	
4	Hou et al. (2025)	Food Hydrocolloids	/	/
5	Patil et al. (2024)	Industrial Crops and Products	/	
6	Hamed et al. (2024)	Journal of Food Measurement and Characterisation	/	
7	Li et al. (2024)	Food Chemistry: X	/	
8	Mesquita et al. (2023)	Food Science and Technology (Brazil)	/	
9	Avci et al. (2023)	Foods	/	
10	Momchilova et al. (2023a)	Journal of Central European Agriculture	/	
11	Ni et al. (2024)	Applied Food Research	/	/
12	Albayrak et al. (2023)	Journal of Food Science and Technology	/	
13	Zhang & Li (2024)	Food Production, Processing and Nutrition	/	
14	Pasrija et al. (2024)	Applied Food Research	/	
15	Akinsola et al. (2023)	Journal of Culinary Science and Technology	/	
16	Briceño-Islas et al. (2024)	Food Chemistry	/	
17	Botella-Martínez et al. (2023)	European Food Research and Technology	/	
18	Difonzo et al. (2024)	LWT	/	/
19	Khazaai et al. (2024)	Bioenergy Research	/	
20	Abbas et al. (2023)	International Journal of Food Properties	/	
21	Charoenphun et al. (2024)	Current Applied Science and Technology	/	
22	Joshi et al. (2024)	Journal of Food Measurement and Characterisation	/	
23	Momchilova et al. (2023b)	Food Research	/	
24	Alkabaa et al. (2024)	Foods	/	

Table 3 (continued)

No.	Study	Journal	Scopus	ScienceDirect
25	Pojjanapornpun et al. (2023)	Journal of the American Oil Chemists' Society	/	
26	Oba & Yıldırım (2024)	Journal of Food Measurement and Characterisation	/	
27	Yang et al. (2024)	Journal of Oleo Science	/	
28	Tarjuelo et al. (2023b)	Foods	/	
29	Visković et al. (2024).	Industrial Crops and Products	/	
30	Sepeidnameh et al. (2024)	Current Research in Food Science	/	/
31	Papatzimos et al. (2024)	Foods	/	
32	Tarjuelo et al. (2023a)	Journal of Functional Foods		/

Data Abstraction and Analysis

One of the primary assessment methodologies used in this study was an integrative analysis, which was used to investigate and synthesise multiple research designs, particularly quantitative methods. The main aim of this approach was to identify appropriate themes and subtopics related to the focus of study. The data collection phase was the first stage in theme development. As shown in Figure 1, a detailed review of 32 publications was conducted to extract claims or information related to the themes under investigation. Subsequently, significant studies on seed oils were assessed, with attention given to the methodologies used and the findings produced.

The next step involved collaboration with co-researchers to identify themes based on the findings in the context of this study. A log was kept throughout the data analysis process to document any analyses, challenges, perceptions or reflections that arose during the interpretation of the data. Lastly, the results were compared to identify any discrepancies in the theme development process. If conflicts regarding the concepts arose, these were discussed and solved through collective deliberation among the authors.

QUALITY OF APPRAISAL

The process of assessing the quality of selected primary studies is an essential step in SLR. Following the guidelines provided by Kitchenham (2007), it is crucial not only to choose relevant studies but also to critically evaluate the quality of the research findings and make a quantitative comparison of the studies. In this review, an approach of quality assessment (QA) based on Abouzahra et al. (2020) was adopted. This method consists of

six key criteria that serve as the basis for evaluating the quality of the studies included in this review.

The QA was based on a scoring procedure that includes three possible ratings for each criterion: “Yes” (Y) for a score of 1, indicating full satisfaction of the criterion; “Partly” (P) for a score of 0.5, suggesting partial fulfilment with some gaps; and “No” (N) for a score of 0, if the criterion is not met at all. The scoring was conducted by three independent experts who evaluated each study based on the following six QA criteria:

- QA1 : Does the study have a clear purpose? The criterion verifies whether the study has a well-defined purpose. A well-defined purpose gives the research a clear direction and guarantees that the scope of the research is well understood.
- QA2 : Does the work clearly convey its interest and usefulness? This examines whether the work clearly explains its relevance and potential implications. It is significant that the research makes its relevance clear to emphasise its contribution to the field and beyond.
- QA3 : Is the research methodology clearly stated? The focus of this criterion is on evaluating the methodology used in the study. A clear and appropriate methodology is key to getting reliable results and ensuring the reproducibility of the study.
- QA4 : Are the approach's concepts explained clearly? This criterion assesses whether the key concepts and theoretical framework of the study are well-defined and explained. Clear definitions are needed to understand the research methodology used.
- QA5 : Does the work get measured and compared to other comparable studies? This verifies if the study contextualises its findings in relation to existing scholarly literature. By benchmarking the study against other similar works, the researcher shows how the current study contributes to the existing body of knowledge and addresses gaps or challenges in the field.
- QA6 : Are there any clear constraints to the work? This criterion examines whether the research clearly states its restrictions. Recognising and discussing the limitations is critical to understanding the boundaries of the research findings and their generalisability.

Each expert evaluated the study independently, and each of their individual scores for every criterion were then combined to produce a total score for the study. The sum of the scores across all experts determined whether the study is of sufficient quality to proceed in the review process. A study must receive a total score greater than 3.0 to be eligible for inclusion in the next stage. The threshold ensures that only studies scoring an acceptable quality are eligible to proceed with the review process. This improves the validity and reliability of reported findings.

RESULTS AND FINDINGS

Based on the QA, Table 4 presents the performance results of the selected primary studies. The review of 32 articles reveals a generally high standard of research, with some areas excelling and others needing improvement.

Most of the studies showed excellent clarity in defining the purpose of their research (QA1), effectively outlining their objectives and the significance of their work. These articles often highlighted the practical implications of their findings, particularly in the context of food science and oil production. However, there were a few papers that lacked sufficient detail on their specific aims, leaving the reader with some ambiguity regarding the scope of the study.

For the explanation of the relevance and applicability of the research (QA2), most of the studies were successful in clearly demonstrating the relevance and usability of their findings, especially in addressing real-world challenges like food product enhancement or developing sustainable production processes. However, some studies could have better connected their findings to broader industry or social requirements, which would have made the research more compelling and impactful.

In terms of methodology (QA3), most articles performed well, with clear explanations of methods used in their research. This made it easy for readers to understand the adopted approach, particularly for studies on complex processes such as lipid emulsions or enzymatic reactions. However, there were some articles that had some gaps in method descriptions, which could make readers unfamiliar with the methods fail to fully understand the processes used.

Most of the studies also did well in defining the concepts behind their approach (QA4), so that major terms and concepts were explained in detail. This was especially crucial in more technical subjects like fatty acid compositions or seed oil extraction methods. However, some studies could have explained some concepts better, especially for specialized or niche subjects, to provide more clarity to less familiar readers.

Regarding the comparison with other similar work (QA5), most studies were able to position their results within the wider scientific literature by citing previous research. This helped to validate their results and highlighted the novelty of their contributions. However, a few studies missed this opportunity, not fully engaging with past literature, which made it harder to assess how their results fit into the existing body of knowledge.

Finally, reporting of the limitations of the studies (QA6) indicated that most of the studies did mention their limitations but these were usually brief and not elaborated. Common limitations mentioned included sample sizes, methodological constraints, and external factors affecting the outcome. A few studies did not discuss their limitations in detail, which could have led to questions about the robustness and applicability of their findings. A more thorough and transparent discussion of limitations would have enhanced the credibility of these studies.

In summary, while the studies generally performed well, with an average score above 75% and some showing more than 80%, there are clear areas for improvement. Most of the studies adequately defined their purposes, defining concepts, and comparing their findings with existing research. However, more in-depth discussions on study limitations and stronger engagement with prior work could further strengthen the overall quality of future research in this field.

Table 4
Quality assessment of the selected articles

Study	QA1	QA2	QA3	QA4	QA5	QA6	Total Marks	Percentage (%)
Piasecka et al. (2023)	1	1	1	1	1	1	6	100
Martínez et al. (2023a)	1	1	1	1	1	1	6	100
Martínez et al. (2023b)	1	1	1	1	1	1	6	100
Hou et al. (2025)	1	1	1	1	1	0.5	5.5	91.67
Patil et al. (2024)	1	1	1	1	0.5	1	5.5	91.67
Hamed et al. (2024)	1	1	0.5	1	0.5	0	4	66.67
Li et al. (2024)	1	1	1	0.5	0.5	0.5	4.5	75
Mesquita et al. (2023)	1	1	1	1	0	0.5	4.5	75
Avci et al. (2023)	1	1	1	1	1	0.5	5.5	91.67
Momchilova et al. (2023a)	1	0.5	1	1	1	0.5	5	83.33
Ni et al. (2024)	1	1	0.5	1	0.5	0.5	4.5	75
Albayrak et al. (2023)	1	1	0.5	0.5	0.5	1	4.5	75
Zhang & Li (2024)	1	1	1	1	1	0.5	5.5	91.67
Pasrija et al. (2024)	1	1	1	1	0.5	0.5	5	83.33
Akinsola et al. (2023)	1	1	1	1	1	1	6	100
Briceño-Islas et al. (2024)	1	1	1	1	1	0.5	5.5	91.67
Botella-Martínez et al. (2023)	1	1	1	1	1	0.5	5.5	91.67
Difonzo et al. (2024)	1	1	1	1	1	0.5	5.5	91.67
Khazaai et al. (2024)	1	1	1	1	1	0.5	5.5	91.67
Abbas et al. (2023)	1	1	1	1	0.5	1	5.5	91.67
Charoenphun et al. (2024)	1	1	0.5	1	0.5	0.5	4.5	75
Joshi et al. (2024)	1	1	1	1	0.5	1	5.5	91.67
Momchilova et al. (2023b)	1	1	1	0.5	1	0.5	5	83.33
Alkabaa et al. (2024)	1	1	1	1	0.5	1	5.5	91.67
Pojjanapornpun et al. (2023)	1	1	1	1	1	1	6	100
Oba & Yıldırım (2024)	1	1	1	1	0.5	1	5.5	91.67

Table 4 (continued)

Study	QA1	QA2	QA3	QA4	QA5	QA6	Total Marks	Percentage (%)
Yang et al. (2024)	1	1	1	1	1	1	6	100
Tarjuelo et al. (2023b)	1	1	1	0.5	1	0.5	5	83.33
Visković et al. (2024)	1	1	1	1	0.5	0.5	5	83.33
Sepeidnameh et al. (2024)	1	1	1	1	0.5	1	5.5	91.67
Papatzimos et al. (2024)	1	1	1	1	1	0.5	5.5	91.67
Tarjuelo et al. (2023a)	1	1	1	1	1	0.5	5.5	91.67

The themes generated were refined to ensure consistency. The process of selection and analysis was carried out by the author and co-authors to verify the validity of the issues identified. An expert review phase was incorporated to confirm the clarity, relevance, and appropriateness of each subtheme, thereby establishing domain validity. Any discrepancies or disagreements in the development of the themes were discussed and resolved among the authors. If inconsistencies in the themes arose, they were addressed collectively. In the final step, the themes were adjusted to maintain consistency. The validity of the issues was further evaluated by two experts, one specialising in chemistry and the other in food technology. This expert review phase played a critical role in confirming the clarity, importance, and adequacy of each subtheme, thereby establishing its domain validity. Any necessary revisions were made based on expert feedback and comments, allowing the themes to be fine-tuned accordingly.

Extraction, Characterisation, and Functional Properties of Seed Oils

This section compiles insights from 13 studies that explored various extraction methods, characterisation techniques, and the functional properties of seed oils. Numerous methods have been researched to enhance the yield of oil from seeds, with each method having its advantages and challenges. Ultrasound-assisted extraction (UAE) has emerged as a promising technique, as demonstrated by Piasecka et al. (2023), who utilised it to extract oil from cranberry seeds. This method, compared to conventional extraction processes, offers a more environmentally friendly approach, minimising harmful impacts. In the study, UAE parameters such as amplitude and extraction time were optimised to achieve the highest oil yield with an improved quality. The oil extracted under optimal conditions exhibited a unique fatty acid profile, with a higher presence of oleic and α -linolenic acids, and demonstrated improved oxidative stability, which could benefit the food and cosmetic industries. The findings suggested that ultrasound-assisted processes had the ability to reduce environmental impacts without sacrificing oil quality.

Innovative methods have also explored seed oils as functional ingredients. Hou et al. (2025) explored the role of pea protein/carrageenan emulsion gels as solid fat substitutes, incorporating various seed oils such as sunflower seed oil and palm stearin. These gels were evaluated for their texture, crystallisation, and sensory properties, providing insight into their potential use in food products like sausages. The study found that emulsion gels made with specific oil phases, particularly palm stearin, had comparable textural properties and crystallisation behaviour to those of animal fats. This opens up the possibility of developing healthier, plant-based fat substitutes that mimic the functional properties of traditional animal fats. Hence, it offers a sustainable and nutritious option for reduced-fat food products.

The versatility of seed oils extends beyond food products, with applications in industrial coatings also being conducted. Patil et al. (2024) focussed on the use of *Madhuca indica* (mahua) seed oil in the synthesis of polyurethane-based anticorrosive coatings. These coatings, derived from renewable sources, exhibited excellent thermal stability and corrosion resistance, which suggests seed oils can replace petroleum-derived materials in industrial applications. The study suggests the suitability of seed oils for the production of green and high-performance materials, which is consistent with growing demand for sustainable and renewable alternatives in various industries.

The potential use of seed oils as functional food additives has been also investigated based on their bioactive components, such as antioxidant and antimicrobial activity. Mesquita et al. (2023) studied papaya seed oil, which is a by-product of fruit processing, to assess the nutritional and bioactive potential. The oil was shown to be rich in oleic acid, a monounsaturated fatty acid, and exhibited high antioxidant activity. However, its antimicrobial properties were less pronounced. This finding is consistent with the broader trend of using waste by-products to produce functional ingredients with beneficial health effects that assist in mitigating agro-industrial waste.

Another significant example is the work by Ni et al. (2024), which involved the use of *Lycium barbarum* seed oil-based oleogels for chocolate production. The inclusion of oleogels in chocolate formulations offers a promising strategy for reducing saturated fat content while retaining the stability and texture of the product. The study proved that *Lycium barbarum* seed oil oleogels significantly improved the storage stability of chocolate by reducing the occurrence of undesirable changes such as surface frosting and the overall shelf life. Such innovations illustrate the potential of seed oils in the production of healthier and more stable food products.

In addition to these developments, significant advances have been made in optimising oil extraction processes for various seed types. Khazaai et al. (2024) applied an Adaptive Neuro-Fuzzy Inference System (ANFIS) model to predict the oil extraction yield from rubber seeds. The study identified key factors such as moisture content and particle size that

influenced the extraction efficiency and hence is important information for the development of extraction procedures. The percentage oil yield from rubber seeds with a high content of unsaturated fatty acids makes this seed a viable feedstock for biodiesel production, which improves the sustainability of biofuel production further.

The development of novel food formulations using seed oils has also been explored in the context of low-fat mayonnaise production. Alkabaa et al. (2024) used a combination of chia seed oil by-product gum and other stabilising agents to create bigels, which were subsequently used in low-fat mayonnaise formulations. The bigels were shown to be a good fat replacer, being able to mimic the texture and consistency typical of full-fat mayonnaise. This study reflects the growing interest in plant-based ingredients to replace traditional fats as healthier alternatives without compromising sensory properties.

The capability of multilayer emulsions in enhancing the stability and oxidative properties of seed oils has been highlighted by Sepeidnameh et al. (2024). By encapsulating grape seed oil in multilayer emulsions, they introduced considerable enhancement in the stability of the oil, which is crucial for its use in food products. The study indicated that increasing the number of layers in the emulsion improved both the physical and oxidative stability of the oil. This makes it a more reliable ingredient for food formulations. This procedure can help reduce the issues due to the inherent instability of certain seed oils and thus enhance their commercial viability.

Health Benefits, Nutritional Value, and Functional Applications of Seed Oils

A total of 10 studies were identified under this theme, which focussed on the nutritional composition, bioactive compounds, and health-promoting effects of seed oils. It has been shown that several oils, such as those from muskmelon, mango, and guava seeds, contain essential fatty acids, polyphenols, and antioxidants, which improve human health (Abbas et al., 2023; Joshi et al., 2024; Pasrija et al., 2024). These oils have been linked to promote may help cardiovascular health by lowering cholesterol levels due to its high linoleic acid content (Pasrija et al., 2024). The high PUFA content of guava seed oil makes it more beneficial than conventional fats in food (Joshi et al., 2024).

Beyond nutrition, seed oils are utilised in food preservation, processing and product development. Seed oils such as those from mango and grape seed oils have been studied for their potential to enhance product quality and prolong shelf life. Mango seed oil, which is rich in palmitic and stearic acids has already been utilised as a substitute for cocoa butter in chocolate. With this substitution, the antioxidant capacity as well as the sensory attributes have improved (Abbas et al., 2023). Similarly, grape seed oil has been used in salad dressings for better oxidative stability and improved sensory qualities, but lower fat content of the product (Joshi et al., 2024). These examples highlight the functional properties of seed oils in developing healthier and more acceptable food products, which are gaining consumer interest due to their health-promoting effects.

Seed oils also play a significant role in non-food industries. A valued ingredient in nutraceutical products is chia seed meal protein hydrolysates obtained from a by-product of chia oil extraction, which exhibit significant antidiabetic and antioxidant activities (Briceño-Islas et al., 2024). This waste-to-value approach, where seed by-products such as protein hydrolysates are utilised for their bioactive properties, shows the sustainable use of seed oils and their derivatives in creating functional food ingredients (Briceño-Islas et al., 2024; Visković et al., 2024).

The health benefits of seed oils are further emphasised in the context of their bioactive compounds, including polyphenols, antioxidants, and essential fatty acids. For instance, the antioxidant potential of pomegranate and grape seed oils has been shown to preserve the quality of fresh-cut fruits and vegetables (Albayrak et al., 2023). The incorporation of these oils into emulsions for electrospray-coating methods demonstrated improved both sensory qualities and antioxidant activity (Albayrak et al., 2023). These findings suggest that the incorporation of seed oils in food processing improves the shelf life of products while also boosting their nutritional value, making them ideal for health-conscious consumers.

Seed Oils in Food Product Development and Fat Replacement

This section reviews 9 studies that investigated the use of seed oils in food formulations, particularly as fat substitutes in meat and bakery products. The use of seed oils as substitutes for animal fats in food product development is gaining significant attention, particularly for its potential health benefits and improved nutritional profiles. Studies have explored the impacts of various seed oils, such as melon, pumpkin, hemp, and chia, as partial or complete substitutes for animal fats in different meat products, such as burgers, sausages, and salami. These substitutions improve the fatty acid composition, reduce saturated fat content, and enhance the overall healthfulness of these foods.

The use of melon and pumpkin seed oils in deer burgers was investigated by Martínez et al. (2023b). They found that these oils improved the fatty acid profile of the burgers by increasing polyunsaturated fatty acids like linoleic acid and also resulted in positive sensory evaluations from consumers. Likewise, Martínez et al. (2023a) substituted pork fat with emulsified chia seed oil in sausages, achieving a softer texture and a more desirable fatty acid profile, which included an increase in linoleic and linolenic acids. These findings align with other studies showing that seed oils can serve as valuable fat replacements while maintaining or improving the sensory qualities of meat products (Botella-Martínez et al., 2023; Hamed et al., 2024).

The nutritional benefits of replacing animal fats with seed oils extend beyond the reduction of saturated fats. Studies have demonstrated that incorporating seed oils in food products can increase levels of beneficial omega-3 and omega-6 fatty acids, which are associated with improved cardiovascular health and reduced inflammation. For instance,

hemp seed oil, as explored by Papatzimos et al. (2024), was shown to replace both animal fats and sodium nitrite in fermented salami, improving the product's nutritional value, sensory characteristics, and shelf life. The substitution of chia and grape seed oils in cooked sausages was observed by Momchilova et al. (2023), who noted significant improvements in the nutritional profile, including higher unsaturated fatty acid content. These studies collectively highlight the potential of seed oils in enhancing the nutritional quality of meat products while offering a healthier alternative to traditional fat sources (Zhang & Li, 2024).

The technological aspects of using seed oils as fat replacers have also been explored, with several studies investigating the effects of these oils on the physical properties of the products. Emulsions of seed oils, such as those made from melon, pumpkin, and hemp, were found to influence the texture and stability of meat products, sometimes leading to slight changes in product hardness, cohesiveness, and moisture retention. For example, the study by Botella-Martínez et al. (2023) demonstrated that the gelled emulsion of hemp seed oil and buckwheat flour was a viable alternative to pork fat in Alheiras, a Portuguese meat product. Although the substitution affected the lipid oxidation rates, leading to increased oxidation in the reformulated samples, the health benefits of the higher unsaturated fatty acid content outweighed the drawbacks. This finding highlights the balance between achieving healthier product formulations and managing the challenges of oxidative stability (Difonzo et al., 2024; Momchilova et al., 2023).

In terms of sensory properties, the substitution of animal fats with seed oils often results in favourable evaluations, although some formulations may require adjustments to texture and flavour to align with consumer preferences. The sensory characteristics of the reformulated products, such as odour, texture, and taste, are crucial for consumer acceptance. Studies by Martínez et al. (2023b) and Avci et al. (2023) found that although the reformulated products often exhibited a softer texture or slight changes in flavour, they were still positively received by consumers. This suggests that the nutritional improvements offered by seed oils, such as lower saturated fat content and higher polyunsaturated fat content, do not negatively impact consumer preferences when appropriately formulated.

DISCUSSION AND CONCLUSION

Recent advancements in oil extraction methods, such as UAE, have shown considerable promise in enhancing both the yield and quality of seed oils. These methods have proven to be more environmentally sustainable compared to traditional techniques. Oils extracted through such processes often exhibit favourable fatty acid profiles and improved oxidative stability. Seed oils have also been studied for their role in developing healthier food formulations, such as plant-based fat substitutes. Emulsion gels made from sunflower and palm stearin seed oils, have similar textural and crystallisation properties as animal fats, suggesting their potential use in lower-fat food products. In industrial

applications, seed oils derived from sources like *Madhuca indica* (mahua) seeds have potential as green alternatives to replace petroleum-derived substances in the production of polyurethane-based anticorrosive coatings. The trend of using waste by-products such as papaya seed oil has further extended to the bioactive potential of seed oils, particularly their antioxidant properties, which contribute to both human health and waste reduction. Seed oils also contribute to food preservation, with specific oils like *Lycium barbarum* improving the storage stability of products such as chocolate through oleogel formation. These innovations help to lower the amount of saturated fat that is present in food while maintaining desired product characteristics. In addition, research into refining extraction methods has led to more efficient and sustainable processes, predicting key factors like particle size and moisture content to optimise oil yields. The incorporation of seed oils into low fat mayonnaise and multilayer emulsions demonstrate the ability of seed oils to provide improved texture and enhanced oxidative stability to food products. Overall, seed oils are paving the way for sustainable, health-promoting solutions across diverse industries.

Due to their high levels of essential fatty acids, polyphenols, and antioxidants, seed oils like muskmelon, mango, guava, and grape seed oils are of very broad benefit to human beings. These oils are especially beneficial for heart health, lowering cholesterol, and reducing inflammation. Muskmelon seed oil, containing high linoleic acid, is related to cardiovascular well-being, while guava seed oil with high polyunsaturated fatty acids is a viable alternative to traditional fats in food processing as a healthy substitute. Beyond their nutritional value, the seed oils also play an important role in food preservation and processing. Mango seed oil has been used as a substitute for cocoa butter in chocolate to improve both its antioxidant content and sensory qualities because it contains high amounts of palmitic and stearic acids. Grape seed oil contributes to the stability and taste of salad dressings, while also reducing fat content. In the pharmaceutical and cosmetic industries, seed oils are incorporated into products due to their bioactive properties. By-products like protein hydrolysates from chia seed meal have considerable health benefits, including antioxidant and antidiabetic effects, thus advancing the nutraceutical field. The sustainable use of these by-products shows the potential for waste reduction while at the same time creating valuable ingredients for functional foods. Seed oils such as pomegranate and grape seed oils have been discovered to contain antioxidant activity that maintains the freshness and quality of fruits and vegetables. This also indicates their significance in improving the shelf life and nutritional value of food products. The increasing volume of studies on seed oils reflects their significance in creating healthier and more sustainable foods, while also stimulating innovation in non-food sectors.

Studies on seed oils like melon, pumpkin, hemp, and chia have shown that replacing animal fats with these oils can improve the fatty acid composition of meat products, including burgers, sausages, and salamis. This substitution reduces saturated fat levels and

increases beneficial polyunsaturated fatty acids, including linoleic acid and omega-3 fatty acids, which are known for promoting cardiovascular health and reducing inflammation. Certain studies have established that emulsified seed oils in food products such as deer burgers and sausages not only enhance their fatty acid profiles but also improve sensory qualities, including texture and taste, which are crucial for consumer acceptance. The incorporation of hemp seed oil in fermented salami has improved its nutritional content, organoleptic properties, and shelf life, while also reducing the need for sodium nitrite. This substitution of animal fats by seed oils has some challenges, particularly concerning oxidative stability, because the high unsaturated fatty acid content will raise the rate of oxidation, which could affect product shelf life. However, the overall health benefits of these seed oils will generally overshadow the stability issues. Sensory properties are another area of concern, as changes in texture and flavor may occur during product reformulation. Although some formulations may require adjustments to meet consumer preferences, studies have found that, when properly formulated, the nutritional improvements do not significantly compromise consumer acceptance. Therefore, replacing animal fats with seed oils presents a promising opportunity to improve the healthiness of meat products without sacrificing, and even enhancing, their consumer acceptability. However, addressing oxidation issues and optimising texture and further optimising the texture and formulation of these products will be essential to fully realise the potential of these oils in the marketplace.

LIMITATIONS AND FUTURE DIRECTIONS

There are a few limitations to consider that may have influenced the comprehensiveness of the findings. It was challenging to draw direct comparisons or general conclusions because of the observable variety in the types of oil, extraction techniques, and health outcome measurements. A significant gap in the literature is the lack of standardised methods to evaluate the health impacts of seed oils in human populations. Many studies have relied on animal models or *in vitro* data, with limited human clinical trials to support health claims. Most research tends to focus on individual components, such as fatty acids or antioxidants, rather than assessing the holistic effects of seed oils. Although some short-term studies have reported encouraging results, there is still limited evidence on the long-term health effects, particularly when seed oils are consumed in large amounts or over extended periods.

Extraction and processing methods are another area of concern. Some of the most common methods include cold-pressed extraction or supercritical carbon dioxide (CO₂) extraction. However, there is little to no agreement on which method maximises the health benefits of seed oils. The refining processes are also of concern since it can impair the nutritional value and reduce the bioactive compounds of the oils. As demand for plant-based functional foods grows, it is crucial to study the best ways these oils can be processed and added into functional foods. The differences in extraction and processing

methods, coupled with factors such as storage conditions and oil stability, can lead to wide variations in quality and efficacy. This calls for more coordinated research efforts to understand how each of these factors influences health outcomes.

Future research should focus on standardising extraction protocols and analytical methods used in evaluating seed oils, which would improve consistency and allow for better cross-study comparisons. Long-term human clinical trials are essential to validate the therapeutic effects of seed oils. These should ideally include diverse population groups, dietary patterns, and health markers. Further exploration into the biochemical mechanisms of action and the synergistic potential of combined bioactive compounds would provide critical insights to support the integration of seed oils into functional foods. Life cycle assessments and economic feasibility studies will be useful in assessing their potential as environmentally friendly alternatives to traditional fats, both in food and industrial applications.

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Serological Survey and Risk Factors of Bovine Anaplasmosis in a Breeding Population of Mafriwal Cattle in Johor, Malaysia

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ABSTRACT

Bovine anaplasmosis is a significant cattle disease with economic implications caused by intrerythrocytic bacteria, *Anaplasma marginale* and *Anaplasma centrale*. In Malaysia, where the dairy industry is growing to meet increasing demands, understanding disease epidemiology is crucial. This cross-sectional study investigated the seroprevalence of bovine anaplasmosis in 242 Mafriwal cattle population in a government commercial dairy farm using commercial eELISA kit and its associations with various risk factors using Chi-square test. The study revealed a high seroprevalence of 79.75%, with lactating cattle having the highest seropositivity (95.08%) among the other management groups. However, no significant association ($p < 0.05$) was found between packed cell volume (PCV) and seropositivity, although a higher proportion of seropositive cattle (82.73%) have a low PCV.

Cattle that were kept in semi-intensive housing had a higher seropositivity (81.87%) than those managed under intensive system (73.33%) but the housing type did not significantly affect the seropositivity. There is no significant correlation between the molecular findings of bovine anaplasmosis and seropositivity, yet polymerase chain reaction (PCR) confirmed 82.14% of seropositive cases and 76.47% of PCR negative samples is seropositive. Seropositivity increase from 68.85% in 2021 to 90.83% in 2022, indicating a potential rise in the prevalence of bovine anaplasmosis over time. This study

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revealed that bovine anaplasmosis is prevalent among Mafriwal cattle population in studied farm, and it is significantly associated with management groups and the year of sampling. Increased monitoring and control measures are needed to curb the spread of bovine anaplasmosis. Further research is warranted to explore additional risk factors and epidemiological aspects, benefiting cattle health and production in Malaysia.

Keywords: *Anaplasma*, bovine anaplasmosis, cELISA, Mafriwal, serological, seropositivity

INTRODUCTION

Anaplasma marginale is the primary cause of bovine anaplasmosis, a common tick-borne disease among cattle. This intracellular haemoparasite poses serious health risks leading to decreased productivity and weight loss. While in severe cases, it causes mortality in the affected cattle population (Abba et al., 2016; Smith, 2015). In general, haemoparasites infection causes notable economic losses to the dairy industry worldwide due to reduced milk production, increased costs associated with treatment of diseases and the consequent effect on the efficiency and profitability of dairy production systems (Bitrus et al., 2018; Hanzlicek et al., 2016; Machado et al., 2015).

Bovine anaplasmosis, also known as gall sickness is a disease caused by the intraerythrocytic bacteria, *Anaplasma marginale*. Meanwhile, *Anaplasma centrale*, is a closely related *Anaplasma* sp. but typically causes milder form of bovine anaplasmosis. It had been used as a live vaccine to protect against *A. marginale* infections in some regions worldwide (Ierardi, 2025). The disease is transmitted through the bites of infected ticks and leads to the development of haemoparasites within red blood cells of the hosts. The infections result in a variety of clinical symptoms, including fever, weakness, inappetence, dehydration, constipation, jaundice, depression, laboured breathing, and abortion, with haemolytic anaemia as a primary symptom of bovine anaplasmosis (Abba et al., 2016; Das et al., 2021; Jaswal et al., 2013). The anaplasmosis infection may persists in the host, making the infected animals as asymptomatic carriers for the disease (Seo et al., 2018). The complexity of bovine anaplasmosis epidemiology is attributed to the multifaceted connections between haemoparasite, host, and vector (Paramanandham et al., 2019).

A gold standard method for the detection of bovine anaplasmosis are the microscopic examination of Giemsa-stained blood smears. However, polymerase chain reaction (PCR), which is a molecular-based diagnostic method, yields high sensitivity and specificity for *Anaplasma* spp. detection. This diagnostic tool provides a rapid and inexpensive diagnosis, and it is particularly useful in animals with low parasitemia (El-Ashker et al., 2015; Jalali et al., 2013). Serological methods, including enzyme-linked immunosorbent assay (ELISA) and immunofluorescence test (IFAT) aids in the identification of subclinical or chronic infection of bovine anaplasmosis, where the pathogen may not be detectable by

microscopic examination or PCR (Ashuma et al., 2014). These methods have become the essential tools to determine the seroprevalence of *A. marginale* in the dairy herds (Curtis & Coetzee, 2021). These assays will provide valuable information for disease surveillance through a rapid and relatively inexpensive screening of large cattle herd. While these tests require some laboratory equipment, such as, incubator and plate reader, and are not directly accessible to farmer, it can be conducted through regional veterinary laboratories. This makes the assay a feasible option for routine herd-level monitoring in many farms setting. Additionally, it had been proven that serology methods yield a higher prevalence for the diagnosis of bovine anaplasmosis (Nur-Amalina et al., 2023). The diagnosis offers valuable epidemiology information to assess the disease prevalence for the development of targeted control measures (Parvizi et al., 2020; Spare et al., 2020). This will reduce the morbidity and mortality, and improve the productivity of the herd, ultimately minimising the economic losses caused by the infections (Scariot et al., 2022).

The connection between seropositivity and parameters like management group, housing system and health status enable researchers to understand the factors effecting the disease development among the Mafriwal population. The susceptibility toward bovine anaplasmosis varies with the age of cattle since older animals are more susceptible than younger cattle (Eleftheriou et al., 2022; Zim et al., 2024). Some studies reported that female cattle are significantly vulnerable to anaplasmosis infection compared to bulls (Atsuwe et al., 2024; Badshah et al., 2023). Additionally, higher seropositivity were reported in cattle managed in larger herd size, with inadequate acaricide treatments and practicing pasture grazing (Selim et al., 2021; Zim et al., 2024). Clinical conditions, such as anaemia, fever, and jaundice, are strongly correlated with bovine anaplasmosis infection (Değirmençay et al., 2022). According to Scariot et al. (2022), mastitis and retained placenta are significantly associated with anaplasmosis infection among dairy cattle. Furthermore, the prevalence of bovine anaplasmosis was reportedly higher in summer since tick population reach their peak activity level at warmer months. Therefore, environmental factor including seasonal changes serve as one of the potential risk factors for bovine anaplasmosis infection (Abdela et al, 2018; Oliveira et al., 2021). Meanwhile, the diagnoses of bovine anaplasmosis in Malaysia yield a high prevalence through microscopic, molecular and serological diagnostic methods (Agina et al., 2021; Bitrus et al., 2018; Nur-Sabrina et al., 2024; Ola-Fadunsin et al., 2018). Therefore, the knowledge of epidemiological factor affecting the susceptibility of Malaysian cattle, particularly Mafriwal, is crucial as this disease are prevalent in Malaysia.

Mafriwal breed was developed to supply the needs for local dairy products by Malaysian consumers. The Department of Veterinary Service (DVS) Malaysia developed a tropicalised dairy breed, Mafriwal, in the 1970s by selectively breeding Sahiwal and Friesian cattle. The Mafriwal cattle population has been maintained in a Malaysian government commercial farm located in the southern region. This breed prevails within

Malaysia's dairy industry because of their ability to tolerate various environmental stressors including their endurance in a tropical climate (Mastura et al., 2019; Panandam & Raymond, 2005). Notwithstanding their tolerance to various environmental stressors, a recent study confirmed the presence of *A. marginale* in the Mafriwal population. Based on their study, parasitism significantly impacts on the milk yield and body weight of Mafriwal cattle (Nur-Sabrina et al., 2024). Despite the widespread prevalence of bovine anaplasmosis, the studies on bovine anaplasmosis in Malaysia remained limited (Hayyan & Nasruddin, 2025). The gap in scientific literature highlighted the need to have a region-specific epidemiological study. Therefore, this study was carried out to determine the seroprevalence rate of *Anaplasma* spp., while exploring the risk factors associated with bovine anaplasmosis among Mafriwal cattle population.

MATERIALS AND METHODS

Study Design and Setting

This cross-sectional observational study was conducted in a breeding population of Mafriwal cattle at a government-operated commercial dairy farm in Johor, a state in the Southern region of Peninsular Malaysia, on December 2021 and June 2022.

Cattle Management

The Mafriwal cattle population in this study was classified into four management groups namely calves below one year old, yearlings, lactating cows, and dry cows. Newly born calves were managed intensively where they were fed with colostrum twice daily at 10% of their body weight, followed by fresh raw milk, fresh fodder, and calf starter pellets at two months old. Weaning occurred at 100 days or until they reached 90 kg. Calves remained under intensive housing until they became yearlings. Yearlings were transitioned semi-intensive housing, where they were released into the paddock with signal (*Brachiaria decumbens*) and guinea (*Panicum maximum*) grasses, practicing a rotational grazing system. Yearlings were supplemented with concentrates including palm kernel cake and molasses once daily. Later, as the calves reached two years old, they were mixed with selected bulls on pasture and subsequently went through gestation, lactation and dry cow groups. Nonetheless, gestating cows were excluded from this study to prevent stress during sampling.

Sample Collection and Storage

Blood samples were obtained from the coccygeal vein of 242 Mafriwal cattle of different management groups namely calves below one year old (n = 60), yearlings (n = 61), lactating cows (n = 61), and dry cows (n = 60). All Mafriwal cattle in the farm except the pregnant

cows were included in the sampling to increase statistical power and to estimate the true seroprevalence of bovine anaplasmosis in the population. The blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-coated blood collection tubes for the purpose of determining the packed cell volume (PCV) of the cattle and for molecular identification of *A. marginale*. The PCV was performed using the fresh samples and the remaining blood samples were aliquoted and placed in 1.5 mL microcentrifuge tube at -20 °C until DNA extraction. The serum samples were collected from serum-separator tubes (SST) for antibody detection using ELISA. Serum samples were kept in 1.5 mL microcentrifuge tube at -20 °C until use.

Blood Sample Analysis

Packed Cell Volume

An aliquot of blood samples collected in the EDTA tubes were drawn into hematocrit capillary tubes by capillary action and sealed at one end with Cristaseal (Hawksley and Son Ltd, United Kingdom). The sealed capillary tubes were centrifuged at 220 Relative Centrifugal Force (RCF) for five minutes and read with a rotoreader (Basripuzi et al., 2018). In this study, PCV values below 0.24 L/L were assigned as "low," while values of 0.24 L/L and above were categorized as "normal" according to Bull et al. (2003).

Polymerase Chain Reaction

The DNA of *A. marginale* and *A. centrale* was extracted from each blood sample using the Geneaid Gsync™ DNA extraction kit according to the manufacturer's instructions (Geneaid Biotech Ltd. New Taipei City, Taiwan). The extracted DNA were stored in 1.5 mL microcentrifuge tube at -20 °C until further use (Nur-Sabrina et al., 2024). The MSP4 gene of *A. marginale* and 16s rRNA gene of *A. centrale* were amplified on a MyCycler™ thermocycler (Bio-Rad, USA) using published primers and thermocyclic profile presented in Table 1 (Shkap et al., 2008).

The DNA products were electrophoresed at 400W/100V for 40 minutes on 2 % agarose gel (Promega Madison, USA) with Tris-acetic acid-EDTA (TAE) buffer, and stained with Midori Green dye (Nippon Genetics, Europe). Visualisation of DNA fragments was performed using the GelDoc™ EZ Imager.

Enzyme-linked Immunosorbent Assay (ELISA)

Samples and reagents preparations for ELISA were performed following the manufacturer's recommended protocol (VMRD, USA). Each undiluted serum sample of 50 µL was added in duplicate to individual wells of a recombinant MSP5-coated plate from Anaplasma Antibody Test Kit, cELISA V2 (VMRD, USA). The undiluted positive and negative

Table 1
Primers used for the amplification of *Anaplasma* spp.

Anaplasma spp.	Targeted gene	Oligonucleotide sequence	Thermocycler profile
<i>A. marginale</i>	MSP4	Reverse: CATCTCCCATGAGTCACGAAGTGGC Forward: GCTGAACAGGAATCTTGCTCCAAG	ID: 95°C/5mins D: 95°C/1mins A: 65°C/2mins E: 72°C/1min No of cycles: 40 FE: 72°C/10mins
<i>A. centrale</i>	16s rRNA	Reverse: CATCTCCCATGAGTCACGAAGTGGC Forward: GCTGAACAGGAATCTTGCTCCAAG	ID: 94°C/5mins D: 94°C/30s A: 58.5°C/30s E: 72°C/1min No of cycles: 39 FE: 72°C/5mins

Note. min = minutes; ID = initial denaturation; D = denaturation; A = annealing; E = extension; FE = final extension; bp = base pair

control sera of 50 µL were run in duplicate and triplicate, respectively. The assay plate was then incubated at 23±2 °C for 60 minutes before the serum samples and controls were discarded. The plate was washed twice with 1:10 diluted washing solution. Then, 50 µL of monoclonal antibody-peroxidase conjugate in 1:100 of dilution buffer was added to each well and incubated at 23±2 °C for 20 minutes. The plate was washed four times. A total of 50 µL of substrate solution was added to each well and incubated for 20 minutes at 23±2 °C. The reaction in each well was stopped by adding 50 µL of stop solution. The optical density (OD) of each well was read at 630 nm on a microplate absorbance spectrophotometer (Bio-Rad, USA).

Test Validation and Results Interpretation

Test validation involved the utilisation of both negative and positive controls. The ELISA assay was considered valid if the mean OD of the triplicate negative controls fell within the range of >0.40 and ≤2.10, and the mean OD of duplicate positive controls exhibited ≥ 30% inhibition.

Results were interpreted based on the percentage inhibition (I%) which was calculated as: $I\% = 100 [1 - (OD_{\text{Sample}} / OD_{\text{Negative control}})]$, where OD_{Sample} was the OD of sample and $OD_{\text{Negative control}}$ was the mean OD of negative control. Serum samples with < 30% of inhibition is considered negative for antibody against bovine anaplasmosis while serum samples with ≥ 30% of inhibition were positive.

Statistical Analysis

Descriptive statistics for categorical variables were performed by calculating frequencies and percentages for each category. Chi-square test was used to analyse the data using R software (version 4.3.1) to determine the associations between different management groups (calves below one year old, yearlings, lactating cows, dry cows), PCV level (low, normal), housing system (intensive, semi-intensive), molecular identification of *A. marginale* (positive, negative) and year of sampling (2021, 2022) with seropositivity at 95% confidence interval.

RESULTS

The findings of this study revealed that 79.75% sampled (193/242) cattle in this population were tested seropositive for bovine anaplasmosis. A significant association between the different management groups and seroprevalence was observed ($\chi^2(3) = 9.98$, $p = 0.019$) in which the lactating group had the highest seroprevalence (95.08%). A total of 115 (82.73%) seropositive cattle were detected with low PCV levels ($n = 139$) while only 78 (75.73%) seropositive cattle were detected with normal PCV levels ($n = 103$). However, there was no statistically significant association between the PCV levels and seropositivity of anaplasmosis ($\chi^2(1) = 2.55$, $p = 0.110$).

Similarly, no significant association was observed between the type of housing systems and the seroprevalence ($\chi^2(1) = 2.04$, $p = 0.153$) although cattle reared in semi-intensive housing ($n = 182$, 81.87%) have a higher seroprevalence compared to intensive housing ($n = 60$, 73.33%). The results showed that 82.14% (115/140) of the PCR-positive samples were found to be seropositive, while 76.47% (78/102) of the PCR-negative samples were found to be seropositive. Nonetheless, there is no significant association between PCR results and seropositivity of the samples towards bovine anaplasmosis ($\chi^2(1) = 1.18$, $p = 0.277$).

In 2021, a total of 84 animals were found to be seropositive, accounting for 68.85% of the sampled population ($n = 122$). In 2022, there were 109 seropositive animals, representing 90.83% of the sampled cattle in the same population ($n = 120$). A highly significant association between the seroprevalence of bovine anaplasmosis and the year of sampling ($\chi^2(1) = 18.10$, $p = 0.000$) is shown in Table 2.

DISCUSSION

This study demonstrates a high seroprevalence (79.75%) of bovine anaplasmosis among the tested Mafriwal cattle population which are kept for commercial dairy production and breeding purposes. In contrast, a lower seroprevalence of anaplasmosis using the same cELISA kit as used in the current study (51.11%) was reported among randomly sampled dairy cattle of various breeds from different farms in Selangor, Malaysia (Bitrus et al., 2018).

Table 2

The association between seroprevalence with age group, PCV, housing type, molecular identification, and year of sampling

Variable		Number of animal's tested	Number of seropositive animals	Percentage (%)	χ^2	df	p
Management group	Calves	60	44	73.33	9.98	3	0.019*
	Yearlings	61	47	77.05			
	Lactating	61	58	95.08			
	Dry	60	44	73.33			
PCV	Low (<0.24 L/L)	139	115	82.73	2.55	1	0.110
	Normal (\geq 0.24 L/L)	103	78	75.73			
Housing type	Intensive	60	44	73.33	2.04	1	0.153
	Semi-intensive	182	149	81.87			
Molecular identification of <i>Anaplasma</i> spp.	Positive	140	115	82.14	1.18	1	0.277
	Negative	102	78	78.47			
Year	2021	122	84	68.85	18.10	1	0.000***
	2022	120	109	90.83			

Note. χ^2 = chi square; df = degree of freedom; p = p-value; * = p-value < 0.05; ** = p-value < 0.01; *** = p-value < 0.001

The seroprevalence of bovine anaplasmosis vary as the management system of each farm affect the seroprevalence of bovine anaplasmosis differently (Ola-Fadunsin et al., 2018).

In the present study, lactating cattle, classified under management groups variable, exhibited the highest seropositivity rate (95.08%). In contrast, other management groups, including calves, yearlings, and dry cows, had relatively lower seropositivity rates. The statistically significant relationship between management groups and seropositivity ($\chi^2(3) = 9.98, p = 0.019$) in the present study, suggested that production stage and physiological status influenced the susceptibility of Mafriwal cattle to anaplasmosis. While management groups are not strictly age categories, lactating and dry cows are generally older animals, whereas calves represent younger cattle. Therefore, the overlap may partially explain the agreement with Bitrus et al. (2018) and Debbarma et al. (2020), which reported high seropositivity observed in cattle greater than 3 years old. However, these studies revealed that the age of animal and the seropositivity are not statistically significant ($p > 0.05$) whereas our study suggested otherwise. These findings are attributed by the differences in study design, classification criteria or epidemiological factors. The use of management

group in the current study likely captured physiological and exposure risks, which may not be reflected when considering age alone. Furthermore, Manap et al. (2024) found that Mafriwal cattle were more susceptible to haemoparasite infections with advancing production stage, in which the yearlings, lactating cows, and dry cows were estimated with higher odds ratio to be infected in comparison to the calves, likely due to cumulative exposure to anaplasmosis or physiological stress. While Atif et al. (2013) suggested that seroprevalence of bovine anaplasmosis increased with age, Ola-Fadunsin et al. (2018) revealed that a higher prevalence was observed in younger cattle. However, the present study suggested that age and production stage play a significant role, in agreement with Vetrivel et al. (2017) in which bovine anaplasmosis was found to be significantly associated with age of dairy cattle. Young calves may receive passive humoral antibodies from their dam through colostrum, whereas cattle over five months old typically gain immunity through exposure to pathogen and clinical infection, building their own active immune response (Vlasova & Saif, 2021). The exposure to infections and immunological progression may explain the lower seropositivity observed in calves and the higher seroprevalence among lactating and dry cows in the present study. Previous findings revealed that the seropositivity in lactating group are higher (60 %) than the peripartum, dry, and pregnant groups (Da Silva & Da Fonseca, 2013). Although transient immunosuppression is well-recognised during pregnancy, lactating cows also experience the transient reduction in immune function during early lactation due to physiological stress of milk production, which makes them more susceptible to the bovine anaplasmosis (Aktas & Özübek, 2017). Moreover, the lactating cows experience various physiological changes and stressors due to the demands of milk production, including nutrient partitioning towards lactation and the production of colostrum. Therefore, it compromises the immune function and increase the susceptibility to anaplasmosis infection. Furthermore, high levels of prolactin and progesterone in lactating cattle can lead to a temporary suppression of the immune system, making them more susceptible to infections like bovine anaplasmosis (Aleri et al., 2016; Debbarma et al., 2020; Vlasova & Saif, 2021).

In addition, no association was observed between the seroprevalence of bovine anaplasmosis and the PCV level in the Mafriwal cattle population ($\chi^2(1) = 2.55$, $p = 0.110$). The PCV were observed to be significantly lower ($p < 0.05$) in cattle with anaplasmosis as it often induces changes in the animal's blood parameters, making hematology and biochemical tests valuable tools for diagnosis (El-Ashker et al., 2015; Bezabih et al., 2017, Abdela et al., 2018; World Organisation for Animal Health [OIE], 2018). However, the non-significant association observed between PCV and seropositivity are because PCV reflect the ongoing parasitaemia. Animal infected with anaplasmosis often become a carrier, maintaining seropositivity without exhibiting haematological changes (Hairgrove et al., 2015). Bovine anaplasmosis causes haemolytic anaemia, where the red blood cell count

in the bloodstream drops, resulting in reduced oxygen-carrying capacity. As a result, the PCV levels in an infected animal's blood decrease. However, mild parasite infections may not result in significant anaemia as shown in animals with stronger infections (Hayyan et al., 2020). Complete blood count (CBC) may reveal characteristics such as anaemia and altered red blood cell indices. The ability of *Anaplasma* spp. to penetrate red blood cells may explain the difference in average PCV values between calves with anaplasmosis and those who are unaffected. The reticuloendothelial system, specifically the spleen, contains macrophages that are principally responsible for removing this pathogen from the organism after it has gone through cycles of replication within erythrocytes (Abdela et al., 2018). Additionally, PCV and serum biochemical analysis help in assessing the severity of the disease.

Although this study revealed that cattle reared in the semi-intensive housing system exhibited a higher seropositivity rate (81.87%) compared to those reared in the intensive housing system (73.33%), the differences was not statistically significant ($p = 0.153$). These findings are in contrast with a study by Sajid et al. (2014), which showed that cattle raised under intensive management system had a significantly lower prevalence of *A. marginale* infection in comparison to the cattle raised in extensive and semi-intensive systems. Bovine anaplasmosis can be transmitted biologically via tick's bite and biting flies; or mechanically via contaminated fomites including needle and surgical equipment; or through transplacental transmission (Aubry & Geale, 2011). Therefore, the farm management and housing system can potentially influence the exposure of the animals to bovine anaplasmosis. Cattle reared in extensive and semi-intensive system are more likely to be in contact with the tick vectors of *Anaplasma* spp. including *Rhipicephalus (Boophilus) microplus* and reservoir animals, particularly the wildlife. Ticks play a significant role as the primary vector for *A. marginale* by sustaining the pathogen in the natural environment while reservoir animals naturally maintain the pathogen and serve as a source of infection (Tucker et al., 2016; Salinas-Estrella et al., 2022). However, cattle reared in the intensive system are kept at a higher stocking density than extensive and semi-intensive cattle, which increases the likelihood of *Anaplasma* spp. infections through the potential mechanical vectors and stress.

Polymerase chain reaction (PCR) is a molecular technique that can be used to detect the presence of *Anaplasma* spp. DNA in the blood samples of cattle. This approach is highly sensitive and specific, making it possible to identify the pathogen even before the onset of clinical signs (Chi et al., 2013). The PCR can directly confirm active infection, and often used as a diagnostic assay (Chi et al., 2013; Singh et al., 2012). In this study, molecular and serological diagnosis showed that 82.14% (115/242) of the Mafriwal cattle population were infected with bovine anaplasmosis. However, the molecular analysis revealed no evidence of association ($\chi^2(1) = 1.18, p = 0.277$) between PCR and serological

results, suggesting possible differences in the detection of current infection versus past exposure or latent carrier states. The presence of antibodies can be detected through serological tests, such as ELISA, which are commonly used for epidemiological studies. However, serological test does not distinguish between past and active infection, leading to discrepancies when compared to PCR tests that detect the pathogen's DNA (Aubry & Geale, 2011; Spare et al., 2020). Previous molecular work on bovine anaplasmosis among Mafriwal cattle population revealed high prevalence of anaplasmosis caused by *A. marginale* and *A. centrale* (Nur-Amalina et al., 2024). Nevertheless, PCR alone does not provide a definitive diagnostic because it only identifies active anaplasmosis and unable to detect past exposure. Therefore, while PCR is a valuable diagnostic tool for identifying active infections, serological results provide a better cumulative exposure history of the disease.

Serological tests, including ELISA, have higher sensitivity for detecting antibodies against *Anaplasma* spp. However, PCR is the preferred method for diagnosing anaplasmosis because it detects active infection and enables better differentiation between *Anaplasma* subspecies (Jalali et al., 2013; Sharma et al., 2015; Shabana et al., 2018). Furthermore, the complex relationship between PCR and seropositivity in bovine anaplasmosis may depend on the stage of infection and the immune status of the animal. During the acute phase of infection, both PCR and seropositivity are expected to be positive. However, it may take a period of time for the immune system produces sufficient levels of antibodies before it can be detected. Therefore, PCR often yield earlier positive results than serology (Hamou et al., 2012). In chronic infections or carrier animals, PCR positivity can persist even when antibody level dropped. This is primarily due to long-term pathogen persistence in host tissues while antibody levels can decrease over time (Aubry & Geale, 2011). In comparison, seropositive animals with negative PCR results may reflect prior exposure to *A. marginale* but the body had already cleared the infection. This aligns with our findings that 76.47% of PCR-negative animals were seropositive, highlighting that the two diagnostic methods detect infection at different stages, which explained the discrepancies of the results. Additionally, although the United State Department of Agriculture's (USDA) cleared and approved the commercial cELISA kit, cross-reactivity has been observed in both *Ehrlichia* genotypes and *Anaplasma phagocytophilum* (*A. phagocytophilum*) (Al-Adhami et al., 2011; Dreher et al., 2005). Overall, both methods have established their role in the diagnosis and monitoring of bovine anaplasmosis with complementing information on cattle infection status (Hansmann et al., 2019; Jalali et al., 2013; Moniuszko-Malinowska et al., 2021).

Temporal trend means the changes and patterns of specific events. In the case of bovine anaplasmosis, it describes the evolution, spread, and fluctuation of the disease over time. This analysis provides additional insights into factors driving the dynamics of bovine anaplasmosis, such as environmental changes, management practices and the effectiveness

of control measures (Leal Filho et al., 2022; Sunder et al., 2019;). Temporal trend analysis of the seroprevalence have revealed some notable patterns. One notable pattern includes the potential increase of disease prevalence over time through the observation of the ascending seroprevalence in the sampled population from 68.85% in 2021 to 90.83% in 2022 with significant associations between seroprevalence and sampling year ($(\chi^2(1) = 18.10, p = 0.000)$). Factors that can be considered affecting this temporal variation include climate change, vector dynamics, varying management practice or other epidemiological factors (Hanzlicek et al., 2016). The study of temporal trends enables researchers to understand the epidemiology of bovine anaplasmosis by detecting significant patterns in bovine anaplasmosis occurrence and predicting future trends. The knowledge assists researchers and relevant individuals or organisations in directing management strategies, implement control measures, and understanding and preparing for potential risks that be posed to cattle populations in the near future.

The findings of this study revealed a high seroprevalence of *Anaplasma* spp. in the studied Mafriwal cattle population. The findings aligned with previous studies reporting that crossbred cattle are highly susceptible to anaplasmosis infection compared to the local breed (Atif et al., 2013; Ola-Fadunsin et al., 2018). These findings concludes that the prolonged exposure of *Anaplasma* spp. for many generations has given the local cattle the ability to develop resistance against the pathogen or vector causing bovine anaplasmosis. Furthermore, the resiliency and hardiness of the local breeds are through the acclimatisation to the specific conditions. Their susceptibility to the infection decreased as they adapt to the local environment through the development of physiological and behavioural traits (Ola-Fadunsin et al., 2018). Contradicting to other local cattle breed, Mafriwal, a Friesian-Sahiwal cross, may have a relatively higher bovine anaplasmosis seroprevalence as a result of different genotypic composition and management conditions. Sahiwal cattle are well-adapted to tropical climates, but Friesian genetics may introduce traits that make them highly susceptible to diseases such as bovine anaplasmosis. A previous study revealed that Friesian-Holstein cattle are genetically more susceptible to tick-borne diseases due to specific BoLA alleles such as DRB32 and DRB316, while Sahiwal cattle carry resistance-associated alleles like DRB314 and 41, contributing to their resilience against infections such as anaplasmosis (Duangjinda et al., 2013).

Cattle seroprevalence of bovine anaplasmosis was high in Mafriwal cattle population in a commercial government farm in Kluang, Johor. Housing systems, PCR result and PCV level were not significantly associated with seropositivity against anaplasmosis; however, seropositivity has shown a significant association with age group and year of samplings. The observed increase in seroprevalence over time highlights the need for improved monitoring and control strategies, such as regular screening and effective vector control. These measures are crucial to reduce economic losses and to enhance the overall health and productivity of cattle herds. In the current study, gestating cows were excluded from

sampling to minimise stress and potential risks to both dam and foetus during pregnancy. Therefore, the effect of pregnancy status of Mafriwal cattle on anaplasmosis infection and the immunity remains an important area for future research. The findings from this study could provide knowledge for the development of management strategies to improve productivity of the cattle.

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Seeds of Health: Maximising Antioxidant Potential from *Phoenix dactylifera* L. Medjool Date Seeds via BBD Optimisation and UPLC-QTOF/MS Metabolite Identification

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ABSTRACT

The growth in date production industries resulted in a significant accumulation of date palm wastes, especially the seeds, which poses environmental issues and escalates processing and storage expenses. Appropriate characterisation of this product would mitigate these issues while adding commercial value through beneficial nutraceutical and pharmaceutical applications. Current research highlights the ultrasonication process parameters required for extracting the maximum yield of antioxidant activity from *Phoenix dactylifera* Medjool seeds utilising Response Surface

Methodology (RSM). A Box-Behnken design has been adopted to determine the impact of 3 extraction variables, namely ethanol (EtOH) concentration (X_A), ultrasonication duration (X_B), and temperature (X_C), on antioxidant activity evaluated through hydroxyl radical (OH^*) scavenging activity. The results portrayed that the experimentally obtained value was consistent with the predicted output, affirming the reliability of the model employed for optimising the extraction methodologies. The extraction at 79% EtOH, for 45 min, at 40°C

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provided the highest antioxidant activity recorded at $87.40\% \pm 1.29$. Two compounds, namely, 1-O-caffeoyl- β -D-glucopyranoside and genistein-7,4'-di-O- β -D-glucoside, categorised to the group of caffeic acid and flavonoid derivatives supported the higher antioxidative response obtained. Our study indicates a suitable technique for extracting Medjool date seeds phytochemicals of therapeutic benefits, applying a modern statistical tool (RSM), which offers a more cost-effective and less labour-intensive optimisation process compared to conventional methods. The outcome also presents bioactive phytochemicals from Medjool date seeds that strengthen the role of this candidate for nutraceutical purposes.

Keywords: Antioxidant, Box-Behnken design (BBD), date seeds, hydroxyl radical scavenging, *Phoenix dactylifera*, response surface methodology (RSM), UPLC-QTOF/MS

INTRODUCTION

Oxidative stress, arising from an imbalance between oxidants and antioxidants or endogenous defence mechanisms within the human body, has been linked to the etiology of various ailments. This includes heart diseases, carcinoma, diabetes, stroke, Parkinson's disease, Alzheimer's dementia, inflammation, arthritis, muscle degeneration, and hyperlipidaemia. This process typically gets initiated with the generation of reactive oxygen species (ROS), particularly free radical species such as hydroxyl radicals (OH^\bullet), superoxide ($\text{O}_2^{\bullet-}$), alkoxy (RO^\bullet), and peroxy (RO_2^\bullet). Oxygen derivatives that are not free radicals, such as hydrogen peroxide (H_2O_2), singlet oxygen (O_2), and hypochlorous acid (HOCl), are also classified as ROS as they can serve as precursors or substrates that lead to the formation of highly reactive free radicals (Patel et al., 2018). These ROS arise from cellular metabolism and exogenous agents, which in turn contribute to the destabilisation and breakdown of cell membranes and set the stage for health challenges (Babior, 2000).

Concurrently, the idea of boosting the self-defence system by consuming synthetically manufactured antioxidants such as propylene glycol (PG), butylated hydroxyanisole (BHA), butylated hydroxyl toluene (BHT), and tert-butylhydroquinone (TBHQ) in food has seen to raise the concerns over its safety and other adverse effects (carcinogenicity) (Singh et al., 2023; Taghvaei & Jafari, 2015). As a result, these factors escalated the quests for exploring novel, effective and cost-effective natural sources of antioxidants, supplanting the artificial alternatives in nutraceuticals, dietary supplements, pharmaceuticals, and cosmetic products.

For centuries, botanical sources have served as the cornerstone of traditional medicinal practices, which continuously introduce innovative therapeutic remedies to mankind up to date. One such plant is the date palm (*Phoenix dactylifera* L.), among the oldest cultivated species, which has been extensively grown in the Arabian Peninsula, North Africa, and the Middle East regions for thousands of years (Chao & Krueger, 2007). This dioecious, perennial, monocotyledonous fruit tree belongs to the *Arecaceae* family (subfamily

Coryphoideae). The species name "*dactylifera*" originates from the Greek word "dactylus," meaning "finger," and the Latin "ferous," meaning "bearing" (Ashraf & Hamidi-Esfahani, 2011). Among the thousands of date varieties cultivated around the world, Medjool dates are particularly famous for their large fruit size, vibrant orange-yellow flesh, and exceptional taste and texture, making them a popular choice in the Islamic world, especially during Ramadan. Medjool dates are also known by several other names, including 'Medjhoor,' 'Medjehuel,' 'Mejhul,' 'Majhoul,' and 'Mejhoul.' These dates are believed to have originated from Morocco's Tafilalet Valley (Errachidia) (Nixon, 1950). Genetically, Medjool dates belong to a primary group of North African date varieties, alongside other well-known types such as 'Deglet Noor' (from Algeria/Tunisia) and various Egyptian cultivars (El-Assar et al., 2005). For years, the focus has been primarily on the fruit, yet the seeds of Medjool date also deserve attention for their potential health benefits, especially as a rich source of antioxidants. These seeds, comprising 10% w/w of total date fruit weight, are often discarded in the industry and are capable of implicating serious environmental concerns if unattended. Beyond their culinary use, date seeds have been traditionally used in ethnobotanical practices for medicinal purposes, such as in Turkey, where they are ground into herbal coffee called "Hurma coffee" to enhance memory (Sekeroglu et al., 2012). Meanwhile, in Algeria, date seeds are commonly used in folk remedies to treat conditions like weakness and gout, and they are also believed to help boost lactation in breastfeeding women (Selmani et al., 2017). These seeds harbour a wealth of bioactive antioxidative compounds, primarily the phenolic acids such as derivatives of hydroxylated benzoic acid (protocatechuic acid, p-hydroxybenzoic acid, gallic acid, and vanillic acid), along with cinnamic acid derivatives (ferulic acid, caffeic acid, m-coumaric, p-coumaric acid, and o-coumaric acid) while rutin, catechin, quercetin, luteolin, and kaempferol were the prominent flavonoids (Al-Farsi & Lee, 2008; Bouhlali et al., 2020). Importantly, without appropriate and optimised extraction conditions, the valuable functional phytochemicals within date seeds may be irrevocably lost.

The valorisation of these agricultural by-products aims to increase their economic and functional value, as they were once limited to use as animal feed. Recently, seeds of date have gained interest as promising sources of bioactive compounds with potential applications in food, pharmaceuticals, and cosmetics. However, many of these by-products remain underutilised due to the absence of effective extraction methods. Several innovative extraction methods have been developed for isolating antioxidant secondary metabolites, with ultrasonic-assisted extraction (UAE) standing out as one of the preferred green technologies that meet modern extraction standards (Shirzad et al., 2017). UAE offers a straightforward, cost-effective, conserves energy, and efficient alternative that not only optimises yield and preserves the quality of extracts, but also scales well to meet industrial demands (Pagano et al., 2021; Rao et al., 2021). On the other hand, it

is essential to acknowledge that extraction conditions play a crucial role in maximising yields and deciding the type and structures of the extracted compounds. Various factors must be carefully considered when using extraction methods, such as the choice and ratio of solvents, extraction temperature, duration, and the solid-to-liquid ratio (Ghasemi et al., 2024). These variables are key to ensuring the full recovery of target compounds while minimising the risk of chemical alterations.

When selecting a reliable mathematical and statistical approach to optimise analytical procedures, Response Surface Methodology (RSM) is widely employed, particularly in the food and medical fields, for refining extraction processes (Fattahi & Rahimi, 2016). RSM offers significant advantages by minimising the number of experimental trials needed while still assessing the relative significance of multiple variables and their interactions, making the process more time and labour-efficient. Among the available Designs of Experiments (DoE), the Box-Behnken Design (BBD) was selected due to its specific suitability for modelling second-order responses, which are the focus of most RSM studies (Ahmed et al., 2022). Additionally, BBD requires only three levels for each factor to construct a second-order regression model, making it a practical choice for this investigation.

The present study focusses on applying RSM, a valuable technique for optimising the antioxidant potential through hydroxyl radical scavenging assay from Medjool date seeds. These seeds were subjected to varying ultrasonic-assisted extraction (UAE) parameters, namely EtOH concentration (50-80%), duration or time of ultrasonication (30-90 min) and extraction temperature (40-70°C). A Box-Behnken design was employed at three-factor and three-level modes.

METHODS

Chemicals and Reagents

Analytical graded chemicals such as ethanol (EtOH), 1,10-phenanthroline monohydrate, ferrous (II) ammonium sulphate hexahydrate, phosphate buffer solution (0.1 M, pH 7.4), and 30% hydrogen peroxide were utilised in this research.

Materials and Chemicals

Date fruits (Medjool) originating from Palestine were locally purchased from licensed vendor, depitted, and cleansed under running water to deter the adherent fleshy tissue. The seeds were then subjected to oven drying at 45°C for 24 hours. Approximately 500 g of seeds were retrieved following the drying process, where these seeds were ground into a powdered form using a heavy-duty crusher and grinder. To achieve uniform particle size, the resulting powder was carefully sifted through a 1-2 mm sieve, ensuring homogeneity.

Ultrasound-assisted Extraction (UAE)

The technique of ultrasound-assisted extraction (UAE) was conducted using an ultrasonic water bath machine (Elmasonic S60 kHz, Elma Hans Schmidbauer GmbH, Singen, Germany). For each run, Medjool date seeds powder (2 g) was added to 100 mL of extraction solvent following the Box-Behnken experimental design outlined in Table 1. Prior to the extraction process, the samples were centrifuged and filtered through a vacuum filtration unit using Whatman No. 1 filter paper. The filtrate solvent was then removed by vacuum drying in a rotary evaporator at 45°C, ensuring the chemical composition and physical properties of the extract were preserved. The resulting crude extracts of Medjool date seeds (MSE) were stored in sealed glass containers at a temperature of -20°C.

Table 1

The BBD suggested conditions and experimental responses obtained using UAE

Run	Independent variables			
	X _A EtOH concentration (%)	X _B Duration (min)	X _C Extraction temperature (°C)	Y Hydroxyl radical scavenging activity (%)
1	80	60	40	88.50
2	50	30	55	66.52
3	65	60	55	76.32
4	50	60	40	66.10
5	65	90	70	82.50
6	80	30	55	78.36
7	50	90	55	59.77
8	65	90	40	69.62
9	80	60	70	85.09
10	65	60	55	78.20
11	65	30	70	76.08
12	80	90	55	81.62
13	65	60	55	77.49
14	65	60	55	76.81
15	50	60	70	74.51
16	65	30	40	79.80
17	65	60	55	75.32

Hydroxyl Radical Scavenging Activity

Antioxidant activity possessed by compounds within MSE was evaluated through the hydroxyl radical (OH•) scavenging assay following a modified method of Mukhopadhyay et al. (2016). To perform the assay, a 3 mM solution of 1,10-phenanthroline was prepared using phosphate buffer (0.1 M, pH 7.4), while a ferrous ammonium sulphate solution with the same concentration was prepared in water. In a multiwell plate, 50 µL of MSE extract at a concentration of 1 mg/mL was combined with 50 µL of ferrous ammonium sulphate. Subsequently, 50 µL of 0.01% H₂O₂ was added to initiate the reaction. The resulting solution was placed in darkness at room temperature for 5 min. Following incubation, 50 µL of 1,10-phenanthroline was added, thoroughly mixed, and further incubated for a duration of 10 min at room temperature. The mixture's absorbance was then recorded at 510 nm with the aid of a microplate reader. The blank solution, containing only ferrous ammonium sulphate, water, and 1,10-phenanthroline, exhibited the highest absorbance. Additionally, an extra blank reagent was prepared, consisting of only 1,10-phenanthroline and the absorbance of this blank was subtracted from the absorbance of all the treatment samples. The scavenging activity of MSE compounds towards hydrogen peroxide was calculated using the provided formula Eq. [1]:

$$\% H_2O_2 \text{ Scavenging Activity} = \frac{A_T}{A_B} \times 100, \quad [1]$$

where A_T is the absorbance of a solution containing the MSE, while A_B is the absorbance of a blank.

Response Surface Methodology Design (RSM)

The present study used a Box-Behnken Design (BBD), utilising a three-level, three-factor full factorial design (3³) to assess the impact of each independent variable: X_A (EtOH concentration, 50-80%), X_B (ultrasonication duration, 30-90 min), and X_C (temperature, 40-70°C) on the response variable (hydroxyl radical scavenging activity) associated with UAE. The variables were coded at three levels (-1, 0, 1), as shown in Table 2, with a sum of 17 experimental runs suggested and conducted as outlined in Table 1. The levels regarding the independent variables were selected and slightly modified based on the optimised values and ranges reported in previous studies. These studies detailed the use of RSM optimisation for ultrasonication-assisted extraction from various date seed varieties and pitaya seeds (Afifi et al., 2017; Alshammari et al., 2024; Niroula et al., 2024; Zulkifli et al., 2020). Subsequently, the acquired data was analysed using RSM to determine the optimal processing condition for each independent variable. The RSM outputs, such as contour

and 3D graphic surface plots, were retrieved to visualise the optimum and most influential variables. The influence of the extraction parameters on hydroxyl radical scavenging values was examined using a second-order polynomial equation Eq. [2], derived from RSM:

$$Y = \alpha_0 + \alpha_1 X_A + \alpha_2 X_B + \alpha_3 X_C + \alpha_{11} X_A^2 + \alpha_{22} X_B^2 + \alpha_{33} X_C^2 + \alpha_{12} X_A X_B + \alpha_{13} X_A X_C + \alpha_{23} X_B X_C \quad [2]$$

where Y represents the response variable (hydroxyl radical scavenging activity); extraction variables include, X_A (EtOH concentration), X_B (duration), and X_C (extraction temperature); X_A^2 , X_B^2 , and X_C^2 defining the square effects; interaction terms were represented by $X_A X_B$, $X_A X_C$, and $X_B X_C$; α_0 stands for the constant coefficient of the model; α_1 , α_2 , α_3 referring to linear effects; α_{11} , α_{22} , α_{33} directing quadratic effects, and α_{12} , α_{13} , α_{23} indicating interaction effects.

Table 2

Independent extraction variables subjected to BBD optimisation and its response

Independent variables	Levels			Dependent variable (Y)	Goal
	-1	0	1		
EtOH concentration (%) (X_A)	50	65	80	Hydroxyl radical scavenging activity (%)	Maximised
Duration (min) (X_B)	30	60	90		
Extraction temperature (°C) (X_C)	40	55	70		

Analysis of variance (ANOVA) was conducted for the response variable, with a significance level set at $p < 0.05$, to determine the significant factors in the model while fitting the data to the mathematical models. The adequacy of the models was assessed using criteria, including model analysis, F-value, lack-of-fit test, and comparing R^2 values (actual- R^2 and adjusted- R^2). According to Jumbri et al. (2015), a regression model with an R^2 value greater than 0.9 defined the response model possessing strong fitness. Additionally, the significance of the corresponding variables is deduced by larger F-values and smaller p -values, indicating higher significance. Design Expert Software (version 13, Stat-Ease Inc., Minneapolis, MN, USA) was accessed for the experimental data analysis.

Validation of the Model

The optimal condition suggested by RSM was validated for the maximum extraction of hydroxyl scavenging antioxidant potential according to the values retrieved, utilising the option of desirability. Consequently, the validation experiment was performed in triplicates.

The experimental result was matched with the model's forecasted value, which authenticates the model's validity. The percentage (%) difference between the predicted and experimental data was calculated as follows Eq. [3]:

$$\% \text{ Difference} = \frac{(\text{Predicted value} - \text{Experimental value})}{\text{Predicted value}} \times 100 \quad [3]$$

Bioactive Phytochemical Identification – UPLC-QTOF/MS

Separation of phytochemicals within the optimised MSE was completed using ultra-high-performance liquid chromatography (UPLC-QTOF/MS). At the same time, the Waters Acquity ultra-performance LC system (Waters, Milford, MA, USA) was used for analysis. Chromatography separation performed on MSE used specific operational criterias, utilising an Acquity UPLC HSS T3 column with dimensions of 100 mm × 2.1 mm × 1.8 μm. . The sample injection volume was set to 1 μL, with the flow rate maintained at 0.6 mL/min. Negative ionisation mode was utilised during the operation. The mobile phase consisted of formic acid (solvent A) and acetonitrile (solvent B), with a gradient elution programmed as follows: 1% B and 99% A at 0 minutes, maintained until 0.5 minutes; transitioning to 35% B and 65% A at 16 minutes; 100% B at 18 minutes; and re-equilibrating to 1% B and 99% A by 20 minutes. Detection was carried out using a Waters Vion IMS QTOF system (Milford, MA, USA). Data collection utilises the mass-to-charge ratio (m/z) range between 50 to 1500, using high-definition mass spectrometry elevated energy (HDMSE) with a scan rate of 0.1 s/scan. Collision energies (CE) stay constant for scans at low energy at 4 eV and gradually increase to 40 eV approaching high-energy scans.

RESULTS AND DISCUSSION

Fitting the Model

The empirical model was assessed through ANOVA (Table 3) to determine its significance and suitability. In general, the relevance of the associated variables becomes more significant with greater F-values and smaller *p*-values. The Fischer variation ratio (F-value) serves as a valid statistical measure of the extent to which the parameters explain the variation in the data relative to its mean (Beeler et al., 2024). The statistical analysis, including the *p*-value and F-value (with a 95% confidence interval) of the current study, indicated a highly significant model (*p* < 0.0001; F = 90.61) was developed from this study. This suggests that there is merely a 0.01% probability that such a large F-value resulted from noise within the model. Adequate precision serves as a measure of the signal-to-noise ratio. Our findings achieved the acceptable minimum threshold of greater than 4 (Chen et al., 2023), a desirable limit that indicates the model was fit. Additionally, an insignificant lack of fit was observed for the model (*p* > 0.6426), suggesting that the model adequately

Table 3

Analysis of variance (ANOVA) tabulated for the fitted quadratic polynomial model of Y (hydroxyl scavenging activity)

Source	Hydroxyl radical scavenging activity (Y)				
	dF ^a	Mean square	SS ^b	F-value	p-value
Model	9	91.98	827.83	90.61	< 0.0001
X _A (EtOH Conc. ^c)	1	555.31	555.31	547.03	< 0.0001
X _B (Duration)	1	6.53	6.53	6.44	0.0388
X _C (Temperature)	1	25.10	25.1	24.72	0.0016
X _A X _B	1	25.06	25.06	24.69	0.0016
X _A X _C	1	34.88	34.88	34.36	0.0006
X _B X _C	1	68.92	68.92	67.89	< 0.0001
X _A ²	1	14.48	14.48	14.27	0.0069
X _B ²	1	48.84	48.84	48.12	0.0002
X _C ²	1	53.90	53.90	53.10	0.0002
Residual	7	1.02	7.11		
Lack of fit	3	0.7443	2.23	0.611	0.6426
Pure error	4	1.22	4.87		
R ²	0.9915				
R ² _{Adjusted}	0.9805				
CV (%)	1.33				
Adequate Precision	36.5388				
Mean	76.04				
Std. dev. ^d	1.01				

^aDegree of freedom; ^bSum of squares; ^cEtOH concentration; ^dStandard deviation

explained the observed response. A model's quality could be measured through the use of a determination coefficient (R^2). A high R^2 value implies that the quadratic model applied parallelly adjusted to the investigated results (Zhang et al., 2023). The R^2 value (ideally nearing 1.00) for the hydroxyl radical scavenging antioxidant activity was determined to be 0.9915, which was very closely matched to the adjusted R^2 value of 0.9805, thereby confirming the reliability of the model. Referring to the adjusted R^2 , only less than 2% of the overall variation remains unexplained by the model. These findings support the likelihood of replicating the data with a high degree of agreement between the observed response and the expected value, as described by Akçay and Anagün (2013). The low coefficient of the variation value ($CV < 10\%$) for hydroxyl radical scavenging activity at 1.33% demonstrated that the models had a preferable accuracy. Ideally, a reduced CV demonstrates limited variability in the mean values, signifying greater precision, reliability, and consistency in the experimental outcomes (Wu et al., 2020). Following this, a second-order polynomial equation (quadratic model) resulted from the regression analysis of the data for hydroxyl radical scavenging response, as illustrated in Eq. [4]:

$$Y = 76.83 + 8.33X_A - 0.9038X_B + 1.77X_C - 1.85X_A^2 - 3.41X_B^2 + 3.58X_C^2 + 2.50X_AX_B - 2.95X_AX_C + 4.15X_BX_C \quad [4]$$

Implication of Extraction Conditions towards Hydroxyl Radical Scavenging Activity

The migration of bioactive compounds from the plant matrix to a solvent can be achieved by modulating their diffusion coefficients through methods such as ultrasonication, suitable extraction temperature, duration of treatment, and solvent concentration, which holds a notable impact on solvent polarity (Wang et al., 2008). The choice of using UAE, the green extraction procedure, arises from the fact that sonicator-based extraction efficiently isolates non-lipid phytochemicals, particularly antioxidants (Sanou et al., 2023). Furthermore, the utilisation of ultrasound proves advantageous due to its ability to disrupt plant cell structures, thereby releasing cellular contents into the extraction medium (Chemat et al., 2017). Among the three key effects of ultrasound (thermal, mechanical, and cavitation), sonic cavitation is the most predominant force in UAE, and it facilitates the mass transfer of bioactive compounds (Das et al., 2022). It works by generating acoustic cavitation in the medium, where minuscule bubbles adhere to solid particles, and upon collapsing, these bubbles rupture cell structures by producing microjets that target cell surfaces, increasing pressure, and accelerating the diffusion of cell components into the solvent (Zahari et al., 2020). In this study, EtOH was chosen as the extraction solvent due to its reduced toxicity relative to other polar organic solvents. In relation to the antioxidant assay performed, while hydrogen peroxide is not a free radical, it reacts with metals such as iron and copper in physiological systems, producing highly reactive hydroxyl radicals (Fenton reaction) that pose significant toxicity to living tissues (Ofoedu et al., 2021). Hence, this assay mimics the production of hydroxyl ROS in our body system and the need to scavenge these free radicals using antioxidants to prevent oxidative stress and destruction towards cells and organs.

Across all the 17 experimental runs in Table 1, the extraction process yielded approximately 9.59-17.05% of extract, with the highest yield observed in Run 3 and the lowest in Run 9. Referring to the ANOVA tabulation (Table 3), it displayed a very highly significant ($p < 0.0001$) linear effect for X_A , EtOH concentration, followed by X_C , temperature ($p < 0.01$) and at a satisfactory level for X_B , duration ($p < 0.05$). These findings highlight that the concentration of EtOH has the most significant impact on the extractability of hydroxyl radical scavenging compounds from MSE. Following closely are temperature and ultrasonication duration. The interaction effects of all variables were also proven to be significant in the decreasing order from X_BX_C , X_AX_C , and X_AX_B . Additionally, smaller p -values were also obtained for all quadratic coefficients (X_A^2 , X_B^2 , and X_C^2). Thus, this

simplified the presence of significant positive linear, interaction, and quadratic effects of all variables tested on the response (hydroxyl radical scavenging activity).

In relation to the constructed regression model, a 2D-contour line (Figure 1a(i), 1b(i) and 1c(i)) and 3D-response graphs (Figure 1a(ii), 1b(ii), and 1c(ii)) were generated for the response by varying two factors within the experimental range while keeping the third-factor constant. The maximum ability to scavenge hydroxyl radicals was achieved under the conditions following experimental run 1 (88.50%), with an EtOH concentration of 80%, ultrasonication duration of 60 min, and a temperature of 40°C. However, the lowest scavenging activity (59.77%) was observed in the experimental run 7, conducted with 50% EtOH for 90 min, and at 55°C, respectively.

Figures 1a (i) and 1a (ii) display the 3D and contour plots illustrating the relationship between hydroxyl radical scavenging activity with EtOH concentration and ultrasonication duration. Notably, as the EtOH concentration gradually increased while maintaining a constant sonication duration, the investigated response exhibited a gradual rise, ultimately reaching its peak at the highest EtOH concentration studied (80% EtOH). Mohamed Ahmed et al. (2020) reported a similar relationship of extraction conditions when studying the TPC content in *Solenostemma argel* Hayne leaves and also for seed-related extractions such as in Sapodilla fruit seeds, which reported 80% EtOH as the preferred solvent concentration for having high TPC value (Aquino et al., 2020). Increasing the sonication duration (while maintaining a constant EtOH concentration (80%)) led to a marginal increase in the hydroxyl radical scavenging activity. Prior to numerous experimentations, the combination of EtOH and water demonstrated greater efficacy in extracting polyphenolic compounds compared to using a single solvent system. The enhanced efficacy was clearly attributed to the adjustment made to the polarity of the solvent, which often modifies the solvent's density, dielectric constant, and viscosity (Elboughdiri, 2018). Despite numerous research findings favouring EtOH concentrations of 50-62% are optimal for extracting high TPC, TFC, and antioxidant potential, including research on date seeds (Afifi et al., 2017; Alshammari et al., 2024; Luo et al., 2018; Zhang et al., 2024), this study advocates for a higher EtOH content of 80% as the preferred solvent concentration for extracting MSE phytochemicals with specific structural arrangements responsible for scavenging hydroxyl radicals. It is crucial to recognise that the antioxidant effectiveness of phenolic compounds depends highly on their specific structural makeup. Apart from this, elevated water content in the solvent was also known to enhance the contaminant solutes to be co-extracted. However, at the cost of reducing the extraction efficiency of phenolic active compounds (Rafi et al., 2020). This phenomenon could potentially account for the observed lower hydroxyl scavenging activity as depicted in the MSE when the content of water to EtOH ratio increased.

The linear increase in the antioxidant activity (hydroxyl scavenging) with increasing EtOH (up to 80%) was observed at low (< 46°C) and also as higher temperatures (64-70°C)

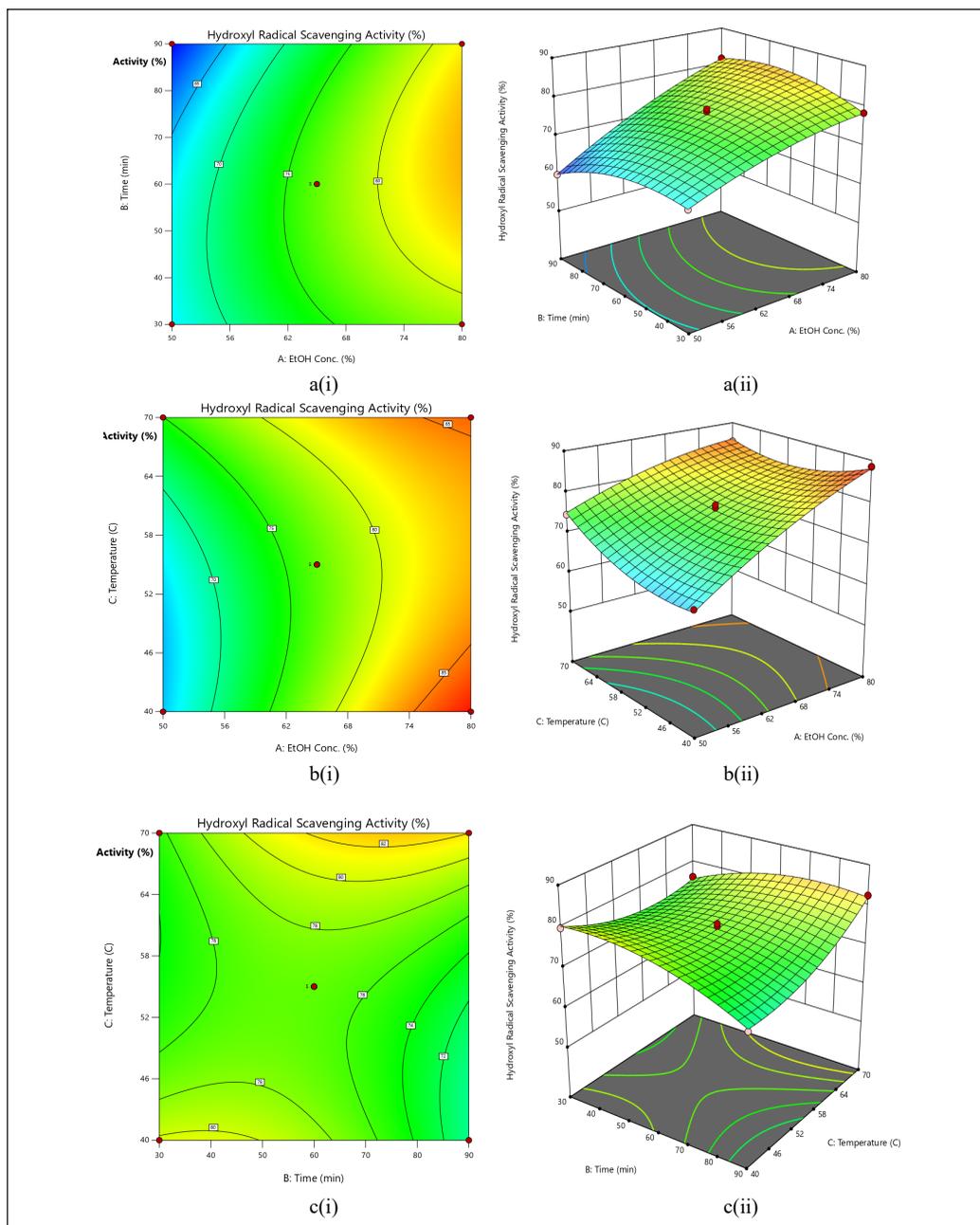


Figure 1. RSM generated contour plot (i) and 3D surface plots (ii) for hydroxyl radical scavenging (Y); (a) effect of EtOH concentration and duration (time); (b) effect of EtOH concentration and temperature; (c) effect of temperature and duration (time)

were approached (Figure 1b(i) and 1b(ii)). This result was supported by a study conducted on the effect of EtOH concentration and temperature on grape cane extraction, which demonstrated a similar trend (Karacabey & Mazza, 2010). Temperature holds a pivotal role in controlling the extraction process by weakening solvent viscosity and surface tension, as well as softening the tissues and disrupting the cell structures, particularly in fragmenting the cell wall and membrane (Zhang et al., 2024; Hossain et al., 2012). Moreover, the factor of heat also hydrolyses the bonds of bound phenolic compounds (phenol-protein or phenol-polysaccharide). These increase the solubility and diffusion coefficient, facilitating the mass transfer of polyphenolic compounds (Liu et al., 2022). The observed improvement in antioxidant activity from MSE with increasing temperature is likely attributed to the heat-induced disruption to the cell structures, which enhances the solubility and extraction of phenolic compounds from the intracellular matrix. Inversely, the enhanced hydroxyl scavenging ability utilising higher EtOH concentration but at lower temperatures may have been attributed to the fact that Medjool date seeds comprised antioxidants that were both heat-sensitive and insensitive. Therefore, maintaining high EtOH concentrations, regardless of whether the temperature is elevated or reduced, may prove effective in improving the extraction of antioxidant compounds from MSE, particularly those responsible for neutralising hydroxyl radicals. However, low temperatures will be preferred when relying on the cost-effectiveness and sustainable processing of date seeds for industries.

It appears that extending the ultrasonication duration (30-90 min) when the highest temperature was used (70°C) in the current study moderately increased the antioxidative potential. This elevates the exposure duration of date seed solids within the solvent at maximum temperature, resulting in the recovery of the highest levels of heat-insensitive antioxidants.

Response Optimisation and Verification of the Model

In this study, numerical optimisation of extraction conditions was conducted on the basis of the initial experimental outcomes, in which our target intended to attain maximum hydroxyl radical scavenging potential from MSE. The optimum extraction conditions suggested through RSM-guided optimisation utilising its desirability function at 0.981 were at 79% EtOH, 45 min, and 40°C, respectively, for achieving up to 87.40% of hydroxyl scavenging potential. Under these optimised conditions, the extraction process produced $11.19\% \pm 0.28$ of extract. The results of the experimental response matched well with the predicted value, with a CV accounting for 0.64%, as shown in Table 4. Zakaria et al. (2021) deemed the experimental results to align with the predicted data only when the CV values were below 5%, which is consistent with the findings in this study. This clearly proves that the RSM model generated is well-fitted for the recovery of antioxidants from MSE via UAE while having a good correlation.

Table 4

Predicted and experimental response values for hydroxyl radical scavenging activity utilising optimised conditions

Response variable (Y)	Predicted value	Experimental value ^a	% Difference (CV) ^b
% Hydroxyl radical scavenging	87.96	87.40 ± 1.29	0.64

^aMean ± standard deviation of triplicates (n = 3); ^bCV: coefficient of variation

Functional Metabolite Identification from MSE - UPLC-QTOF/MS

The optimised EtOH extract of MSE was fractionated by chromatography for secondary functional metabolites. The compounds presumably in charge of the antioxidative activity discussed in Table 4 and Figure 1 in MSE were tentatively listed in Table 5 and attached with their UPLC-QTOF/MS chromatograms (Figure 2). These compounds belong to the group of bioactive caffeic acid and flavonoid derivatives. Plenty of compounds from similar groups have been reported in other scientific studies exploring Medjool date seeds (Khallouki et al., 2018; Salomón-Torres et al., 2019). However, this study introduces additional findings by identifying 1-O-caffeoyl-β-D-glucopyranoside and genistein-7,4'-di-O-β-D-glucoside also to be present in MSE.

1-O-caffeoyl-β-D-glucopyranoside was known to exhibit potent antioxidative activity, as reported by Deng et al. (2016) and Braham et al. (2005), investigated in cape gooseberry, *Physalis pubescens* L. and *Moricandia arvensis*, respectively. This correlated with the high antioxidative activity detected in our current study. The second compound listed as the genistein-7,4'-di-O-β-D-glucoside is classified as a flavonoid derivate. According to De la Parra et al. (2016), genistein is generally an important phytochemical used as an anti-cancer. This liaised with the report released by Subarmaniam et al. (2023), which listed genistein-7,4'-di-O-β-D-glucoside isolated in *A. paniculata* ethanolic extract exhibits anti-cancer potential. Alongside this, the same compound was reported to be found in *Saphoro japonica* L. and noted an excellent protective effect against erythrocyte hemolysis (Wang et al., 2019; Qi et al., 2007). These results revealed that flavonoid and caffeic acid derivatives are health-benefit-contributing compounds in Medjool date seeds, strengthening the arguments for the potential therapeutic usage of these seeds.

Table 5

Identified bioactive compounds from optimised MSE using UPLC-QTOF/MS corresponding to the antioxidative properties

Identified compound name	Classification	Molecular formula	Natural mass (Da)	Observed m/z	Retention time, RT (min)
1-O-caffeoyl-β-D-glucopyranoside	Caffeic acid derivative	C ₁₅ H ₁₈ O ₉	342.09508	341.0869	5.92
Genistein-7,4'-di-O-β-D-glucoside	Flavonoid	C ₂₇ H ₃₀ O ₁₅	594.15847	593.1521	10.14

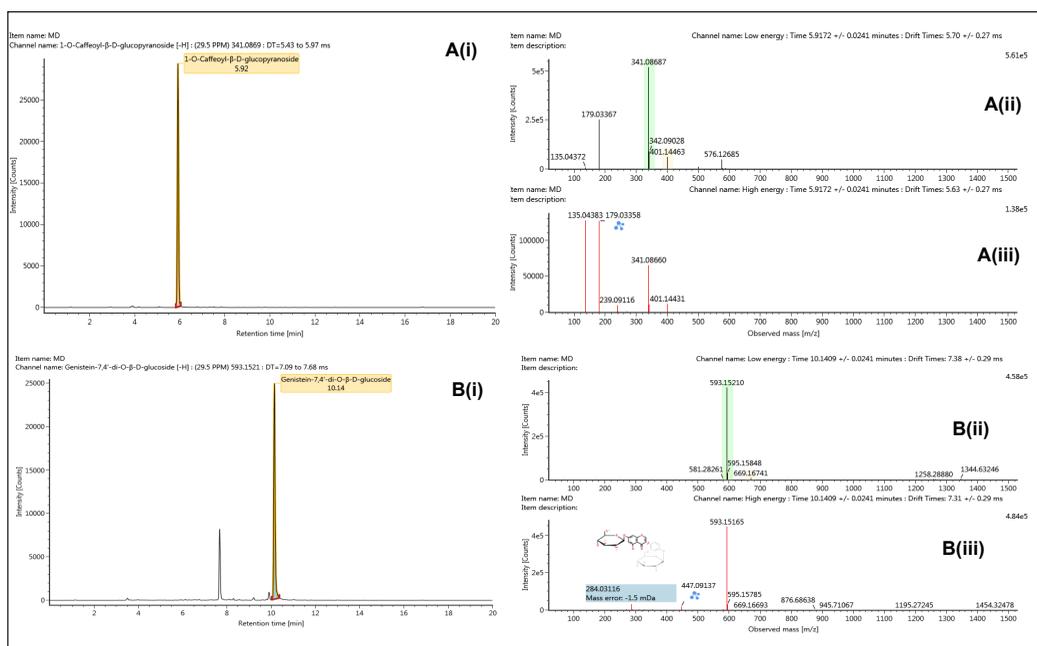


Figure 2. (i) UPLC-QTOF/MS chromatograms, (ii) low collision energy mass spectra (MS), and (iii) high collision energy mass spectra (MS), of: (A) 1-O-caffeoyl-β-D- glucopyranoside, (B) genistein-7,4'-di-O-β-D-glucoside identified from MSE

CONCLUSION

Optimisation of antioxidant activity (hydroxyl radical scavenging potential) from *P. dactylifera* Medjool seeds extracts was successfully determined, employing RSM. The optimised conditions for the maximum retrieval of hydroxyl scavenging were achieved at 79% EtOH with a lower temperature at 40°C, ultrasonicated for 45 min. Ethanol concentration and temperature were the crucial factors significantly affecting the antioxidant activity derived from MSE. The identification of functional antioxidative phytochemicals within the extracted Medjool date seeds signifies the scavenging activity observed. This investigation holds potential implications for the large-scale extraction of Medjool date seeds by boosting the extraction methodologies and producing nutraceutical and pharmaceutical-rich constituents.

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Effects of Foliar Application of Micronutrients on Nutrient Uptake, Fruit Yield, and Quality of Xa Doai Oranges

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ABSTRACT

Xa Doai orange is a commercially valuable cultivar in Central Vietnam, yet its productivity and fruit quality are often limited by micronutrient deficiencies. This study aimed to evaluate the effects of foliar application of zinc sulfate ($ZnSO_4 \cdot 7H_2O$), manganese sulfate ($MnSO_4$), and boric acid (H_3BO_3) at varying concentrations (0%, 0.4%, 0.6%, 0.8%, and 1.0%) on nutrient uptake, fruit yield, and quality attributes of Xa Doai orange. The experiment was conducted over one growing season (2022–2023) in a randomised complete block design with five treatments and three replications. Foliar sprays were applied twice times during critical growth stages: fruit set, and fruit development. Each plot consisted of nine trees, and data were collected on leaf nutrient content, yield components, and juice quality parameters. The results showed that the 0.8% treatment (T4) significantly improved leaf nutrient concentrations (45.04 mg/kg Zn, 49.62 mg/kg Mn, 51.79 mg/kg B), fruit weight (221.79 g), and yield (39.55 kg/tree) compared to the control ($P \leq 0.05$). Juice content, total soluble solids (12.92 %), and vitamin C (468.00 mg/L) were also highest at this concentration. No significant changes in soil pH or residual micronutrients were observed. These findings indicate that foliar spraying of Zn, Mn, and B at 0.8% is an effective, environmentally safe strategy to enhance productivity and fruit quality in Xa Doai orange. Further studies are needed to assess long-term effects and economic viability under diverse agroecological conditions.

Keywords: Foliar application, micronutrients, nutrient uptake, Xa Doai oranges, yield, quality

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INTRODUCTION

Citrus fruits, especially oranges, are among the most economically significant fruit crops globally due to their nutritional value and consumer demand (Hussain et al., 2021; Liu et al., 2012). In Vietnam, the

Xa Doai orange (*Citrus sinensis* L.) is a premium local cultivar well known for its distinctive aroma, juicy texture, and high sugar content. It plays a vital role in the agricultural economy of Nghe An Province, particularly in Quy Hop District, where it has traditionally accounted for a large share of total orange production. However, recent years have witnessed a marked decline in orange cultivation area—from 4,734.84 hectares in 2021 to just 1,849.33 hectares by August 2023 (Nghe An Department of Crop Production and Plant Protection [NADCPPP], 2023). This decline is largely attributed to reduced productivity, declining fruit quality, increased disease pressure, and poor soil fertility management.

One of the most pressing agronomic challenges is the widespread deficiency of essential micronutrients such as zinc (Zn), manganese (Mn), and boron (B) in orange orchards, particularly in regions with acidic soils and prolonged intensive farming systems such as those found in Nghe An. These deficiencies are becoming increasingly common due to soil leaching, excessive liming, and the limited application of micronutrient fertilisers (Alva & Tucker, 1999; Srivastava et al., 2015; Hiền et al., 2019). Although required in small quantities, these micronutrients are essential for enzymatic activation, cell division, sugar metabolism, photosynthesis, and hormone regulation (Hänsch & Mendel, 2009; Sahu et al., 2020; Mukherjee & Bordolui, 2022). Micronutrient deficiencies can lead to disrupted nutrient balance, resulting in reduced fruit set, poor fruit quality, and lower vitamin C content (Walli et al., 2022; Khatun et al., 2024).

Foliar application of micronutrients has emerged as an effective strategy to correct deficiencies and improve nutrient use efficiency, especially in perennial fruit trees like citrus. Several studies have demonstrated the beneficial effects of foliar micronutrient sprays on citrus and other fruit crops. For example, Ashraf et al. (2013) and Razzaq et al. (2013) reported increased yield and juice quality in citrus following foliar application of zinc. Similarly, foliar application of Mn and B improved photosynthesis, fruit quality, and vitamin C levels in Kinnow and sweet orange (Ilyas et al., 2015; Walli et al., 2022). Despite these findings, few studies have examined the optimal combinations and concentrations of micronutrients in Xa Doai oranges, especially under the ecological conditions of North Central Vietnam. Most farmers still rely on traditional knowledge or general fertiliser recommendations without scientific validation, leading to suboptimal results and inefficient resource use.

Given this context, the present study was undertaken with the following objectives:

1. To evaluate the effects of foliar application of Zn, Mn, and B at varying concentrations on the nutrient uptake efficiency of Xa Doai orange leaves;
2. To assess their impacts on fruit yield and quality attributes such as juice content, total soluble solids (TSS), titratable acidity (TA), and vitamin C content; and
3. To determine the optimal foliar micronutrient concentration that enhances both productivity and fruit quality under the agro-climatic conditions of Quy Hop District, Nghe An Province.

This research is geographically focussed on the tropical monsoon climate of North Central Vietnam and targets Xa Doai orange, a cultivar of high local and commercial importance with limited scientific data available. The findings are expected to provide agronomically relevant recommendations for micronutrient management in citrus farming systems, support sustainable production practices, and contribute to the scientific understanding of foliar nutrition strategies for fruit crops. Furthermore, this study may serve as a foundation for future research on nutrient interactions, economic returns of micronutrient use, and long-term effects on soil health and fruit quality.

MATERIALS AND METHODS

Experimental Site and Plant Material

The experiment was conducted from December 2022 to December 2023 at a commercial Xa Doai orange orchard in Quy Hop district, Nghe An province, Vietnam (19°19'N, 105°11'E). The site is located in a tropical monsoon climate zone, with annual rainfall of approximately 1,800 mm, average temperatures ranging from 15°C to 36°C, and relative humidity between 70–90%.

The soil at the study site was classified as loamy (45% sand, 30% silt, and 25% clay) with slightly acidic pH (6.03–6.43), moderate organic matter (2.1–2.5%), and low levels of available micronutrients (Zn, Mn, B). The orchard consisted of five-year-old Xa Doai orange trees grafted onto sour orange rootstock. Trees were planted at 4.0 m × 5.0 m spacing, with consistent orchard management practices applied throughout the study, including drip irrigation, pruning, weed control, and pest management.

Experimental Design and Treatments

The experiment was laid out in a randomised complete block design (RCBD) with five treatments and three replications. Each experimental plot had an area of 180 m² (12 m × 15 m), and nine trees were planted per plot, totalling 135 trees for the entire experiment. Details of the treatment combinations are presented in Table 1.

Table 1
Micronutrient concentrations in foliar spray treatments

Treatment	Foliar spray concentration (%)
T ₁ (control)	Water only
T ₂	0.4% ZnSO ₄ ·7H ₂ O, MnSO ₄ , H ₃ BO ₃
T ₃	0.6% ZnSO ₄ ·7H ₂ O, MnSO ₄ , H ₃ BO ₃
T ₄	0.8% ZnSO ₄ ·7H ₂ O, MnSO ₄ , H ₃ BO ₃
T ₅	1.0% ZnSO ₄ ·7H ₂ O, MnSO ₄ , H ₃ BO ₃

The micronutrient solution was a mixture of ZnSO_4 , MnSO_4 , and H_3BO_3 , diluted to the appropriate concentrations. Treatment concentrations were chosen based on prior studies and field trials in citrus nutrition (Hiên et al., 2019).

Treatments were randomly assigned within blocks. The same trees were used throughout the study for all measurements.

All treatments were applied in addition to the base fertiliser regimen commonly used by local farmers, which included 1.5–2 kg of lime, 40–60 kg of cow manure, 200–250 g N, 150–200 g P_2O_5 , and 100–120 g K_2O per tree annually.

Foliar Application Procedures

Micronutrient solutions were freshly prepared before each application using distilled water. A 15-litre knapsack sprayer with a cone-type nozzle was used for foliar spraying. Each tree received approximately 2 litres of solution per spray.

Spraying was performed at twice key growth stages: fruit set (early May), and fruit development (mid-August). Sprays were applied early in the morning (6:30–8:00 AM) under favourable environmental conditions (ambient temperature 24–28°C, RH ~70%, wind speed <5 km/h) to maximise foliar absorption and reduce drift.

Soil Sampling and Analysis

Soil samples were collected twice: before treatment application (December 2022) and after treatment application (December 2023). Four soil cores were taken from the topsoil (0–20 cm) beneath the tree canopy using a soil auger, and composited into one sample per plot (15 composited samples per sampling time). Samples were air-dried, sieved through a 2-mm mesh, and stored in sealed plastic bags. Soil pH was determined in a 1:2.5 soil-to-water suspension using a pH metre.

Micronutrients (Zn, Mn, B, Fe) were extracted using DTPA (Lindsay & Norvell, 1978) and measured using atomic absorption spectrophotometry (AAS) (Jones, 2001; 2002).

Leaf Sampling and Analysis

Leaf samples were collected twice: in December 2022 (before treatment) and December 2023 (after treatment). From each tree, 20 fully expanded, aged 3 to 5 months, non-fruiting leaves were sampled from the fourth node of current-season shoots, with sampling done from all four canopy directions.

Leaves were washed with deionised water, oven-dried at 65°C to constant weight, ground into fine powder, and digested using a 2:1 mixture of HNO_3 : HClO_4 . The digested samples were filtered, diluted, and analysed for Zn, Mn, and B concentrations by AAS (Soil and Plant Analysis Council [APAC], 2000).

Data Collection and Analysis

Fruit samples were collected in December 2023 at the commercial maturity stage. The following parameters were evaluated using standard procedures:

1. Number of fruits per tree : All marketable fruits from each tree were counted manually at harvest and recorded as the total fruit number per tree.
2. Average fruit weight (g) : After counting, 30 harvested fruits from each tree were weighed using a digital balance (± 0.01 g accuracy). The average fruit weight was calculated as the total fruit weight divided by the number of fruits.
3. Fruit diameter (cm) : The transverse diameter of each sampled fruit was measured at its widest point (equatorial region) using a digital Vernier caliper (± 0.01 cm precision). Three fruits per tree were measured, and the average was used.
4. Peel thickness (cm) : Peel thickness was measured at three equidistant points around the equator of each fruit using the same caliper. The average of the three readings per fruit was used for analysis.
5. Total fruit yield per tree (kg) : The total fresh weight of fruits harvested per tree was recorded using a portable digital balance and expressed in kilogrammes.
6. Juice content (% by weight) : Each sampled fruit was manually juiced using a stainless-steel juicer. The juice was filtered and weighed. Juice content was calculated using the formula:

$$\text{Juice content (\%)} = \frac{\text{Juice weight}}{\text{Fruit weight}} \times 100$$

7. Total soluble solids (TSS) : A few drops of the freshly extracted juice were placed on the prism of a digital refractometer (ATAGO PAL-1, Japan), and the reading was recorded at 20°C.
8. Titratable acidity (TA, % citric acid) : Ten millilitres of filtered juice were diluted with 50 mL of distilled water and titrated against 0.1 N NaOH using phenolphthalein as an indicator (Boland, 1995). The endpoint was determined by the appearance of a persistent pink colour. Titratable acidity was calculated and expressed as % citric acid using the formula:

$$\text{TA (\% citric acid)} = \frac{\text{Volume of NaOH} \times \text{Normality} \times 0.064}{\text{Volume of juice}} \times 100$$

where 0.064 is the milliequivalent weight of citric acid.

9. Vitamin C content (mg/100 mL) : Vitamin C was determined using the 2,6-dichlorophenolindophenol (DCPIP) dye titration method. Ten millilitres of juice were titrated with standard DCPIP solution until a persistent pink endpoint was observed. The vitamin C content was calculated based on a standard ascorbic acid curve and expressed in mg/100 mL of juice.

All data were analysed using SPSS version 26.0. One-way analysis of variance (ANOVA) was conducted to evaluate treatment effects, and mean differences were separated using the Least Significant Difference (LSD) test at a 5% significance level ($P \leq 0.05$).

RESULTS AND DISCUSSION

Effect on Yield Components

The yield components of Xa Doai orange responded positively to foliar application of micronutrients, particularly at higher concentrations (Table 2). Among these components, the number of fruits per tree ranged from 177.44 to 180.44, showing no statistically significant differences among treatments ($P > 0.05$). This indicates that foliar micronutrient application had a limited effect on fruit set in this specific context. Similar results were reported by Hasani et al. (2012), who found that foliar Zn and Mn applications did not significantly affect fruit number in pomegranate. However, other studies have documented different outcomes. Singh et al. (2018) observed a significant increase in fruit count in sweet orange when treated with a foliar combination of 0.5% Zn, 0.7% B, and 0.7% Cu. Likewise, Bhanukar et al. (2021) and El-Gioushy et al. (2021) reported improved fruit number and overall productivity in citrus under specific micronutrient combinations.

Table 2
Effects of foliar application of micronutrients on the yield components and yield of the Xa Doai oranges

Treatment	Number of fruits/tree	Fruit weight (g/fruit)	Fruit diameter (cm)	Peel thickness (cm)	Yield (kg/tree)
T1	178.78 ^a	192.23 ^b	7.18 ^a	0.37 ^a	34.35 ^c
T2	176.89 ^a	199.70 ^b	7.40 ^a	0.36 ^a	35.33 ^b
T3	180.44 ^a	209.03 ^b	7.49 ^a	0.35 ^a	37.69 ^a
T4	178.22 ^a	221.79 ^a	7.52 ^a	0.32 ^a	39.55 ^a
T5	177.44 ^a	221.56 ^a	7.50 ^a	0.32 ^a	39.25 ^a
LSD _{0.05}	6.80	10.62	0.87	0.24	2.16
CV%	4.00	7.70	1.80	9.90	6.10

Note. Different letters within the columns indicate significant differences at $P \leq 0.05$

These contrasting findings underscore that the impact of foliar micronutrient application on fruit number is context-dependent, likely influenced by species, cultivar, baseline soil fertility, and environmental conditions.

In contrast to fruit number, fruit weight showed significant and consistent improvement with increasing foliar concentrations. Treatment T4 (0.8%) produced the heaviest fruits at 221.79 g, followed closely by T5 (1.0%) at 221.56 g. These values represent an increase of approximately 17.6% over the control (188.59 g), and the difference was statistically significant ($P \leq 0.05$). This enhancement is biologically meaningful, as heavier fruits directly contribute to higher market value and total yield. The low coefficient of variation ($CV < 10\%$) and confirmed LSD values indicate high consistency and reliability of the treatment effects. These findings are in agreement with Bhanukar et al. (2021), who reported increased fruit weight in Blood Red oranges with 1.0% $ZnSO_4$ application, and Bhalariao et al. (2020), who found that $ZnSO_4 + MnSO_4$ foliar application improved fruit mass in sweet oranges.

Although fruit diameter also increased slightly from 7.18 cm in the control (T1) to 7.52 cm in the 0.8% treatment (T4), the differences were not statistically significant. However, this positive trend may be biologically meaningful and can be attributed to the physiological roles of zinc and boron in fruit development. Zinc activates key enzymes involved in protein and carbohydrate metabolism, facilitating better assimilate translocation to the fruit, while boron enhances sugar transport and cell wall expansion, contributing to increased fruit volume. Such micronutrient-driven processes improve cell division and elongation in the fruit's pericarp, potentially leading to larger diameters over time. Similar trends have been reported in other citrus studies, where foliar application of Zn and B, either individually or in combination, resulted in increased fruit diameter in sweet orange, mandarin, and related cultivars (Kamei et al., 2019; Makhoul et al., 2019; Mann et al., 1985; Zoremfluangi et al., 2019).

Peel thickness decreased gradually with higher foliar concentrations, from 0.37 cm in the control to 0.32 cm in T4 and T5. Although the difference was not statistically significant, thinner peel is a favourable trait for juice yield and consumer acceptance. This trend is supported by findings of Singh et al. (2018) and Luxmi et al. (2024), where reduced peel thickness was recorded in citrus under micronutrient spraying.

The most substantial improvement was recorded in total fruit yield per tree, where T4 (0.8%) achieved a mean yield of 51.79 kg/tree—an increase of 50.8% compared to the control (34.35 kg/tree). This difference was statistically significant ($P \leq 0.05$) and is of practical importance to commercial growers. The increase in yield is attributed to a combination of higher fruit weight and juice content, possibly resulting from improved nutrient availability and enhanced physiological processes such as photosynthesis and sugar translocation. Similar improvements were reported by Nandita et al. (2022),

who found that combined foliar application of Zn, B, Cu, and Fe significantly improved sweet orange yield.

In summary, while fruit count per tree remained unchanged, foliar application of Zn, Mn, and B—particularly at 0.8%—enhanced fruit weight, yield, and peel traits, demonstrating its agronomic effectiveness for improving Xa Doai orange productivity under the conditions of Nghe An Province.

Impact on Fruit Quality

Fruit quality is a critical determinant of consumer preference and market value. In this study, foliar application of micronutrients (Zn, Mn, B) at increasing concentrations positively affected key quality attributes of Xa Doai oranges, including juice content, total soluble solids (TSS), titratable acidity (TA), TSS/TA ratio, and vitamin C content (Table 3).

The juice content ranged from 41.34% in the control (T1) to a maximum of 51.55% in T4 (0.8%), representing a 24.7% increase. This improvement was statistically significant ($P \leq 0.05$) and is particularly important for juice yield and consumer satisfaction. These findings are supported by Razzaq et al. (2013) and Singh et al. (2018), who observed similar increases in juice content in citrus fruits following foliar Zn and B application.

TSS, an indicator of sugar concentration and sweetness, also improved significantly with increasing foliar concentrations. TSS increased from 10.3 in T1 to 12.92 % in T4—a 25.4% enhancement—indicating better carbohydrate accumulation and flavour development. This difference was statistically significant ($P \leq 0.05$) and aligns with the findings of Bhalerao et al. (2020), Hasani et al. (2012), and Walli et al. (2022), who demonstrated that foliar application of Zn and Mn enhances TSS in citrus and other fruit crops by improving photosynthetic efficiency and sugar translocation.

Table 3
Effects of foliar application of micronutrients on the quality attributes of Xa Doai oranges

Treatment	Juice content (%)	TSS (%)	TA (%)	TSS/TA	Vitamin C (mg/L)
T1	48.35 ^c	10.30 ^c	0.45 ^a	22.72 ^d	426.33 ^c
T2	48.57 ^c	11.16 ^d	0.39 ^a	28.94 ^c	432.11 ^{bc}
T3	53.04 ^{ab}	11.57 ^c	0.39 ^a	29.89 ^c	445.56 ^b
T4	53.82 ^a	12.92 ^a	0.36 ^a	36.36 ^a	468.00 ^a
T5	50.39 ^{bc}	12.37 ^b	0.38 ^a	32.98 ^b	445.78 ^b
LSD _{0.05}	3.01	0.44	0.13	1.46	17.50
CV%	8.80	5.70	4.80	7.30	6.00

Note. Different letters within the columns indicate significant differences at $P \leq 0.05$

Although titratable acidity (TA) did not differ significantly among treatments (range: 0.31–0.36%), the TSS/TA ratio, which serves as a flavour balance index, was highest in T4 (36.36), compared to 28.03 in the control. A higher TSS/TA ratio reflects better sweetness-acidity balance, a key determinant of fruit palatability and marketability. Singh et al. (2018) and Zoremthlengi et al. (2019) reported similar improvements in citrus taste profile with foliar micronutrient combinations.

Vitamin C content, a vital nutritional quality trait and antioxidant marker, also increased significantly under foliar treatment. The control recorded 426.33 mg/L, while T4 reached 468.00 mg/L—an enhancement of 9.8%. This aligns with studies by Ali et al. (2014) and Ilyas et al. (2015), which found that zinc and manganese foliar sprays enhance ascorbic acid synthesis due to their roles in enzymatic co-factors and oxidative metabolism.

Together, these results confirm that foliar application of Zn, Mn, and B at 0.8% concentration optimizes fruit quality in Xa Doai oranges. The improvements are not only statistically significant ($P \leq 0.05$) but also biologically and commercially meaningful, particularly for high-value speciality citrus such as Xa Doai, where flavour, juice yield, and nutritional value are key competitive advantages.

Soil and Plant Nutrient Status

Soil Properties

Soil analysis conducted before treatment application (December 2022) and after treatment application (December 2023) revealed no statistically significant differences in key parameters, including pH and DTPA-extractable Zn, Mn, B, and Fe concentrations (Table 4). The soil pH remained stable around 6.03–6.43, while available micronutrient levels showed only slight, non-significant variation across treatments. These results suggest that foliar application of micronutrients did not contribute to nutrient accumulation or chemical changes in the soil, which is consistent with the mode of application where nutrients are absorbed directly through leaf surfaces. Similar findings were reported by Bhalerao et al. (2020) and El-Gioushy et al. (2021), who also observed minimal impact of foliar micronutrient application on soil properties in citrus orchards.

From an environmental perspective, foliar fertilisation presents a lower risk of soil contamination and nutrient runoff, especially under intensive citrus management on sloping or sandy soils. However, repeated foliar application over multiple seasons could lead to localized accumulation in the phyllosphere or shallow soil layers due to leaf wash-off during rain events. Therefore, we recommend periodic soil testing and nutrient budgeting to prevent potential overuse and ensure long-term sustainability.

Table 4
Chemical properties of top-soils (0 to 20 cm) before and after treatment

Treatment	pH		Zn (mg/kg)		Mn (mg/kg)		B (mg/kg)		Fe (mg/kg)	
	Before	After	Before	After	Before	After	Before	After	Before	After
T ₁	6.14 ^a	6.25 ^a	2.01 ^a	1.91 ^a	9.40 ^a	10.93 ^a	0.38 ^a	0.36 ^a	31.17 ^a	34.07 ^a
T ₂	6.30 ^a	6.19 ^a	2.00 ^a	1.95 ^a	9.73 ^a	9.13 ^a	0.39 ^a	0.37 ^a	36.63 ^a	39.17 ^a
T ₃	6.03 ^a	6.34 ^a	2.03 ^a	1.98 ^a	10.93 ^a	11.97 ^a	0.37 ^a	0.36 ^a	34.07 ^a	36.17 ^a
T ₄	6.26 ^a	6.43 ^a	2.31 ^a	2.16 ^a	8.13 ^a	10.43 ^a	0.40 ^a	0.38 ^a	39.17 ^a	36.63 ^a
T ₅	6.21 ^a	6.20 ^a	2.14 ^a	2.08 ^a	8.97 ^a	9.00 ^a	0.39 ^a	0.38 ^a	35.33 ^a	34.02 ^a
LSD _{0.05}	0.30	0.46	0.49	0.26	3.96	4.39	0.12	0.20	8.16	5.88
CV%	2.60	3.90	12.30	6.70	9.10	14.70	3.20	3.50	12.20	11.50

Note. Different letters within columns indicate significant differences at $P \leq 0.05$

Table 5
Effects of foliar application of micronutrients on leaf nutrient content of the orange tree

Treatment	Zn (mg/kg)		Mn (mg/kg)		B (mg/kg)		Fe (mg/kg)	
	Before	After	Before	After	Before	After	Before	After
T ₁	21.15 ^a	19.25 ^c	21.68 ^a	22.96 ^d	30.24 ^a	31.61 ^c	65.62 ^a	60.19 ^c
T ₂	20.92 ^a	41.82 ^b	22.68 ^a	44.43 ^c	31.12 ^a	46.69 ^d	66.05 ^a	68.93 ^b
T ₃	22.68 ^a	42.46 ^b	21.82 ^a	47.15 ^b	30.81 ^a	48.31 ^c	67.56 ^a	73.63 ^a
T ₄	21.91 ^a	45.04 ^a	21.57 ^a	49.62 ^a	31.43 ^a	51.79 ^a	64.22 ^a	75.04 ^a
T ₅	20.27 ^a	43.30 ^b	20.77 ^a	46.46 ^b	29.62 ^a	50.67 ^a	69.53 ^a	76.38 ^a
LSD _{0.05}	3.63	1.69	2.23	1.75	3.49	1.66	4.46	3.98
CV%	9.00	2.30	5.50	2.20	6.10	1.09	3.60	3.20

Note. Different letters within the columns indicate significant differences at $P \leq 0.05$

Leaf Nutrient Uptake

Significant differences were observed in leaf nutrient concentrations among treatments (Table 5). The highest leaf Zn, Mn, and B concentrations were recorded in T₄ (0.8%): 45.04 mg/kg for Zn, 49.62 mg/kg for Mn, 51.79 mg/kg for B, and 75.04 mg/kg for Fe—substantially higher than in the control (T₁), and above the sufficiency thresholds for citrus as reported by Alva and Tucker (1999).

The elevated concentrations of Zn, Mn, B, and Fe in leaf tissues were closely associated with notable improvements in yield and fruit quality attributes—particularly higher fruit weight, juice content, total soluble solids (TSS), and vitamin C levels (Walli et al., 2022). Similar correlations between foliar micronutrient uptake and enhanced productivity in citrus and other fruit crops have also been documented in previous studies (Bhalerao et al., 2020;

El-Gioushy et al., 2021; Hasani et al., 2012), further supporting the positive relationship between nutrient absorption efficiency and agronomic performance.

This enhanced nutrient uptake efficiency may be attributed to favourable conditions during foliar application, such as increased leaf permeability and stomatal conductance in the early morning under moderate relative humidity (Maity et al., 2021). Bhalerao et al. (2020) also emphasised that timing and environmental conditions during spray application are critical for maximising absorption and utilisation efficiency. Moreover, the physiological functions of these micronutrients play a crucial role in supporting plant metabolism and reproductive success. Zinc promotes auxin biosynthesis and activates numerous enzymes involved in protein metabolism (Nayan & Fouzi, 2023; Kumar et al., 2024); manganese is essential for chloroplast development, the water-splitting reaction of photosystem II, and the regulation of oxidative stress (Uthman et al., 2022); while boron facilitates sugar transport, stabilises cell walls, and plays a pivotal role in pollen germination and fruit set (Razzaq et al., 2013; Singh et al., 2018; Walli et al., 2022).

These mechanisms together contributed to enhanced photosynthesis, assimilate partitioning, and reproductive development, ultimately resulting in greater fruit biomass and better internal quality. Comparable physiological responses to foliar micronutrient treatments have been documented by Zoremfluangi et al. (2019), who reported improved leaf nutrient content and fruit characteristics in Khasi mandarin under combined Zn–Mn–B foliar application.

CONCLUSION

This study confirmed that foliar application of micronutrients—zinc (Zn), manganese (Mn), and boron (B)—significantly improved nutrient uptake, yield components, and fruit quality of Xa Doai orange under the agroecological conditions of Nghe An Province, Vietnam. Among the tested treatments, the 0.8% foliar concentration (T4) was the most effective. The application of ZnSO₄, MnSO₄, and H₃BO₃ at 0.8% increased fruit weight by 17.6% and total yield by 50.8% compared to the control. While the number of fruits per tree remained statistically unchanged, significant improvements were recorded in juice content (+24.7%), total soluble solids (TSS, +25.4%), and vitamin C content (+9.8%). These quality attributes are crucial for consumer preference and processing value. Elevated leaf concentrations of Zn, Mn, and B confirmed improved uptake efficiency without causing accumulation in soil or altering soil pH, highlighting the environmental safety of foliar application. These findings suggest that spraying at 0.8% during key stages—fruit set, and fruit development—can be a practical strategy to boost both yield and quality in Xa Doai orange cultivation.

However, this study was conducted over a single growing season and limited to one cultivar. Further research should assess the long-term effects, economic returns, and

optimisation of application frequency and timing under varied conditions. Overall, the results indicate that foliar micronutrient application is a promising and sustainable approach to improving orange production in central Vietnam.

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Field Assessment of Plant Growth Performance and Residue Persistence of Saponin-based Molluscicide Formulations

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ABSTRACT

This study evaluated the plant growth performance and environmental residue behaviour of saponin-based molluscicide formulations under field conditions. The findings were compared against two commercial chemical molluscicides: niclosamide and fentin acetate. Two saponin formulations, emulsifiable concentrate (EC) and wettable powder (WP) of saponin-based molluscicides, were applied at varying concentrations on rice crops, alongside chemical and water controls. Growth parameters, including plant height, tiller number, biomass, and harvest index, were recorded, while molluscicide residues were analysed in rice tissues, soil, and water using HPLC. Results showed no significant differences in growth performance among treatments. However, niclosamide exhibited the highest residue levels and most prolonged half-lives across all matrices, raising concerns over persistence and potential food safety risks. In contrast, saponin formulations demonstrated faster degradation with half-lives below 10 days and reduced environmental persistence. Despite detectable residues in polished grains, saponin is considered safe for human consumption due to

its natural occurrence and rapid biodegradability. The findings supported the potential of saponin-based molluscicides as environment friendly alternatives to synthetic chemicals in rice cultivation. Further studies are recommended to improve formulation efficacy and understand their biochemical interactions with target pests.

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INTRODUCTION

Pomacea maculata Perry, also known as the black apple snail, is one of the major pests that has drastically affected farmers in Malaysia's rice industry for decades. Due to their tremendous reproduction rate, they can now be abundantly spotted in all paddy fields throughout Peninsular Malaysia, igniting great concern in rice production and quality. Subsequently, they could trigger a greater threat to rice production due to their large size and great appetite (Arfan et al., 2014; Burks et al., 2011; Mokhtar et al., 2024). Various methods have been implemented to reduce the population of this pest, with chemical control being the most effective way to minimise them.

As technology in the agriculture industry advances, most farmers have decided that it is much easier to chemically control the apple snails' population in rice-growing areas by applying molluscicides to targeted areas. Additionally, chemical control is the most preferred approach in controlling pests due to its easy application, low labour costs, cost-effectiveness, and ability to kill pests quickly (Yigit & Velioglu, 2020). Metaldehyde, some carbamates such as methiocarb, niclosamide, and iron phosphate plus chelating agent were widely used as active ingredients in molluscicides (Andrews et al., 1982; Calumpang et al., 1995; Zhang et al., 2011). In Taiwan, metaldehyde, niclosamide, and triphenyltin acetate have been widely utilised for eradicating this invasive rice pest (Cheng & Kao, 2004; Wu et al., 2010). Applying chemical molluscicides reduced the number of apple snails but are highly toxic to non-target organisms such as fish and affected the environment (Roonjho et al., 2022; San Martín et al., 2008).

To assess potential phytotoxicity from molluscicides, key growth parameters (plant height, tiller number, and all rice cultivation stages) were monitored (DoA, 2008). Paddy cultivation has six stages. These include planting day (1-5 days), tillering stage (15-40 days), panicle initiation stage (40-69 days), heading stage (70-89 days), ripening stage (90-104 days), and harvest stage (105-112 days). These stages are identified and monitored through growth parameters, which are practical and fundamental in assessing the growth and tracking the health of the plants. They have also been used to estimate the final yield and act as a guide in decision-making regarding a suitable approach for the studied organism. These parameters are vital for assessing potential phytotoxicity caused by molluscicides on paddy plants by measuring plant height, number of tillers, fresh and dry weight of paddy straws and grains, total above-ground biomass, and harvest index. Phytotoxicity may affect plants' vigour by stunting the growth, leaf discolouration, chlorosis, necrosis, or yield decline (Dias, 2012).

Up to the present, rice research has focussed more on developing sustainable agricultural practices and restoring functional food webs for integrated pest management and nutrient cycling (Schmidt et al., 2015). However, the extensive use of pesticides has negatively impacted human and other life forms. Variations in pesticide residue levels in

raw agricultural commodities are affected by several parameters, such as geographical locations, weather conditions, growing season, and growth rates (Fujita & Iijima, 2013). Ideally, a pesticide must be lethal to the targeted pests, but not to non-target species, including humans. Measuring pesticide residue levels in crops and aquatic life is vital for ensuring food safety. All assessed food must not exceed the maximum residue level (MRL) in the Codex, a guideline of the food code established by the FAO. Detecting and identifying various pesticide residues is becoming a significant public health concern and a vital procedure to be acknowledged as a guarantee of food safety (Hou et al., 2013; Teló et al., 2017; Zhang et al., 2011). While saponins are known, information on their field residue persistence and direct comparison with synthetic pesticides in a rice ecosystem is still lacking.

MATERIALS AND METHODS

Chemicals and Reagents

All standard compounds (saponin, niclosamide and fentin acetate) were purchased from the Thermo Fisher Scientific with more than 97% purity. HPLC grade acetone, acetonitrile (MeCN), and methanol (MeOH), analytical standard sodium chloride (NaCl), anhydrous magnesium sulphate (anhydrous MgSO₄), and Orthophosphoric acid were also acquired from the Thermo Fisher Scientific.

Treatments

Saponin emulsifiable concentrates, EC (T1), saponin wettable powder, WP (T2), two selected commercial molluscicides; niclosamide (T3) and fentin acetate (T4) as positive controls, and water (T5) as negative control, were used as treatments. All molluscicides were applied following the recommended rate, with five treatments.

Method Validation of Molluscicide Compounds

Quantification of saponin in molluscicide-containing saponin (Sapputra), niclosamide, and fentin acetate from commercial molluscicide compounds was done by referring to respective methods with modifications (Dong et al., 2017; Sun et al., 2012; Yang et al., 2003). The separation of saponin was executed using a C18 column, and the detection wavelength was set at 210 nm. The injection volume was 20 µL. A C18 column was used to quantify fentin acetate, and the detection wavelength was at 220 nm. The injection volume was 10 µL. Niclosamide was quantified using a C18 column with a 202 nm detection wavelength. The injection volume was 10 µL. For the recovery percentage, the values of spiked and unspiked samples were compared to evaluate the recovery and matrix effect in samples, accordingly. The percentage recoveries were determined by using the formula (Xu et al., 2012; Zaidon et al., 2019):

$$\text{Recovery percentage (\%)} = \frac{C_{\text{spiked}} - C_{\text{unspiked}}}{C_{\text{added}}} \times 100\% \quad [1]$$

where C_{spiked} is the concentration of the target analyte measured in a spiked sample, C_{unspiked} is the concentration of the target analyte measured in a blank sample, and C_{added} is the known concentration added to the sample.

Preparation of Stock Standard Solution

A stock standard solution of saponin, niclosamide, and fentin acetate was prepared at a concentration of 1000 ppm by dissolving 0.05 g of the respective standard in 10 mL of deionised water (saponin) and 10 mL of acetonitrile (niclosamide and fentin acetate) in a 50 mL volumetric flask. Then, intermediate working standard solutions were prepared by diluting the stock solutions in deionised water to obtain respective standards of 100 ppm. Finally, serial dilutions of the working standard solutions were prepared to obtain seven calibration solutions (50, 25, 10, 5, 1, 0.5, and 0.1 ppm) in deionised water. All the standard solutions were kept in scintillation vials at 4°C in the refrigerator.

Sample Preparation

The rice plants were ground into powder form using a table-type smashing machine. This step was crucial as it increased the surface area of the materials and helped facilitate solvent penetration into the cells. The material that passed through an 80-mesh screen was used for extraction purposes. The powder was kept in an airtight plastic bag in a refrigerator at 6 ± 1 °C with 5% to 85% relative humidity for further analysis. Using a soil sampling apparatus, 200 g of soil sample was collected randomly at ten different sampling points in each plot with a zero to ten cm depth (Ma et al., 2012; Zhang et al., 2011). Stone and plant debris were manually removed, and the soil samples were stored at -20 °C for further analysis. A 100 ml water sample was collected at intervals for each plastic container. Water samples were filtered using filter paper and concussion extraction before being reconstituted in 5 mL HPLC methanol for HPLC analyses (Huang et al., 2013).

Extraction of Rice and Soil Samples

Rice (1 g) and soil samples (5 g) were weighed separately in a 50 mL Teflon centrifuge tube, and an adequate standard was added to these blank samples. The centrifuge tubes with spiked samples were vortexed for 1 min. They were allowed to stand for 2 hours at room temperature to distribute and interact the molluscicide evenly with the sample matrix. 10 mL of acetonitrile was added to each centrifuge tube. After the tube was shaken vigorously for 10 min, 1 g NaCl and 4 g anhydrous MgSO_4 were added to the centrifuge tubes and again shaken for 5 min. Followed by centrifugation (4000 rpm, 5 min) of the sample containing centrifuge tubes, 1.5 mL of the upper layers (acetonitrile) supernatant was transferred to a 2 mL centrifuge tube containing 150 mg of MgSO_4 and 75 mg of Florisil for rice and 150

mg of MgSO_4 and 75 mg of C18 for soil samples, accordingly. Afterwards, the centrifuge tubes were vortexed for 1 min and centrifuged for 5 min at 5000 rpm. Finally, the upper acetonitrile layer was filtered from the individual tube through a $0.22 \mu\text{m}$ hydrophilic PTFE filter and transferred to an autosampler vial for HPLC analysis (Kaium et al., 2018).

Then, a 10 mL water sample was added to 50 mL Teflon centrifuge tubes, and the standard was added to the water samples. The centrifuge tubes were vortexed for 1 min and allowed to stand for 20 min at room temperature. Next, 10 mL of acetonitrile was added to each centrifuged tube and vortexed vigorously for 2 min. After the addition of 4 g MgSO_4 and 1 g NaCl, the tubes were immediately vortexed vigorously for 2 min and centrifuged for 5 min at 4000 rpm. Lastly, 1.0 mL of the supernatant (acetonitrile) from each tube was transferred into an autosampler vial with a $0.22 \mu\text{m}$ hydrophilic PTFE filter for HPLC analysis.

Data Collection and Analysis

Growth quality data, including plant height, number of tillers, fresh and dry weight of paddy straw and grain, total above-ground biomass, harvest index, and soil and water pH, were recorded during the field assessment. Total above-ground biomass and harvest index were derived by using the following formula (Saito et al., 2023):

$$\text{Total above-ground biomass (kg/ha)} = \frac{\text{Dry weight of grains}}{\text{(kg/ha)}} + \frac{\text{Dry weight of straws}}{\text{(kg/ha)}} \quad [2]$$

$$\text{Harvest index} = \frac{\text{Dry weight of filled grains (kg/ha)}}{\text{Total above-ground biomass (kg/ha)}} \quad [3]$$

Residue level, degradation rate of molluscicides, half-lives, and maximum residue level (MRL) were recorded afterwards. The degradation rate refers to the time required for 50% of the initial dose of pesticides to dissipate or break down into simpler compounds or metabolites in the environment (Kah et al., 2007). It is also referred to as the first-order half-life or DT50. Degradation rate and half-lives of molluscicides were calculated using the following formula (Graebing et al., 2004):

$$C_t = C_0 e^{-kt} \quad [4]$$

Where:

C_t = concentration of pesticide at time t

C_0 = initial concentration of pesticide

k = degradation rate constant (in time^{-1})

t = time (typically in days).

Meanwhile, the degradation rate constant (k) was obtained using the formula:

$$k = \frac{\ln(C_0) - \ln(C_t)}{t} \quad [5]$$

Half-life was calculated as follows:

$$t_{1/2} = \frac{\ln(2)}{k} \quad [6]$$

The statistical significance between molluscicide treatments was interpreted by an Analysis of Variance (ANOVA) test using the Statistical Analysis System 9.4 (SAS).

RESULTS AND DISCUSSION

All graphs showed linear and reproducible calibration curves, with the R^2 values nearly approaching 1 (saponin: 0.9971, niclosamide: 0.9991, and fentin acetate: 0.9991). These values indicated that the responses of the HPLC detector towards residues of all compounds were good, where the linearity factors were at $R^2 > 0.99$. The retention times obtained from HPLC analysis for saponin, niclosamide, and fentin acetate were 2.9, 4.3 and 5.3 min, respectively, showing a slight difference compared to reference retention times. However, Sun et al. (2017) explained that the actual chromatogram may vary from the reference chromatogram due to factors such as the type of columns used, flow rates, and the mobile phase. These factors may contribute to the difference between measured and predicted retention time. In addition, the recovery of saponin for grains, leaves and stems, water and soil ranged from 58% to 118% whereas for niclosamide, the percentage values ranged from 71.3% to 113.9%, respectively, as shown in Table 1. Meanwhile, for fentin acetate, all samples' recovery ranged from 71.3% to 94.1%. The recovery results were acceptable, compared to the guidelines for residue analysis quality control (Corley, 2003; Fitri et al., 2017). The percentage of recovery values accepted at the global stage was between 60% and 140% (Pihlström et al., 2021). It is recommended that when outliers are identified, they should be excluded from the statistical calculation of the mean, SD and RSD, with justification and statistical significance. Nevertheless, all individual recovery data (including those excluded) should be reported (Fitri et al., 2017). Hence, the extraction methods were validated and used to determine paddy, soil, and water compound residues.

No significant ($p \leq 0.05$) differences were observed among treatments for all measured growth parameters (plant height, tiller count, biomass, and harvest index) at any sampling stage (Table 2). The average plant height ranged from 126.7 to 118 cm for all treatments, respectively. However, the plant treated with niclosamide (T3) gave the lowest reading in plant height. Paddy plants treated with the negative control treatment (T5) showed the highest number of tillers, with 24 tillers during 110 DAA. This was followed by T2

Table 1

The average recoveries of molluscicides in rice plants, soil and water (value within the range 60-140% is considered as reliable (Pihlström et al., 2021))

Molluscicides	Spiking level (ppm)	Mean value of molluscicides recovery (%)			
		Leaves	Grain	Soil	Water
Saponin	0.5	78	58	89.6	80.1
	1	76	119	71	82.1
	5	118	64.7	104	94.0
Niclosamide	0.5	71.3	75.7	113.9	94.9
	1	76.8	81.3	90.2	96.5
	5	83.2	84.5	93.9	112.8
Fentin acetate	0.5	94.1	86.1	82.1	70.9
	1	88.7	91.2	81.9	71.3
	5	92.2	84.7	80.5	79.4

(saponin WP) and T4 (fentin acetate), with 22 and 21 tillers, respectively, indicating better branching and potential productivity for these treatments. Meanwhile, the lowest number of tillers was detected in the sample treated with niclosamide, 17 tillers. There were no significant differences between treatments for fresh and dry weight of paddy straw and grains, the total above-ground biomass, and harvest index.

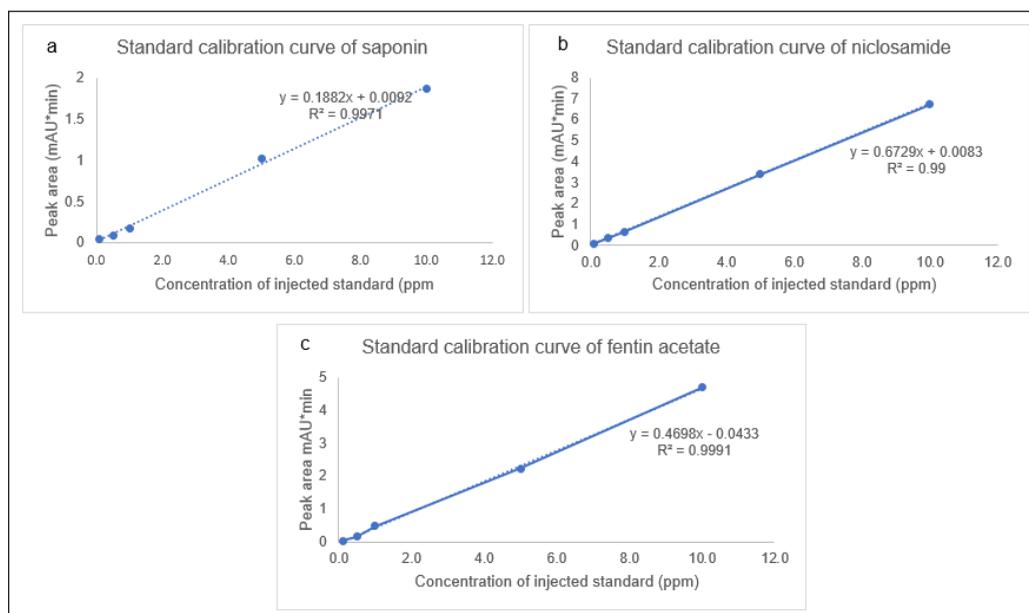


Figure 1. Standard calibration curve of (a) saponin, (b) niclosamide, and (c) fentin acetate

Ramli et al. (2019) stated that observing paddy growth through plant height, width of leaves, number of leaves, and number of tillers is crucial in determining the harvest yield. Overall, good performance of paddy growth contributed to high grain yield harvested (Kamarulzaman et al., 2017; Ramli et al., 2018). As mentioned in Table 2, the highest plant height and number of tillers were exhibited in T5 (water, negative control), but it gained almost a similar yield compared to other treatments. Tillering capacity may influence the yield potential of a paddy cultivar (Krishnan et al., 2011; Yoshida, 1973). Generally, paddy plants with more tillers may experience a greater inconsistency in the nutrient distribution among tillers, leading to a dissimilar pattern in grain development and yield between tillers (Krishnan et al., 2011; Yoshida, 1973).

Residue levels in paddy straws and grains, soil, and water samples collected from the field assessment were determined by HPLC and shown in Table 3, 4, and 5, respectively. These results were based on the findings of Aktar et al. (2009), in which pesticide residues were discovered in soil, air, surface, and groundwater across the plant cultivations. Subsequently, the residue levels decreased over time as the volatilisation of the compound took place. During harvest, which was at 110 DAA, plants treated with niclosamide (T3) exhibited the highest residue with 86.0 ppm in paddy straw, followed by T2 (74.3 ppm), T4 (66.0 ppm), and T1 (48.0 ppm). The highest declination regarding residue dissipation was observed in T1 (saponin EC), as it lost 2,091.7 ppm throughout the assessments, from 30 to 110 DAA. This declination was probably due to a more volatile compound than other molluscicide compounds.

Referring to Table 3, compound residues were determined in unpolished and polished grain. Residue levels in unpolished grain at 110 DAA showed T2 (90.0 ppm) having the highest residue, followed by T1 (72.7 ppm), T3 (72.0 ppm), and T4 (71.3 ppm). However, these measurements were statistically similar, with no significant residual level differences across the treatments. Polished grain residues showed a general reduction compared to unpolished grain. Treatment 3 (37 ppm) had the highest residue, followed by T2 (26.7 ppm), and then T4 (21.0 ppm). Apart from that, the half-life data reflected how long it took for the pesticide residue to degrade to half of its initial concentration. Both positive controls represented the most prolonged half-life recorded during the assessment, in which T4 (fentin acetate) had an approximate half-life of 70 days, followed by niclosamide (T3) at 32 days. For saponin formulations, both showed a short half-life of less than ten days.

Detecting pesticide residues in food commodities has significantly hindered international trade (Bajwa & Sandhu, 2014). The migration of pesticide compounds into the grains may occur based on their chemical characteristics, the concentration of pesticides used and other biotic and abiotic conditions. According to recommendations, pesticides should only be present on the outer layer and eliminated during grain milling, mostly in coproducts such as bran and husk (Dors et al., 2011). In this assessment, molluscicide residues still existed

Table 2
Growth data of paddy plants and grains between molluscicide treatments

Treatment	Plant												Grain			Harvest index	Total above biomass (kg/ha)	
	Average plant height (cm)						Average no of tillers						Fresh weight (kg)	Dry weight (kg)	Fresh weight (kg)			Dry weight (kg)
	30 DAA	60 DAA	90 DAA	110 DAA	30 DAA	60 DAA	90 DAA	110 DAA	30 DAA	60 DAA	90 DAA	110 DAA						
T1	46.8 a	93.8 ab	111.9 a	118.6 a	11 a	19 bc	19 bc	19 bc	19 bc	1.32 a	0.59 a	0.71 a	0.46 a	0.44 a	1.04 a			
T2	44.4 a	89.5 b	116.6 a	121.3 a	13 a	22 ab	22 ab	22 ab	22 ab	1.45 a	0.52 a	0.65 a	0.45 a	0.46 a	0.97 a			
T3	45.1 a	95.3 a	114.2 a	118.0 a	11 a	17 c	17 c	17 c	17 c	1.14 a	0.40 a	0.77 a	0.45 a	0.53 a	0.85 a			
T4	46.6 a	95.8 a	115.9 a	119.4 a	12 a	21 abc	21 abc	21 abc	21 abc	1.37 a	0.54 a	0.87 a	0.66 a	0.55 a	1.2 a			
T5	48.5 a	91.8 ab	118.8 a	126.7 a	13 a	24 a	24 a	24 a	24 a	1.07 a	0.48 a	0.93 a	0.56 a	0.54 a	1.04 a			

Note: Means comparison followed by the same letters are insignificant among types of molluscicides against residue level at $p \leq 0.05$, according to Tukey's. *Note that DAA is the day after the application. (T1= Saponin EC, 23 333.33 ppm, T2= Saponin WP, 23 333.33 ppm, T3= Niclosamide EC, 3 300.00 ppm, T4= Fentin acetate WP, 01 118.5 ppm, T5= Water)

Table 3

Average amount of residue, half-life, and degradation rate of paddy straw, polished, and unpolished grains from field assessment

Treatment	Residue in paddy straw (ppm)		Unpolished grain (ppm)	Polished grain (ppm)	Half-life (day)	Degradation rate constant (days ⁻¹)
	30 DAA	110 DAA	110 DAA	110 DAA		
T1	2109.7 a	48.0 b	72.7 a	18.0 bc	9	0.08
T2	1632.3 a	74.3 ab	90.0 a	26.7 ab	8	0.09
T3	1724.7 a	86.0 a	72.0 a	37.0 a	32	0.02
T4	1574.0 a	66.0 ab	71.3 a	21.0 ab	70	0.0099
T5	0 b	0 c	0 b	0 c	-	-

Note: Means comparison followed by the same letters are insignificant among types of molluscicides against residue level at $p \leq 0.05$, according to Tukey's. *Note that DAA is the day after the application. (T1= Saponin EC, 23 333.33 ppm, T2= Saponin WP, 23 333.33 ppm, T3= Niclosamide EC, 3 300.00 ppm, T4= Fentin acetate WP, 01 118.5 ppm, T5= Water)

in the Polish grain and exceeded the MRL permitted in food. The MRLs for niclosamide and fentin acetate in rice were 2 mg/kg and 5 mg/kg, respectively (China Food and Drug Administration, 2017). As for Malaysia, if there is no established national MRL, CODEX MRLs will be first adopted, followed by MRLs established by the Association of Southeast Asian Nations (ASEAN). If neither administration has established MRLs, a default MRL of 0.01 ppm will be applied. Hence, it can be concluded that all compound residues obtained from this study exceeded the MRL stated by the administration, which is not suitable for human consumption in the long term. However, even though saponin residues are high in the grains, it is considered safe to be consumed as saponin is rapidly dissipated and broadly found in many plants used for the daily diet and medicinal purposes for humans (Oleszek & Oleszek, 2020; Sharma et al., 2023).

Data from Table 4 demonstrated the soil sample's residue level, half-life, and degradation rate constants. There were significant differences between treatments at 7, 30 and 110 DAA. The soil treated with niclosamide (T3) had a high initial residue at 7 DAA (6,012.3 ppm). At 110 DAA, the residue value was high at 1,373.7 ppm, showing slower degradation. Additionally, the long half-life of 35 days and the low degradation rate constant (0.02) indicated a slower degradation compared to T1 and T2, suggesting the persistence of niclosamide in the soil. On the contrary, T4 (fentin acetate) had the highest residue (6,981 ppm at 7 DAA) but experienced a sharp decline from 110 DAA to 1,093 ppm. Despite the high initial residue, the relatively short half-life of 11.3 days and a high degradation rate constant (0.06) suggest a faster breakdown than other treatments.

Meanwhile, the residue values in water significantly differed between treatments at 30, 60 and 110 DAA, as shown in Table 5. As per result, saponin WP (T2) residue was slightly

Table 4
Average amount of residue, half-life, and degradation rate of soil sample from field assessment

Treatment	Soil residue (ppm)					Half-life (day)	Degradation rate constant (day ⁻¹)
	7 DAA	30 DAA	60 DAA	90 DAA	110 DAA		
T1	4 975.0 b	4 389.7 b	3 110.0 a	1 689.7 a	431.3 c	13	0.05
T2	5 413.0 ab	3 700.7 b	2 431.7 a	1 413.7 a	978.0 b	14	0.049
T3	6 012.3 ab	4 801.0 b	2 723.7 a	2 211.0 a	1 373.7 a	35	0.02
T4	6 981.0 a	6 596.7 a	3 310.7 a	2 134.3 a	1 093.0 ab	11	0.06
T5	0 c	0 c	0 b	0 b	0 d	-	-

Note: Means comparison followed by the same letters are insignificant among types of molluscicides against residue level at $p \leq 0.05$, according to Tukey's. *Note that DAA is the day after the application. (T1= Saponin EC, 23 333.33 ppm, T2= Saponin WP, 23 333.33 ppm, T3= Niclosamide EC, 3 300.00 ppm, T4= Fentin acetate WP, 01 118.5 ppm, T5= Water)

Table 5
Average amount of residue, half-life and degradation rate of water sample from field assessment

Treatment	Water residue (ppm)					Half-life (day)	Degradation rate constant (day ⁻¹)
	7 DAA	30 DAA	60 DAA	90 DAA	110 DAA		
T1	8 065.7 a	5 078.7 b	3 261.7 b	1 837.7 a	326.7 ab	19	0.035
T2	6 911.3 a	5 765.0 b	3 402.3 b	1 919.0 a	707.3 a	17	0.04
T3	7 688.7 a	6 051.7 ab	3 988.0 b	2 297.7 a	684.0 a	25	0.02
T4	8 140.7 a	7 369.0 a	5 667.7 a	3 214.0 a	459.3 ab	11	0.07
T5	0 b	0 c	0 c	0 b	0 b	-	-

Note: Means comparison followed by the same letters are insignificant among types of molluscicides against residue level at $p \leq 0.05$, according to Tukey's. *Note that DAA is the day after the application. (T1= Saponin EC, 23 333.33 ppm, T2= Saponin WP, 23 333.33 ppm, T3= Niclosamide EC, 3 300.00 ppm, T4= Fentin acetate WP, 01 118.5 ppm, T5= Water)

higher than T1 at 110 DAA, suggesting slower breakdown at later stages despite its faster rate in the early period. T3 (niclosamide) showed the slowest degradation, with 684 ppm remaining by 110 DAA. The long half-life of 25 days and a low degradation rate constant of 0.02 suggest a much slower degradation than other treatments. Meanwhile, T4 initially had the highest residue (8 140.7 ppm at 7 DAA) but showed rapid degradation, with 459.3 ppm remaining at 110 DAA. It exhibited the fastest degradation among other treatments, with the shortest half-life of 11 days and the highest degradation rate constant of 0.07.

As given in Tables 4 and 5, the residue in soil was relatively higher than the residue in water at 110 DAA. This condition proves the dissipation theory of chemical compounds from water to soil in most field studies regarding various crops, making the soil more acidic and infertile in the long term. Similar results were obtained in a study by Tang et al. (2018) in which pyrethroid (synthetic organic insecticide) residues were relatively higher in sediments than in surface water. As explained by Vryzas (2018), the dissipation of pesticides can be induced physically, chemically and biologically, including volatilisation, absorption by soil colloids or transported off-site through surface runoff and leaching. These processes may influence the quantity of each pesticide detected in soil, sediments, or water bodies. According to Zhang et al. (2012), pesticide residues in soil raise several potential harmful effects, such as adverse impact on subsequent crops and groundwater contamination. Pal et al. (2006) also stated that pesticide residues may affect non-target soil microorganisms, thus impairing the pesticide degradation process and eventually inducing its persistence in soil.

Biopesticides are biodegradable, which enables them to decompose rapidly and does not severely affect surface and ground water (Alliance, 2015). This statement is based on the findings from this field assessment, which found that saponin residues were lower than niclosamide residues in paddy straws, grains, soil, and water. In addition, Frank et al. (2002) suggested that the half-life of niclosamide should be 2 to 5 times longer in dry soil compared to those in moisture-maintained soil. This is probably the cause of the persistence of niclosamide in dry conditions, as there is a low risk of water leaching and runoff of materials. Likewise, the half-life of soil exposed to fentin acetate obtained from field assessment was 11 days, which is similar to the study done by Yen et al. (2001), who discovered a shorter half-life of fentin acetate of 8.3 to 19.4 days in clay loam soil. In contrast, Paton et al. (2006) assessed the half-life of fentin acetate to be 27 and 33 hours in non-sterile soil. In the past, as suggested by Loch et al. (1990), the half-life of fentin acetate ranged from 47 to 140 days, depending on the soil type. Hence, it is vital to monitor the dissipation of pesticides in soil and water due to their significant and severe impacts towards paddy growth, as they can hinder some key parameters that may impose a decline in grain yield afterwards. The mechanism behind the effect of saponin molluscicides towards apple snails using biochemical approaches must be further studied for better understanding and substantial evidence on the biochemical pathways of saponin intervention towards the internal system of apple snails.

CONCLUSION

All growth and residue parameters of rice exposed to different concentrations of saponin-based molluscicides were assessed. From the result obtained in this experiment, it can be concluded that niclosamide (T3) demonstrated the highest amount and most prolonged half-life of compound residues in paddy straw, polished grains, soil, and water. Both saponin formulations exhibited a higher dissipation rate as they lost 50% of their residue in less than 10 days, compared to niclosamide and fentin acetate, which need more time to dissipate by half. Therefore, saponin-based formulations are suitable as an alternative approach to control the apple snails and minimise the application of chemical molluscicides for rice cultivation in Malaysia. Additional research is recommended to refine saponin-based formulations further, optimising their concentrations, and application methods for different agricultural settings. This will help maximise pest control efficacy while reducing costs and labour inputs for farmers.

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Two New Species of *Pandanus* Subg. *Rykia* Sect. *Rykia* (Pandanaceae) from Sarawak, Borneo

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ABSTRACT

Two new *Pandanus* species are herein described: *Pandanus mansio* Haziah, Raffi, Shabdin & Meekiong and *P. sakek* Haziah, Raffi, Shabdin & Meekiong. These species were discovered in Sematan, Lundu, Kuching, Sarawak. *Pandanus mansio* closely resembles *Pandanus leuconotus* but differs by its taller stature, longer and narrower leaves with an unarmed mid-section of the leaf margin, pinkish-orange to green leaf base and ovoid cephalium with long peduncle. Meanwhile, *P. sakek* is morphologically similar to *P. kamiae* but distinguishable by its shorter, narrower and subcoriaceous leaves and smaller cephalium bearing longer styles, including falcate styles at apical drupes. With these additions, Sarawak now harbours five species of *Pandanus* subg. *Rykia* sect. *Rykia*.

Keywords: Biodiversity, Malaysia, pandan, *Pandanus mansio*, *Pandanus sakek*

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INTRODUCTION

Pandanaceae is a monocotyledon plant family widely distributed across the paleotropical region, comprising five main genera: *Pandanus*, *Freycinetia*, *Benstonea*, *Martellidendron* and *Sararanga* (Buerki et al., 2012). The family is distributed across Western Africa, Madagascar, Southeast Asia, the Pacific Islands and Australasia, with approximately 800 species recorded to date (Pandanaceae Project, 2020; POWO,

2025). Despite its diversity, the Pandanaceae of Borneo is currently represented by an outdated species inventory compiled by Stone (1970a, 1993), with no additional taxa recorded since then, except for the reclassification of several Bornean species from *Pandanus* to the newly described genus *Benstonea* (Buerki et al., 2016; Callmander et al., 2012) and the revisions of several others as synonyms (Callmander et al., 2020; Keim et al., 2011). Therefore, to improve understanding of this ecologically significant plant group, a series of field excursions have been conducted since 2022, focusing on documenting the species diversity of Pandanaceae in Western Sarawak. These efforts have led to the discovery of two morphologically distinctive taxa from previously recorded species in literature and herbarium collections. One of the species holotypes was collected from a domesticated specimen. It is well known that the ethnic communities in Sarawak have maintained a long-standing relationship with forests for their provisioning services. One of the practices involves the collection of various plant species for multiple purposes. In certain cases, such practices have led to the domestication of specific plant species (Jones et al., 2016). This is a case in point for the present study, whereby newly identified species were discovered from a domesticated species.

Both described species belong to *Pandanus* subg. *Rykia* sect. *Rykia* (De Vriese) Kurz (1867), based on its shared morphological characteristics such as leaves with unarmed apical ventral pleats and conspicuous tessellate reticulation, purplish-coppery leaf sheaths and spines, solitary or occasionally spicate cephalia with stout style which are generally forked stigmas (Nadaf & Zanan, 2012; Stone, 1983b, 1993). The *Pandanus* subg. *Rykia* section *Rykia* is known as one of the taxonomically complex groups within the genus *Pandanus*, due to its unresolved species boundaries and overlapping morphological traits among the species (Stone, 1970b; Stone, 1972). An earlier study has attempted to address these challenges through morphological, anatomical and phylogenetic analysis, aiming to clarify species relationships within the group (Rahayu et al., 2011). In addition, several species from this section have been revised and newly described (Callmander & Buerki, 2018; Nadaf et al., 2011; Zanan & Nadaf, 2012a, 2012b), yet no taxonomic updates from this section have been made for Sarawak within the Borneo region. To date, only three species from this section have been reported in Sarawak: *Pandanus albifrons* B. C. Stone (Teisher & Brazillian Flora Group, 2025), *Pandanus dictyotus* B. C. Stone (Stone, 1983c; Stone, 1993) and *Pandanus kamiae* B. C. Stone (Beentje & Callmander, 2023). The addition of these two newly described taxa increases the number of known species from subg. *Rykia* sect. *Rykia* to five, marking significant contributions to the diversity of Pandanaceae flora in Sarawak. In this study, the descriptions and taxonomic notes of these two new species are presented.

MATERIALS AND METHODS

Field sampling was conducted in Kuching Division, in which individuals bearing fruits (cephalia) were photographed and collected following the techniques outlined by Stone (1983a) and subsequently preserved as herbarium specimens based on the protocols listed by Bridson and Forman (1998). The specimens were deposited in the Herbarium Universiti Malaysia Sarawak. Species identifications were made by referring to *Pandanus* herbarium voucher specimens examined in the Sarawak Herbarium (SAR) and the Herbarium of Universiti of Malaya (KLU), as well as online databases such as Global Plants on JSTOR (<https://plants.jstor.org>), Global Biodiversity Information Facility GBIF (<https://www.gbif.org/>) and Tropicos Pandanaceae Project (<http://legacy.tropicos.org/Project/Pandanaceae>). Identification of the species was also aided by reference to manuscripts prepared by Beentje and Callmender (2023), Holttum and St. John (1962), and Stone (1983b; 1983c; 1993). Morphological comparisons were also conducted with closely resembling species, in which their accepted names were validated using Plants of the World Online databases (<https://powo.science.kew.org/>). Provisional conservation status of the new species were assessed by estimating the species extent of occurrence (EOO) and area of occupancy (AOO) using the Geospatial Conservation Assessment Tool, GeoCAT (<https://geocat.iucnredlist.org/>). The extinction risk was determined based on Criterion B and D by IUCN (2012) and guidelines provided by IUCN Standards and Petitions Committee (2024). Subsequently, taxonomic data including species distribution, habitats, ethnobotanical uses and taxonomic keys to *Pandanus* subg. *Rykia* sect. *Rykia* of Sarawak were presented.

RESULTS AND DISCUSSION

Taxonomic Treatment

Pandanus mansio Haziah, Raffi, Shabdin & Meekiong *sp. nov.* (Figure 1)

Type : MALAYSIA: Sarawak, Kuching, Lundu, Sematan, 83 m alt., 10 July 2023, *Haziah Musa et al. PH050* (holotype SAR; isotype Herbarium Universiti Malaysia Sarawak).

Diagnosis : *Pandanus mansio* can be distinguished from *P. leuconotus* by having taller shrub with longer and narrower leaves (vs. smaller shrub with shorter and wider leaves in *P. leuconotus*), unarmed margins in the mid-part of the leaf (vs. dentate throughout the leaf), pinkish orange to green (vs. conspicuously white) leaf basal and ovoid cephalia with longer peduncle (vs. oblong cephalia with short peduncle in *P. leuconotus*) (Table 1).

Description : Decumbent shrub pandan up to 4 meters tall. **Stems** are erect or prostrate and ascending with persistent old leaves and small, blunt thorns, and bear few short aerial roots. **Leaves** linear-oblongate, *ca.* 312–368 cm × 6.0–6.8 cm, pinkish orange to purple when young and green at maturity, tessellate-reticulate venations (conspicuous when dry), subcoriaceous; apex slightly cucullate, attenuate with cauda *ca.* 8–14 cm long; marginal spines near base to middle part with antrorse spines 2–5 mm long, 13–65 mm apart, purplish bronze, higher up completely unarmed, near apex spines less than 1 mm long, 2–5 mm apart, brownish pink; midrib spines near base with generally retrorse, single spines (occasionally antrorse, biforked or clustered with two or three spines), 5–10 mm long, irregularly spaced, 5–40 mm apart, higher up spines sparsely arranged, near apex with single spines less than 1 mm long, 3–28 mm apart, randomly disposed and denser towards distal end; leaf sheath amplexicaul, green, 6.5–8 cm × 10.5–14 cm wide at base. **Infructescence** terminal, solitary, erect on a straight, trigonous peduncle, pink in early stages of fruiting and gradually turns to green at maturity, 16–22 cm × *ca.* 2 cm diameter; peduncle with more than four leafy bracts, brown, deciduous, chartaceous with slightly cucullate leaf apex with no flagellum. **Cephalia** bearing more drupes, *ca.* 240 drupes in immature fruits, lesser drupes *ca.* 100 drupes in mature fruit; oblong in early stages of fruiting, which gradually turns to ovoid at maturity; *ca.* 10–13 cm × 7.5–8 cm, shorter and wider in mature fruit, pale orange to sage green. **Drupes** cuneate-oblong, 21–30 mm × 7.8–11 mm, 6 angled (rarely 5 angled); pileus 8–13 mm × 10–14 mm, *ca.* 2–5 mm deep; apical drupes convex with deeper pileus, lateral drupes truncate with hexagon pyramidal, pale orange to sage green. Style bony, 4–6.7 mm long, generally simple, occasionally biforked (but more frequently occurred in immature cephalia), bright orange to green; stigma 1.6–3.3 mm long, reddish brown. **Endocarp** ellipsoid, 4–7 mm × 2–4.5 mm; mesocarp apical and basal fibrous. **Male inflorescence** unknown.

Distribution and habitat : *Pandanus mansio* is only known from Sematan, Lundu, and Kuching, where it grows within the fragmented and degraded freshwater swamp forests in the lowlands.

- Provisional conservation assessment** : *Pandanus mansio* was only known from a single locality where its original habitat had been severely fragmented due to oil palm plantations. The species faces a continuing decline in habitat quality due to land conversion and habitat alteration, with fewer than 10 mature individuals recorded. Its extent of occurrence (EOO) was estimated to be less than 1 km² due to its small and restricted distribution, while its area of occupancy (AOO) was estimated at 4 km². Therefore, we provisionally assigned this species as Critically Endangered (CR) under IUCN criteria B1ab(iii,v)+B2ab(iii,v);D.
- Phenology** : Fruiting in July.
- Etymology** : The epithet '*mansio*' refers to the plant's local name, known by the Bidayuh Salako ethnic group as *sakek mansio*.
- Uses and vernacular name** : The leaves of *Pandanus mansio* were utilised for handicrafts purposes (Haziah et al., in prep) by the Bidayuh Salako people in Kampung Pueh and are known as *sakek mansio* by the locals.
- Notes** : The new species, *Pandanus mansio*, closely resembles *P. leuconotus*, an endemic species from Sabah, particularly during its juvenile stages. Juvenile individuals of *P. mansio* are morphologically nearly indistinguishable from mature plants of *P. leuconotus*, sharing several traits, including a purplish tinge near the leaf bases, cucullate and abruptly cuspidate leaf apices with either absent (Figure 1B) or short flagella measuring up to 5 cm. However, even at early development stages, *P. mansio* displayed two distinguishing features that remained consistent throughout its development stages, such as the absence of spines at the mid-section of the leaf margin, similar to *P. dictyotus* (Stone, 1983c) and the lack of conspicuous white leaf bases, which was a distinguishing feature present in *P. leuconotus* (reminiscent of *P. albifrons*). Although it could be argued that the leaf bases' colouration (absence or presence of white leaf bases in *Pandanus*) may vary due to environmental factors (Menzies et al., 2016), *ex situ* observations of *P. mansio* over a one-year period showed consistent pinkish colouration (Figure 2), despite being transplanted into different environmental conditions. Furthermore, herbarium specimens of *P. leuconotus* consistently exhibited striking white leaf bases, which further validated that these are two distinct taxa.

Pandanus mansio diverged more from *P. leuconotus* as it matured, forming a taller shrub reaching up to 4 meters in height, compared to the shorter *P. leuconotus*, which grew up to 2.5 meters tall. Leaf morphology also differed between the two taxa, with *P. mansio* bearing exceptionally longer and narrower leaves compared to *P. leuconotus*, as well as more prominent spines at the basal midrib, measuring up to 10 mm, nearly double the length observed in *P. leuconotus*. As for the cephalia, both *P. mansio* and *P. leuconotus* are morphologically similar, with nearly identical drupes dimension, style length and style shapes (having generally simple styles and rarely biforked styles). However, *P. mansio* can be readily distinguished by its longer peduncle, reaching up to 22 cm, in comparison to the approximately 9 cm long peduncle in *P. leuconotus*. Aside from that, *P. mansio* possessed ovoid cephalia, in contrast to the oblong cephalium of *P. leuconotus*.

It is important to note that while immature cephalia of *P. mansio* exhibited an oblong shape similar to the mature cephalia of *P. leuconotus*, they differed in style behaviour. In *P. mansio*, biforked styles occurred more frequently in immature cephalia compared to mature ones, though simple styles have a relatively higher proportion overall. This pattern suggests that style behaviour may vary interspecifically, thus might pose a degree of difficulty in species identification, especially for the taxonomically challenging group, such as the subgenus (subg.) *Rykia* sect. *Rykia*. Aside from the difference in ratio of simple to biforked styles, the variation in cephalia dimensions and drupe number between different development stages was also important to take into account. The current study corroborates Stone (1970b), who noted that the dimensions of cephalia tend to decrease as it matures. However, while Stone assumed that the number of drupes remains constant across development stages, field observations from this study suggested that mature cephalia could bear fewer drupes, approximately 100 to 150 less than the immature cephalia, offering significant taxonomic insights in species delimitation, especially in the subg. *Rykia* sect. *Rykia*.

It is also noteworthy that *P. mansio* displayed unusual spine morphology and arrangement compared to other *Pandanus* species, characterised by the occasional presence of antrorse, biforked spines (Figure 1I) or clusters of two to three spines (Figure 1H) along the basal midrib of the leaves. These structures were consistently observed in both fertile individuals (with mature and immature cephalia) of *P. mansio*, and more prominently occurred on the nearest leaf to the infructescence. Although such traits may not be visible in sterile individuals, their presence may offer additional diagnostic value in distinguishing *P. mansio* from other members of *Pandanus* subg. *Rykia* sect. *Rykia*. A detailed comparison between *P. mansio* and *P. leuconotus* is provided in Table 1.

Additional : MALAYSIA: Sarawak, Kuching, Lundu, Sematan, 83 m alt., 10 July 2023,
specimens Haziha Musa et al. PH051 (Herbarium Universiti Malaysia Sarawak!);
examined PH052 (Herbarium Universiti Malaysia Sarawak!); ***P. leuconotus* (Type):**

MALAYSIA: Sabah state, Kota Kinabalu, Poring, base of Mt. Kinabalu, near the Hotsprings, 31 March 1977, *B. C. Stone 12906* (holotype KLU!; isotypes PH: barcodes PH00018284; BISH: barcodes BISH1011549, barcodes BISH1011548; L: barcodes L 0050564); *P. cf. leuconotus*: MALAYSIA: Sabah state, Sandakan-Telupid Rd, 83rd mile, Base of Bukit Tangkunan, swampy forest, 100 m alt., 31 March 1977, *B. C. Stone 12897* (KLU!).

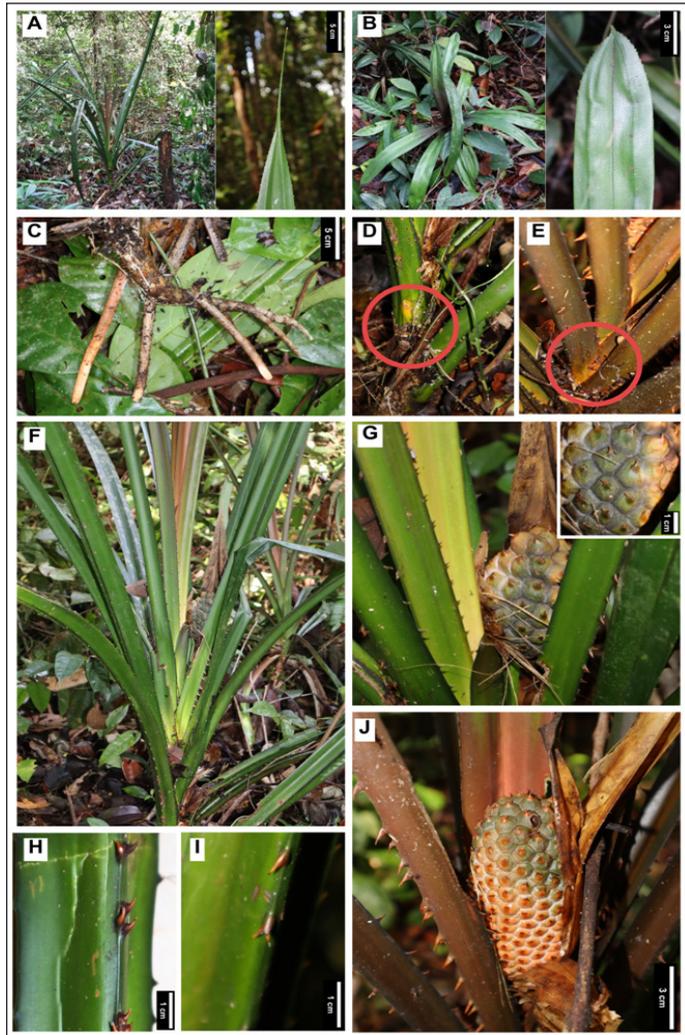


Figure 1. Pandanus mansio Haziah, Raffi, Shabdin & Meekiong, *sp. nov.* **A.** Growth form of the mature plant with a close-up at the leaf apex. **B.** Habit of a young sterile plant with a close-up leaf apex. **C.** Slender stem bearing a few short prop roots. **D.** Green leaf sheath of a mature plant (as shown in the circle). **E.** Pinkish orange leaf sheath of the plant at early reproductive stages (as shown in the circle). **F.** Inflorescence of mature plant. **G.** Cephalium of a mature plant showing ovoid-shaped cephalia with close-up style and stigma (inset). **H.** Abaxial midrib basal spines clustered in groups of two to three. **I.** Biforked spine at the midrib basal of mature leaves. **J.** Cephalium at the early stages of fruiting, showing oblong cephalia



Figure 2. *Ex situ* conservation of *Pandanus mansio* (PH052b) showing consistent colouration of the leaf bases, matching those from its original locality, one year after transplantation at the UNIMAS Arboretum

Table 1

Comparison between *Pandanus mansio* Haziah, Raffi, Shabdin & Meekiong, sp. nov. and *Pandanus leuconotus* B. C. Stone. Note: *Pandanus leuconotus*' descriptions were based on Stone (1983b; 1983c; 1993) and specimens examined as listed

Characteristics	<i>P. mansio</i>	<i>P. leuconotus</i>
Leaves		
Shape	Linear–oblanceolate, leaf erect or upright	Broadly loriform, leaf arcuate or curved
Dimension	312–368 cm × 6–6.8 cm	120 cm × 9.5 cm
Length of cauda	8–14 cm	Approximately 7.5 cm
Marginal spines	Present at apical and basal, with the middle part completely unarmed	The entire margin dentate throughout
Midrib basal spines	Generally retrorse and single spines, occasionally antrorse, biforked or clustered of two to three spines, which is prominent in mature fertile plant, 5–10 mm long.	Retrorse, single spines throughout, 4–5.8 mm long.
Colour of leaf sheath	Pinkish orange (juvenile) to green (mature)	Conspicuously glaucous white
Infructescence		
Peduncle length	16 to 22 cm	Approximately 9 cm
Cephalia		
Shape	Ovoid	Oblong
Dimension	10–13 cm × 7.5–8 cm	10–12.5 cm × 6–7 cm
Styles		
Styles shape	Generally simple, occasionally biforked (but more frequently occurred in immature cephalia)	Generally simple, rarely biforked

Pandanus sakek Haziah, Raffi, Shabdin & Meekiong *sp. nov.* (Figure 3)

Type : Malaysia, Sarawak, Kuching, Lundu, Sematan, domesticated, 41.15 m alt., 24 October 2022, *Haziah Musa et al. PH014* (holotype SAR; isotype Herbarium Universiti Malaysia Sarawak).

Diagnosis : *Pandanus sakek* is morphologically similar to *P. kamiae* but can be distinguished by its shorter and narrower leaves (vs. longer and wider leaves in *P. kamiae*), subcoriaceous leaf (vs. coriaceous and stiff leaf), smaller cephalia with longer styles (vs. longer cephalia with shorter styles), which bear predominantly biforked styles (vs. mostly simple styles) and falcate style at apical drupes, which curved away from the axis (vs. deflected towards the distal of cephalia) (Table 2).

Description : Acaulescent shrub pandan up to 4 meters tall. **Stems** are erect, short and unbranched. **Leaves** lanceolate, 254–270 cm × 8 cm, green, slightly waxy, tessellate–reticulate venations (conspicuously when dry), subcoriaceous, green; apex attenuate with cauda *ca.* 9–14 cm long; marginal spines near base to middle part with antrorse spines 4–5 mm long, 13–20 mm apart, green with orangish tip, higher up with retrorse spines, 1 mm long, 4 mm apart, white; midrib spines near base with retrorse spines, 3–5 mm long, 10–45 mm apart, orangish brown, higher up with antrorse spines, less than 1 mm long, 5 mm apart, white; leaf sheath amplexicaul, orangish brown, 7 cm × 19 cm wide at base. **Infructescence** terminal, solitary, erect on a straight, trigonous peduncle, green, *ca.* 35 cm long × 2 cm diameter; peduncle with more than six leafy bracts enclosing the cephalia, lanceolate to ovoid; inner bracts brown and caducous; outer bracts yellow to green, persistent. **Cephalia** bearing *ca.* 600 drupes, oblong, 19 × 8.8 cm, green. **Drupes** cuneate–oblong, 28–31 mm × 8.8 mm–10 mm, apical drupes longer and wider than lateral, 6–angled (rarely 5–angled); pileus *ca.* 9 × 12 mm, 3.3–8.6 mm deep; apical drupes convex with deeper pileus, lateral drupes truncate with hexagon pyramidal to slightly hemispherical pyramidal, green. **Style** *ca.* 5.5–7.0 mm long, bony, shining, biforked, horn–like style, rarely a simple style except for one–third from the distal part of the cephalia covered with simple, falcate style curving outwards from the axis, green; stigma *ca.* 3–4 mm long, brown. **Endocarp** ellipsoid, 8 × 4.8 mm; mesocarp apical fibrous and fleshy; basal mesocarp highly fibrous. **Male inflorescence** unknown.

- Distribution and habitat*** : This species is a domesticated species and can be commonly found within the settlement area of the Bidayuh Salako community in Kampung Pueh.
- Provisional conservation assessment*** : The populations of the domesticated *Pandanus sakek* were found within the settlement area in Sematan. Although its wild population have not yet been determined, the local informants reported that the species is believed to have originated from the vicinity area of Sematan (R. Panchar, personal communication, June 16, 2025). Therefore, until a clearer understanding of the species' wild population is obtained, it is appropriate to provisionally assign *P. sakek* under the Data Deficient (DD) category.
- Phenology*** : The fruiting occurs in October.
- Etymology*** : The epithet *sakek* is derived from its vernacular name, which generally refers to pandan species in the Bidayuh Salako language.
- Uses and vernacular name*** : *Pandanus sakek*, commonly known by the names *sakek*, *sakek benang* and *sakek tanam*, has been utilised for different purposes such as handicrafts and medicinal uses by the Bidayuh Salako ethnic in Kampung Pueh (Haziah et al., in prep).
- Notes*** : *Pandanus sakek* is morphologically similar to *Pandanus kamiae*, sharing an acaulescent shrub habit, similar leaf sheath colour and solitary oblong cephalia. However, it can be distinguished by several traits such as its shorter, narrower lanceolate leaves (vs. longer, wider linear leaves in *P. kamiae*) and subcoriaceous leaf (vs. coriaceous and stiff leaf in *P. kamiae*). Its cephalia are relatively smaller but bear longer styles and lack the flattened, depressed base characteristics present in *P. kamiae*. The style shapes further differentiate the two species, in which *P. sakek* styles are predominantly biforked, whereas *P. kamiae* styles are mostly simple (or subulate) as described in Stone (1970b). A distinctive feature of *P. sakek* is the presence of falcate simple styles at the apical drupes, which curve away from the axis, in contrast to deflecting simple styles oriented distally in *P. kamiae* or most of *Pandanus* subg. *Rykia* sect. *Rykia*. This trait remained consistent and was observed in immature cephalia (not collected) of *P. sakek* (Figure 3G), highlighting its diagnostic characteristics.

Additional specimens examined : MALAYSIA: Sarawak, Kuching, Lundu, Sematan, Kampung Pueh, 18 m alt., 2 March 2022, *Haziah Musa et al. PH004* (Herbarium Universiti Malaysia Sarawak!); *P. kamiae*: MALAYSIA: Sarawak, Samarahan, Simunjan, Sabal Forest Reserve, 60 m alt., 23 February 2022, *Haziah et al. PH002* (SAR!); Kuching, Lundu, Sematan, 39.2 m alt., 29 February 2024, *Haziah et al. PH070* (Herbarium of Universiti Malaysia Sarawak); Sarawak, Bako National Park, Jalan Telok Paku, less than 15 m alt., 18 January 1977, *B. C. Stone 13485* (KLU!); Johore, Johore-Timor-Jemaluang Forest Reserve, Cpr. 17, in logged forests, under ca. 61 m alt., 8 July 1968, *Y. K. KAM KLU-9521* (holotype PH: barcodes PH00018277, PH00018276; isotype BISH: barcodes BISH1011516, BISH1011517); Johore, Mersing to Kluang Road Mile 29, 50 m alt., August 1982, *B. C. Stone 15306* (KLU!); Kuala Lumpur, University of Malaya, Rimba Ilmu, 30 March 1982, *B. C. Stone 15131* (KLU!).

Table 2

Comparison between Pandanus sakek Haziah, Raffi, Shabdin & Meekiong, sp. nov. and Pandanus kamiae B. C. Stone. Note: Pandanus kamiae's descriptions were based on Stone (1970b; 1983b), Beentje and Callmander (2023) and specimens examined as listed

Characteristics	<i>P. sakek</i>	<i>P. kamiae</i>
Leaves		
Shape	Lanceolate	Linear
Dimension	254–270 cm × 8 cm	400–800 cm × 6–10 cm
Leaf texture	Subcoriaceous, not stiff	Coriaceous, stiff
Cephalia		
Dimension	19 cm × 8.8 cm	23–27 cm × 9–13 cm
Style		
Length	ca. 5.5–7.0 mm long	2–6 mm
Shape (lateral drupe)	Flattened depressed base absent, simple or biforked styles, mostly biforked	Flattened depressed base present, simple or biforked styles, mostly subulate
Shape (apical drupe)	Simple style, falcate curving away from the axis	Simple style, deflected towards the distal

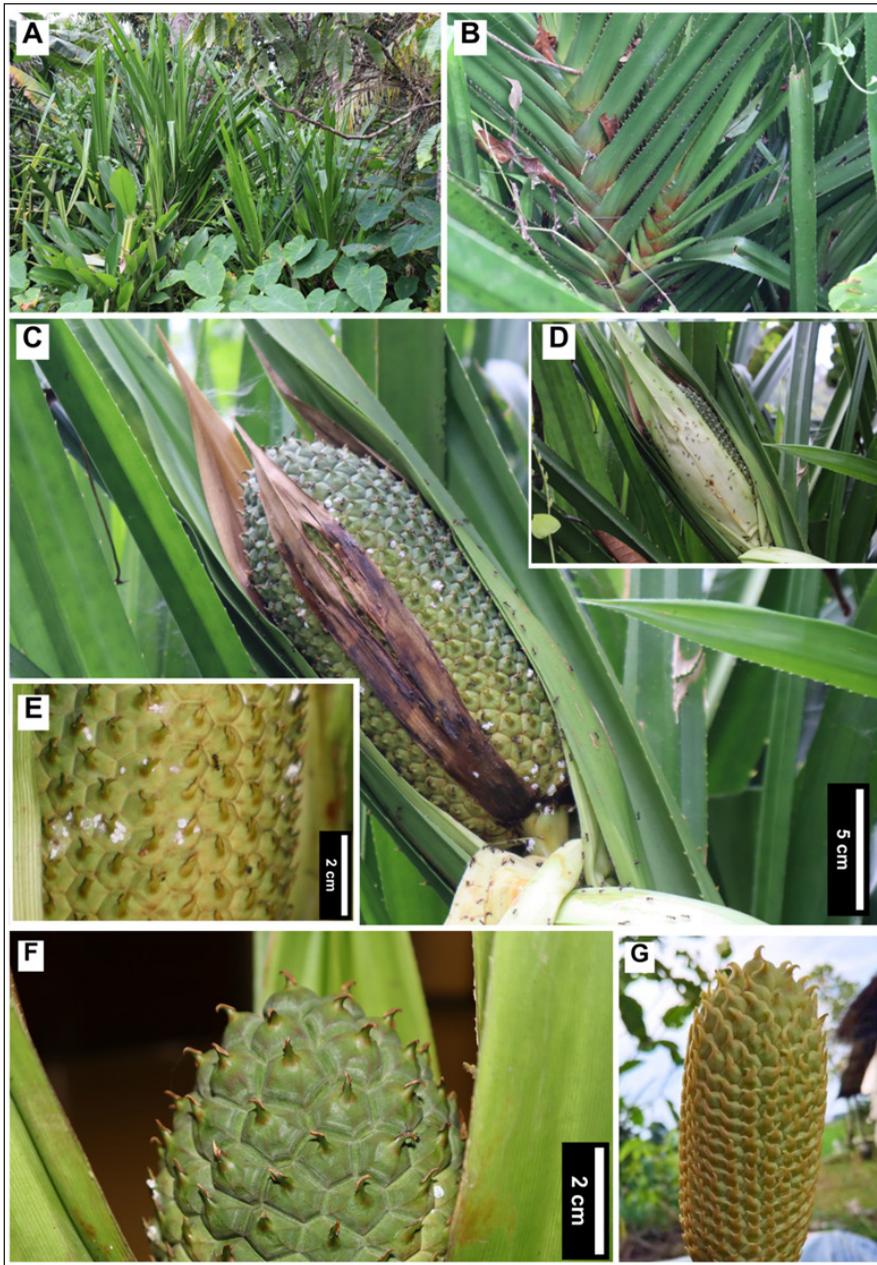


Figure 3. *Pandanus sakek* Haziah, Raffi, Shabdin & Meekiong, *sp. nov.* A. Habit. B. Orangish brown leaf sheath. C. Ellipsoid oblong cephalia. D. Cephalia covered with leafy bracts. E. Close-up of lateral drupes showing style and stigma of *P. sakek*. F. Close-up of apical drupes showing falcate style pointing away from the axis. G. Falcate style at the apical drupes of immature cephalium. Note: Image 3G is credited to Rita Panchar

Key to *Pandanus* (Subg. *Rykia* Sect. *Rykia*) from Sarawak, Borneo

- 1a. Acaulescent shrub with the absence of prop roots..... 2
- 1b. Stemmed shrub with the presence of a few short prop roots..... 3
- 2a. Subcoriaceous and less stiff leaves measuring up to 270 cm long, cephalia with falcate apical drupes deflected away from the axis..... *Pandanus sakek*
- 2b. Coriaceous and stiff leaves measuring up to 800 cm long, cephalia with apical drupes deflected towards distal.....*P. kamiae*
- 3a. Leaves with conspicuously white leaf sheath and marginal spines dentate throughout the leaf.....*P. albifrons*
- 3b. Leaves with no white leaf sheath and marginal spines only present at the apical and basal parts of the leaf, the middle part unarmed.....4
- 4a. Ovoid cephalia, 10–13 cm long, 7.5–8 cm wide, with 21–30 mm long, 7.5–8 cm wide drupes.....*P. mansio*
- 4b. Sub-ellipsoid cephalia, 7 cm long, 4.5 wide, with 16–21 mm long, 5–10 cm wide drupes..... *P. dictyotus*

CONCLUSION

This study formally describes two new species from *Pandanus* subg. *Rykia* sect. *Rykia*, *Pandanus mansio* and *Pandanus sakek* further enrich the documented diversity of Pandanaceae in Sarawak. These findings acknowledged the taxonomic complexity within subg. *Rykia* sect. *Rykia*, particularly the slight morphological variations in leaves, cephalia and styles shapes across different developmental stages, offers new insights into species delimitation of this poorly understood group. Beyond taxonomy, the discoveries also carry important conservation implications in which both species were found outside protected areas, highlighting the urgency of further field surveys to clarify their distribution, ecological preferences and conservation status. With the addition of *P. mansio* and *P. sakek*, the total number of *Pandanus* species recorded in Sarawak has now increased to 15 species in total, reflecting the region’s rich species diversity and reinforcing the need for continued botanical exploration, particularly in unexplored regions of Borneo Island.

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Possibilities of Applications and the Interest in Adopting Biochar as a Renewable Source of Nutrients to Increase Crop Productivity and Improve Soil Fertility of Small-scale Farms in Narathiwat Province, Thailand

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ABSTRACT

This study assessed the potential application of biochar as a renewable nutrient source to enhance soil fertility and crop productivity in small-scale farming systems in Narathiwat Province, Thailand. A field experiment using five treatments—control (no amendment), rice husk biochar, rubber wood biochar, oil palm bunch biochar, and raw organic residues—was conducted on baby corn (*Zea mays* L. var. *saccharata*) grown in sandy soil. Oil palm bunch biochar significantly improved plant height, biomass, and yield compared to the other treatments, while also increasing soil organic matter and nutrient availability. A complementary survey of 30 local farmers revealed that 30% had already adopted biochar, and 43.33% expressed interest in its future use. Among the interested group, 73% believed adoption could increase if simpler application methods and more accessible equipment were developed. These findings demonstrate the promise of oil palm bunch biochar as an effective and sustainable soil amendment, particularly for improving sandy soil conditions in smallholder farming systems.

Keywords: Baby corn, biochar, farmer adoption, sandy soil, soil fertility

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INTRODUCTION

Thailand is one of the countries rich in natural resources, and agriculture is a key sector that supports the economy. Agricultural production from Thailand is not only for domestic consumption but also contributes significantly to global food production. Thailand ranks as the second-largest exporter of rice in the global market.

Moreover, the Thai government has supported innovation and new technologies to enhance agricultural productivity. However, increased agricultural activity has also led to the rapid accumulation of agricultural residues (Koul et al., 2022). In 2023, Thailand generated substantial waste from three major economic crops: rice, para rubber, and oil palm. Rice production amounted to 25.80 million tons, resulting in 5.16 million tons of rice husks. Para rubber, a key export commodity, contributes to approximately 500,000 rai of replanting annually due to declining latex yields in trees over 25 years old, producing around 8 million tons of rubber sawdust. Additionally, oil palm production generated approximately 80,000 tons of oil palm bunches annually. If not managed properly, this accumulation of crop waste poses serious threats to ecosystems and contributes to climate change (Boluda-Verdú et al., 2022). To address these challenges, research shows that agricultural waste can be converted into biochar (Lin et al., 2021; Rathore et al., 2021; Rathod et al., 2023; Van Nguyen et al., 2022; Wang & Wang, 2019a). Biochar, a carbon-rich material produced via pyrolysis of organic residues such as rice husks, sawdust, and oil palm bunches at temperatures above 300°C in the absence of oxygen, can be used to improve soil fertility and environmental health (Panwar et al., 2019; Patel et al., 2021).

Applying biochar to soil has been shown to improve soil structure, fertility, and water-holding capacity, and to boost crop yields with minimal environmental impact (Dai et al., 2020; Hien et al., 2021; Mylavarapu et al., 2013; Neogi et al., 2022; Piash et al., 2023; Siedt et al., 2021; Verheijen et al., 2019; Wang et al., 2022). This potential is especially valuable in Narathiwat Province, where many farmers operate on sandy soils with low fertility. According to the Land Development Department (2024), about 22,135 rai of land in the province is affected by poor soil conditions, exacerbated by long-term chemical fertiliser use. Biochar, being rich in stable carbon, can remain in the soil for extended periods, offering long-term benefits (Agegnehu et al., 2016; Das et al., 2020; Li et al., 2017; Majumder et al., 2019; Qian et al., 2020; Samoraj et al., 2022). Furthermore, crop residues from para rubber, oil palm, and rice, which are abundantly available in the region, can be utilised for biochar production, reducing waste and reliance on chemical fertilisers. Although many studies have reported the agricultural benefits of biochar (Jiang et al., 2021; Maroušek & Trakal, 2022; Mohammadi et al., 2017; Neogi et al., 2022; Rombel et al., 2022; Vochozka et al., 2016; Wang et al., 2022), few have explored its adoption by farmers. This research thus aims to fill that gap by evaluating the effects of different types of biochar on crop performance and by analysing farmers' awareness, interest, and adoption of biochar technology.

This study focuses on baby corn (*Zea mays* L. var. *saccharata*), a crop with a short growth cycle, economic importance for small-scale farmers, and high sensitivity to soil fertility, making it an ideal test crop. The research objectives are to measure the effects of various biochar types on baby corn growth and yield in sandy soil, and to assess farmer

acceptance of biochar use. The findings are expected to provide insights that support sustainable agricultural practices, economic viability, and environmental protection for small-scale farmers in Narathiwat Province.

MATERIALS AND METHODS

Comparison of Different Waste of Biochar on Plant Growth, Yield, and Soil Fertility

The purpose of this experiment was to find the best waste residue that increases the growth and yield of baby corn.

Experimental Design and Treatments

This study used a completely randomised design (CRD). Seven treatments and four replications were examined in this experiment.

- Treatment 1: Control (sandy soil)
- Treatment 2: Rice hull biochar
- Treatment 3: Rubber sawdust biochar
- Treatment 4: Oil palm bunch biochar
- Treatment 5: Rice hull
- Treatment 6: Rubber sawdust
- Treatment 7: Oil palm bunch

Treatment 2-7 was prepared using sandy soil well mixed with different materials at a ratio of 1:1 (by weight).

Baby corn (*Zea mays* L. var. *saccharata*) seeds were obtained from the Department of Agriculture, Thailand. Seeds were germinated in nursery trays and transplanted after 10 days. Baby corn seedlings were planted in each plot in triplicate. After 1 week, only one seedling was planted in each pot and watered at field capacity. Fertiliser was applied as a compound Fertiliser N:P₂O₅: K₂O, 15:15:15, 50 kg/rai before planting, and 46-0-0, 50 kg/rai, 25 d after planting.

The biochar used in this experiment was prepared by pyrolysis of rice hulls, rubber sawdust, and oil palm bunches at 450°C for 3 hours in a limited oxygen environment, following standard biochar preparation protocols.

Data Collection

The measured plant growth indexes and yield included:

Plant Height - The plant height was measured regularly every 1 week from ground level to the highest part of the leaf.

Number of Leaves - The number of leaves was counted manually each week during the vegetative growth period until 55 days.

Stem Diameter was measured 5 cm above the soil surface using a digital vernier calliper.

Stem Fresh-Dry Weight - At the end of the experiment (55 days), aboveground biomass was collected and weighed. The stem was then oven-dried at 80°C for 72 h, and the dry weight was recorded.

Root Length - At 55 days, the longest root from each plant was measured from the base to the tip.

Yield Weight - Yield per corn was recorded using an electronic balance, measured both before and after husking.

Soil Collection and Analysis

Soil used was the Ban Thon soil series: Bh (sandy, siliceous, superactive, ortstein, isohyperthermic, Typic Haplorthods) collected from Mueang District, Narathiwat, at 0–15 cm depth. Samples were air-dried and sieved through a 2 mm sieve. Chemical analysis included pH (pH meter), organic matter (Walkley and Black), total N (Kjeldahl), available P (Bray II), and available K (NH₄OAc, pH 7.0) following Sparks et al. (2020).

Statistical Analysis

The data were analysed using SPSS software. One-way ANOVA and Duncan's Multiple Range Test (DMRT) were used to determine significance at $p < 0.05$. For descriptive survey data, frequency and Pearson's correlation were used.

The Adoption and Barriers of using Biochar by Farmers

Data Collection and Analysis

A field survey of 30 farmers in Narathiwat Province was conducted to assess biochar adoption. A standardised questionnaire with 13 items across four dimensions—economic, social, environmental, and barriers to adoption—was administered using a Likert scale (3 = very important; 2 = important; 1 = unimportant).

Descriptive statistics such as frequency and percentage were used, and Pearson correlation analysis was performed to explore associations.

Guideline for the Utilisation of Biochar in Sandy Soil

Data Collection and Analysis

Content analysis was used to interpret farmer feedback regarding biochar adoption and to identify practical guidelines and challenges.

RESULTS AND DISCUSSION

Effect of Biochar on Plant Growth, Yield, and Soil Fertility

Plant Growth

On average, the tallest plant (144.56 cm) was observed in the oil palm bunch, but not in the rice hull biochar and oil palm bunch biochar. The shortest plants (38.22 cm) were observed in the control treatment. The number of leaves per plant was higher in response to oil palm bunch biochar (12.66 leaves), but the minimum average number was observed in the control treatment (8.44 leaves). The highest average stem diameter was observed for the oil palm bunch biochar (2.22 mm), but the lowest average number was observed in the control treatment (0.99 mm). The maximum mean stem fresh and dry weights were obtained with palm bunch biochar (8.43 and 2.20 g), but the minimum average number was obtained with the control treatment (0.27 and 0.10 g). The longest average root length was observed for rice hull biochar (59.17 cm), but the shortest average number was observed in the control treatment (28.78 cm). The effect of biochar on plant growth presented in Table 1 showed that the treatment that was applied with raw waste material

Table 1
The effects of the biochar on the plant growth

Treatment	Height (cm)	Leaf no. (leaves/pot)	Stem diameter (mm)	Stem fresh weight (g)	Stem dry weight (g)	Root length (cm)
Control	38.22 ± 1.52 ^d	8.44 ± 0.32 ^d	0.99 ± 0.03 ^c	0.27 ± 0.04 ^d	0.10 ± 0.03 ^c	28.78 ± 1.20 ^c
Rice hull biochar	135.99 ± 5.29 ^a	11.98 ± 0.46 ^b	2.01 ± 0.10 ^b	5.57 ± 0.80 ^c	1.27 ± 0.09 ^b	59.17 ± 2.50 ^a
Rubber sawdust biochar	78.89 ± 3.41 ^b	10.22 ± 0.37 ^c	1.45 ± 0.09 ^c	2.13 ± 0.33 ^d	0.27 ± 0.05 ^c	33.62 ± 1.70 ^c
Oil palm bunch biochar	132.89 ± 4.93 ^a	12.66 ± 0.52 ^a	2.22 ± 0.15 ^a	8.43 ± 0.90 ^a	2.20 ± 0.15 ^a	54.89 ± 2.20 ^b
Rice hull	61.99 ± 2.78 ^c	10.11 ± 0.40 ^c	1.49 ± 0.09 ^c	1.73 ± 0.30 ^d	0.40 ± 0.06 ^c	48.50 ± 1.90 ^b
Rubber sawdust	54.99 ± 2.22 ^c	9.44 ± 0.36 ^c	1.22 ± 0.07 ^d	1.07 ± 0.22 ^d	0.13 ± 0.03 ^c	31.12 ± 1.39 ^c
Oil palm bunch	144.56 ± 5.63 ^a	11.55 ± 0.45 ^b	2.22 ± 0.12 ^a	6.93 ± 0.85 ^b	2.13 ± 0.11 ^a	46.69 ± 2.11 ^b
C.V. (%)	8.91	4.38	4.55	19.65	25.23	11.95
F-test	**	**	**	**	**	**

Note. Numbers followed by the same letter in the same column are not significantly different at the 99% level (DMRT). F-test: ** = significant at $p < 0.01$

and biochar (Treatment 2-7) as an amendment significantly increased plant height, number of leaves, stem diameter, stem fresh and dry weight, and root length when compared with the control. This indicates that the application of biochar improves plant growth (Table 1).

Plant Yield

Table 2 shows the effect of biochar on yield. The individual weight was significantly higher in oil palm bunch biochar, followed by rice hull biochar and oil palm bunch. The types of organic wastes of these plants, rice hulls, and rubber sawdust, can increase plant growth, but this growth does not develop in plant yield. The control, rice hull, and rubber sawdust treatments produced no yield.

The use of biochar enhanced growth parameters and resulted in the highest plant yield. Analysis of average baby corn yield before and after peeling. The best crop performance in terms of yield was observed among the treatments of all biochar treatments that enhanced growth and yield, whereas the lowest yield was recorded with the control. However, all raw wastes, namely rice hull and rubber sawdust, had no effect on yield. However, all of these treatments were significantly higher than that of the control. Biochar amendment significantly increased crop growth, productivity, and soil nutrition. (Abiven et al., 2015; Lychuk et al., 2015; Manirakiza & Seker, 2020). Moreover, Meyer et al. (2021) investigated the effects of biochar and found that it enhanced the soil organic content, soil water storage capacity, and nitrogen content, while also exceeding the average yield of 7%. Applying biochar made from forest biomass to beet fields in Labrador, Canada, improved yields from 2.9 to 11.4 mg ha^{-1} , resulting in an average annualised net return over variable costs of \$4953 ha^{-1} , four times the average return on potato production of \$965.48 ha^{-1} (Keske et

Table 2
The effects of the biochar on the plant yield

Treatment	Yield before peel (g/corn)	Yield after peel (g/corn)
Control	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d
Rice hull biochar	5.50 ± 0.66 ^b	1.50 ± 0.24 ^b
Rubber sawdust biochar	1.20 ± 0.29 ^c	0.89 ± 0.21 ^c
Oil palm bunch biochar	7.90 ± 0.82 ^a	2.30 ± 0.36 ^a
Rice hull	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d
Rubber sawdust	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d
Oil palm bunch	4.50 ± 0.56 ^b	1.30 ± 0.22 ^b
C.V. (%)	39.92	33.01
F-test	**	**

Note. Numbers followed by the same letter in the same column are not significantly different at the 99% level (DMRT). F-test: ** = significant at $p < 0.01$

al., 2020). It has been demonstrated that biochar offers agricultural ecosystems a number of advantages, including increased yields (Katterer et al., 2016).

Soil Fertility

The initial soil values are listed in Table 3. The pH of the soil ranged from 5.0 to 5.9 among different biochars. pH of all treatments was in a range of 5.0-6.8 for tropical soil (Udo & Ogunwale, 1977). The resulting electrical conductivity content in the soil was in the range of 0.02-0.04 mScm⁻¹ and remained below the limit of the EC value for agricultural soil, 4 dSm⁻¹ (Christiansen, 1977). Organic matter content ranged from 2.11 to 2.65%, showing that it was optimum at the critical level between 1.5 and 3.5% for soils in the tropics. The control group had the lowest score (1.16%). Total N varied between 0.08-0.16%, showing that it was lower than the critical level between 0.3 and 0.6% for soils in the tropics (Bremner & Mulvancy, 1982). Available P content extended between 22.0 and 138.0 mg kg⁻¹, showing that it was greater than the critical level between 10 and 15 mg/kg (Adeoye & Agboola, 1985). The available K content ranged from 7.0 to 30.0% and was lower than the critical level of 60-90 mg kg⁻¹ (Adeoye, 1986).

Application of biochar increased soil pH, OM, N, and P values compared to the other treatments. This indicates that OM and P are not required for optimal crop production. However, soil requires the addition of nitrogen for optimal crop production. Direct fertilisation impacts and an increase in soil nutrient stocks, particularly phosphorus (P), have been demonstrated for biochar (Biederman & Harpole, 2013; Gul & Whalen, 2016; Shepherd et al., 2017; Zhang et al., 2017).

Table 3
Soil analysis before planting

Treatment	pH	EC (mScm⁻¹)	OM (%)	Total N (%)	Avail. P (mg kg⁻¹)	Avail. K (mg kg⁻¹)
Control	5.1 ^d	0.03	1.67 ^d	0.08 ^c	22.00 ^f	17.00 ^b
Rice hull biochar	5.4 ^e	0.03	2.11 ^c	0.11 ^{abc}	91.00 ^d	30.00 ^a
Rubber sawdust biochar	5.1 ^d	0.02	2.11 ^c	0.11 ^{abc}	29.00 ^e	8.00 ^{dc}
Oil palm bunch biochar	6.4 ^a	0.04	3.36 ^a	0.17 ^a	138.00 ^b	9.00 ^d
Rice hull	5.5 ^b	0.03	2.06 ^c	0.10 ^{abc}	99.00 ^c	7.00 ^e
Rubber sawdust	5.3 ^e	0.02	3.29 ^a	0.16 ^{ab}	22.00 ^f	9.00 ^d
Oil palm bunch	6.3 ^a	0.04	2.33 ^b	0.12 ^{abc}	183.00 ^a	15.00 ^c
C.V. (%)	14.77	2.23	12.37	11.71	1.32	1.46
F-test	**	ns	**	*	**	**

Note. Numbers followed by the same letter in the same column are not significantly different from the DMRT test at the 99% level

Table 4
Soil analysis after planting

Treatment	pH	EC (mScm ⁻¹)	OM (%)	Total N (%)	Avail. P (mg kg ⁻¹)	Avail. K (mg kg ⁻¹)
control	5.0 ^a	0.03 ^c	1.16 ^d	0.06	12.00 ^c	4.00 ^g
Rice hull biochar	5.7 ^b	0.06 ^b	1.84 ^c	0.09	68.00 ^b	434.00 ^a
Rubber sawdust biochar	5.2 ^a	0.03 ^c	1.91 ^c	0.10	12.00 ^c	33.00 ^f
Oil palm bunch biochar	5.7 ^b	0.15 ^a	2.65 ^a	0.13	85.00 ^a	90.00 ^c
Rice hull	5.9 ^b	0.05 ^{bc}	1.85 ^c	0.09	51.00 ^d	123.00 ^b
Rubber sawdust	5.2 ^a	0.05 ^{bc}	2.30 ^b	0.12	14.00 ^c	68.00 ^c
Oil palm bunch	5.7 ^b	0.06 ^b	1.91 ^c	0.10	56.00 ^c	83.00 ^d
C.V. (%)	17.00	18.5	17.46	11.7	2.70	5.78
F-test	**	**	**	ns	**	**

Note. Numbers followed by the same letter in the same column are not significantly different from the DMRT test at the 99% level

Table 4 shows the soil analysis after baby corn was harvested; the pH of the soil ranged from 5.0 to 5.7 among different biochars. pH of all treatments was in a range of 5.0-6.8 for tropical soil (Udo & Ogunwale, 1977). The resulting electrical conductivity content in the soil was in a range of 0.03-0.15 mScm⁻¹ and remained below the limit of the EC value for agricultural soil, 4 dS/m⁻¹ (Christiansen, 1977). Total N varied between 0.06-0.13%, showing that it was lower than the critical level between 0.3 and 0.6% for soils in the tropics (Bremner & Mulvaney, 1982). Available P content extended between 12.0 and 85.0 mg kg⁻¹, showing that it was greater than the critical level between 10 and 15 mg kg⁻¹ (Adeoye & Agboola, 1985). The available K content ranged from 4.0 to 434% and was greater than the critical level of 60-90 mg kg⁻¹ (Adeoye, 1986).

The soil was analysed before and after the baby corn was harvested. Soil increased the availability of OM, N, and P after biochar application. Moreover, increased soil organic matter content (Van Zwieten et al., 2007; Bera et al., 2016; Lashari et al., 2013; Van Zwieten et al., 2010). The application of biochar to tropical soils makes the soil more fertile. However, K decreased because much of the K in the biochar plots could have been lost for plant uptake.

The Adoption and Barriers of using Biochar by Farmers

According to farmer field surveys in the sandy soil of Narathiwat Province, most farmers were interested in using biochar (43.33%), followed by farmers who decided to adopt the practice of applying biochar in their fields (30.00%). However, 26.67% of farmers hesitated to use biochar in croplands (Table 5). Most farmers in this area are small-scale farmers and have experience in crop cultivation for a long time. Similar to Latawiec et al. (2017),

farmers who had operated agricultural enterprises for 10-20 years were the most familiar with biochar (40%), while people who had operated agricultural businesses for 5-10 years were the least familiar (17%). Small-scale farmers in Brazil are more likely to embrace innovation because their operations yield greater profits than those of larger farmers (Table 5).

Table 6 presents Pearson correlation coefficients (r) between the biochar adoption and economic, social, environmental, and barrier factors with interest in biochar adoption in farmer fields at $p < 0.05$. can be classified into three groups of farmers with various reasons to use biochar.

Table 5
Interest in the adoption of using biochar by farmers in Narathiwat Province

Interest adoption	Frequency	Percent
Interest	13	43.33
Practice (adopted)	9	30.00
Hesitate	8	26.67
Total	30	100

Table 6
The correlation of economic, social, environmental, and barrier factors with interest in biochar adoption in farmer fields

Factors	Interest in adopting		
	Interest	Adopted	Hesitate
Economic value			
Creating a value-added agricultural waste product	0.107	0.347	-0.480*
Save chemical fertiliser cost	0.404*	0.400**	-0.867**
Increase income, yield	0.389**	0.342	-0.789**
Purchase of agricultural waste materials and processing them to make money	0.007	0.376*	-0.398**
Social value			
Biochar creates local jobs	0.121	-0.064	-0.070
Enhance food security	0.112	0.492**	-0.635**
Improve livelihoods and reduce poverty for farmers	0.260	-0.050	-0.240
Environmental value			
Biochar improves soil health in terms of enhancing the physical, chemical, and biological properties of the soil	0.586**	0.040	-0.699**
Utilising available agricultural waste biomass	0.340	0.173	-0.561
Carbon sequestration	0.569**	-0.277	-0.350
Barriers to adoption			
Insufficient practical knowledge about biochar application	0.086	-0.308	0.223
Production methods are complicated	-0.538**	-0.065	0.67**
Limited access to necessary equipment	-0.339	-0.011	0.391*

Note. *Significant at five levels of probability ($p < 0.05$)

First group: Farmers who were interested in using biochar showed that economic value was positively correlated with saving chemical costs (0.404*) and increasing income and yield (0.389**). Environmental values were positively correlated with biochar, which improved soil health by enhancing the physical, chemical, and biological properties of the soil (0.586**) and carbon sequestration (0.569**). However, barriers to adoption were negatively correlated with complicated production methods (-0.538**). Biochar must be profitable for farmers to be used extensively as a soil amendment. One of the primary elements encouraging farmers to adopt new techniques is yield gains, as they directly improve financial performance (Azzi et al., 2019). Moreover, selling biochar as a product or using or selling the heat and other energy generated during pyrolysis are two other ways for farmers to make money using a biochar system (Azzi et al., 2022).

In the second group, farmers willing to accept biochar and apply it in practice showed that economic value was positively correlated with saving chemical costs (0.400**), and farmers could purchase agricultural waste materials and process them to make money (0.376*). Regarding social value, being positively correlated with biochar can enhance food security.

The last group, farmers, was hesitant to use biochar in their field; it showed that economic value was negatively correlated with creating a value-added agricultural waste product (-0.480*), saving chemical costs (-0.867**), increasing income and yield (-0.789**), and purchasing agricultural waste materials and processing them to make money (-0.398**). Social value was negatively correlated with enhanced food security (-0.635**). Barriers to adoption were positively correlated with complicated production methods (0.67**) and limited access to necessary equipment (0.391*).

Guideline for the Utilisation of Biochar in Sandy Soil

Guideline for Interest Group

The farmers in this group considered the economic and environmental values, including saving costs of commercial fertiliser and biochar, which can increase income and yield, as well as carbon storage in soil. However, farmers are concerned that the biochar method is complicated. Therefore, the government should support farmers with training methods or transfer new technologies for biochar production in an easy way. However, an alternative way to improve sandy soil for farmers in this group in the first way is to use oil palm bunches to improve soil; however, in treatment 7, the application of oil palm bunches increased plant height not significantly with the biochar of rice hull and oil palm bunches. Then, they exchange experiences with farmers who adopt biochar in their fields. Next, we show the advantages or compare the growth and yield of biochar and raw oil palm bunches and assess the interest in adopting biochar again. Compared to conventional crops,

biochar was shown to be more beneficial when applied to sandy soils and intensive crop production systems (such as those that produce fruits and vegetables). In some regions, it has been demonstrated that using biochar increases yields, giving farmers an extra revenue stream (Jansen, 2023).

Guideline for Adopted Group

The farmers in this group are ready to practice applying biochar in their field because they accept the economic and social value, including saving on chemical fertiliser costs and purchasing agricultural waste materials and processing them to make money. This encourages them to utilise para-sawdust biochar, followed by oil palm bunches and rice hulls, because para-rubber is the main economic crop in Narathiwat Province, followed by oil palm and rice. Therefore, every year, this agricultural waste is easily found in this area, and the price for transporting it is low.

Guideline for Hesitant Group

Farmers in this group are most concerned about economic value, including creating value-added agricultural waste products, saving chemical costs, increasing income and yield, and purchasing agricultural waste materials and processing them to make money. Social value farmers were negatively correlated with enhanced food security. Barriers to the adoption of this group were production methods that were complicated and had limited access to the necessary equipment. To overcome barriers to the adoption of biochar, the same interest group was the government, which should support farmers with training methods or transfer new technology for the production of biochar in an easy way and also support equipment or credit access to them. Typically, in the local context of Narathiwat Province, a great number of the farmers applied more chemical fertilisers in the long term, and most of the farmers prefer to use chemical fertilisers rather than organic fertilisers to preserve crop yield because they think the use of biochar requires more labor time and machine cost compared with the use of chemical fertilisers, which have high nutrient content and faster growth; however, biochar increases crop yield slowly in the short term. Therefore, organisations should provide financial incentives to adopt biochar to address these challenges. Shackley et al. (2016) discussed the societal effects of biochar attractiveness in relation to food security, economics, health, and energy efficiency. According to their findings, social and cultural factors are the primary obstacles to using biochar in agriculture. The socio-cultural challenges identified included farmers' mistrust, a lack of biochar, a lack of equipment for producing biochar, people's opposition to new development ideas, and a lack of knowledge about the qualities of biochar. According to several studies, biochar is more likely to be cost-effective in underdeveloped nations because of lower production costs (kilns and

stoves) and the possibility of higher yield increases in tropical and sandy soils (Dickinson et al., 2015; Pratt & Moran, 2010).

CONCLUSION

From the overall results, treatment with raw material and biochar (Treatment 2-7) as an amendment resulted in the highest plant height, number of leaves, stem diameter, stem fresh and dry weight, and root length when compared with the control. In contrast, the treatment control showed the lowest overall growth response and yield performance in plants. Thus, it can be concluded that the application of biochar has a significant effect on plant growth, yield, and soil quality. The probability of using biochar in terms of adoption is 30% of farmers will use biochar in their fields because they believe that biochar has economic and environmental value in terms of saving chemical fertiliser costs, purchasing agricultural waste materials and processing them to make money, and enhancing food security. Moreover, 43% of farmers are interested in applying biochar in their fields, but they are concerned about production methods being complicated and having limited access to necessary equipment. This means that if the solution for the method and equipment of biochar is 73% probable, farmers will use biochar in sandy soils. This is the first study in Narathiwat Province and one of the first in sandy soil, showing a relationship between applying biochar to soil on plant growth and yield and farmers' interest in adopting it. This is a possible choice for sustainable agricultural management of their farms. This information will help small grower groups in Narathiwat Province.

LIMITATION

A limitation of this study was the lack of data on the number of baby corn ears per plant, which is an important yield trait. Future research should include this measurement to fully assess crop performance under different biochar treatments.

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The Effect of Dry Skin Extract of Tobacco Stems (*Nicotiana tabacum* var. Virginia) on the Armyworm Insect Pest (*Spodoptera litura* F.) and Its Predators on Potato Plants (*Solanum tuberosum* L.)

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ABSTRACT

This study aims to determine the effect of various concentrations of dry tobacco stem bark extract (*Nicotiana tabacum* var. Virginia) on armyworm pests (*Spodoptera litura* F.) and the presence of predators on potato plants (*Solanum tuberosum* L.). The experiment was conducted in Sembalun Village, East Lombok Regency, from July to September 2024. The parameters observed were pest population, intensity of pest attacks, presence of pest enemies, number of tubers, and tuber weight. This study used a Randomised Block Design consisting of 6 treatments (T) with four replications, namely T0 (control), T1 (abamectin), T2 (tobacco extract 30 ml/1000 ml water), T3 (tobacco extract 40 ml/1000 ml water), T4 (tobacco extract 50 ml/1000 ml water) and T5 (tobacco extract 60 ml/1000 ml water). The results showed that the use of botanical pesticides from dry tobacco stem bark can reduce the population and intensity of armyworm attacks. Application with a concentration of 30 ml/L water (T2) had a different effect compared to the control treatment

(T0). The higher the concentration used, the higher the ability to suppress pest populations and the intensity of pest attacks. The percentage of dry tobacco bark yield at a concentration of 60 ml/L water (T5) did not differ significantly from the chemical pesticide abamectin (T1) in suppressing the population and intensity of attacks by armyworms (*Spodoptera litura* F.).

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INTRODUCTION

Modern agriculture faces significant challenges, including pest management, particularly for food crops like potatoes (*Solanum tuberosum* L.). Potatoes are a vital agricultural commodity, serving as a food source and a globally valuable economic commodity. Potatoes are known to play a crucial role in agriculture, from households to large-scale industries in Indonesia, contributing to the production of flour and chips (Haverkort et al., 2023; Mulyono et al., 2017). Furthermore, potatoes are a staple food in many countries and also provide essential nutrients. However, potato production is often affected by various pests that can cause significant losses, including attacks by armyworms (*Spodoptera litura* F.) (Chakrabarti et al., 2022).

The armyworm (*Spodoptera litura* F.) is a well-known leaf-eating pest that is highly detrimental to farmers. This pest is polyphagous, meaning it damages not only food crops but also plantation crops. Furthermore, armyworm larvae can also reduce crop yields (Chisonga et al., 2023). Armyworm larvae often attack plants, especially their leaves, in large groups. This pest is active at night, so larval attacks usually occur at this time. The damage profile usually includes holes in the leaves, leaving only the leaf veins (Supartha et al., 2021).

Chemical pesticides are often the primary control method used by farmers, but continued reliance on their use can raise environmental and health concerns. Excessive reliance on synthetic insecticides can lead to pest resistance, ecological imbalances, and the death of non-target organisms (Rani et al., 2021). In response, there is growing interest in developing environmentally friendly alternatives that are biodegradable and leave minimal residues. Botanical pesticides derived from plants may offer a solution, offering several advantages, including their ability to degrade in the environment, their effectiveness, and their safety for non-target organisms (Khursheed et al., 2022). One promising source is the dried bark of tobacco (*Nicotiana tabacum* var. Virginia), which contains nicotine, a compound toxic to insects (Sarjan et al., 2021). This agricultural byproduct, often considered waste, can be reused as a plant-based insecticide while enriching soil organic matter. Sarjan et al. (2019) have formulated a liquid form of this product marketed as *Nabati BT Virginia* (Virginia tobacco stem), which has been shown to be effective against Lepidoptera pests on vegetable crops.

Repellents can act against various plant pests. Sarjan et al. (2021) stated that tobacco contains nicotine, which is toxic to insects, making it effective as an insecticide. Tobacco stems that have not been used optimally (often considered waste) can actually be used as a plant insecticide. Additionally, tobacco stems also have the potential to serve as a source of organic matter, providing essential nutrients to plants. Sarjan et al. (2019) also developed a formulation in the form of a liquid solution, which they used as a prototype for commercialisation under the pesticide brand *Nabati BT Virginia* (Virginia Tobacco Bar).

Currently, this pesticide is recommended for controlling pests of the order Lepidoptera (e.g., caterpillars) on various vegetable crops.

On the other hand, a promising approach to controlling armyworm pests on potato plants is to use natural enemies, such as predators, which can reduce the population and intensity of these pests' attacks. Predators of armyworms include: *Oxyopes javanus* Thorell (Nazli & Butt, 2020), *Lycosa pseudoannulata* (Luo et al., 2024), *Paederus fuscipes* (Chamankar et al., 2023), *Rhinocoris sp.* (Xuan Lam et al., 2024), *Andralus sp.* (Bayu et al., 2022), *Coranus sp.* (Mhlanga et al., 2022), *Vespidae* (Otis et al., 2023) and *Solenopsis geminata* (Lee et al., 2021). The presence of predators in agricultural land is influenced by several environmental factors, including biotic and abiotic factors (Sarjan, 2018). According to Furlong and Zalucki (2010), the amount of food (prey) or insect pests is an important factor that is positively related to predator population. This can be interpreted as the population density of insect pests increases, the predator population also tends to increase and vice versa. Therefore, expanding the use of botanical pesticides in the field and assessing the impact of natural enemies, such as predators, on controlling the population and intensity of armyworm pest attacks is very important. Hence, a study was conducted with the title "The Effect of Dry Skin of Tobacco Stems (*Nicotiana tabacum* var. Virginia) on Armyworm Pests (*Spodoptera litura* F.) and their predators on potato plants (*Solanum tuberosum* L.)"

RESEARCH METHODS

Time and Place of Research

This research was conducted in Sembalun Village, Sembalun District, East Lombok Regency, West Nusa Tenggara, from July to September 2024.

Experimental Design

This research employed the experimental method. Meanwhile, the design used was a Randomised Group Design with six treatments. The six treatments used consisted of:

1. T0 (control)
2. T1 (abamectin)
3. T2 (tobacco extract 30 ml/1000 ml water)
4. T3 (tobacco extract 40 ml/1000 ml water)
5. T4 (tobacco extract 50 ml/1000 ml water)
6. T5 (tobacco extract 60 ml/1000 ml water)

Research Implementation

The research implementation is based on the work of Sarjan et al. (2021), which includes land preparation, plot and bed preparation, planting, extraction of a compound from

tobacco stem waste, and application of the tobacco stem waste extract. Land preparation is done using a hoe by loosening 5 hectares of land. Then plots and beds are prepared. In the experimental area, six treatment plots were prepared, followed by four replication plots for each, resulting in a total of 24 treatment plots. In 1 treatment, it consists of 2 beds. The length of the bed is 3 metres by 1 metre.

Meanwhile, the bed spacing and the distance between replicates are 50 cm each, with a planting distance of 30 cm x 60 cm. Two rows of potato plants were planted in each bed, with each row consisting of 13 plants, resulting in a total of 26 plants in one bed and 52 in one treatment. Next, drill a planting hole with a depth of about 5-10 cm and an inter-plant spacing of 30 cm. Planting is done by placing potato seeds in the prepared planting holes. One potato seed is placed in each hole with the sprout facing upwards, and then covered with soil. Tobacco stem waste extract is prepared by soaking 1 kg of tobacco bark in a bucket of 2.5 litres of clean water. The two ingredients are then stirred and kneaded by hand for 30 minutes, after which they are left to rest for 24 hours. After soaking for 24 hours, the water is filtered to separate the bark from the extract, which is then used for its intended purpose. Next, add 100 grams of Wings Dab Soap to the extract and stir for 5-10 minutes. After the Wings Soap dissolves, filter it again into a clean container, and the extract is ready to use.

Plant care includes fertilisation, irrigation, embankment, and weeding. Fertilisation is carried out twice: the basic fertilisation co-occurs with planting, and the additional fertilisation takes place when the potato plants are 21 days old. Urea and NPK are used as fertilisers. Irrigation of potato plants is typically done once a week, or more frequently depending on the weather, water, and soil conditions in the potato-growing environment. The cultivation is done three times, namely at 4 WAP, then at 6 WAP, and one week before harvesting the potato plants. Weeding was done three times, specifically when the potato plants were at 5 WAP, then at 7 WAP, and finally at 10 WAP, as evident from the condition of the weeds present at the time of the survey.

Observation

Pest observations were conducted 8 times, namely at 3 WO, 4 WO, 5 WO, 6 WO, 7 WO, 8 WO, 9 WO, and 10 WO. Pest observations were conducted using *yellow sticky traps*, *yellow pan traps*, and *pitfall traps*, which were installed in the morning between 07:00 and 09:00 Central Indonesia Time and left for 24 hours before being withdrawn in the morning. Insects caught in *yellow sticky traps* were counted directly in the field. Insects caught in *yellow pan traps* and *pitfall traps* were filtered and picked up using a brush to prevent damage to their body parts. The insect is then placed in a bottle filled with 70% alcohol and taken to the laboratory for identification and analysis. The parameters observed in this study were pest population, intensity of pest infestation, presence of pest enemies, number of potato tubers, and weight of potato tubers.

Data Analysis

The observational data in this study were analysed using ANOVA with a significance level of 5%. Prior to the analysis, an assumption test was conducted, followed by an Honest Significant Difference (HSD) test at a significance level of 5%. A regression test was then conducted to determine the relationship between population size and pest attack intensity.

RESULTS AND DISCUSSION

Morphological Characteristics and Symptoms of Armyworm Larvae Attacks

The armyworm's body has black spots that give it a dark appearance, but if you look closely, you can see brownish lines on the sides of its body (Figure 1). Adult armyworms are about 5 cm long and usually attack plants at night, while these caterpillars hide in the soil during the day. Armyworms undergo complete metamorphosis into egg, larva, pupa, and imago stages. Its life cycle lasts about 20 days before it develops into a cocoon (pupa) and finally a butterfly. The butterfly is a dark grey moth, and the female can produce up to 2,000 eggs or more. Eggs are usually laid in clusters on the underside of leaves and hatch within 3 to 5 days (Yulasari, 2016).

Symptoms of armyworm infestation begin in the larval stage, where the larvae attack and damage the leaves, leaving behind remnants of the upper epidermis and leaf veins (Figure 1). This causes the plant to lose leaves. Armyworms begin attacking plants in the early vegetative stage, and their population peaks when the plants are 38 days old. The caterpillar population then increased again after the plants were 73 days old. Infested young plants retard their growth and can lead to plant death in severe infestations. Pests that attack during the flowering stage and early pod formation can significantly reduce crop yields and potentially even hinder the harvest. The damage that occurs in plants aged 10 days after planting (DAT) is 12.5%, while damage in plants aged more than 20 days after planting can exceed 20%.



(a)



(b)

Figure 1. (a) Armyworm (*Spodoptera litura* F.) and (b) Attack symptoms

Average Population and Attack Intensity of Armyworm Larvae (*Spodoptera litura* F.)

The results of further tests on the population and intensity of armyworm infestation showed significantly different results between the control and other treatments. The results of the analysis of variance are shown in Table 1.

Based on Table 1, the average population of armyworm pests in the T0 (control) treatment showed a significant difference compared to other treatments. This was because the T0 (control) treatment did not receive any pest control measures, resulting in a higher population of armyworms in the T0 treatment compared to the other treatments. The abamectin treatment (T1) was significantly different from the control treatment (T0) and 30 ml/L (T2). However, the abamectin treatment (T1) using chemical insecticides did not show any significant differences from the 40 ml/L (T3), 50 ml/L (T4), and 60 ml/L (T5) treatments. According to Khurshed et al. (2022), the higher the concentration of tobacco stem extract used, the more metabolite compounds it contains, so the active ingredient content also increases, and the toxic effect of plant biopesticides becomes stronger. The nicotine content in tobacco extract has a significant impact on its effectiveness as an insecticide. The nicotine contained in tobacco extract has a significantly effective insecticide effect. Nicotine, found in tobacco, can be found throughout the tobacco plant and is known to kill various types of organisms, including armyworms. Furthermore, Kaminski et al. (2020) in their research stated that nicotine is an alkaloid compound that occurs naturally in tobacco plants. This compound affects the pest's central nervous system and causes death in armyworm larvae. Besides nicotine, tobacco also contains various other chemical compounds, including acids, alcohols, aldehydes, ketones, alkaloids, amino acids, carbohydrates, esters, and terpenoids.

The average attack intensity in the control treatment (T0) was significantly different from the other treatments. This is because the control treatment (T0) is not controlled. The treatment with abamectin (T1) up to the 60 ml/L treatment (T5) showed no significant

Table 1

*Average population and intensity of attacks by armyworms (*Spodoptera litura* F.)*

Treatment (T)	Population (Individuals)	Intensity (%)
T0 (control)	8.00 ^a	16.85 ^a
T1 (Abamectin)	1.72 ^c	2.22 ^b
T2 (30ml/L)	4.13 ^b	6.64 ^b
T3 (40ml/L)	3.88 ^{b^c}	6.20 ^b
T4 (50ml/L)	3.56 ^{b^c}	5.84 ^b
T5 (60ml/L)	1.66 ^c	2.24 ^b
HSD 5%	1.98	4.14

Note. Numbers followed by the same letter in the same column do not differ significantly in the 5% HSD test

differences. This is because tobacco contains saponin compounds that can inhibit the taste receptors in the mouth of the larvae, resulting in the armyworm larvae not receiving taste stimuli. Saponin also has a bitter taste that causes larvae to be reluctant to feed (Arbaiatusholeha et al., 2016). Thus, the intensity of attacks in the treatment with plant extracts from tobacco stems could compensate for the results obtained in the abamectin (T1) treatment, which used chemical insecticides.

Average Tuber Number and Weight of Potato Tubers (*Solanum tuberosum* L.)

Based on the results of further tests on the number and weight of potato plant tubers, this is shown in Table 2.

Based on Table 2, the average tuber number and tuber weight of potato plants in the control treatment (T0) were significantly different from the other treatments, with an average tuber number of 7.03 and tuber weight of 0.30 kg. This is believed to be due to armyworm attacks on potato leaves, which disrupt the photosynthesis process and ultimately produce only a small number of tubers. This statement is in line with the opinion of Daudi et al. (2021) in their research, which explains that leaf damage caused by armyworms negatively impacts and disrupts photosynthesis, which in turn leads to reduced crop yields. However, the extent of crop losses depends on the severity of the leaf damage and the plant's growth stage at the time of infestation.

Treatment with abamectin (T1), a chemical insecticide, was not significantly different from treatment with 60 ml/L (T5), but treatment with abamectin (T1) was significantly different from treatment with 20 ml/L (T2), 30 ml/L (T3), and 40 ml/L (T4). This is related to the population and intensity of attacks in abamectin (T1) and 60 ml/L (T5) treatments, which were not significantly different. The population size and intensity of attacks depend on the condition of the plant's structure, specifically its leaves. Under healthy leaf conditions, the rate of photosynthesis can be maximised, allowing the plant to produce

Table 2

*Number of tubers and tuber weight of potato plants (*Solanum tuberosum* L.)*

Treatment (T)	Number of Tubers (Grains)	Tuber Weight (kg)
T0 (control)	7.03 ^c	0.30 ^c
T1 (20ml/L)	11.04 ^a	0.53 ^a
T2 (30ml/L)	9.41 ^b	0.43 ^b
T3 (40ml/L)	9.41 ^b	0.45 ^b
T4 (50ml/L)	9.54 ^b	0.45 ^b
T5 (60ml/L)	10.5 ^{ab}	0.50 ^{ab}
HSD 5%	0.71	0.05

Note. Numbers followed by the same letter in the same column do not differ significantly in the 5% BNJ test

sufficient assimilates that meet its needs and are stored in the tubers. (Saragih, 2019) states that leaves are among the plant organs that function as photosynthetic organs because they contain chlorophyll, a pigment that absorbs light energy and performs photosynthesis to produce sugar. The larger the leaf surface area, the more chlorophyll is present, indicating a higher rate of photosynthesis. By increasing the rate of photosynthesis, plant growth and yield will be faster.

The Ability of Botanical Pesticides of Dry Tobacco Stem Skin to Suppress the Population and Attack Intensity of Armyworm

Based on Table 3 above, the ability to suppress plant pests using the dry peel of tobacco stems (*Nicotiana tabacum* var. Virginia) under abamectin (T1) chemical insecticide treatment resulted in a 78.50% reduction in the average population of armyworm pests, with an average attack intensity of 86.82%. Under the treatment of plant pesticides, namely 20 ml/L (T2), 30 ml/L (T3), 40 ml/L (T4), and 60 ml/L (T5), respectively, the ability to suppress plant pesticides in tobacco stems increased by 20 ml/L (T2) on average. Suppressed the population by 48.37 % and suppressed the average attack intensity by 60.59%. In the 30 ml/l (T3) treatment, the average suppressed population was 51.50%, and the average suppressed attack intensity was 63.20%. In the 40 ml/l (T4) treatment, the average suppressed population was 55.50%, and the average suppressed attack intensity was 65.34%. In the 50 ml/l (T5) treatment, the average suppressed population was 79.25%. The average attack intensity was reduced by 86.70%. Thus, it can be said that the ability of herbal pesticides to suppress the dry skin of tobacco stems (*Nicotiana tabacum* var. Virginia) is *dependent* on the population and intensity of armyworm pest infestation (*Spodoptera litura* F.), exceeding 50%. At the highest concentration, the suppression effect

Table 3
Plant pesticides on the skin, dry tobacco stems, and the ability to increase tuber yield

Treatment (T)	Suppression Ability		Ability to Increase the Yield	
	Population	Intensity (%)	Number of Tuber	Tuber Weight
T1 (Abamectin)	78.50 ^b	86.82 ^a	56, 81 ^a	74.75 ^a
T2 (20ml/l)	48.37 ^c	60,59 ^c	33.66 ^c	42.62 ^c
T3 (30ml/l)	51.50 ^d	63.20 ^d	33.80 ^d	48.85 ^d
T4 (40ml/l)	55.50 ^c	65.34 ^c	35.51 ^c	49.18 ^c
T5 (50ml/l)	79.25 ^a	86.70 ^b	43.75 ^b	65.24 ^b
HSD 5%	-	-	-	-

reached 79.25%. Based on the research results of Syahwal (2023), it was found that the ability of plant pesticides from paitan plants to suppress the population and intensity of armyworm pest infestations exceeded 50%. At the highest concentration, namely 60 ml/L of paitan extract, the population and intensity of armyworm infestation reach 80%. It can be said that comparing the suppression ability of plant pesticides from dry tobacco skin and those from paitan plants does not make much difference.

Related to the ability of plant pesticides from dried tobacco stem skin (*Nicotiana tabacum* var. Virginia) to suppress the population and intensity of armyworm infestation in the treatment T1, which is 78.50% and 86.82 %, respectively, can increase the number of tubers by 56.81 % and tuber weight by 74.75 %. In addition, the average population and attack intensity in treatment T2 were 48.37% and 60.59%, respectively, resulting in a 33.66% increase in tuber number and a 42.62% increase in tuber weight. For treatment T3, the average population and intensity were 51.50% and 63.20%, respectively. The results showed that the number of tubers was 33.80%, and the tuber weight was 48.85%. In treatment T4, the average population and attack intensity were 55.50% and 65.34%, respectively, resulting in a 35.51% increase in the number of tubers and a 49.18% increase in tuber weight. In treatment T5, the average population and attack intensity were 79.25% and 86.70%, respectively, resulting in a 43.75% increase in the number of tubers and a 65.24% increase in tuber weight.

Development of the Armyworm Population (*Spodoptera Litura* F.) Weekly Observations

Based on observations of the armyworm (*Spodoptera litura* F.) population, each observation was repeated eight times on potato plants, yielding the population data presented in the graph (Figure 2).

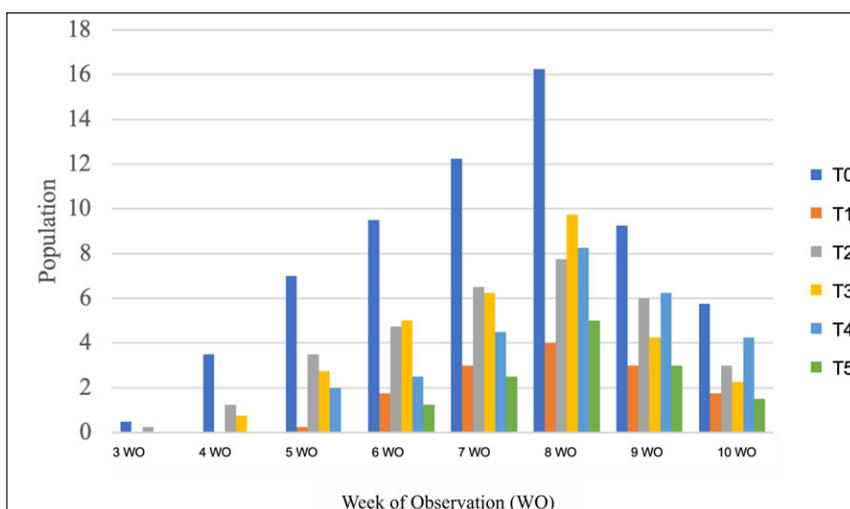


Figure 2. Number of armyworm (*Spodoptera litura* F.) populations per week

Based on Figure 2, it is a graph of the evolution of the armyworm population in each week of observation (WO). At the first observation (3 WO), the armyworm population began to emerge. Then the population increased until the highest population was reached at the sixth observation (8 WO) with an average population of 16.25 in the control treatment (T0). This is believed to be due to the abundant availability of food, as the quality of available food has a significant impact on the growth and development of the armyworm population. Pest population increases can be influenced by extrinsic and intrinsic factors (Supartha et al., 2021). Extrinsic factors include environmental factors such as food availability, natural enemies, climate, space, and competition. Intrinsic factors, on the other hand, include high adult fecundity and a short life cycle. Alvarez and Rodríguez (2024) also explained in their findings that dense plant leaves can serve as protection for various insects from sunlight and attacks by natural enemies. Differences in plant age also influence insect pest behaviour, as these pests tend to be attracted to areas with high fertility or abundant food sources.

In the seventh (9 WO) and eighth (10 WO) observations, the armyworm population decreased. This is because the potato plant has entered the generative phase, which is characterised by a decrease in nutrient content in the potato leaves. This finding is consistent with the research of Syahwal et al. (2024), which stated that the decline in pest populations was caused by reduced food availability, particularly in plant leaves. This condition is characterised by many leaves starting to turn yellow, wilt, dry out, and eventually fall off.

Development of the Attack Intensity of the Armyworm (*Spodoptera litura* F.) Every Observation Week

Based on observations of the attack intensity of the pest *Spodoptera litura* F., each observation was conducted eight times on potato plants, resulting in the attack intensity data shown in the graph (Figure 3).

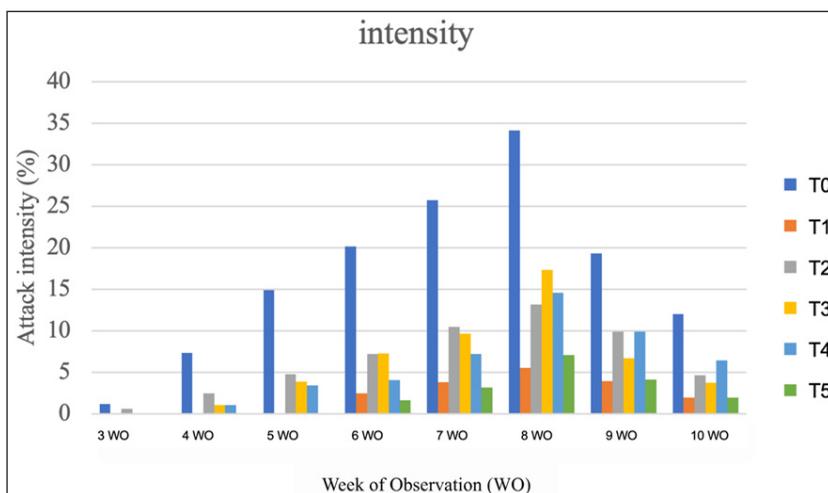


Figure 3. The intensity of attacks by armyworm pests (*Spodoptera litura* F.) per week

Based on Figure 3 above, it is a graph of the evolution of armyworm pest attack intensity in each week of observation (WO). At the first observation (3 weeks after planting), the attack intensity of armyworm pests began to appear. Then it increased until the highest attack intensity was reached at the sixth observation (8 WO) with an average attack intensity of 23.55% in the control treatment (T0). This is due to the increasing number of pest populations, which will lead to an increasing intensity of attacks caused. According to Palit et al. (2016), the greater the pest population, the higher the intensity of attacks caused by armyworm pests. The high intensity of armyworm pest attacks is also due to this pest being polyphagous. This corresponds to the conditions in the field, where other host plants, such as tomatoes, shallots, spinach, and cabbage, are present. Therefore, when other host plants are present, armyworm pests remain, increasing the intensity of their attacks.

From the seventh observation (9 WO) to the eighth observation (10 WO), the intensity of the armyworm pest attack decreased. The reason for the decrease in attack intensity is that the potato plants have entered the generative phase, and food availability is reduced, as evidenced by the fact that the leaves of the potato plants start to fall.

Main Predator Species in Controlling Armyworm Pest Populations (*Spodoptera litura* F.)

Based on Table 4, the main predators identified in this study included spider species from the Salticidae family, followed by the Black Ant (*Dolichoderus thoracicus*) and the Wasp (*Dusona douragia*). The highest predator abundance was observed in T0 (Control) with 22 individuals, while the lowest population was recorded in T1 (Abamectin), with only 2 individuals. Notably, Salticidae spiders were found in all treatments, but their populations were significantly higher in T2 (30 ml/L) compared to the chemical control (T1). This suggests that the botanical pesticide tobacco stem, at low concentrations, may be less harmful to predatory spider populations than synthetic alternatives.

Table 4 shows that as the concentration of tobacco extract increased (from T2 to T5), the predator population showed a gradual decline (15 individuals in T2 to 7 in T5). This decline is likely due to two factors: increased toxicity at high doses and a significant decrease in prey availability (armyworm).

Based on Table 4, the main predator identified in this investigation is a spider species from the family Salticidae. The average population of Salticidae spiders found in the field was 30 individuals. According to Oberg (2007), spiders are one of the important predators in plant ecosystems. As general predators, spiders have diverse prey and play an important role in reducing and preventing natural pest infestations in crop production. Spiders also help increase biodiversity in agroecosystems. Apart from this, the main predators found in the field include black ants (*Dolichoderus thoracicus*), with a total population of 25 individuals, and wasps (*Dusona douraguia*), with a total population of 16 individuals.

Table 4
Main predators of field armyworm pests (*Spodoptera litura* F.)

No.	Predator Species	T0	T1	T2	T3	T4	T5	Total Population (Individuals)	Pictures
1.	Spider family Salticidae	9	1	6	5	5	4	30	
2.	Black Ants (<i>Dolichoderus thoracicus</i>)	8	1	5	5	4	2	25	
3.	Wasp (<i>Dusona douraguia</i>)	5	0	4	3	3	1	16	
Total		22	2	15	13	12	7	71	

The increase in the number and diversity of predators is not always accompanied by an increase in the number of pests they prey on. The research results of Aprilizah (2006) found that a large population of predators is not always compensated for by an increase in the number of pests they hunt. Begon et al. (1986) also found that this phenomenon is caused by the type of interference (mutual interference) from other predators, which can reduce the level of predation. This shows that predator efficiency does not always increase with increasing predator diversity.

Relationship Between Population Size and Intensity of Armyworm Pest Infestation (*Spodoptera litura* F.)

Results of the regression analysis between population variables and attack intensity by armyworm pests (*Spodoptera litura* F.)

Based on Figure 4, the regression analysis plot for all treatments shows that the equation $Y = 2.3075x - 2.1611$ can be interpreted as meaning that for each additional individual of the pest *Spodoptera litura* F., the intensity of the attack caused by this pest increases by 2.3075%, with a coefficient of determination of 98%. This shows that the degree of relationship between population and attack intensity has a powerful influence.

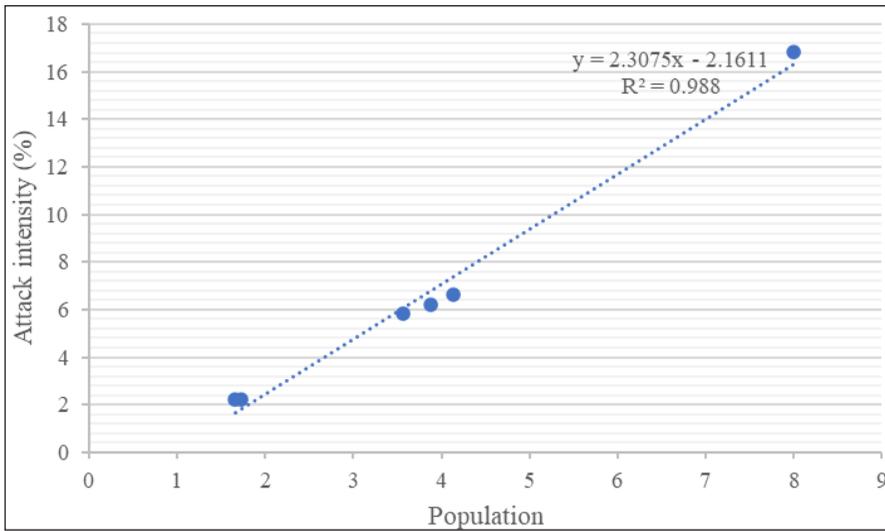


Figure 4. Regression analysis of the relationship between population size and attack intensity

The Relationship between Population Suppression Ability and the Intensity of Attacks on Potato Tuber Yields.

Results of regression analysis between population variables and the intensity of attacks by armyworms (*Spodoptera litura* F.) on potato crop yields.

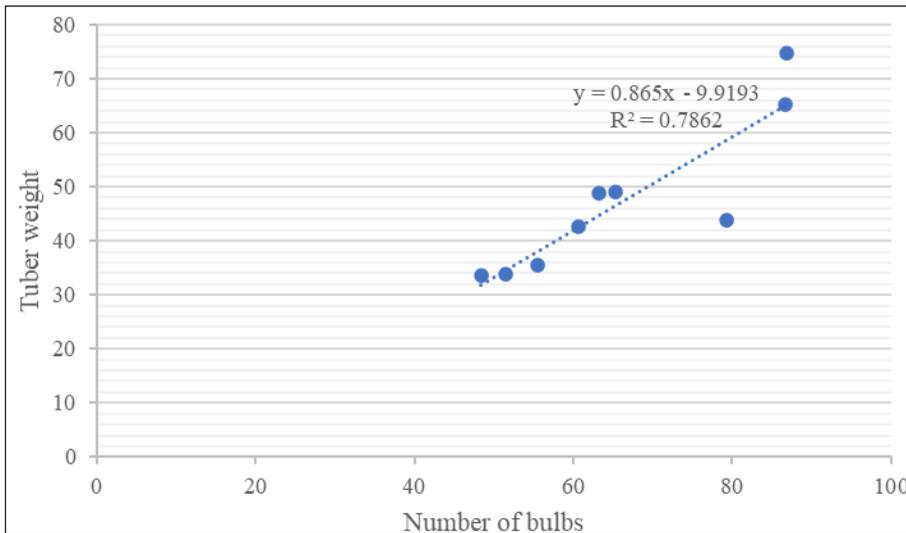


Figure 5. Regression analysis between the ability to suppress populations and the intensity of attacks on potato tuber yields

Based on Figure 5, the regression analysis diagram of suppression ability shows that the equation $Y = 0.865 - 9.9193$, with an R-squared value of 0.78, can be interpreted as meaning that for each additional suppression ability of botanical insecticides, the yield of potatoes increases by 0.865%, with a coefficient of determination of 78%. This shows that the degree of relationship between pressing ability and the number of tubers yields has a strong influence. It is clear that the higher the concentration used, the greater the ability to suppress pest population and intensity, and the higher the crop yield.

CONCLUSION

The findings of this study indicate that herbal pesticides derived from dried tobacco stem husks play an important role in reducing armyworm attacks, which have implications for increasing crop yields. The results of the study specifically showed that the application of a water concentration of 30 ml/l (T2) showed a significant difference in effectiveness compared to the control treatment (T0). Furthermore, there was a clear trend indicating that higher extract concentrations had an effect on increasing efficacy in suppressing pest populations and increasing the percentage of tuber yields. It is worth noting that a water concentration of 60 ml/l (T5) of the extract showed a comparable effect to the chemical pesticide abamectin (T1) in controlling armyworm populations (*Spodoptera litura* F.) and in increasing potato (*Solanum tuberosum* L.) yields. In addition, this study also identified the main natural predators of armyworm pests in the field, including Salticidae spiders, black ants (*Dolichoderus thoracicus*), and wasps (*Dusona douraguia*), which can contribute to the biological control of this pest. Broadly speaking, these results highlight the potential use of herbal pesticides as a viable and sustainable alternative to chemical pesticides in agricultural practices.

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Improving Maize Yield, Biomass, and Selected Chemical Properties of an Acid Soil Using Transformed Chicken Dung

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ABSTRACT

Ultisols and Oxisols are acidic soils with high concentrations of Al (Aluminium) and Fe (Iron), making them unsuitable for agriculture without intervention, which contributes to Brunei's low agricultural self-sufficiency. Sustainable agro-waste management can enhance soil quality and crop productivity. With the increasing number of poultry farms, better management of chicken dung produced is crucial for maintaining soil fertility and enhancing maize production. This study aimed to improve selected soil chemical properties, maize yield, and above-ground biomass of maize plants by repurposing chicken dung as an organic amendment (OA). A field trial was conducted using a Random Complete Block Design with five treatments: (T1) chemical fertiliser only; (T2) 100% OA + chemical fertiliser; (T3) 75% OA + chemical fertiliser (T3); (T4) 50% OA + chemical fertiliser; and (T5) 25% OA + chemical fertiliser. Maize yield (test crop), maize plants' above-ground biomass, and soil samples were collected and analysed using standard procedures. Results suggest that the OA treatments enhanced maize fresh cob yield and maize plants' above-ground biomass compared with chemical fertiliser alone by 89.42% and 50.95%. Moreover, the OA increased soil exchangeable Al³⁺ and H⁺, whereas soil nitrate, P, and Na⁺ decreased. However, no significant differences were observed in soil pH, total organic carbon, total N, K, NH₄⁺, Mg, and Ca²⁺.

Further field trials may reveal the long-term soil, maize fresh cob yield, and above-ground maize plants' biomass improvements. This study provides a novel approach to restoring Brunei's degraded agricultural soil by integrating organic amendments and promoting sustainable waste management.

Keywords: Crop productivity, low pH soils, organic amendment, soil quality

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INTRODUCTION

Soil serves as a medium for several purposes, including building construction, agroforestry, and animal and crop production. However, the natural state does not guarantee the best ecological functions for these purposes. In the case of agriculture, the tropics primarily have Ultisols and Oxisols (von Uexküll & Mutert, 1995), which are highly acidic with pH values below 5.5. Thus, they are relatively unfavourable or infertile for production agriculture without suitable intervention, such as applying lime, organic matter, and chemical fertilisers. Because Ultisols and Oxisols have high concentrations of Al and Fe, they are noted for causing nutrient imbalances such as low P in addition to reducing critical biological activities and microbial communities (Agegnehu et al., 2019; Osman, 2018).

The traditional practice of liming and the use of chemical fertilisers may enhance soil acidification and further deteriorate soil health (Cai et al., 2019). Additionally, these traditional practices are deemed uneconomical and unsustainable for the environment because, for example, the liming effect is temporary; thus, compelling farmers to apply significant amounts of lime every growing season, and this practice is expensive, time-consuming, and laborious. Although agricultural lime is easy to handle, over-liming has its risks, and such risks are listed as follows: (i) further increased soil pH causing a decrease in solubility of nutrients, (ii) molybdenum toxicity, (iii) micronutrient deficiency, and (iv) disruption of soil structure by reducing the stability and drainage, and (v) liming effect is temporary (Osman, 2018; Weil & Brady, 2016). Moreover, excessive application of chemical fertilisers high in P and N causes environmental issues such as the eutrophication of water bodies.

The use of manure as an ingredient for organic fertilisers or soil amendments is suggested to be more sustainable for soil productivity and management, food production, cost-effective, and can increase crop yield by improving soil fertility compared with synthetic fertilisers such as compound N-P-K (nitrogen-phosphorous-potassium) or straight N, P, and K fertilisers (Ali et al., 2023; Zhang et al., 2020). It is worth stressing that the excessive use of chemical fertilisers for improving soil productivity decreases soil pH and worsens the initial problem. In comparison with plants treated with chemical fertiliser only, combining organic soil amendments and chemical fertilisers enhances soil chemical properties, nutrient uptake, and crop yield (Ali et al., 2023). A study by Cai et al. (2019) revealed that manure positively impacted maize yields more than synthetic fertilisers. In addition, the study demonstrated that manure and interactive manure with synthetic fertiliser application could prevent soil acidification, other to increasing soil pH and improving soil structure. The production of organic fertilisers or soil amendments from poultry manure suggests the possibility of slow-release properties causing long-term improvement of soil and crop productivity (Purnomo et al., 2017). The timely release of nutrients prevents nutrient loss; therefore, the plants can receive sufficient nutrients to enhance their growth,

which can be translated into the crop yield. Similarly, the application of chemical fertilisers amended with organic amendments showed significant improvement in maize yield (Ch'ng et al., 2017). The study further revealed minimal N loss between the plant-soil system, an improvement in soil pH, nutrient availability, and a reduction of Al and Fe, leading to an improvement of nutrient uptake in the rooting zone of the maize plants. Adebayo et al. (2017) reported that the growth of *M. oleifera* was significant when poultry manure, cow dung manure, organo-minerals, and NPK were applied. Nonetheless, using poultry manure was superior in terms of providing optimum crop growth qualities, which translates into increasing crop yield. Furthermore, the biochemical transformation of poultry wastes offers benefits, particularly in nutrient recycling and application to agricultural soils, and this approach reduces environmental degradation (Ali et al., 2023; Drózdź et al., 2020; Obi et al., 2016) because poultry waste has a variety of naturally occurring beneficial microorganisms that contribute positively to ecological nutrient cycles, such as recycling C, N, P, and other elements present in the poultry by-product (Williams, 2013).

In Brunei, there has been a yearly increase in the broiler and egg industry. The annual increase is the main reason for attaining 100% SSL in the broiler sector, 100% SSL in the egg sector, 94.60% SSL in the day-old chicks' sector, and 80.10% SSL in the fertilised eggs sector in Brunei (Department of Agriculture and Agri-food, 2023). In return, these sectors generate more waste, which could cause environmental problems, such as increasing waste received by Brunei's landfills. Nonetheless, biodegradable wastes such as chicken dung have valuable nutrients that could be recycled, thus regenerating agricultural wastes into beneficial by-products that can improve Brunei soils' physical, chemical, and biological properties. The broiler and egg wastes can be turned into organic fertilisers or soil amendments through composting or co-composting of the wastes. Several studies have provided results on using organic amendments in improving soils' chemical, physical, and biological properties in a manner that translates into increased yield of crops (Drózdź et al., 2020; Gržinić et al., 2023; Mironiuk et al., 2023).

Higher crop yield not only enhances food production but also provides raw materials for animal feed, supporting the livestock and poultry industry. Despite its potential, this approach has yet to gain widespread adoption in Brunei. At the same time, this approach enables effective and efficient waste management and utilisation, a practice that has yet to gain full traction in Brunei. To this end, regenerating poultry manure into value-added products such as organic amendments will support waste and soil management in Brunei and simultaneously improve the crop industry's sufficiency rate. Therefore, this study aimed to improve selected chemical properties of soil, maize yield, and above-ground maize plants' biomass production using transformed organic amendment from chicken dung.

MATERIALS AND METHODS

Soil and Organic Amendment Characterisation

The area used in this study is located at Hasmit Enterprise, Kampung Batong, Brunei (4.83788° N, 114.82173° E). Soil from the area was collected at 0-20 cm using an auger. The sampling area was 22 m x 71 m, and 10 soil samples were randomly taken. Afterwards, the soil samples were air-dried, ground, and sieved to pass a 2-mm sieve, after which they were analysed for pH and electrical conductivity (EC) using a pH and electrical conductivity meter (Ohaus, AQUASEARCHER™ AB33M1 Bench Meter) (FAO, 2021a, 2021c). Organic matter and total organic carbon were determined using the Walkley-Black method (FAO, 2019). The soil total P, K, Mg, and Ca were extracted using the aqua regia method (Moursy et al., 2020), where 20 mL of aqua regia (1:3 of HCl: HNO₃) was used to digest 2 g of the soil. Afterwards, the concentration of extracted K, Mg, and Ca was determined using an atomic absorption spectrophotometry (AAS) (AA-7000, Shimadzu), and total P following the molybdenum blue method in conjunction with ultraviolet-visible spectrophotometry (UV-Vis) (Cary 60 UV-Vis Spectrophotometer, Agilent). Similarly, the soil available P and exchangeable base cations such as K, Mg, Ca, and Na were extracted using the Mehlich 1 extraction method (Tan, 2005). Thereafter, the extractants were analysed using AAS for the exchangeable cations and UV-vis for available P, after the blue colour was developed by following the method described by Murphy and Riley (1962). The soil total N and exchangeable NH₄⁺ were determined using the Kjeldahl method (FAO, 2021b; Lee et al., 2017) (Vapodest 450, Gerhardt), and exchangeable NO₃⁻ was determined following the sodium salicylate method (Monteiro et al., 2003) using a UV-Vis Spectrophotometer (UV-2700, UV-VIS Spectrophotometer, Shimadzu). The soil exchangeable acidity, Al³⁺, and H⁺ were extracted using 1 M KCl, after which they were determined using the acid-base titration with 0.01 M NaOH and 0.01 M HCl (Rowell, 1994).

The organic amendment was produced by mixing 25 kg of dried chicken dung with 1.25 kg of corn feed, 0.5 L of molasses, and 5% chicken manure slurry, following the procedure of Omar et al. (2021) with slight modification. The chicken manure slurry was made by mixing fresh chicken manure with water, which was a source of microbes. The corn feed was added to provide energy for the microbes, and the molasses provided carbohydrates, for example, glucose. For the first four weeks, the amendment was turned once every week. The corn feed and molasses were topped up during the first turning only to provide sufficient nutrients for the microbes. Seven boxes as replicates of the co-composting process were produced to ensure repeatability and error minimisation. The co-composting process lasted for 50 days, and the ambient and compost temperatures were monitored every 48 hours (7 AM and 5 PM) using a digital thermometer. The organic amendment

produced was analysed for pH, electrical conductivity, organic matter, and total organic carbon following the procedures cited previously. Aqua regia was used to extract P, K, Mg, and Ca, after which AAS was used to analyse the concentration of K, Mg, and Ca, whereas P was analysed using the molybdenum blue method. The Kjeldahl method was used to determine total N.

Field Trial

A field experiment was conducted at Hasmit Enterprise, Kampung Batong, Brunei. The test crop used for this study was maize because of its sensitivity to low pH soils with high Al and Fe ions but low in organic matter. Land preparation was done in stages following standard procedures. Firstly, the land used was cleared by removing weeds manually, after which it was harrowed three times, such that the soil broke up for even seedbed establishment. The experimental field was designed with three blocks in a Randomised Complete Block Design. Each block had five plots to accommodate the treatments used in this field trial (five treatments x three blocks). Thus, a total of 15 plots were constructed, and the size of each plot was 3.2 m (width) x 5.6 m (length), where a total of 56 maize seeds were planted per plot. The Dolomite application was performed a day for equilibration before the organic amendment was broadcast. The dolomite applied was based on the rate of 6 t ha⁻¹ as recommended by the *Jabatan Pertanian & Agrimakanan* (2023). The organic amendment was evenly applied to each plot according to its designated treatments. A total of five treatments were evaluated in triplicate (Table 1). The organic amendments applied were based on the rates of 5 t ha⁻¹ (based on a planting density of 27,777 plants ha⁻¹) (Johan et al., 2021). The organic amendment rate was varied as 100%, 75%, 50%, and 25% of the recommended 5 t ha⁻¹. Chemical fertilisers N-P-K (15-15-15) were applied at a rate of 60 kg N ha⁻¹ (Omar et al., 2020). The fertiliser was broadcast in two equal splits: (500 g NPK) and (500 g NPK) at 10 and 28 days after sowing (DAS), respectively. The treatments evaluated are presented in Table 1.

Standard procedures were used to monitor the maize plants until they were harvested at 73 DAS. The maize fresh cobs and shoots (leaves and stems) were harvested by cutting the stems at 0.1 mm from the soil surface. This was followed by collecting soil samples in each plot at 0 -20 cm. After harvesting, the cobs, leaves, stems, and soil were collected and processed for laboratory analysis. To assess total above-ground fresh biomass, the cobs, leaves, and stems were separated and weighed accordingly. The prepared soils were analysed for pH, EC, organic matter, total organic carbon, exchangeable acidity, Al³⁺, H⁺, P, and base cations (K, Mg, Ca, and Na), and total N, P, K, Mg, and Ca using the method previously cited.

Table 1

Description of treatments, rate of organic amendment, chemical fertiliser, and dolomite used

Treatments Code	Description	Organic Amendment rate	Chemical Fertiliser Rate	Dolomite Rate
T1	Soil + chemical fertiliser + dolomite	-	1 kg	10.75 kg
T2	Soil + 100% organic amendment + chemical fertiliser + dolomite	9 kg	1 kg	10.75 kg
T3	Soil + 75% organic amendment + chemical fertiliser + dolomite	6.75 kg	1 kg	10.75 kg
T4	Soil + 50% organic amendment + chemical fertiliser + dolomite	4.5 kg	1 kg	10.75 kg
T5	Soil + 25 % organic amendment + chemical fertiliser + dolomite	2.25 kg	1 kg	g

Statistical Analysis

Analysis of variance (ANOVA) was used to identify the treatment effect, and Tukey's HSD test was used to differentiate the treatment means at $p \leq 0.05$. This statistical analysis was performed using the Statistical Analysis System (SAS) On Demand for Academics (Version 9.4).

RESULTS AND DISCUSSION

Characteristics of the Soil and Organic Amendment

The soil collected from Hasmit Enterprise was analysed for its selected chemical properties. Table 2 presents the selected chemical properties of the soil before planting. The soil had a low pH value of 5.1 with high exchangeable acidity. This indicates it is a strongly acidic soil (USDA NRCS, 1998). A reason for this result is that 1 mole of Al^{3+} can release three moles of H^+ during aluminium hydrolysis in water, whereas in the case of Fe^{2+} , it releases two moles of H^+ during its hydrolysis in the soil. These chemical reactions contribute to the acidity of the soil (Weil & Brady, 2016). The low P is related to its fixation by the Al and Fe ions. Fixation of P by these acidic ions leads to the formation of insoluble phosphate, and this process makes P unavailable for plants. Meanwhile, the soil also showed low EC, indicating there are insufficient nutrients available in the soil, partly because of leaching and weathering. As the exchangeable basic cations, such as K, Ca, Mg, and Na, are leached out of the soil profile, they are replaced by acidic cations, Al^{3+} , Fe^{2+} , and H^+ in the soil to further make the soil acidic if no intervention, such as liming or application of organic amendment or soil conditioners, is practised. Although the total soil nutrients were relatively high, they are not plant-available due to fixation or adsorption to the soil's colloids. Furthermore, the nitrogen profile also reveals there were more NH_4^+ than NO_3^- due

to suppression of nitrification, which is expected from acid soil due to reduced activity of nitrifying bacteria and archaea in acid or low pH soils (Ni et al., 2023).

The organic amendment produced from the chicken was analysed for its selected chemical properties, and the results indicate it is of good quality (Table 3). The high pH of the organic amendment suggests that it is neutral and has a liming effect on the

Table 2
Selected chemical properties of the soil before conducting the field trial

Variables	Value Obtained \pm SD
pH in water	5.10 \pm 0.03
Electrical conductivity (mS cm ⁻¹)	0.0061 \pm 0.0004
Exchangeable Acidity (cmol kg ⁻¹)	1.57 \pm 0.12
Exchangeable Al ³⁺ (cmol kg ⁻¹)	0.18 \pm 0.02
Exchangeable H ⁺ (cmol kg ⁻¹)	1.39 \pm 0.10
Total organic carbon (%)	2.17 \pm 0.039
Organic matter (%)	3.74 \pm 0.12
Total N (%)	0.23 \pm 0.01
Total P (ppm)	7468.90 \pm 104.26
Total K (ppm)	7150.35 \pm 41.79
Total Ca (ppm)	763.33 \pm 102.14
Total Mg (ppm)	722.09 \pm 40.90
Available P (ppm)	2.03 \pm 0.72
Available K ⁺ (cmol kg ⁻¹)	39.62 \pm 1.91
Exchangeable NH ₄ ⁺ (ppm)	16.62 \pm 1.57
Available NO ₃ ⁻ (ppm)	1.93 \pm 0.19
Exchangeable Ca ²⁺ (cmol kg ⁻¹)	0.094 \pm 0.003
Exchangeable Mg ²⁺ (cmol kg ⁻¹)	1.39 \pm 0.12
Exchangeable Na ⁺ (cmol kg ⁻¹)	0.08 \pm 0.00

Table 3
Selected chemical properties of the organic amendment produced from chicken dung

Variables	Organic Amendment \pm SE
pH in water	7.19 \pm 0.05
Electrical conductivity (mS cm ⁻¹)	8.99 \pm 0.07
Total organic carbon (%)	34.24 \pm 0.44
Organic matter (%)	59.02 \pm 0.76
Total N (%)	4.29 \pm 0.05
Total P (ppm)	8701.43 \pm 184.14
Total K (ppm)	59987.50 \pm 764.13
Total Mg (ppm)	2708 \pm 0.58
Total Ca (ppm)	78708.33 \pm 441.00

soil because of the presence of basic cations such as K, Mg, and Ca. Based on its EC, the organic amendment may be rich in dissolved salt, translating into high levels of plant-available nutrients that are non-toxic to plants. The organic amendment shows a high level of total organic carbon and organic matter, implying that it comprises highly decomposed organic by-products, which can improve soil structure, water retention, nutrient reservoir for essential nutrients, and biological activities, including beneficial microorganisms. The overall total nutrient content of organic amendment possesses substantial quantities of N, P, K, Mg, and Ca, resulting from the diverse nutrient sources from the raw materials used. To conclude, the results obtained demonstrate that the organic amendment was mature and stable for use (Ch'ng et al., 2013; Omar et al., 2021).

Selected Soil Chemical Properties, Maize Yield, and Above-ground Biomass

The soil pH at the end of the field trial showed differences between T1, T3, and T5, but T3 and T5 (Organic amendment with chemical fertiliser) decreased the soil pH compared with the treatment with chemical fertiliser only (T1). Nitrification and ammonification cause H^+ production and the presence of organic acid from mineralisation, one of the major contributors to soil acidification and low buffering capacity. This is consistent with the findings of Duşa et al. (2023) that different combinations of organic amendment and chemical fertiliser caused a decrease in soil pH during autumn. Similar to the finding of Angelova et al. (2013), compost caused a slight decrease in pH because of nitrification and mineralisation of organic materials to produce organic acids. Zaki et al. (2018) explained that there was a decline in the pH of the soil with N-P-K fertiliser, compost cover crop, and straw mulch was because of nitrification to nitrates, decomposition of residues in the straw mulch treatment, and acidification through nitrogen-fixation in the rooting zones of legumes. A study conducted by Chong et al. (2022) resulted in reduced soil pH between the experiment's incubation times; however, the pH increased from day 60 of incubation. The decrease in soil pH was due to high soil buffering capacity; nevertheless, soil pH increases because of the constant dissolution of calcium silicate. It is also true that long-term application of organic amendment can improve the overall soil pH in acid soil (Cai et al., 2019; Ch'ng et al., 2017). The fact that the soil's electrical conductivity, regardless of treatments were similar suggests that the relatively high electrical conductivity of the organic amendment did not have adverse effects on the soil and the maize plants because the soil's electrical conductivity was below the threshold of $>2.0 \text{ mS cm}^{-1}$ (Ch'ng et al., 2017; Chong et al., 2022; Johan et al., 2021).

The high exchangeable acidity, Al^{3+} , and H^+ are highly related to the changes in soil pH because of the negative correlation between soil pH, exchangeable acidity, Al^{3+} , and H^+ (Dai et al., 2021). The organic amendment influenced the soil exchangeable acidity and Al^{3+} . There was a reduction of soil exchangeable acidity and Al^{3+} in the plot with

T2 but an increase in T3, T4, and T5 (Table 3). The reduction of the soil exchangeable acidity and Al^{3+} in the plot with T2 is related to the protonation and proton (H^+) exchange between the organic amendment and the soil, precipitation of soluble Al and Fe ions, and using organic amendment with high concentrations of base cations (K, Mg, Ca, and Na) (Ch'ng et al., 2017; Dai et al., 2021; Rusli et al., 2022). Nevertheless, an increase in soil exchangeable acidity can be caused by the release of H^+ from the decomposition of organic matter (Agegnehu et al., 2019), which is highly available in the organic amendment. Similarly, the increase in soil acidity was caused by the liming effect of the organic amendment, thus enabling the replacement of acidic cations onto the soil colloid and release into the soil solution (Toluwase Oreoluwa et al., 2020). This study suggests that long-term application of organic amendment with the aid of any liming materials can minimise the slow liming effect of organic amendment only during the first cycle of planting, and application of the organic amendment for three to five planting cycles could provide sufficient organic matter to buffer the soil pH (Ali et al., 2023; Ch'ng et al., 2017).

There were no differences between T1 (chemical fertiliser only) and T2, T3, T4, and T5 (organic amendment with chemical fertiliser) for the soil total organic carbon and organic matter, total N, ammoniacal-N, and nitrate-N, and because of the soil's natural characteristics, applying dolomite was essential during the first planting stage. However, this may have caused a priming effect, which further mineralises the organic matter and reduces the total organic carbon in the soil, a practice that also leads to a reduction in soil nitrogen (Sae-Tun et al., 2024). A study by Ali et al. (2023) revealed similar results. They reported no differences among treatments because of the low N in the organic amendment. However, the residual effect of the organic amendment during the two cropping cycles improved soil total C, available nitrate, ammoniacal-N, and CEC, and reduced exchangeable acidity and Al^{3+} . The high content of the organic matter in organic amendment enables better retention of organic N by slowly mineralising the soil organic N to soil inorganic N for efficient plant uptake (Omar et al., 2020), suggesting that using organic amendment has a greater long-term effect without inducing nutrient loss (Diacono & Montemurro, 2010).

The overall results for total and available nutrients of the soil indicate that organic amendment improved some of the selected chemical properties of the soil. The organic amendment did not influence the soil K and Mg. Although the organic amendment showed a high total K value, the supplied K appears to be fixed due to the initial conditions of the acid soil. Several factors may influence K fixation in the soil such as: (1) the nature of the soil colloids, (2) the levels of previous potassium additions and removals, (3) wetting and drying, (4) freezing and thawing, and (5) the presence of excess lime (influence of pH) (Weil & Brady, 2016). A study conducted by Roy et al. (2023) indicates that the rate of K fixation increased with higher K application rates but decreased as fixation sites became more saturated. This is because K fixation occurs when the concentration of K in the

Table 4
Treatments effects on the selected chemical properties of the soil after the field trial

Treatments	pH in Water	EC (mS cm ⁻¹)	cmol kg ⁻¹		
			Exch. Acidity	Exch. Al ³⁺	Exch. H ⁺
T1	5.49 ^a ± 0.04	0.020 ^a ± 0.0009	0.56 ^c ± 0.08	0.045 ^{bc} ± 0.02	0.51 ^c ± 0.06
T2	5.12 ^{ab} ± 0.04	0.017 ^a ± 0.0013	0.58 ^c ± 0.02	0.035 ^c ± 0.02	0.54 ^c ± 0.00
T3	4.97 ^b ± 0.07	0.021 ^a ± 0.0023	0.88 ^b ± 0.07	0.08 ^{ab} ± 0.01	0.80 ^b ± 0.06
T4	5.07 ^{ab} ± 0.19	0.016 ^a ± 0.0005	1.04 ^{ab} ± 0.01	0.11 ^a ± 0.02	0.92 ^{ab} ± 0.08
T5	4.74 ^b ± 0.06	0.022 ^a ± 0.0010	1.08 ^a ± 0.11	0.10 ^a ± 0.02	0.98 ^a ± 0.09

Treatments	TOC (%)	OM (%)	Total N (%)	Exch. NH ₄ ⁺ (ppm)	Available NO ₃ ⁻ (ppm)
T2	2.23 ^a ± 0.08	3.84 ^a ± 0.10	0.22 ^a ± 0.015	18.83 ^a ± 3.32	4.97 ^a ± 0.98
T3	2.34 ^a ± 0.06	4.03 ^a ± 0.13	0.23 ^a ± 0.005	52.06 ^a ± 18.83	5.11 ^a ± 0.49
T4	2.07 ^a ± 0.14	3.56 ^a ± 0.13	0.22 ^a ± 0.010	26.58 ^a ± 4.60	4.11 ^a ± 0.15
T5	2.51 ^a ± 0.19	4.32 ^a ± 0.32	0.26 ^a ± 0.001	27.69 ^a ± 3.32	8.15 ^a ± 0.83

Note. Means within the column with different letter(s) indicate significant differences between treatments by Tukey's HSD test at $p \leq 0.05$

soil solution rises; therefore, soils with higher native K content fix less K compared with soils with lower K content. However, there was a significant difference in the soil total and available P, total Ca, and exchangeable Na. As for soil total P, substantial differences were caused by the treatments, where T3 caused the highest total P concentration, whereas T2 and T1 had the lowest soil total P concentration. For soil available P, there were significant differences in the plots with T1 (Chemical fertiliser only), T2, T3, T4, and T5 (Organic amendment with chemical fertiliser), and T3, T4, and T5 (organic amendment with chemical fertiliser), with T3 plot having the highest concentration of available P and the T1 plot having the lowest concentration. Significant differences existed in the total Ca of the plot with chemical fertiliser (T1) and the soil with the organic amendment with chemical fertiliser (T2 and T5). The plot with T5 had the highest concentration of total Ca, but those with T2 and T1 had similar concentrations. However, the soil exchangeable Ca was not significantly affected by the treatment differences. The soil exchangeable Na showed significant differences among the treatments. As shown in Table 5, the T5 plot had the highest Na concentration, followed by T2, T1, T3, and T4 plots. Although chemical fertiliser treatment had the highest soil nutrient availability in some variables, the surge in the availability of these base cations was due to the inherent high content of the base cations in the organic amendment (Table 2). This is because chemical fertilisers are more soluble, making it easier for plant uptake (Zaki et al., 2018); at the same time, nutrients are prone to leaching from the soil (Cai et al., 2019). However, the co-application of organic amendment and chemical fertiliser can prevent nutrients from leaching. It has been demonstrated co-application of organic amendment and inorganic fertiliser can retain ammoniacal-N because of the high content of cations such as K, Ca, and Mg, the ability of organic matter to adsorb ammoniacal-N, and the high CEC of the compost is retained by clay minerals (Omar et al., 2021). Co-application of organic amendment and chemical fertilisers and/or lime increased the soil available P because organic matter mineralisation releases organic acids, which have a high affinity for Al and Fe ions because of their highly negatively charged sites. This reaction decreases P fixation; at the same time, the organic amendment is a good source of available P because of a higher P concentration than needed for mineralisation (Fekadu et al., 2018; Rusli et al., 2022). The overall characteristics of the organic amendment, which include the decomposition of organic matter and the release of humic substances to the soil, have a positive effect on the soil's exchangeable basic cations and can influence soil health and aggregate stability (Rusli et al., 2022). As dolomite was initially applied during the land preparation, and with the addition of organic amendment and chemical fertiliser, this also increased the soil total Ca (Islam et al., 2021).

Table 5

Effects of treatments on the soil Total P, K, Ca, and Mg after the field trial

Treatments	ppm			
	Total P	Total K	Total Ca	Total Mg
T1	67.84 ^{ab} ± 11.67	628.75 ^a ± 187.75	518.75 ^b ± 18.85	542.70 ^a ± 86.62
T2	62.83 ^b ± 10.45	668.44 ^a ± 1.81	612.50 ^b ± 37.50	401.69 ^a ± 70.94
T3	120.41 ^a ± 5.89	717.38 ^a ± 130.74	754.17 ^b ± 117.33	502.25 ^a ± 83.00
T4	82.75 ^{ab} ± 3.46	660.38 ^a ± 27.38	870 ^{ab} ± 94.19	476.21 ^a ± 40.12
T5	90.91 ^{ab} ± 8.59	494.76 ^a ± 62.13	1233.30 ^a ± 158.33	304.69 ^a ± 40.69

Note. Means within the column with different letter(s) indicate significant differences between treatments by Tukey's HSD test at $p \leq 0.05$

The overall finding suggests that increasing the rate of organic amendment did not result in a proportional increase in total above-ground maize biomass. However, there were differences in the maize fresh cobs' weight. Treatments 2 caused the highest fresh cob yield, followed by T4, T3, T5, and T1 (Table 6), suggesting that the combined use of the organic amendment and inorganic fertiliser was more effective than using the inorganic fertiliser only. The study conducted by Omar et al. (2020) revealed similar results where the co-application of chemical fertiliser and organic amendment, and additional clinoptilolite zeolite improved the maize yield because of the mineralisation of organic N to inorganic forms, beneficial for maize uptake. This is also consistent with the findings of Islam et al. (2021), who reported that co-application of lime and organic amendment increased crop yield, nutrient uptake, and soil nutrient availability. Similarly, Cai et al. (2019) suggest that the application of manure has a major role in improving the soil environment for regulating crop growth, especially nutrient availability and the community of soil microbes. Zaki et al. (2018) demonstrated that compost and mulch can increase plant height, cob weight, and biomass. In a related study, the organic amendment and inorganic fertiliser enhance physiological processes in plants, which include effective translocation of nutrients for grain formation and improvement in the stomata conductance and photosynthesis rate of the crop (Ali et al., 2023); these physiological processes enhance the overall crop development and growth.

An improvement in the soil nutrient availability in the plots with the organic amendment caused the overall improvement in the maize plants' growth and development. Table 7 shows that the fresh weight of the maize plants' leaves

Table 6
Effects of treatments on the soil available P and exchangeable K, Ca, and Na after the field trial

Treatments	ppm				
	Available P	Exchangeable K	Exchangeable Ca	Exchangeable Mg	Exchangeable Na
T1	6.54 ^b ± 1.02	60.62 ^a ± 9.03	490.80 ^a ± 60.08	111.81 ^a ± 4.34	39.16 ^{bc} ± 0.61
T2	7.55 ^b ± 0.49	70.93 ^a ± 1.86	349.48 ^a ± 110.79	111.96 ^a ± 1.39	43.27 ^b ± 1.10
T3	17.29 ^a ± 1.82	75.95 ^a ± 13.32	340.96 ^a ± 68.74	106.88 ^a ± 1.30	39.29 ^{bc} ± 1.35
T4	7.37 ^b ± 0.14	56.46 ^a ± 5.20	314.91 ^a ± 36.25	113.64 ^a ± 1.35	34.25 ^c ± 1.04
T5	10.09 ^b ± 2.32	63.49 ^a ± 3.96	199.99 ^a ± 12.09	111.36 ^a ± 2.15	50.90 ^a ± 2.60

Note. Means within the column with different letter(s) indicate significant differences between treatments by Tukey's HSD test at $p \leq 0.05$

Table 7
Effects of the chemical fertiliser and different amounts of organic amendments on maize fresh cob yield and above-ground biomass of maize plants

Treatments	Fresh Weight (kg/plot)			
	Cob	Leaves	Stem	Total Weight (leaves + stem)
T1	0.83 ^c ± 0.09	1.10 ^b ± 0.12	2.27 ^b ± 0.43	3.37 ^b ± 0.54
T2	7.85 ^a ± 0.45	1.45 ^{ab} ± 0.25	3.95 ^{ab} ± 0.25	5.40 ^{ab} ± 0.50
T3	4.20 ^{bc} ± 1.25	1.65 ^{ab} ± 0.05	4.07 ^a ± 0.19	5.67 ^a ± 0.24
T4	5.55 ^{ab} ± 0.65	1.93 ^a ± 0.03	4.93 ^a ± 0.54	6.87 ^a ± 0.57
T5	2.33 ^{bc} ± 0.32	1.70 ^{ab} ± 0.15	3.77 ^{ab} ± 0.27	5.47 ^{ab} ± 0.33

Note. Means within the column with different letter(s) indicate significant differences between treatments by Tukey's HSD test at $p \leq 0.05$

significantly differed for T4 (50% organic amendment with chemical fertiliser) and T1 (Chemical fertiliser only). The fresh weight of the leaves of the maize plants was higher with T4 (1.93 kg/plot) than the other treatments, and T1 had the lowest fresh weight of leaves (1.10 kg/plot). Similarly, the fresh weight of the stem and the total weight of the maize plant (Leaves + stem) were significant for T1 (Chemical fertiliser only), T3, and T4 (Organic amendment with chemical fertiliser). The mineralisation of organic matter to inorganic forms amplified the soil nutrient availability for plant uptake and other effects of compost, such as improved microbial activity (Saleem et al., 2017). Treating the soil

with the organic amendment populates the soil with beneficial microbes, which is valuable for crop growth. A study by Su et al. (2022) showed the presence of microbes beneficial for tomato growth and positively correlated with the shoot and total dry weight of the tomato. The organic amendment may also have improved the physical structures of the treated soil, enabling better water and nutrient absorption, improving root penetration and aeration, and thus, better growth and development of the maize plant (Ch'ng et al., 2014; Yaqoob et al., 2020).

CONCLUSION

The overall study demonstrated that the co-application of organic amendment and chemical fertiliser can improve some of the chemical properties of the acid soil in the present study. The organic amendment significantly increases soil total and available P, total Ca, and exchangeable Na. Moreover, the approach improves maize fresh cob yield and above-ground biomass of the maize plants. This study suggests organic amendment regains soil health and alleviates P fixation in acid soils that translates into enhanced growth and development of maize plants. The outcome of this present study provides an opportunity to improve the Brunei soil condition of low P availability, crop production, and effective poultry waste management and utilisation. However, this study is limited by one planting cycle, which may restrict the research findings. This limitation does not diminish the study's overall result. Still, it opens the way for to improving the research, such as conducting two planting cycles to understand the residual effect and assess the sustainability of the amendment and the uptake of P in plants.

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Species Composition and Genetic Characterisation of *Acetes intermedius* and *Acetes japonicus* from the Coastal Waters of Terengganu, Malaysia

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ABSTRACT

This study investigates the diversity and species composition of *Acetes* sp. from selected coastal waters of Terengganu, Malaysia. Morphological and molecular identification using mitochondrial DNA COI genes were employed to differentiate species and assess genetic variation. The results revealed the presence of two species, *Acetes intermedius* and newly discovered *Acetes japonicus* in Terengganu waters. *A. intermedius* was found in four locations, Pengkalan Gong Batu, Jeti Marang, Jeti Seberang Takir, and Rhu Muda, while *A. japonicus* was more prevalent in two locations, Pantai Air Tawar and Pengkalan Gong Batu. Genetic analysis identified 10 haplotypes in *A. intermedius* and 8 haplotypes in *A. japonicus*, indicating a relatively high level of genetic diversity within both species. These findings contribute to enhancing our knowledge of *Acetes* diversity along the eastern coast of Peninsular Malaysia, serving as a foundation for future investigations into the ecology and fisheries management of this significant genus.

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INTRODUCTION

The genus *Acetes* (Sergestidae) comprises a group of small shrimps inhabited the coastal and estuarine waters across the Indo-Pacific

waters (Omori, 1975). Known for their ecological significance and economic value, *Acetes* species are frequently harvested for use in various regional fisheries, particularly in the production of shrimp paste and as an ingredient in local cuisine (Food and Agriculture Organisation of the United Nations [FAO], 2008; Vase et al., 2021). Despite their widespread occurrence in Malaysian waters, studies on *Acetes* have primarily focussed on the west coast of Peninsular Malaysia, where aspects such as population dynamics and ecology (Arshad et al., 2012; Arshad et al., 2007; Amani et al., 2011a; Amani et al., 2011b; Nurul Amin et al., 2009; Nurul Amin, 2008; Nurul Amin et al., 2008) and environmental impacts (Rahouma et al., 2012; Rahouma et al., 2013) have been well documented. However, relatively low knowledge of the diversity and species composition of *Acetes* in the east coast region, particularly in Terengganu waters.

Eight out of the sixteen described *Acetes* species were recorded in Malaysian coastal waters: *Acetes erythraeus* Nobili, 1906, *Acetes indicus* H. Milne Edwards, 1830, *Acetes intermedius* Omori, 1975, *Acetes japonicus* Kishinouye, 1905, *Acetes serrulatus* Krøyer, 1859, *Acetes sibogae* Hansen, 1919, *Acetes vulgaris* Hansen, 1919 and *Acetes omori* Hanamura, Imai & Hardianto, 2024 (Amani et al., 2011a, 2011b, 2011c; Arshad et al., 2012, 2013, 2014; Hanamura et al., 2024; Nurul Amin, 2011; Nurul Amin et al., 2012; Omori, 1975). Most studies on *Acetes* have concentrated on the west coast of Peninsular Malaysia, addressing topics such as population dynamics and ecological aspects (Amani et al., 2011a, 2011b; Arshad et al., 2012; Arshad et al., 2007; Nurul Amin et al., 2009; Nurul Amin, 2008; Nurul Amin et al., 2008) as well as environmental impacts (Rahouma et al., 2012). Molecular studies on *Acetes* remain limited, primarily focussing on the west coast of Peninsular Malaysia (Aziz et al., 2010; Maktar, 2013; Wong et al., 2017; Wong, 2013) and Sarawak (Hassan & Othman, 2021). In contrast, research on *Acetes* species in Terengganu has been sparse, with only a few studies investigating heavy metal content (Rahouma et al., 2013). Earlier works by Omori (1975) and Pathansali (1966) documented the presence of *Acetes* species in this region. However, no recent studies have examined the species composition of *Acetes* in Terengganu.

According to the report by the Department of Fisheries Malaysia, the total catch of *Acetes* in Malaysia experienced significant fluctuations from 2013 to 2023. It started at 36,207 tonnes in 2013, increasing to a peak of 43,001 tonnes in 2015. Subsequently, there was a decline until 2021 (41,968 tonnes), followed by a slight recovery in 2022 (27,957.48 tonnes) and 2023 (33,620.14 tonnes). The overall *Acetes* landings in Terengganu have been very low from 2013 to 2023. From 2013 to 2021, there were no recorded *Acetes* landings except for a small catch of two tonnes in 2015. A slight increase in landings was observed in 2022 (0.2 tonnes) and 2023 (2.4 tonnes). The overall trend for *Acetes* landings in both Malaysia and Terengganu is similar, with an initial increase followed by a decline and then a recent recovery. Both Malaysia and Terengganu have seen an increase in *Acetes* landings in recent years (2021-2023). The west coast of Malaysia is the main source of *Acetes*, with Perak generally leading in production but in 2013, Selangor surpassed

Perak as the top producer (Department of Fisheries Malaysia, 2013; 2014; 2015; 2016; 2017; 2018; 2019; 2020; 2021; 2022; 2023).

The goal of the present study is to address the knowledge deficiency by examining the diversity and species composition of *Acetes* in Terengganu waters, combining both morphological and molecular approaches. By identifying the species present and analysing their distribution, this research seeks to contribute valuable information to the understanding of *Acetes* diversity on the east coast of Peninsular Malaysia and provide a baseline for future studies on the ecological and fisheries management of this important genus.

MATERIALS AND METHODS

Acetes Sampling

During the present study, samples of *Acetes* were collected with the help of local fishermen from Pengkalan Gong Batu, Jeti Seberang Takir and Jeti Merang in April 2023, while from Pantai Air Tawar and Rhu Muda in February 2024 (Figure 1). *Acetes* shrimps were collected from inshore push nets and bottom trawling. All geographic locations were listed using their original language names (Table 1). *Acetes* samples were purchased from the local fishermen. The samples were immediately kept in 4% formalin and absolute ethanol for morphological identification and molecular identification, respectively. The samples were then taken to the laboratory for further analysis.

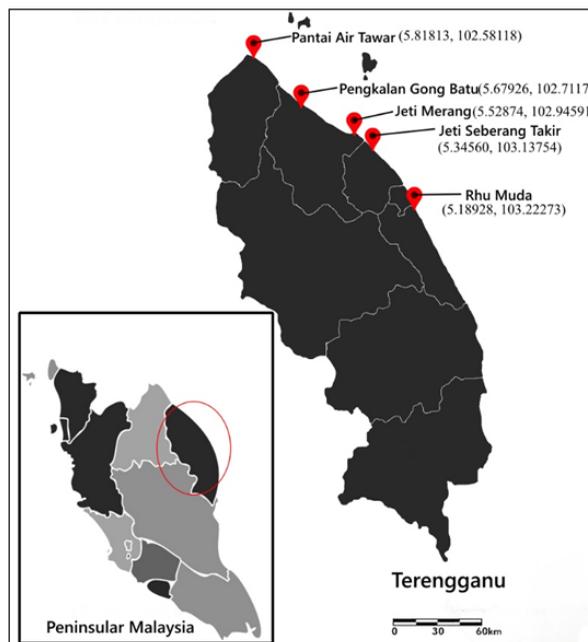


Figure 1. Sampling location of *Acetes* in Terengganu waters

Table 1
Sampling locations of Acetes in Terengganu waters

Sampling Location (Abbreviation)	Coordinate	Sampling Method
Pantai Air Tawar (PAT)	(5.81813, 102.58118)	In-shore
Pengkalan Gong Batu (GB)	(5.67926, 102.71175)	Off-shore
Jeti Merang (M)	(5.52874, 102.94591)	Off-shore
Jeti Seberang Takir (ST)	(5.34560, 103.13754)	Off-shore
Rhu Muda (RM)	(5.18928, 103.22273)	Off-shore

Species Identification, Sex Ratio, and Species Composition

Acetes were examined using a stereo microscope (Olympus SZ51, Japan) and identified according to Omori's (1975) keys and terminology. Antennular, carapace and total length of each *Acetes* were measured. The images were captured using a compound microscope (Leica Microsystems DM750, Germany) attached with a camera (Leica Microsystems ICC50 E, Germany). Drawings were made using a digital inking method using Krita drawing software in Samsung Galaxy Tab S7 FE. The petasma, genital coxae and clasping spines on the lower antennular flagellum were present in males in accordance with Omori (1975). Species composition was determined by calculating the percentage of each *Acetes* species among 30 randomly selected tails from each location (N=30) (Simões et al., 2013).

Statistical Analysis

Chi-square tests were used to determine the sex ratio for each species (Sokal and Rohlf, 1995) and were carried out using Minitab 17. The significance level was set at $P < 0.05$.

DNA Extraction, Amplification, and Sequencing

A total of 55 individuals were used in the molecular study, as shown in Table 2. Following morphological identification, *Acetes intermedius* and *A. japonicus* were identified from the Terengganu samples. For molecular analysis, 10 specimens of each species from every location were selected. However, there were insufficient *A. intermedius* samples from GB and RM for analysis due to a lack of specimens in the collected samples.

DNA was extracted using PrimeWay Genomic DNA Extraction Kit (Apical Scientific Sdn. Bhd., Malaysia). The extraction methods were carried out according to the manufacturer's manual. The amplification of the 634 bp mitochondrial DNA cytochrome c oxidase subunit I (COI) gene was performed using PCR using the universal primer LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al., 1994).

Each PCR reaction mixture contained 12.5 μ L of MyTaq™ Red Mix (Bioline), 0.5 μ L of each primer (Integrated DNA Technologies Pte. Ltd., Singapore), 2 μ L of DNA template

Table 2

Sampling locations, number of samples and field voucher of *Acetes* for molecular study

Species	Sampling Locations*	No. of Samples	Field Voucher
<i>Acetes intermedius</i>	GB	7	GBI2-GBI5, GBI7-GBI9
	M	10	M1-M10
	ST	10	ST1-ST10
	RM	8	RM1-RM5, RM7, RM9, RM10
<i>Acetes japonicus</i>	PAT	10	PAT1-PAT10
	GB	10	GBJ1-GBJ3, GBJ6-GBJ10, GBI1, GBI6

and the volume was filled to 25 μ L with ultra-pure water. The PCR protocols was carried out using Applied Biosystems Veriti 96-well Thermal Cycler (Thermo Fisher Scientific, USA) according to the following profile: initial denaturation at 94°C for 60 seconds, followed by five cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 90 seconds, and extension at 72°C for one minute which was followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 90 seconds, and extension at 72°C for 60 seconds and a final extension at 72°C for five minutes (Costa et al., 2007; Hebert et al., 2003; Wong et al., 2017). The PCR products were submitted to Apical Scientific Sdn. Bhd. for Sanger sequencing.

Phylogenetic Analysis

The sequences were aligned using Multiple Alignment using Fast Fourier Transform (MAFFT) and queried in the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) GenBank. The aim was to assess the sequence similarity between current study sequences and existing GenBank entries (Norshida et al., 2021). In order to avoid false positives, only top matches with $\geq 94\%$ sequence similarity were retained (Khaleel et al., 2020). Haplotype (h) and nucleotide (π) diversities (Nei, 1987) were calculated using DnaSP v.6 (Rozas et al., 2017) based on segregating sites (S). Analysis of molecular variance (AMOVA) and pairwise F_{ST} was conducted in ARLEQUIN v3.5 (Excoffier & Lischer, 2010) to assess genetic population differentiation, using 1000 permutations. Two neutrality tests, Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997), were examined in ARLEQUIN v3.5 to determine the deviation of studied populations from genetic equilibrium (Kamarudin et al., 2017).

A maximum likelihood (ML) tree was constructed using IQ-TREE (Trifinopoulos et al., 2016) under the best-fit substitution model selected by Bayesian information

criterion (BIC) in ModelFinder (TIM+F+G4) (Kalyaanamoorthy et al., 2017). The bootstrapping was set for 1000 replications. A maximum parsimony (MP) tree was inferred using a heuristic search with TBR optimisation in PAUP (Swofford, 2021). The dataset was subjected to 1000 bootstrap replications. Nodal support values were retrieved using non-parametric bootstrapping with 1000 replicates and 10 random addition sequence replicates. *Belzebub faxoni* (Borradaile, 1915), the outgroup, was set as the root for all trees (GenBank Accession Number: KY449077), which follows the latest global phylogeny (Simões et al., 2023; Vereshchaka et al., 2016; Vereshchaka, 2017). Other sequences retrieved from NCBI GenBank included *Acetes japonicus* (acc. no: HQ630564, KF977240, OP420186 and OP420185), *A. indicus* (acc. no: LC804564 - LC804567), *A. omorii* (acc. no: LC804570 - LC804574), *A. spiniger* (acc. no: LC804568 and LC804569), *A. serrulatus* (acc. no: LC804579, HQ630498 and HQ630499) and *A. sibogae* (acc. no: HQ630582 and HQ630583). The tree was visualised using FigTree (Rambaut, 2018).

RESULTS AND DISCUSSION

Taxonomic Accounts

The "Material Examined" sections detail the number of specimens examined, which are broken down by sex. Additionally, the total length (in millimetres) is provided, where this measurement is taken from the tip of the rostrum to the apex of the telson.

Acetes intermedius Omori, 1975

Material Examined from Terengganu, Malaysia. – Pengkalan Gong Batu (GB): 6 ♂, 12.70-18.80 mm, 7 ♀, 15.20-20.20 mm Jeti Merang (M): 17 ♂, 13.40-24.70 mm, 13 ♀, 15.20-27.80 mm; Jeti Seberang Takir (ST): 16 ♂, 17.30-22.30 mm, 14 ♀, 16.40-23.70 mm; Rhu Muda (RM): 23 ♂, 14.60-23.70 mm, 7 ♀, 15.50-22.80 mm (Figures 2 and 3).

Diagnosis – Male antennular flagellum consisted of 11-13 segments and a single clasping spine (Figure 2). The clasping spine exhibited a row of small teeth, differing from the large teeth described by Omori (1975). The main branch's first segment bore 3 marginal spinules, while the segment opposing the clasping spine tip had 4 spinules. The clasping spine was longer, and the number of setae was fewer than those reported by Omori (1975). Similar to Omori (1975), the petasma was equipped with 3 large falcate hooks and several smaller ones. The large hooks exhibited a gradual increase in size towards their distal end. There was no clasping spine present on the female antennular flagellum. Petasma was absent in the female. The anterior part of the third thoracic sternite displayed a pair of protuberances (Figure 3). Unlike the significantly concave sternite described by Omori (1975), the present specimen showed only a slight concavity in the median part. The apex of the telson was truncated. In general, females tend to be larger than males in

terms of all three measurements, suggesting potential sexual dimorphism. However, in GB, the males have a slightly larger average carapace length. *A. intermedius* from M tends to be larger in all measurements compared to other locations. ST also shows relatively larger sizes compared to RM and GB. This indicates potential geographical variations in *A. intermedius* size. The total length ranges in the current study were generally consistent with those reported by Omori (1975). There was a slight overlap in the female size ranges, but the male size range in the current study was slightly narrower.

Distribution – Melaka (Klebang Besar), Terengganu (Seberang Takir and Marang) and Sarawak (Bintulu and Miri).

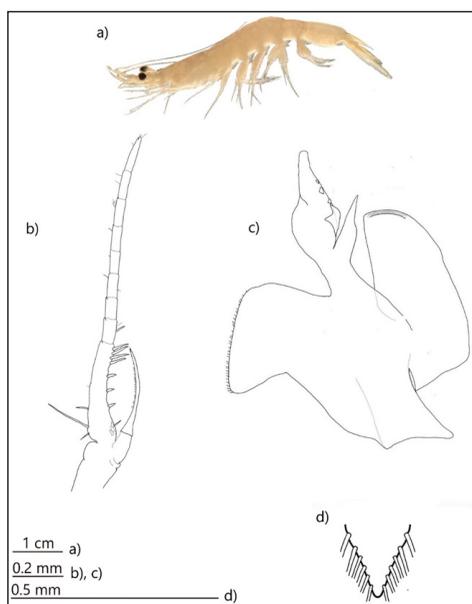


Figure 2. *Acetes intermedius*. Male: a) dorsal view; b) lower antennular flagellum; c) petasma; d) apex of telson. a), d) from Rhu Muda; b) from Jeti Merang; c) from Jeti Seberang Takir

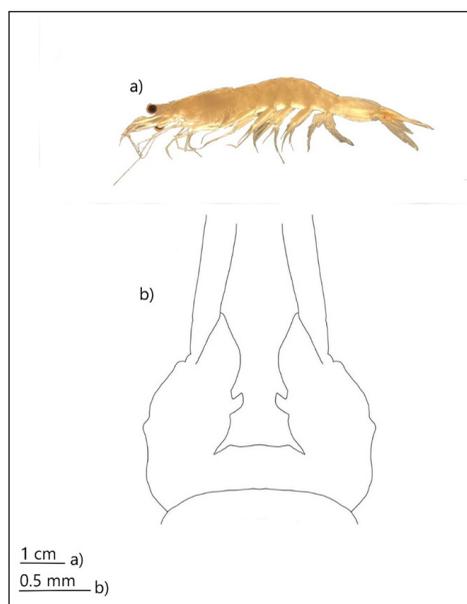


Figure 3. *Acetes intermedius*. Female: a) dorsal view; b) third thoracic sternite, ventral view. a) from Rhu Muda; b) from Jeti Seberang Takir

Acetes japonicus Kishinouye, 1905

Synonym: *Acetes cochinensis* Rao, 1970; *Acetes dispar* Hansen, 1919

Material Examined from Terengganu, Malaysia. – Pantai Air Tawar (PAT): 10 ♂, 8.60-12.20 mm, 20 ♀, 7.90-17.30 mm; Pengkalan Gong Batu (GB): 14 ♂, 12.30-22.80 mm, 3 ♀, 14.90-15.10 mm (Figures 4 and 5).

Diagnosis – The male has 9-11 segments of the antennular flagellum, with a double clasping spine (Figure 4). The longer clasping spine has a row of teeth on its inner surface near the tip. The first segment of the main branch has a single basal spinule, while the segment opposite the clasping spine tip has 3-4 spinules. The remaining segments lack

minute spinules, unlike those described by Omori (1975). The distal part of the capitulum of the petasma is bulbous and has numerous hooks of varying sizes. The processus ventralis extends beyond the capitulum, unlike in Omori's (1975), which was shorter. These differences may be related to the stage of development (Omori, 1975). There was no clasping spine present on the female antennular flagellum. Petasma was absent in the female. The third thoracic sternite of females were produced posteriorly as shown in Figure 5, with a deeper emargination on the posterior margin than described by Omori (1975). The apex of the telson was rounded. Males tend to be slightly larger than females in terms of antennular length and carapace length, while there is no significant difference in total length. However, this trend is not consistent across all locations. *A. japonicus* from GB tends to be larger in all aspects compared to other locations. PAT has the smallest average size for all measurements. This indicates potential geographical variations in *A. japonicus* size. The total length range in the current study was significantly smaller than that reported by Omori (1975), especially for the winter populations.

Distribution – Melaka (Klebang Besar), Perak (Kuala Gula, Kuala Kurau, Kuala Sepetang and Matang Mangrove Forest Reserve), Pulau Pinang (Gelugor, Batu Maung, Bagan Ajam, Teluk Bahang and Pantai Bersih), Kedah (Kuala Sala, Sungai Udang, Tanjung Dawai and Sungai Merbok), Perlis (Sungai Berembang and Sungai Baharu), Sarawak (Miri) and Johor (Pontian).

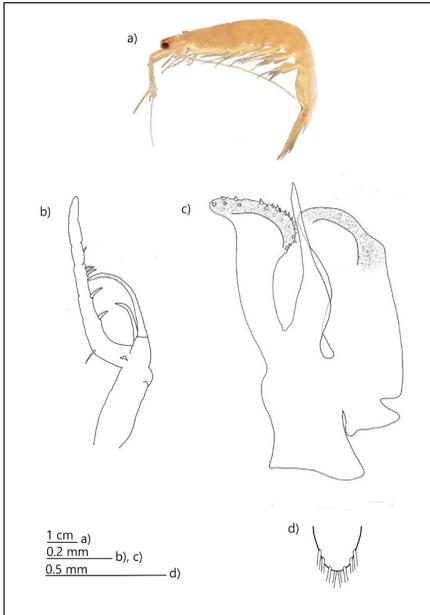


Figure 4. *Acetes japonicus*. Male: a) dorsal view, b) lower antennular flagellum; c) petasma; d) apex of telson. a), b), c) from Pengkalan Gong Batu; d) from Pantai Air Tawar

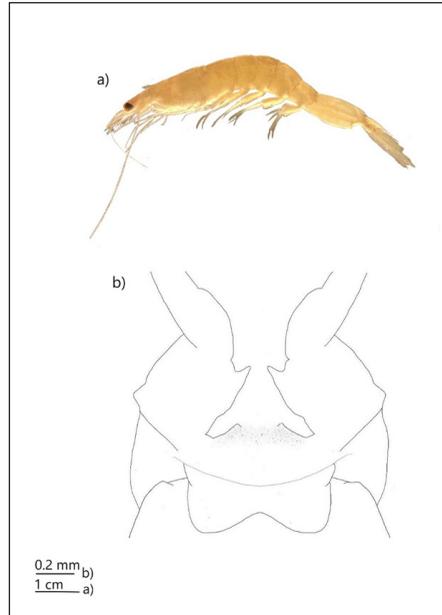


Figure 5. *Acetes japonicus*. Female: a) dorsal view, b) third thoracic sternite, ventral view. a), b) from Pengkalan Gong Batu

Species Composition

The species composition of *Acetes* in the study is shown in Figure 6. *Acetes intermedius* is the dominant species, with a composition of 100% in locations ST, M and RM. *A. japonicus* is present in locations GB and PAT, with a composition of 43.33% in GB and 100% in PAT. The species composition varies across locations. While *A. intermedius* is dominant in most areas, *A. japonicus* is present in GB and PAT.

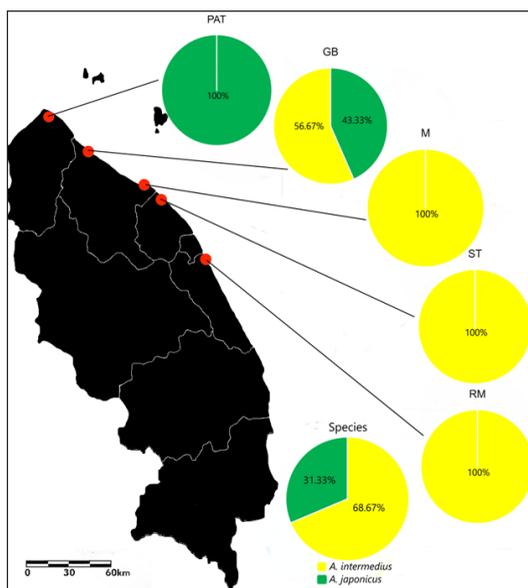


Figure 6. The species composition of *Acetes* in Terengganu waters

Sex Ratio

A mean sex ratio was observed with 0.74♀:1♂, favouring males as in Table 3. Pianka (2011) suggested a balanced sex ratio in dioecious organisms; however, Wenner (1972) found that crustaceans often exhibit skewed sex ratios due to factors such as differential growth, migration patterns, sex changes, and feeding habits. The factors should be further investigated to determine their roles which resulted in the observed sex ratio. Our results align with those of Amin et al. (2008) for *A. intermedius* and Arshad et al. (2008) for *A. vulgaris*.

Table 3

Number of males and females of *A. japonicus* and *A. intermedius*, with the resulting sex ratio and χ^2 tests

Species	F	M	Total	Sex Ratio (♀: ♂)	χ^2 Test	P-value
<i>A. japonicus</i>	23	24	47	0.96:1	0.02	>0.05
<i>A. intermedius</i>	41	62	103	0.66:1	4.28	<0.05
Total	64	86	150	0.74:1	3.23	>0.05

Molecular Analysis

Genetic Diversities

The haplotype composition, segregating sites (S), number of haplotypes (N_{hap}), haplotype (gene) diversity (h), and nucleotide diversity (π) for *Acetes intermedius* were tabulated in Table 4, and *A. japonicus* were tabulated in Table 5. Ten haplotypes are present for *A. intermedius*, as in Table 4. The Ai1 haplotype occurred most in four sampling locations. Samples from RM recorded the highest haplotype and nucleotide diversities with 0.8095 and 0.0085, respectively. The high haplotype and low nucleotide diversities resulted from a fast population growth from a small ancestral population, allowing for an increase in haplotype diversity through mutations, but limiting the accumulation of significant sequence differences (Chanthran, et al., 2020).

There were eight haplotypes present for *Acetes japonicus*, as in Table 5. The Aj1 haplotype was the most common and occurred in two sampling locations. Samples from GB recorded the highest haplotype (0.8667) and nucleotide (0.0038) diversities. The overall genetic diversity in the present study was 0.5898, surpassing the 0.5386 reported by Wong et al. (2017). Similarly, the nucleotide diversity in the current study, 0.0026, was

Table 4
Haplotype and molecular diversity in *Acetes intermedius*

Haplotype	Sampling Locations*				Total
	GB	M	ST	RM	
Ai1	5	8	6	3	22
Ai2	1		2		3
Ai3			2		2
Ai4	1				1
Ai5				1	1
Ai6		1			1
Ai7		1			1
Ai8				2	2
Ai9				1	1
Ai10				1	1
n	7	10	10	8	35
S	2	5	3	14	21
N_{hap}	3	3	3	5	10
h	0.5238	0.6444	0.7556	0.8095	0.6706
π	0.0009	0.0028	0.0017	0.0085	0.0043

Note. *Abbreviations for sampling locations: refer to Table 1 [n : number of sequences; S : number of segregating sites; N_{hap} : number of haplotypes; h : haplotype diversity; and π : nucleotide diversity]

Table 5
Haplotype and molecular diversity in *Acetes japonicus*

Haplotype	Sampling Locations		Total
	PAT	GB	
Aj1	8	5	13
Aj2		1	1
Aj3		1	1
Aj4		1	1
Aj5		1	1
Aj6		1	1
Aj7	1		1
Aj8	1		1
<i>n</i>	10	10	20
<i>S</i>	4	10	13
<i>N_{hap}</i>	3	6	8
<i>h</i>	0.3778	0.8667	0.5898
π	0.0013	0.0038	0.0026

Note. *Abbreviations for sampling locations: refer to Table 1 [*n*: number of sequences; *S*: number of segregating sites; *N_{hap}*: number of haplotypes; *h*: haplotype diversity; and π : nucleotide diversity]

notably higher than the 0.0010 found in the earlier study. In comparison to a previous study by Wong et al. (2017), which identified only two haplotypes of *A. japonicus* from three sampling locations, the current study shows a significantly higher genetic diversity in *A. japonicus*. A larger sample size in the current study might have revealed more haplotypes and increased the overall genetic diversity.

Population Differentiation

The AMOVA of every *Acetes* species were shown in Table 6. The F_{ST} value for *Acetes japonicus* was low (0.0584) and not significant ($P > 0.05$). This shows that there was a small genetic differentiation among the populations.

The percentage variation within the populations of *A. japonicus* was high (94.16%). This suggests that the majority of genetic variation occurred within the populations. In contrast to a previous study by Wong et al. (2017), which reported a significantly higher within-population variation (120.28%) and a significantly lower among-population variation (-20.28%), the current study found a higher percentage of variation among populations (5.84%) and a lower within-population variation (94.16%). It is noteworthy that the F_{ST} values in both studies were not significant, suggesting that the observed genetic differences among populations were not statistically significant. The F_{ST} value for *A. intermedius* was

Table 6
AMOVA of Acetes species

Species	Source of Variation	df	Sum of Squares	Variance Components	Percentage Variation	F _{ST} Value	P-value
<i>Acetes japonicus</i>	Among populations	1	0.900	0.0344	5.84	0.0584	P>0.05
	Within populations	18	10.000	0.5556	94.16		
	Total	19	10.900	0.5900			
<i>Acetes intermedius</i>	Among populations	3	12.860	0.389	28.11	0.2811	P<0.05
	Within populations	30	29.875	0.9959	71.89		
	Total	33	42.735	1.3852			

higher (0.2811) and significant ($P < 0.05$). This suggests moderate genetic differentiation among populations of *A. intermedius*. The percentage variation within populations of *A. intermedius* was lower (71.89%), which indicates that a smaller proportion of genetic variation is found within populations compared to *A. japonicus*. *A. japonicus* exhibits a lower F_{ST} than *A. intermedius*, suggesting greater gene flow and less genetic divergence, which confirms no variations among populations of *A. japonicus* (Jose et al., 2022).

The pairwise F_{ST} analysis of *Acetes intermedius* in Table 7 revealed a low genetic differentiation between the studied populations. The genetic similarity observed between the populations was high, which implies significant gene flow, which shares genetic material likely due to migration.

Tajima's D and Fu's Fs neutrality tests were shown in Table 8. All the Neutrality Tests in Table 8 were significant for *Acetes japonicus*, while *A. intermedius* showed only significance in Tajima's D. The negative values of Tajima's D, -1.5426, and Fu's Fs, -4.8112, of *A. japonicus* were indicative of population expansion. The negative value of Tajima's D and Fu's Fs suggests an excess of rare alleles in the population that is often

Table 7
The pairwise F_{ST} of Acetes intermedius

	GB	M	RM	ST
GB	-			
M	-0.02661	-		
RM	0.05185	0.03536	-	
ST	-0.0453	0.01688	0.03962	-

Note. *Abbreviations for sampling locations: refer to Table 1 (P<0.05)

Table 8
Neutrality statistics (Tajima's D and Fu's Fs)

	Tajima's D	Tajima's D P-value	Fu's Fs	Fu's Fs P-value
<i>A. japonicus</i>	-1.5426	0.0250	-4.8112	0.0000
<i>A. intermedius</i>	-1.6600	0.0290	0.1290	0.0645

shown as a recent population bottleneck (Eckshtain-Levi et al., 2018; Jose et al., 2022). However, the statistical significance of these findings differs when the P-value for Tajima's D, 0.0250, and for Fu's Fs, 0.0000, suggests a statistically significant deviation from neutrality. These results contrast with a previous study by Wong et al. (2017); Tajima's D, 1.4754, and Fu's Fs, 1.2350, were not significant.

Phylogenetic Tree

Phylogenetic analyses of *Acetes* samples from Terengganu using maximum likelihood (ML) and maximum parsimony (MP) methods yielded consistent tree structures, supporting the division of the genus into two primary clades: *Acetes intermedius* and *Acetes japonicus* (Figure 7). These findings aligned with morphological species identification. Both clades exhibited strong statistical support, with bootstrap values exceeding 70% for both ML and MP. However, subclades within these clades displayed lower bootstrap values, suggesting that a larger sample size might be necessary to enhance their statistical support (Hassan and Othman, 2021).

Notably, an individual of the *A. japonicus* specimen from Malaysia (HQ630564) clustered with the Terengganu *A. japonicus* group, suggesting a genetic connection between the two populations. *A. japonicus* reveals two clades, *A. japonicus* 1 and *A. japonicus* 2, which potentially suggest cryptic species. The *A. japonicus* (1) individuals from PAT and GB shared haplotypes, indicating gene flow between these populations. Similarly, *A. intermedius* individuals from GB, M, ST, and RM also exhibited evidence of gene flow. These results are in agreement with previous studies on *A. americanus* (Brazil 1) (Simões, et al., 2023) and *A. erythraeus* (Sarawak) (Hassan & Othman, 2021), which also demonstrated the presence of genetic variation and cryptic lineages within *Acetes* species.

The population's homogeneity may be attributed to the widespread dispersal of planktonic larvae and the absence of factors that restrict gene flow (Simões, et al., 2023). More detailed research is required to establish the cryptic species status of *A. japonicus*. This would involve combining in-depth morphological analyses with molecular techniques that utilise both mitochondrial and nuclear DNA markers (Kerkhove, et al., 2019).

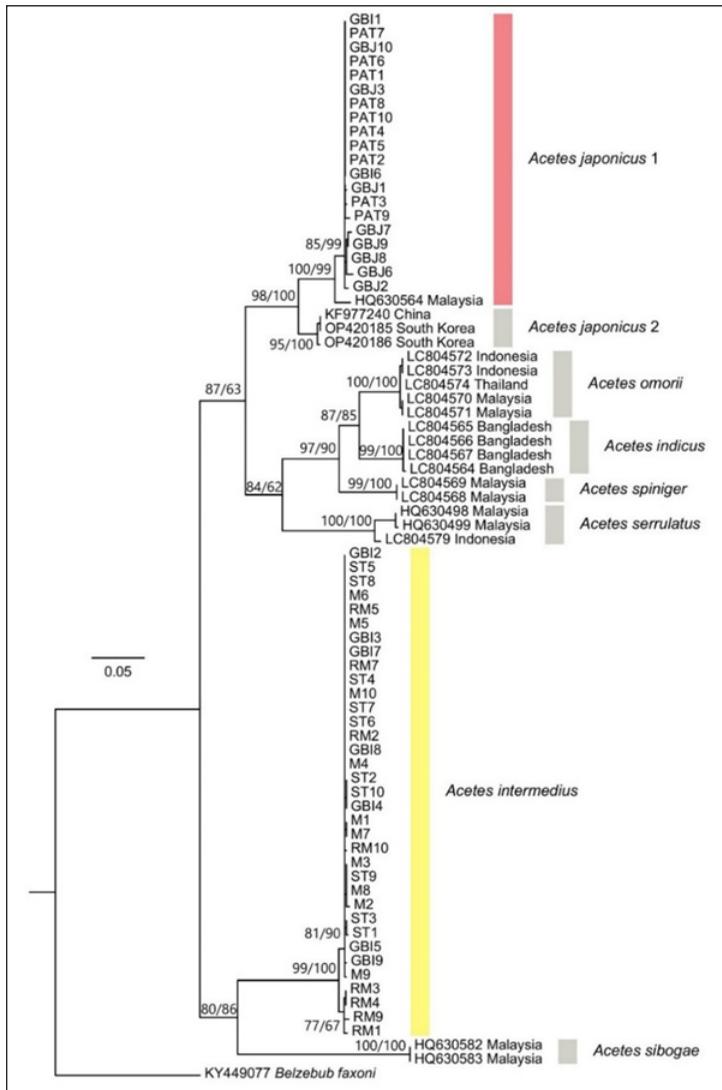


Figure 7. Bootstrap 50% majority rule consensus ML tree of *Acetes intermedius* and *A. japonicus* from Pantai Air Tawar, Pengkalan Gong Batu, Jeti Merang, Jeti Seberang Takir and Rhu Muda with species of *Acetes* acquired from GenBank, *Belzebub faxoni* as outgroup. The values at the node represent ML (%) and MP (%)

CONCLUSION

In conclusion, two *Acetes* species were identified from five *Acetes* landing sites in Terengganu, which are *Acetes intermedius* and *A. japonicus*. *A. japonicus* recorded in the current study is the new record on the east coast of Peninsular Malaysia. *A. intermedius* dominated the *Acetes* population in Terengganu and was present in all sampling sites except for PAT, while *A. japonicus* is only present in GB and PAT. Phylogenetic analysis revealed two distinct clades: *A. japonicus* and *A. intermedius*. Genetic diversity was higher in

A. intermedius with 10 haplotypes compared to 8 in *A. japonicus*. The findings presented in this study represent a preliminary exploration. Further research is suggested to expand upon these results and consider broader sampling locations.

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Optimising Pepper (*Capsicum Annuum* L.) Seedling Growth with the Synergistic Effects of Seaweed Waste Media and Beneficial Microbes Application

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ABSTRACT

In pepper cultivation, seed quality is the main factor affecting plant productivity. The study aims to determine the best composition of seaweed waste seedling media and the mycorrhizal application to enhance pepper seedling growth in the nursery. The study used a Completely Randomised Factorial design and four replications. The first factor is the composition of seedling media: (1) Control (soil + manure), (2) Seaweed waste seedling media without microbes, (3) Seaweed waste seedling media

+ microbes. The second factor is mycorrhizal application: (1) with mycorrhizal application, (2) without mycorrhizal. The research findings indicate that the application of mycorrhizal fungi across three types of seedling media, including standard operating procedure (SOP), non-microbial seaweed, and seaweed with microbes, can enhance pepper seedling growth, chlorophyll content, mycorrhizal infection in roots, and nutrient levels in the seedling media. There is a synergistic effect among microbial consortia, with the addition of mycorrhizae improving pepper seedling growth. Seaweed waste media combined with microbial consortia

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and applied with mycorrhizae is recommended as an adequate soil substitute for enhancing pepper seedling growth.

Keywords: Mycorrhizal, microbes, nursery, pepper, seaweed waste, seedling media

INTRODUCTION

Pepper (*Capsicum annum* L.) is a horticultural commodity with many benefits and high economic value. Capsaicin is the main bioactive compound responsible for the spicy taste of pepper fruit. Capsaicin has various health benefits, including antirheumatic, antibacterial, anti-inflammatory, antirhinitis, and analgesic. Capsaicin also plays an essential role in improving immunity to manage diabetes, lowering cholesterol levels, high blood pressure, and obesity, inhibiting the spread of cancer, treating anaemia, improving eyesight, and supporting hair growth (Chakrabarty et al., 2017; Chung & Campbell, 2016; Idaryani et al., 2021; Saleh et al., 2018; Sanati et al., 2018).

In pepper cultivation, seed quality is a crucial factor that affects plant productivity. The process includes sowing, nursery, and field transfer. Each stage of seedling transfer increases stress, weakens seedlings, and inhibits growth. Efforts to overcome this include sowing pepper seeds in seedling media that can be directly transplanted to the field without disturbing the seedlings. Seaweed waste can be used as a seedling medium, reducing stress during seedling transfer and increasing seedling vigour in the seedling phase. Seaweed waste is an abundant natural resource but has yet to be optimally utilised. Seaweed waste from seaweed blooms and hydrocolloid extraction causes environmental pollution (Dang et al., 2023). The seaweed extraction industry produces liquid and solid waste, with an estimated 89% of the extracted seaweed's Weight ending up as solid waste. Solid waste from the extraction of *Gracilaria* sp. and *Sargassum* has excellent potential to be used as a seeding medium because it has a composition that includes perlite, cellulose, agar, micro and macro minerals, and growth regulators (auxin 191 ppm; GA3 509.5 ppm; cytokinin-kinetin 244.5 ppm, and cytokinin-zeatin 70.5 ppm) (Basmal et al., 2020; Basmal et al., 2019). Perlite itself consists of SiO₂ (75%), Al₂O (14.8%), Fe₂O (1.5%), CaO (0.9%), and K₂O (5.8%), Na₂O (2.9%), and MgO (0.1%) (Cojocararu et al., 2023). Seaweed extraction solid waste can also be a soil conditioner (Ammar et al., 2022; Shang et al., 2023). Application of *Gracilaria* sp. (Pramanick et al., 2016; Prihastanti & Haryanti, 2022) and *Sargassum* sp. (Yusuf et al., 2021) significantly increased plant growth and production. Cocopeat can be added as a filler to a planting medium to increase its water-holding capacity. Cocopeat has advantages such as its light, porous structure, high water holding capacity, neutral pH, natural sterility, and good nutrient retention capacity (Tiwari, 2015).

Adding a microbial consortium to seaweed seed media is one effort to increase the vigour of pepper seeds. The microbial consortium works synergistically to improve the

balance of the soil ecosystem, nutrient availability, and plant resistance to disease and environmental stress. Phosphate- and potassium-solubilising bacteria that can dissolve P and K include *Pseudomonas cepacia*, *P. malei*, *Bacillus mycoides*, *B. subtilis*, *Azotobacter*, *Rhizobium*, *Cyanobacteria*, and *Escherichia*. These bacteria can also produce organic acids that dissolve phosphate and potassium, and enzymes such as phosphatase and phytase break down organic compounds containing phosphate (Patel & Patel, 2022; Rawat et al., 2021).

Arbuscular Mycorrhizal Fungi (AMF) are potential microbes that are widely used in cultivated plants to increase nutrient availability, production, and bioactive content of plants (Sharma et al., 2017; Singavarapu et al., 2024). Mycorrhizal inoculation of pepper plants with 75% irrigation conditions did not reduce pepper production (Calvo et al., 2024). Mycorrhiza increases the production of pineapple crown cuttings and improves seed quality, as measured by root dry Weight, chlorophyll content, root infection, and phosphatase enzyme activity (Putri et al., 2020). The provision of AMF to pepper plants can increase several growth parameters (number of leaves, height, stem diameter, dry Weight of the upper part of the plant) and production (number of fruits, fruit weight), root biomass, plant P content, and chlorophyll (Utari & Rachmawati, 2022) This study determines the composition of seaweed-waste seedling media and the optimal provision of mycorrhizal inoculum to increase the growth of pepper seedlings in the nursery.

MATERIALS AND METHODS

Preparation of Plant Materials and Seedling Media Formulation

The pepper seeds used were of the Kencana variety, suitable for lowland areas. The formulation materials for the seedling media included dried and ground agar-extraction waste (*Gracilaria* sp.) obtained using a hammer mill and dried *Sargassum* sp. cut into *Sargassum* chips. *Sargassum* contains various bioactive compounds, including perlite, cellulose, agar, micronutrients (potassium, nitrogen, phosphorus, calcium, magnesium), and macronutrients; plant growth regulators such as Auxin (191 ppm), GA3 (509.5 ppm), Kinetin (244.5 ppm), and Zeatin (70.5 ppm) (Basmal et al., 2019). The extraction of *Gracilaria* sp. had the macronutrients N (0.20%), P (0.12%), K (0.17%), C-organic (10.96%), Na (0.66%), Ca (0.61%), Mg (0.09%), CEC (13.5 me/100g), N/C ratio of 54:7, as well as the micronutrients Cu (4.80 ppm), Fe (0.24 ppm), Zn (8.42 ppm), Mn (57.58 ppm), B (32.32 ppm). Solid waste also contained growth hormones, including auxins (191 ppm), gibberellin/GA3 (509.5 ppm), kinetin (244.5 ppm), and zeatin (70.5 ppm) (Basmal et al., 2020). Other ingredients included fish meal, molasses, and hydrogel as components of the media mixture. Cocopeat, with a 10-12% moisture content, was used as the filler for the seedling's media.

Table 1
Seedling media formula from seaweed waste

No.	Seedling Media Materials (%)	Composition
1	Agar (<i>Gracilaria</i>) waste	40
2	<i>Sargassum</i>	51
3	Fish flour	7
5	Hydrogel	1
	Total	100

Preparation of *Bacillus* sp, *Pseudomonas* sp, and *Trichoderma* Microbes

The microbes used in this study are *Bacillus* sp., *Pseudomonas* sp., and *Trichoderma* sp. These are known to produce organic acids that solubilise phosphorus and potassium, synthesise phytohormones (Indole-3-acetic acid), secrete enzymes such as phosphatase and phytase, and exhibit antagonistic effects against soil-borne pathogens (Hernández-Rodríguez et al., 2008; Patel & Patel, 2022; Rawat et al., 2021; Yao et al., 2008). The microbes were propagated from a single lyophilised ampoule.

The microorganisms were dissolved in 50 ml of nutrient broth (N.B.) media for *B. subtilis* and *P. fluorescens* and incubated for 24 hours. For the propagation of *Trichoderma* sp., 50 ml of potato dextrose broth (PDB) media was used and incubated for 72 hours. The next stage involved propagating the microbes in a mixture of liquid *Sargassum* extract, fish silage, and molasses. This incubation lasted 15 days at room temperature (23-32°C). Subsequently, the microbes were mixed to form a microbial consortium, which was then added to the media formulation as presented in Table 1.

Mixing seedling media

All the ingredients for the seedling media formulation (Table 1) were mixed until homogeneous. For the microbial seedling media, *Bacillus* sp. (5.4×10^7 CFU/ml), *Pseudomonas* sp. (2.35×10^8 CFU/ml), and *Trichoderma* sp. (2.15×10^4 CFU/ml) were added to the mixture and stirred until well combined. Each medium was then mixed with cocopeat as a filler in a 10:90 ratio and stirred until homogeneous. The physical properties, nutrient content, and plant growth regulators (PGRs) of the seedling media are presented in Table 2.

Forming the seedling media

The seedling media is placed in gauze and then compacted using a hydraulic press with a pressure of 3-4 tons until the dimensions are 1.58 cm in diameter and 9 cm in height.

Table 2

Physical characteristics, nutrient content, and plant growth regulators (PGR) for seaweed seedling media

No	Physical characteristics, nutrient content, and PGR for seaweed seedling media	Value
1	Water Content (%)	35.03
2	Ash Content (%)	11.07
3	Water Holding Capacity (%)	7.83
4	Evaporation (%)	29.95
5	Protein (%)	1.71
6	N Content (%)	1.05
7	P ₂ O ₅ Olsen (ppm)	566.50
8	K ₂ O Morgan (ppm)	9047.0
9	K (cmol c/kg)	16.92
10	Auxin (mg /l)	0.111

Propagation of Mycorrhizal Consortium

Mycorrhizal consortium cultivation and harvesting refer to Patil et al. (2022) and Cahyani et al. (2022), with modifications in zeolite preparation. The zeolite media was washed repeatedly until the rinse water was clear. Corn seeds were planted in the zeolite in polybags sized 15 cm × 30 cm, then inoculated with a mixture of three mycorrhizal genera: *Glomus*, *Gigaspora*, and *Acaulospora*, which have been tested and are compatible and able to enhance the vigour and growth of pepper plants. The inoculum was applied to a depth of approximately 5 cm in the planting holes. The corn plants were maintained until approximately 3 months old. Low-phosphorus liquid fertilisers (organic and inorganic) were applied with watering. Harvesting was performed when the plant's vegetative growth was at its maximum, and the early generative phase was achieved by cutting off the top of the plant and then drying it for approximately 2 weeks. The dried roots were separated from the stem base, cut into pieces, and mixed with the seedling medium, which already contained the mycorrhizal population.

Sowing and Maintenance in the Nursery

Pepper seeds are soaked in water for 2 hours to promote germination. The seedling medium, prepared according to the treatment, is dipped in water for approximately 10 seconds, thereby expanding it up to 4 times its initial volume. After that, one pepper seed is sown in each seedling media hole to a depth of 3 cm. For the control treatment, seeds are sown in polybags with a soil and manure mixture (2:1). Mycorrhiza application is given at 25 grams of Mycorrhiza + carrier (300 spores) according to the treatment after the seeds have been sown. The seedlings are placed on nursery racks in the nursery shed until the plants are 1.5 months old. Watering is done as needed.

Research Design

The study uses a Completely Randomised Design (CRD) in a factorial arrangement with 4 replications. The first factor is three seedling media compositions: K= Control (soil + manure), B0= Seaweed waste seedling media without microbes, B1= Seaweed waste seedling media with microbes.

The second factor is two mycorrhiza applications: M0 = Without Mycorrhiza, and M1 = Mycorrhiza consortium. The number of samples per experimental unit is 10, resulting in a total of 240 plants.

Observations

The parameters observed include plant height, number of leaves, leaf length and width, stem diameter, root length, wet and dry root weight, wet and dry plant weight, chlorophyll content (SPAD), mycorrhizal infection in the roots, and nutrient content of the media at the end of the observation using a Palintest device. The obtained data will be analysed using an F-test at a 5% significance level. If the calculated F-value exceeds the F-table value, the analysis will proceed with Duncan's Multiple Range Test (DMRT) at a 5% significance level.

RESULTS

Plant Growth and Chlorophyll Content

Table 3 shows an interaction between seedling media composition treatments and mycorrhizal application on pepper plant growth (plant height, number of leaves) and chlorophyll content 1.5 months after sowing. The best plant height and number of leaves were achieved with the combination of soil + manure media with mycorrhizal application

Table 3

Interaction between seedling media composition and mycorrhizal application on plant height, number of leaves, and chlorophyll content of 1.5-month-old pepper plants

Treatment	Plant Height (cm)	Number of leaves	Chlorophyll Content (SPAD)
KM0	11.70 c	9.74 c	53.72 b
KM1	20.39 a	14.95 a	62.75 a
B0M0	4.26 e	3.37 e	27.73 c
B0M1	17.20 b	12.63 b	51.89 b
B1M0	8.10 d	6.69 d	31,95 c
B1M1	17.49 b	12.76 b	56,44 b
CV (%)	5.989	7.759	6.554

Note. Numbers followed by the same letter in the same column are not significantly different according to the Duncan 0.05% test

(KM1), followed by the combination of seedling media seaweed waste (microbial consortium) with mycorrhizal application (B1M1) and the combination of seaweed waste media (without microbes) with mycorrhizal application (B0M1). The application of microbial consortium (*Bacillus* sp., *Pseudomonas* sp., and *Trichoderma* sp.) (B1M0) alone on seaweed media without the addition of mycorrhiza did not affect the growth of pepper seedlings. Combining seaweed-waste seedling media and a microbial consortium with mycorrhizal inoculation at planting significantly increased pepper growth compared with the control (KM0). These results indicate compatibility among consortium microorganisms, thereby synergistically enhancing growth.

Table 4 shows that leaf length, leaf width, stem diameter, and root length were not affected by the interaction between media composition and mycorrhizal application. However, these variables were influenced by individual factors, namely media composition and mycorrhizal application. Regarding media composition alone, the soil + manure media (K) yielded the best results for leaf length, leaf width, stem diameter, and root length compared to the seaweed waste media without microbes (B0) and the seaweed waste media with a microbial consortium (B1). The soil + manure media (K) significantly improved the growth of leaf length, leaf width, stem diameter, and root length of pepper seedlings in the nursery, attributed to the larger media volume compared to the seaweed waste media, which ensures that essential macro-nutrients for the plants remain high until the plants are 1.5 months old.

Table 4

The influence of a single factor of seedling media composition and mycorrhizal application on leaf length, leaf width, stem diameter, root length, and root wet Weight

Treatment	Leaf length (cm)	Leaf Width (cm)	Stem diameter (mm)	Root length (cm)	Roots Dry Weight (g)
Seedling media composition					
K	4.89 a	2.45 a	2.03 a	11.10 a	0.05
BO	3.77 b	1.82 c	1.59 b	8.89 b	0.04
B1	4.16 b	2.11 b	1.69 b	8.36 b	0.03
Mycorrhiza application					
M0	3.03 b	1.59 b	1.21 b	7.30 b	0.019 b
M1	3.53 a	2.67 a	2.33 a	11.61 a	0.063 a
CV (%)	10.783	9,685	11.711	12.793	41.920

Note. Numbers followed by the same letter in the same column are not significantly different according to the Duncan 0.05% test

Table 5 shows that mycorrhizal application on each type of seedling media, whether soil + manure (KM1), seaweed waste media without microbes (B0M1), or seaweed waste media with microbes (B1M1), improves both wet and dry weights of pepper plants. The

mycorrhizal application significantly enhances root nutrient uptake across all media compositions.

Table 5

Interaction between the composition of the seedling medium and the application of Mycorrhiza to wet weight (roots, stems) and dry weights (stems, leaves)

Treatments	Wet Weight (g)				Dry Weight (g)		
	Root	Stem	Leave	Total	Stem	Leave	Total
KM0	0.21 c	0.26 cd	0.46 c	0.98 c	0.03 c	0.06 c	0.10 c
KM1	0.75 a	1.43 a	2.46 a	5.32 a	0.15 a	0.31 a	0.56 a
B0M0	0.07 c	0.10 d	0.08 d	0.28 d	0.03 c	0.03 c	0.08 c
B0M1	0.69 ab	0.93 b	1.51 b	3.37 b	0.13 b	0.19 b	0.37 b
B1M0	0.19 c	0.31 c	0.51 c	1.12 c	0.02 c	0.04 c	0.07 c
B1M1	0.52 b	0.86 b	1.40 b	3.11 b	0.12 b	0.17 b	0.34 b
CV (%)	26.496	16.867	15.345	16.053	15.145	14.878	12.049

Note. Numbers followed by the same letter in the same column are not significantly different according to the Duncan 0.05% test

The effect of mycorrhizal application on seaweed waste media with microbes on plant and root growth of pepper seeds can be seen in Figure 1. The seedling media with mycorrhizal inoculation supported greater plant and root growth than the control media without mycorrhizae.

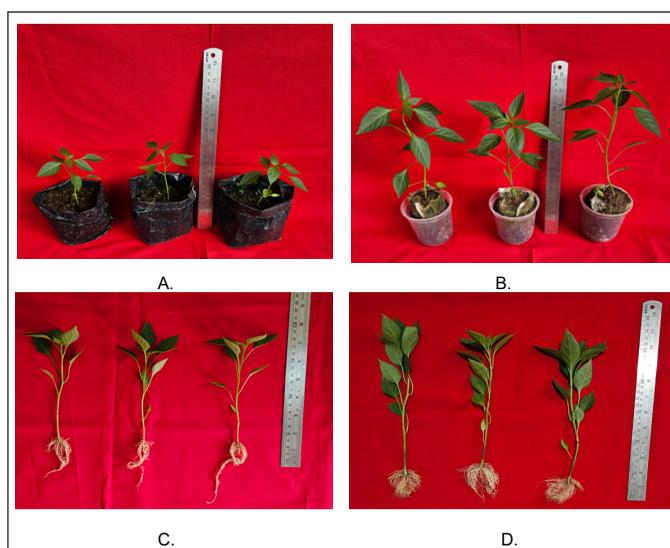


Figure 1. Plant and root growth of pepper peppers aged 1.5 months with (A and C) Soil + manure (control) seedling media, (B and D) Seaweed + consortium microbial seedling media, with mycorrhizal application

Mycorrhizal Infection Percentage

Figure 2 illustrates that the addition of Mycorrhiza significantly increased the percentage of roots infected with Mycorrhiza. The figure also demonstrates that chilli roots can naturally be infected by Mycorrhiza, as observed in the KM0 treatment. In contrast, no root infections were found in plants grown in seaweed medium. In the soil + manure medium without added Mycorrhiza, 5% of chilli roots were naturally infected. The presence of indigenous Mycorrhiza can slightly inhibit the growth and infection of introduced Mycorrhiza, as they are well-adapted to the local environment (Verbruggen et al., 2013). This adaptation results in a smaller increase in the percentage of infected roots in soil media (37.5%) compared to seaweed media (56.3% and 72.9%).

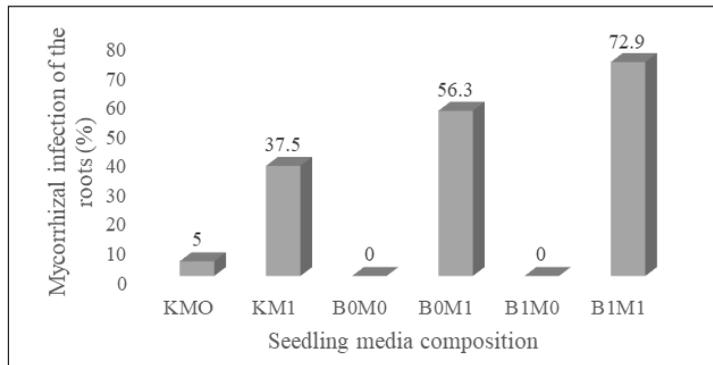


Figure 2. Percentage of mycorrhizal infection in the roots of pepper seeds aged 1.5 months

Nutrient Content of Media

The macro-nutrient content of various seedling media at the end of the seedling stage is shown in Table 6. The table indicates that the Control and seaweed waste seedling media

Table 6

Macronutrient content in various compositions of seedling media at the end of the pepper plant nursery was measured by the Palintest

Nutrient (mg/l)	KM0	KM1	B0M0	B0M1	B1M0	B1M1
N	1.5	0.3	Ttd	3.1	2.6	2.6
NO ₃	6.6	1.3	Ttd	13.7	11.5	12.4
P	38.5	57	21.5	10.5	23	16
K	430	360	445	385	455	455
CaCO ₃	915	931	722,5	868	751	938.75
Mg	222.5	238	175	211	183	231.25
S	45	16.5	0	35	10	10

contain essential macronutrients for plant growth. At the end of the seedling stage, the seedling media seaweed waste, especially the BIM1, had higher levels of macro-nutrients (N, K, Ca, and Mg) compared to soil and manure media. These nutrients are crucial for promoting pepper seedling growth in the nursery.

DISCUSSION

Applying mycorrhiza in each type of seedling media in soil and manure (control), and seaweed waste media without or with microbes can increase plant height and the number of chilli leaves. Table 3 shows that the application of mycorrhiza significantly increased plant height, leaf number, and chlorophyll content in chilli plants, both in soil and in seaweed media. Nutrients in the seedling media support plant growth, while mycorrhizal fungi increase the absorption of root nutrients; this was observed across all media compositions. These findings indicate that adding mycorrhiza facilitates the formation of a beneficial symbiosis with chilli roots. Mycorrhizal fungi colonise plant root systems and form an extensive hyphal network, producing an external mycelial layer around the roots. This condition increases contact between plant roots and the growing medium. Mycorrhizal fungal hyphae can expand their network widely throughout the soil, allowing fungi to access nutrients outside the root area (Shi et al., 2023). Arbuscular mycorrhizal fungi (AMF) have the ability to increase the surface area of roots by effectively producing intraradical and extraradical mycelium for better utilisation and effective transportation of nutrients and water (Tyagi et al., 2017). The hyphal diameter ranges from 2 to 20 μm , with the capacity to absorb nutrients from an area of 25 cm around the roots for translocation (Jansa et al., 2008). In addition, mycorrhizal fungal hyphae form a linear surface that goes across soil pores, increasing the pathway for water flow, thereby enhancing the plant's ability to absorb groundwater (Allen, 2007).

The results of the study showed that AMF significantly increased total nitrogen (N) by 0.03%-0.13% and available phosphorus (P) in ultisol soil from 9.00 ppm to 17.50 ppm. The positive role of Mycorrhiza applied to eggplant plants at 20 g per polybag was reported to provide better eggplant plant growth (Ferdin et al., 2023). In cayenne pepper plants with a dose of 10 g in sandy media for 3.5 months, it was reported to increase plant height 3.3 times and leaf area 4.5 times compared to plants without Mycorrhiza (Yudaswara et al., 2018). In addition, application to plants using Andisol soil media with a mycorrhizal dose of 10 g per plant produced the highest plant height, stem diameter, and number of productive branches at the age of 45 days (Matondang et al., 2020).

Leaf chlorophyll is an essential indicator of leaf greenness and is commonly used to assess nutrient deficiencies and monitor changes in chlorophyll levels (Ali et al., 2016). High chlorophyll content indicates that nutrient availability in the seedling medium is well maintained during the seedling and nursery stages. Increased chlorophyll formation in

leaves indicates increased photosynthetic activity, so plant growth (height and number of leaves) increases. The highest chlorophyll content in chilli leaves was obtained in plants using a combination of soil and manure media with the provision of mycorrhiza (KM1), followed by a combination of seaweed waste media and microbial consortium with the provision of Mycorrhiza (B1M1). However, this is equivalent to combining seaweed-waste media without microbes with mycorrhiza media (B0M1) and to soil- and manure-based media without mycorrhiza (KM0). The highest results in the number of leaves, plant height, number of fruits, fruit weight, and leaf chlorophyll content in large chilli plants were reported to be produced by applying 15 g of Mycorrhiza with daily watering intervals and mycorrhiza inoculation at a dose of 100 g per plant increased leaf chlorophyll content, higher than the control, due to increased plant metabolic processes where the absorption of nitrogen and phosphorus in the leaves increased (Astiari et al., 2021).

Several other studies have mentioned a significant correlation between chlorophyll and leaf nitrogen content in various crops (Kalacska et al., 2015; Wang et al., 2014). Fertiliser application, especially nitrogen, positively impacts chlorophyll content, leaf area index (LAI), and leaf dry weight. Bassi et al. (2018) observed that fertilisation could increase nitrogen remobilisation in low nitrogen availability to support safflower growth, accelerate leaf ageing, and reduce chlorophyll content. As seen in Table 3, the control treatments (KM0 and KM1) showed the highest values for plant height, number of leaves, and chlorophyll content compared to the seaweed waste media treatment, which was predicted due to the difference in media volume. In the control treatment, the media volume was 10 x 15 cm, whereas in the seaweed-waste media treatment, it was 5 x 10 cm. As a result, at the end of the observation period (1.5 months after sowing), the nutrient content in the control media was still sufficiently available.

Meanwhile, treatment of seaweed waste media with a smaller volume resulted in nutrient levels that were insufficient for the 1.5-month observation period, both with a microbial consortium (M1) and without microbes (M0) and did not increase growth. Another possible cause of suboptimal growth is the high tannin content of the cocopeat used as a filler in the seaweed-waste media. Cocopeat has the advantage of having a high water retention capacity and slow degradation (V. D. S. Handayani et al., 2023). Water is gradually released from the planting medium, providing nutrients to plants over an extended period. However, cocopeat also contains toxic tannins and can inhibit plant root growth. Efforts to reduce tannins in cocopeat by soaking it in clean water before use as a plant nursery medium are suboptimal.

The single factor of mycorrhizal application significantly affected the growth of leaf length, leaf width, stem diameter, and root length in chilli seedlings (Table 4). Mycorrhizal fungi enhance plant growth by facilitating nutrient and water uptake, particularly under stressful environmental conditions such as drought. Mycorrhizal hyphae can explore soil

pores beyond the reach of root hairs, thereby increasing water absorption by plants and ultimately increasing productivity in water-deficient conditions (Calvo et al., 2024). Direct contact between the seedling media and mycorrhizae in the planting hole can increase root infection by mycorrhizae and the absorption of phosphorus (P), which is very important for plant growth (Ginting et al., 2018). The statistical analysis showed an interaction between media composition and mycorrhizal application on wet Weight (roots, stems, leaves, total) and dry Weight (stems, leaves, total biomass). The best results for fresh Weight (roots, stems, leaves, total biomass) and dry Weight (stems, leaves, total biomass) were achieved with a combination of soil and manure media with mycorrhizal application (KM1), followed by seaweed waste media without microbes with mycorrhizal application (B0M1), and seaweed waste media and microbial consortium with mycorrhizal application (B1M1) (Table 5).

A new mycorrhizal infection can be observed in the roots of chilli plants two weeks after inoculation. The percentage of mycorrhizal infection in chilli seedlings aged 1.5 months after planting in various seedling media showed the highest value in the B1M1 treatment (72.9%), followed by B0M1 (56.3%) and KM1 (37.5%) (Figure 2). The percentage of mycorrhizal infection in B0M1 and B1M1 showed a high level of root expansion by mycorrhizae (51 - 75%). Root infection with mycorrhizae is considered more effective in nutrient-poor soils, where its presence benefits plants (Adetya et al., 2019; Ginting et al., 2018). B1M1 treatment combines mycorrhizal consortium application with other microbial consortia, including *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Trichoderma* sp., to enhance phosphorus and potassium uptake. This treatment is classified as a Plant Growth-Promoting Rhizobacteria (PGPR) treatment. *Bacillus subtilis* plays a role in IAA production, phytase activity, and antibiotic production (Yao et al., 2008). *Pseudomonas fluorescens* plays a role in IAA production and shows antagonism to *Fusarium verticillioides* (Hernández-Rodríguez et al., 2008b).

Mycorrhizal fungi can interact with various bacterial species, and this interaction occurs in the root zone and within fungal hyphae. The mycorrhizosphere can expand the rhizosphere by increasing the extent of fungal mycelium. This expanded area helps increase nutrient absorption, soil stability, and water retention efficiency (Ramasamy et al., 2011). The nutrient source in the B1M1 planting medium is seaweed and coconut coir (cocopeat). The addition of a microbial consortium (including *Bacillus* sp., *Pseudomonas* sp., *Trichoderma* sp., and *Mycorrhiza*) synergistically improves soil ecosystem balance, increases nutrient availability, and enhances plant resistance to disease and environmental stress. *Bacillus* sp. and *Pseudomonas* sp. produce organic acids that dissolve phosphate and potassium and enzymes such as phosphatase and phytase that break down organic compounds containing phosphate, thereby increasing nutrient availability. *Trichoderma* spp. are biological agents and plant growth stimulants. This mechanism involves a symbiotic

relationship with plant roots, mediated by direct interactions between fungal hyphae and fungal conidia. These conidia grow around the root system and are planted in the surrounding area (Charisma et al., 2012). Arbuscular Mycorrhiza Fungi and *Trichoderma harzianum* have been shown to increase the growth of mustard greens in clay and sandy soils (Ginting et al., 2018).

Seaweed extract enhances cold resistance, improves nutrient uptake, controls phytopathogenic fungi, stimulates seed germination and root growth, and increases nutrient absorption (Brownlee et al., 2012; Čmiková et al., 2024; Lomartire et al., 2021; Nabti et al., 2017). Cocopeat, comprising 75% of the media mix, has proven to be the most effective medium for the growth and performance of *Chrysanthemum* in pots. Its aggregate stability and potassium content make cocopeat an ideal alternative potting medium for chrysanthemums (Singh et al., 2015). Combining cocopeat with other composts is also identified as an excellent growing medium, serving as a nutrient source and soil substitute for vegetable crops (Utari & Rachmawati, 2022). Based on this study, seaweed waste combined with coconut coir (cocopeat) can be used as a soil substitute for seeding media. Seaweed provides essential micronutrients and growth hormones (auxin, cytokinin, gibberellin) that stimulate root and shoot growth. Cocopeat contributes high water-holding capacity, good aeration, and stable physical structure, making it ideal for root development. The combination of seaweed and cocopeat creates a growth medium that is a nutrient-rich, lightweight, and water-retentive medium, ideal for seedling growth, which improves root development, nutrient availability, and water holding capacity, leading to enhanced seedling growth (Handayani et al., 2004; Singh et al., 2015; Tiwari, 2015). Adding a microbial consortium can maximise its function as a nutrient-rich planting medium. In addition, it can be packaged in compact, lightweight containers, facilitating transport, storage, and use.

CONCLUSION

The results of this study demonstrate that using seaweed waste combined with mycorrhizae as a planting medium leads to optimal seedling growth, as indicated by enhanced pepper seedling development, increased chlorophyll content, higher mycorrhizal root infection, and improved nutrient levels in the growing medium. There is a synergistic effect among microbial consortia, with the addition of mycorrhiza improving pepper seedling growth. Additionally, seaweed-waste media combined with microbial consortia and mycorrhizae are recommended as a suitable soil substitute to enhance pepper seedling growth.

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AUTHOR CONTRIBUTIONS

Devi Rusmin, Ireng Darwati, Jamal Basmal, Octivia Trisilawati, Melati, and Eliza Mayura conceived the idea of research; Rudi Suryadi, Muchamad Yusron, Raden Vitri Garvita, and Rinta Kusumawati assisted in field and laboratory studies; Devi Rusmin, Ireng Darwati, Jamal Basmal, Rudi Suryadi, Eliza Mayura and Melati analysed the data and wrote the manuscript; and Devi Rusmin, Jamal Basmal, Rinta Kusumawati, Octivia Trisilawati, Rudi Suryadi and Melati were involved in language improvement and final editing of the manuscript.

DATA AVAILABILITY

Data will be made available on request.

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Transcriptome Profiling of Oil Palm (*Elaeis guineensis* Jacq.) in Phosphorus Deficiency

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ABSTRACT

Phosphorus (P) limitation conditions could be lessened by developing oil palm progenies with increased P absorption efficiency using molecular approaches. The molecular-assisted selection method can be applied to select elite palms with high nutrient-uptake efficiency. The aim of the study was to identify key genes that were differentially expressed under P-deficient conditions. A molecular marker could be developed from the RNA-seq results. RNA-seq was applied for prolific and non-prolific genotypes grown under low- and high- P dosage for 180 days, and their specific morphological characters were also observed. Root length, shoot length, and P-content characteristics

showed significant differences among genotypes under both P dosages. Heat-maps were employed to visualise the RNA sequencing results. In total, 2,905 DEGs involved in P deficiency in oil palm were identified. The GO terms were subsequently divided into 74 sub-categories. Phosphorylation, protein phosphorylation, transferring phosphorus-containing groups, alcohol group as acceptor, and nitrogenous group as acceptor were the five major subcategories related to P deficiency. Five genes were selected based on their response to P deficiency, the up-regulated genes consisted of *NRR1* and *AP4S1*; and the down-regulated consisted of *PTI/STPs*,

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PPD7, and *PPR* genes. These five genes are suggested to be validated using RT-qPCR to determine their expression in the next study. Hence, this research provides a comprehensive understanding of the physiological processes involved in the oil palm seedling response to P deficiency. The results may be used for the development of biomarkers for the rapid selection of candidate palms in oil palm breeding programmes.

Keywords: DEGs; oil palm, P-deficient, RNA-sequencing, transcriptome profiles

INTRODUCTION

Complex NPK is the second fertiliser which the most applied fertiliser in the agricultural industry worldwide. Phosphorus (P) consumption was approximately 12.74 million tons per year (Statista Research Department, 2024). Phosphorus scarcity is a major problem in agricultural lands worldwide, resulting in a decline in crop yields and posing substantial risks to global agriculture (Saputra et al., 2023). In fact, phosphorus is abundant in the lithosphere. Nevertheless, plants can only absorb inorganic orthophosphate (Pi) from soils. This form of P is sluggish and insoluble, dispersed in soils. This situation leads to P's inadequacy in the agricultural industry. Soil microbiota are also sensitive to P availability. They can either compete with plants or engage in mutualistic symbiosis, such as through mycorrhizal associations, to increase plants' phosphorus absorption efficiency (Abobatta et al., 2023). In addition, the application of P fertilisers is often inefficient, with only 15-25% of the applied P being absorbed by plant roots; the remainder is lost through leaching, contributing to soil degradation and water eutrophication (Khan et al., 2023).

P plays an indispensable role in plant physiological processes in improving abiotic stress tolerance, such as heavy metal toxicity, salinity, drought, heat, waterlogging, and elevated CO₂ levels (George et al., 2016). Plants respond to phosphorus availability through specific signalling cascades, modification of root architecture and alteration of stomatal morphology (Shen et al., 2011). Furthermore, by enhancing phosphorus absorption, plants can endure diverse abiotic stresses (Malhotra et al., 2018). Understanding plants' strategies to recognise and respond to phosphorus availability is critical in order to increase productivity and improve crop stress tolerance. Phosphate or phosphate esters are essential for numerous physiological and metabolic processes, including DNA synthesis, phospholipid biosynthesis, cell division, and energy metabolism (Isidra-Arellano et al., 2021). Inadequate phosphorus severely affects fruit production, root development, and vegetative growth, which eventually diminishes yields (Deng et al., 2018).

Development of an oil palm genotype with high phosphorus uptake efficiency using molecular approaches might mitigate the P limitation condition (Faucon et al., 2015). Molecular markers can be used to select elite oil palms with high P uptake efficiency.

However, the molecular mechanism involved in oil palm response to P deficiency is not fully understood. One of the potential approaches that can be used to develop molecular markers for certain characters is through transcriptome profiling by RNA sequencing (Maryanto et al., 2021). This approach has been applied in wheat, rice, and maize. A functional analysis of P deficiency in rice, conducted to understand the biological regulatory systems underlying P-insufficiency tolerance, has been reported (Wissuwa, 2001). In this study, RNA from oil palm seedlings grown under P starvation was sampled and subjected to RNA sequencing, and seedlings grown under adequate P supply were used as the control.

The oil palm response to P deficiency is complex, involving numerous sensing and signalling pathways (Kong et al., 2021). In their research, Kong et al (2021) did not classify between efficient and non-efficient genotypes. Therefore, in this study, the efficient and non-efficient genotypes were used as samples. Previous research found that some phenotypic traits differed significantly among genotypes. This result opens the possibility of improving oil palm P-deficiency tolerance. Progeny A and C were identified as tolerant to P deficiency (prolific), whereas progeny B and D were identified as non-tolerant (non-prolific) based on several genotypic traits (data unpublished). Accordingly, this study aimed to identify P-deficiency-responsive differentially expressed genes (DEGs) in oil palm. The findings may contribute to a clearer understanding of oil palm adaptive responses to P limitation and support oil palm genetic improvement efforts.

METHODS

Plant Materials

Three-month-old Tenera (Dura x Pisifera) seedlings from four oil palm genotypes were used. Genotypes A and C were classified as P-deficiency-tolerant (prolific), and genotypes B and D were classified as P-deficiency-sensitive (non-prolific). The planting materials were classified according to several genotype traits (internal report).

Hydroponic Trial and Morphological Measurement

Seedling which showed uniform plant height were selected and planted in a greenhouse with a culture solution of a hydroponic system. The system had dimensions of 6m × 3m × 1m and comprised six lines with 5cm spacing between them. A total capacity of 120 planting holes, spaced 15cm apart, each with a diameter of 5 cm (Maryanto et al., 2021). The temperature was controlled at 28-30°C. The nutrient solution was prepared following the Hoagland method with several modifications (Hoagland & Arnon, 1950). The seedlings were grown in 1.57% v/v (low-phosphorus/LP), and the control was grown in 14.02% v/v (high-phosphorus/HP). The nutrient solution was replaced biweekly to ensure adequate nutrient availability and was continuously aerated using an air pump.

The experimental design consisted of four oil palm genotypes (A, B, C, and D) x two P-dosages (low- and high-P) x five replicates x 2 repetitions with a total of 80 oil palm seedlings. P treatments were applied for 180 days. Morphological characteristics consisting of root and shoot length were measured biweekly, while P-content was measured at 180 days after transplanting (DAT). The leaf samples for RNA sequencing were collected at 180 DAT and put immediately in RNA Later (Thermo Scientific, Massachusetts, USA).

RNA Extraction, cDNA Library Preparation and Transcriptome Sequencing

Total leaf RNA was isolated by the commercial RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) based on the protocol. Extracted RNA was quantified using a NanoDropTM 2000c Spectrophotometer (Thermo Scientific, Massachusetts, USA). RNA that fulfils the requirement for sequencing was preserved by RNAsable (Biomatrica, California, USA) and sent to Novogene Co., Ltd., Beijing, China (Sahara et al., 2023). Total samples for RNAseq were 16 libraries, consisting of two samples for each genotype and for both P dosages.

The RNA was then measured using the 2100 Bioanalyzer Instrument (Agilent Company, USA) for quantifying RNA concentration, rRNA ratio, and RNA integrity number. mRNA was isolated from the total RNA using magnetic beads with Oligo (dT). The mRNA is then fragmented into short fragments and used as a template for cDNA synthesis. Short fragments were purified and resolved with EB buffer for end reparation and poly (A) addition. The short fragments were connected with adapters, and the cDNA was sequenced using the Illumina NextSeq 2000 sequencer platform (Maryanto et al., 2021).

Data Analysis

Raw RNA quantification was analysed using Kallisto software. Expression normalisation was counted based on transcript per million (TPM) (Bray et al., 2016). The DESeq2 was used for DEGs analysis (Love et al., 2014). The Plant Transcriptional Regulatory Map Database was used for gene ontology (GO) enrichment (Tian et al., 2020). The annotation of DEGs for describing biological processes, molecular functions, and cellular components was analysed using Microsoft Excel.

RESULTS AND DISCUSSION

Morphological Characters

P concentration is affected by plant morphology (Figure 1). Seedlings grown in low-P possessed shorter roots and shoots compared to seedlings grown in high-P.

Plants launched a series of adaptive morphological, biochemical, and physiological approaches to survive in P deficiency. Alteration of plant architecture and root growth is a well-known morphological response to P-deficiency, including reduction of root and

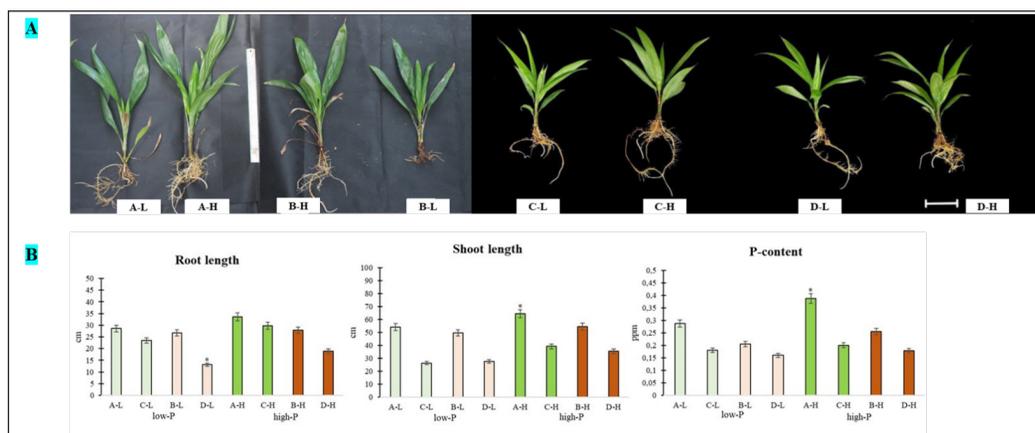


Figure 1. Morphological performance of seedlings at high- and low- P (A) and quantitative data of morphological characters (B). Notes: A-L= prolific progeny in low-P; A-H = prolific progeny in high-P; B-H= commercial prolific progeny in high-P; B-L = commercial prolific progeny in low-P; C-L= non-prolific progeny in low-P; C-H = non-prolific progeny in high-P; D-L= commercial non-prolific progeny in low-P; D-H = commercial non-prolific progeny in high-P; asterisk symbol (*) = significantly different ($p < 0.05$)

shoot length. Moreover, a decay of P content in plants treated with P-deficiency has been reported in another plant. In this study, the prolific genotypes had relatively better growth compared to non-prolific genotypes in low P conditions based on the observations of those three parameters. This might be caused by P homeostasis in prolific areas being better than in non-prolific genotypes. P transfer is arising from older to younger leaves during P-deficiency.

The modification of shoot length observed in this study is a well-documented morphological response to P deficiency. P is an essential element for oil palm growth at both the cellular and whole-plant levels (de Bang et al., 2021), playing a crucial role in cell division and cell enlargement. Consequently, the reduction in shoot length under P-deficient conditions is primarily caused by restricted cell division and limited cell enlargement (Malhotra et al., 2018).

The adaptive response by prolific genotypes under severe P-deficiency is better than that of non-prolific genotypes. The enhancement of root length is a plant endeavour to discover more P in the environment (Fang et al., 2024). Roots function as a sink tissue under P-deficient conditions, promoting root proliferation and more extensive soil exploration. Furthermore, the total phosphorus content in oil palm organs was consistent with the applied treatments: palms receiving low-P doses exhibited reduced tissue P concentrations, whereas those supplied with optimal P levels showed higher P accumulation. This is perhaps caused by P absorption harmonised with the P availability in the cultivation media.

Transcriptome Profile High- and Low- Phosphorus

Transcriptome profiling is addressed to increase the thoughtfulness of the biological regulatory process of P deficiency in oil palm (Maryanto et al., 2021). The Illumina NextSeq 2000 sequencer was used, resulting in a total nucleotide of approximately 18.77 Gb in size. From 151,368,907 total paired final raw reads, approximately 143,904,945 total net reads were obtained (Table 1). The DEGs of seedlings treated in low-P and high-P were shown in the heatmaps (Figure 2 and Figure 3). The heatmap shows two major clusters on the x-axis. The first cluster comprised non-prolific genotypes (B, D), whereas the second was of prolific genotypes (A, C). One group of DEGs showed higher expression in the prolific but lower expression in the non-prolific, while the other group of DEGs showed higher expression in the non-prolific but lower expression in the prolific genotypes. This result exhibited the differences in transcriptome profiles of prolific and non-prolific at the P treatment.

In total, 2,905 DEGs were identified, and their distribution was shown in a volcano plot (Figure 4). The y-axis indicated the significant gene expression level between samples, which was quantified by the p-value, whereas the x-axis denoted the fold change (\log_2 fold change) of the DEGs between the treatment and control. Furthermore, red dot colour indicated upregulated genes, while green dot colour indicated downregulated genes, and black dot colour indicated genes which have similar expression in both samples (Figure 4). Genes located on the outer line with $|\log_2FC| > 1$ and a p-value < 0.05 were assumed to be differentially expressed. The p-value indicates the statistical significance of differentially expressed genes (DEGs) relative to the total transcriptome of all samples, reflecting the reliability of DEG abundance compared with overall transcript levels. Meanwhile, the \log_2 fold change (\log_2FC) represents the magnitude of expression differences of DEGs between samples.

Furthermore, PCA is a technique used to assess relationships among samples and to leverage all collected data. In this study, grouping of samples and correlations among parameters were determined based on transcripts per million (TPM) of the differentially expressed genes (DEGs). PCA clustered the samples into two major groups: the prolific genotype (blue) and the non-prolific genotypes (another colour), based on TPM values. These results indicate that transcriptome profiles of prolific genotypes were more similar to each other and distinct from those of non-prolific genotypes under both low-P and high-P treatments (Figures 5A and B).

Functional Classification Based on Gene Ontology

Three main gene ontologies (GO) are commonly used in gene categorisations, namely, biological process, cellular component, and molecular function. The obtained DEGs were grouped into 74 GOs, comprising 44 GOs in biological processes (59.45%), 28 GOs

Table 1
 Statistics of sequencing data with two P dosage in prolific and non-prolific genotypes

Genotype	P-dosages	Raw reads (paired-end)	Raw nucleotides (bp)	Clean reads (paired-end)	Clean nucleotides (bp)	Percentage of high-quality reads (%)	mapped reads	Percentages of mapped (%)
A (prolific)	LP	21,478,971	6,017,660,359	20,448,575	5,537,984,536	95.20	17,027,891	83.27
C (prolific)	LP	16,441,596	4,600,215,676	15,692,765	4,246,713,393	95.45	14,453,806	92.10
B (non-prolific)	LP	20,330,623	5,576,442,686	19,396,304	5,147,507,641	95.40	15,277,804	78.77
D (non-prolific)	LP	16,718,461	4,599,918,591	15,941,377	4,249,937,494	95.35	13,787,189	86.49
A (prolific)	HP	17,602,780	4,700,069,354	16,652,153	4,292,824,438	94.60	12,082,653	72.56
C (prolific)	HP	17,842,632	5,012,887,986	17,000,235	4,614,693,227	95.28	15,193,341	89.37
B (non-prolific)	HP	23,742,360	6,590,035,757	22,540,702	6,039,166,363	94.94	18,751,400	83.19
D (non-prolific)	HP	17,211,484	4,700,006,818	16,232,834	4,324,293,845	94.31	14,229,163	87.66
Total		151,368,907	41,797,237,227	143,904,945	38,453,120,937	-	120,803,246	-

Note. LP = 1.57 % v/v (low-P); HP = 14.02% v/v (high-P)

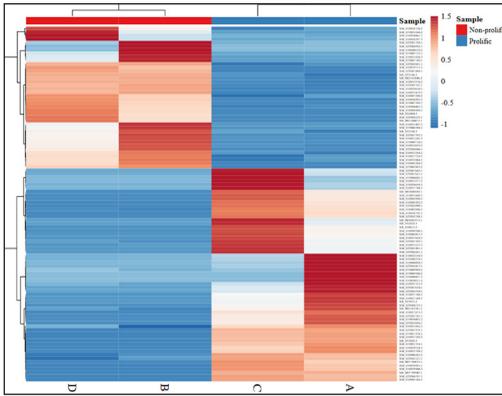


Figure 2. Heat-map of transcriptome profile of prolific and non-prolific samples at 1.57 % v/v (LP) dosage, notes: A, C = prolific genotypes; B, D = non-prolific genotypes; blue-colour = low-expression; red-colour = high-expression

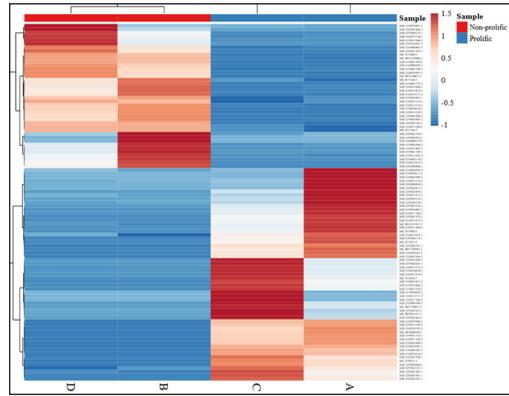


Figure 3. Heat-map of transcriptome profile of prolific and non-prolific samples at 14.02% v/v (HP) dosage, notes: A, C = prolific genotypes; B, D = non-prolific genotypes; blue-colour = low-expression; red-colour = high-expression

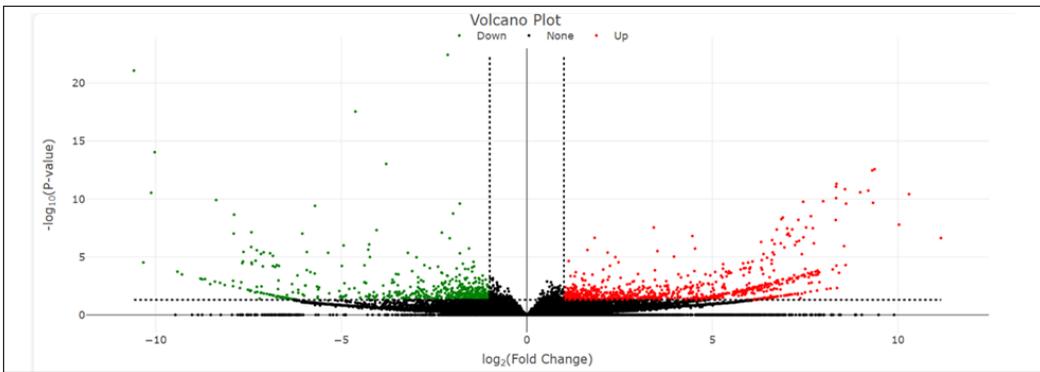


Figure 4. Volcano plots of DEGs, notes: red dot colour = upregulated; green dot colour = downregulated; and black dot colour = genes with similar expression in both samples

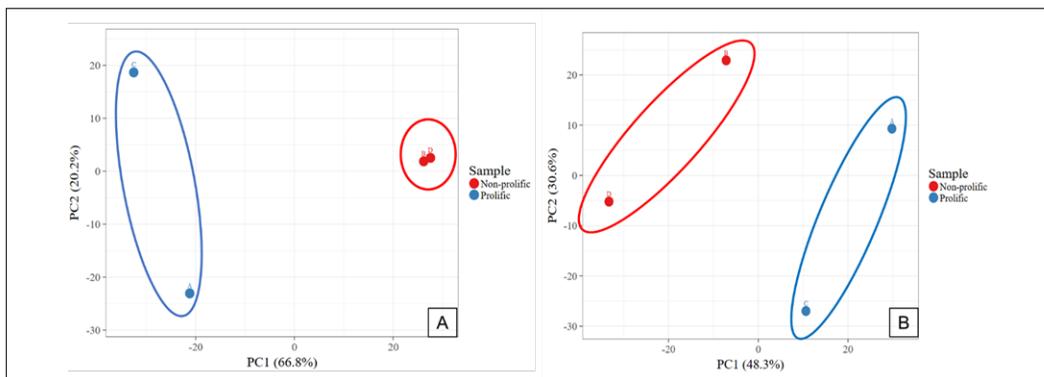


Figure 5. PCA of prolific and non-prolific genotypes based on the transcript per million (TPM) in low-P (A) and high-P (B), prolific (blue) and non-prolific (red)

in molecular functions (37.83%), and 2 GOs in cellular components (2.70%) (Figure 6). The highest gene numbers in biological process, molecular function and cellular component were response to stimulus, binding, and integral component of plasma membrane, respectively. Furthermore, the top five GO classes associated with P deficiency consisted of phosphorylation, protein phosphorylation, transferring P-containing groups, phosphotransferase activity, alcohol group as acceptor, and phosphotransferase activity-nitrogenous group as acceptor. These results indicated that the appearance of these five classes is in accordance with P as a limitation factor during hydroponic trials in oil palm seedlings at the main nursery stages.

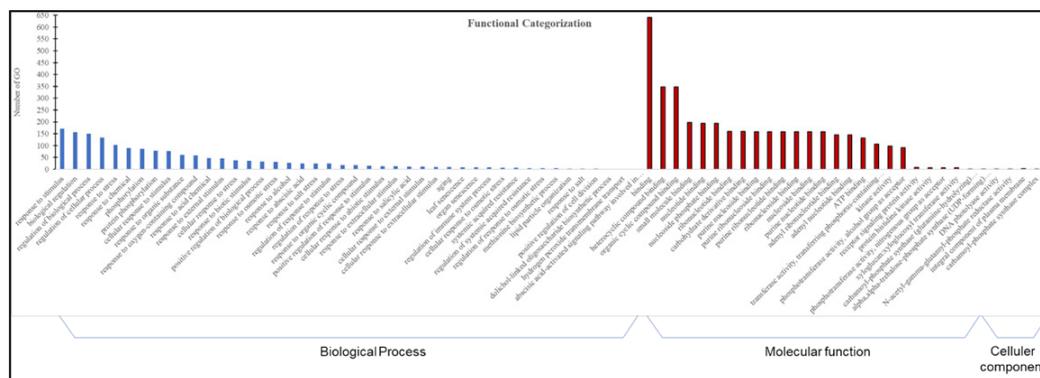


Figure 6. Functional categorisation related P deficiency in oil palm seedlings

Differentially Expressed Gene of High- and Low- Phosphorus

The number of genes involved in a particular treatment is usually shown by a Venn diagram. According to the Venn diagram, the DEGs involved in low-P, high-P and both P dosages were 1,348, 1,465 and 92 DEGs, respectively (Figure 7A). Furthermore, among 92 DEGs involved in both treatments, 45 were upregulated, and 47 were downregulated. Then, particular DEGs were selected for further analysis based on a p-value of less than 0.05 and a q-value of less than 1.00, as well as sorted by highest to lowest log2FC. The highest transcript of log2FC was observed in protein MLN51 homolog (XM_029262920) (Figure 7B). The function of this gene is to stimulate both ATPase and RNA helicase activities. While the lowest transcript was uncharacterised LOC105042954 (Figure 7B). The function of this gene in response to P stress in other crops is unclear (Meng et al., 2024).

The 92 DEGs were then screened based on the highest p-value and q-value, and continued with sorting by the highest to lowest log2FC value. From this selection criteria, 15 DEGs were selected (Table 2). Finally, five genes were chosen based on their physiological response to P condition, consisting of *NRR repressor homolog 1* (XM_010909200, ID-04), *AP-4 complex subunit sigma* (XM_010916464, ID-07),

serine/threonine protein phosphatase (XM_010909466, ID-10), *psbP domain-containing protein 7* (XM_010940137, ID-11), and *pentatricopeptide repeat-containing protein* (XM_010907539, ID-14) (Table 2).

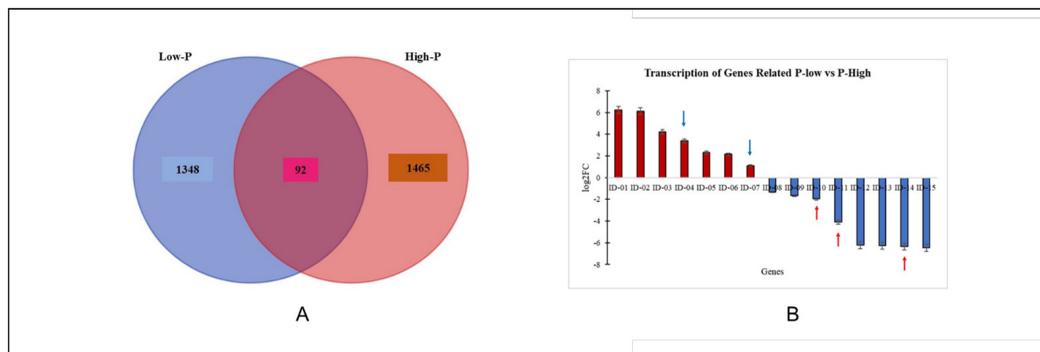


Figure 7. The number of genes involved in particular treatment of oil palm seedlings (A) and transcription level of genes responding to P deficiency (B), notes: red colour= up-regulated; blue colour= down-regulated; ID 01, 02, 03...etc. = geneID

There are two model responses of genes to nutrient deprivation. The first are early responsive genes, which respond very quickly and are often non-specific, and the second are slow responsive genes, influenced by long-term stress, which are considered to be more specific for nutrient deficiency. The classification of the five selected genes was not yet known. Stress-responsive genes can be classified into two groups: early responsive genes, which react rapidly and are often non-specific, and slow responsive genes, which are influenced by long-term stress and are considered more specific to nutrient deficiencies. The classification of the five selected genes in this study has not yet been determined. The first selected gene, *NRR* repressor homolog 1, is known to play a key role in plant defence responses against pathogens and various stresses. In rice, *NRR* undergoes alternative splicing, producing two 5'-coterminal transcripts, *NRRa* and *NRRb*. The expression of *NRR* in rice roots is notably affected by macronutrient deficiencies, particularly nitrogen and phosphorus. These transcripts, *NRRa* and *NRRb*, function as regulatory elements controlling root architecture in response to nitrogen or phosphorus deficiency (Zhang et al., 2012). The *NRRa* embraces an extra CCT domain at the C-terminus. Knockdown of both *NRR* by RNAi enhanced rice root growth. By contrast, over-expression of *NRRa* in rice revealed significantly stunted root growth. These results show that *NRR* played negative functions in rice root growth (Zhang et al., 2012). In this research, the expression of *NRR* was upregulated in P deficiency trials (Table 2). High expression of *NRR* would be possible and would exhibit significantly retarded root growth in oil palm. The finding corresponded with root length observations. The roots of seedlings grown in low-P were shorter than the roots of seedlings grown in high-P (Figure 1B).

Table 2
List of selected differential expression genes based on the highest to lowest of *p*-value, *q*-value and *log2FC*

Code	ID_Gene	Description	pvalue	qvalue	log2FC	Genes	Function
ID-01	XM_029262920	protein MLN51 homolog	0,044	1	6,23	MLN51	MLN51 stimulates both ATPase and RNA helicase activity
ID-02	XR_003801506	uncharacterised LOC105049634	0,049	1	6,11	uncharacterised LOC105049634	not characterised
ID-03	XM_010915242	kinesin-like protein KIN-4C	0,046	1	4,22	KIN4C	Microtubule-dependent motor protein plays a role in the control of the oriented deposition of cellulose microfibrils
ID-04	XM_010909200	NRR repressor homolog 1	0,038	1	3,40	NRR1	Play a role in a plant's defence response to biotic and abiotic stresses
ID-05	XM_010935203	NAC domain-containing protein 71	0,046	1	2,33	NAC071	plays a role in the tissue reunion of wounded inflorescence stems
ID-06	XM_010924311	ferritin-4	0,041	1	2,15	FER4	involved in iron homeostasis and mitochondrial iron trafficking
ID-07	XM_010916464	AP-4 complex subunit sigma	0,046	1	1,10	AP4S1	involved in plant immunity and vacuolar protein sorting
ID-08	XM_019852988	histidine protein methyltransferase 1 homolog	0,040	1	-1,31	METTL18	controlling the biosynthesis of other amino acids, growth and development of plant embryos, chelation transport of metal ions.
ID-09	XM_010933404	protein DEHYDRATION-INDUCED 19 homolog 2	0,042	1	-1,66	DI19-3	a nuclear transcriptional activator that helps plants tolerate drought stress

Table 2 (continued)

Code	ID_Gene	Description	pvalue	qvalue	log ₂ FC	Genes	Function
ID-10	XM_010909466	serine/threonine protein phosphatase	0,036	1	-1,95	PTI/STPs	enzymes that remove phosphate from threonine and serine residues in proteins
ID-11	XM_010940137	psbP domain-containing protein 7	0,035	1	-4,10	PPD7	involved in photosynthesis, defence responses, growth and development of plant
ID-12	XM_010910002	uncharacterised LOC105034740	0,048	1	-6,22	uncharacterised LOC105034740	not characterised
ID-13	XM_010926490	uncharacterised LOC105047534	0,047	1	-6,26	uncharacterised LOC105047534	not characterised
ID-14	XM_010907539	pentatricopeptide repeat-containing protein At2g13600	0,041	1	-6,36	PPR	plant growth and development
ID-15	XR_003800558	uncharacterised LOC105042954	0,043	1	-6,47	uncharacterised LOC105042954	not characterised

The second gene, which is upregulated, is *AP4SI*. *AP* complexes are important regulators of intracellular protein transport. *AP* complexes also accomplish essential tasks in controlling various physiological processes and protein sorting in plants. *AP* also engaged in plant immunity and vacuole protein sorting in response to environmental stress. *AP-1* activity leads to hindered growth, male and female sterility, and cytokinesis imperfections. *AP* demonstrated significantly retarded growth of *Arabidopsis* in P deficiency condition (Wang et al., 2023). The expression of *AP4SI* corresponded with the observed root and shoot phenotypes. In this study, seedlings grown under low-P conditions exhibited shorter roots and shoots compared with those grown under high-P conditions (Figure 1B).

The third selected gene, *PTI/STPs*, displayed a downregulated expression pattern. Serine/threonine protein phosphatases (*STPs*) are enzymes found in eukaryotes that play key roles in signal transduction and regulation of various cellular processes (Bajsa et al., 2011). *STPs* are important enzymes in plant dephosphorylation, a process that removes phosphate from serine and threonine residues on proteins. These phosphatases play crucial roles in different plant processes, including stress responses, development, and hormonal signalling. In *Arabidopsis*, *STPs* may eliminate phosphate from threonine and serine residues in proteins as a response to P deficiency. The serine/threonine protein phosphatase enzyme is controlled by *PTI/STPs* (Pais et al., 2009). The expression of *PTI/STPs* was in line with the plants' P-content. *STPs* would eradicate phosphate from threonine and serine that might be used in other metabolic reactions, thus triggering low P-content in leaves of the oil palm seedlings (Figure 1B). The expression of *PTI/STPs* may be responsive to P availability, as it is involved in plant signalling processes.

The fourth selected gene, *PPD7*, encodes a *PsbP* domain-containing protein, which is a key component of the oxygen-evolving complex (OEC) in chloroplasts and plays a crucial role in photosynthesis. Studies in *Arabidopsis* have shown that *PsbP* homologs have distinct and essential functions in maintaining photosynthetic electron transfer. In this study, *PPD7* was downregulated under P-deficient conditions (Table 2). A low transcript of *PPD7* indicated that its activity is affected by P deficiency. *PPD7* activity is influenced by the photosynthesis rate. A low transcript of the gene would diminish the rate of photosynthesis, thus inhibiting plant growth (Ishihara et al., 2007). P is known to have an essential function in photosynthesis and carbohydrate production, even though the effect of P on crops' photosynthesis is species-specific (Thuynsma et al., 2016). The expression of *PPD7* in this study corresponded with the observed morphological traits, as roots and shoots of seedlings grown under low-P conditions were shorter than those of seedlings grown under high-P conditions (Figure 1B). This suggests that *PPD7* may be particularly responsive to P availability.

The fifth selected gene, *PPR*, encodes a pentatricopeptide repeat-containing protein, which is involved in transcriptional, post-transcriptional, and translational regulation within

organelles (Meng et al., 2024). Proteins containing the PHD finger motif, including some PPR proteins, have been reported to participate in responses to abiotic stresses as well as in plant growth and development, as observed in black cottonwood (Quan et al., 2023). The expression of PPR may therefore be specifically responsive to P availability. Hence, this result suggested that five genes need to be validated as marker candidates related to P deficiency in oil palm using the RT-qPCR approach in the next study.

CONCLUSION

Understanding the basic molecular mechanisms involved in P regulations is important for developing P-use-efficient genotypes. Specific morphology characters consisted of root length, shoot length, and P-content parameters, which indicated a significant difference under the two conditions. Transcriptome profiling can be applied to identify key genes participating in the P deficiency response of oil palm seedlings. The total identified DEGs were 2,905. A total of 1,348, 1,465, and 92 DEGs were identified in low-P, high-P, and both conditions, respectively. Forty-five DEGs (49%) out of 92 DEGs identified in both treatments were upregulated, and 47 (51%) DEGs were downregulated in P-deficient conditions. Five genes were selected for further analysis based on their function in P regulation in other plants, including nutrition response and root growth (*NRR*) repressor homolog 1 (XM_010909200), *AP-4 complex subunit sigma* (XM_010916464), *serine/threonine protein phosphatase* (XM_010909466), *psbp domain-containing protein 7* (XM_010940137) and *pentatricopeptide repeat-containing protein* (XM_010907539). Five genes need to be analysed using the RT-qPCR approach for validation as marker candidates related to P deficiency in oil palm in the next study.

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Direct PCR-Based Detection of Pork Adulteration in Food Products Using ND4 and Cytb Markers

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ABSTRACT

The adulteration of edible processed meat has become widespread, as low-quality meat is added. One of the most prevalent instances is the use of an adulterated meat product containing pork, which caused problems for Muslims because of their dietary restrictions. Food safety needs a quick, easy, and specific DNA-based detection method. Therefore, the present study sought to detect pork contamination utilising the direct-PCR method with NADH dehydrogenase 4 (ND4) and cytochrome b (Cyt-b) primers in fresh pork and commercially processed meat products. The lysis buffer recipe was optimised using two temperatures and three incubation periods. Conventional PCR and electrophoresis were used to confirm DNA detection. Three brands of meatballs, sausages, and corned beef obtained from several markets in Malang City, Indonesia, were examined following optimisation. With high levels of DNA concentration and purity as well as visible DNA bands, the incubation temperature of 95°C for 5 minutes clearly exhibited the best results. All ND4 and Cyt-b primers amplified pork DNA in the majority of the processed samples, except for some Cyt-b primers, which did not amplify DNA in certain corned beef products. The direct-PCR technique is clearly a straightforward, fast, inexpensive, and reliable method for the detection of pork adulteration in processed meat products and could also be applied for halal food authentication.

Keywords: Adulteration, direct PCR, ND4, Cyt-b, pork detection

INTRODUCTION

Food adulteration is the modification of food products by the addition of inferior or unauthorised materials. This case is a serious issue in the food industry, especially in the

meat industry (El Sheikha et al., 2017). Many meat products are often economically mixed with lower-priced, poorer-quality, and unsafe meats to increase profitability (Valand et al., 2020). Pork is frequently adulterated due to its muscle texture and fibre arrangement, which appear similar

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to those of beef (Yang et al., 2018; Li et al., 2023). Adulteration of pork is prohibited by Islamic law regarding the halalness of food in a product (Song et al., 2019).

To determine the presence of pork in food products and thus the halalness of a product, a detection method is required, one of which is a DNA-based method employing the PCR or Polymerase Chain Reaction method. PCR is a type of enzymatic synthesis that amplifies particular DNA fragments that will become the target DNA. The PCR method is frequently used because it is direct, has high sensitivity and specificity, and is inexpensive (Alikord et al., 2018). The primer is a key aspect in the success of the PCR process (genetic marker). Several primers, including mitochondrial DNA cytochrome-b/Cyt-b (Indriati & Yuniarsih, 2019), 12S rRNA (Cahyadi et al., 2017), D-Loop (Karabasanavar et al., 2017), and NADH dehydrogenase-4 (ND4), have been employed successfully in detecting pork DNA fragments (Kusnadi et al., 2021). The primers utilised in this work were Cyt-b and ND4, which have previously successfully detected pork DNA from processed meat products (Kusnadi et al., 2020; Kusnadi et al., 2024). To our knowledge, there is no evidence that ND4 can identify pig DNA fragments, and no references compare ND4's performance to that of Cyt-b.

DNA isolation is typically performed before the PCR process to isolate pure DNA. According to Gargouri and Kacem (2018), a variety of DNA extraction protocols involving phenol-chloroform-isoamyl alcohol and others are effective but time-consuming and require many steps. Direct PCR is an alternative approach to combine the cell lysis step with DNA amplification in one reaction (Guan et al., 2019). The lysis buffer approach can be utilised to shorten the time required for DNA isolation while also being cost-effective. Using the lysis buffer will lyse the cell wall, allowing the components within the cell to escape. This is referred to as the direct-PCR approach (Gupta, 2019). Therefore, an effective detection method for identifying pork contaminants is urgently needed and critical for food safety (Ha et al., 2017).

A limited study has shown the capability of direct PCR detection of pork DNA using Cyt-b and ND4 primers. Furthermore, the optimisation of lysis buffer conditions in direct PCR applications has not been thoroughly investigated to improve detection consistency in commercial products. Hence, the current study used the direct PCR method and two primers to detect pork DNA to avoid adulteration of processed meat products while reducing the time and expense required.

MATERIALS AND METHODS

Time and Location

The present study was carried out at the Biology and Molecular Laboratory, Central Laboratory of Life Sciences (LSIH), Universitas Brawijaya, Malang, Indonesia. The study was carried out between May and September 2021.

Sample Collection and Preparation

Pork meat and processed meat items such as meatballs, sausages, and corned pork were used as samples in this study. This study's sample was obtained from street vendors, traditional markets, and modern marketplaces in Malang City. Each of the four samples was weighed (0.25 g), wrapped in aluminium foil, and labelled according to the sample type. Samples were then stored at -20°C for further analysis.

Sampling Test

Lysis buffer and incubation conditions were tested upon sampling by direct PCR. The temperature of 95°C for 5 min was selected as the optimal lysis temperature in all samples (three brands × three product types × three replicates). The DNA was then measured for concentration and quality before amplification with ND4 and Cyt-b primers and then visualised using electrophoresis.

Direct PCR (Cell Lysis)

The direct-PCR approach bypasses the need for DNA isolation, as does the normal PCR method, and instead employs a lysis buffer to obtain DNA from cells. Weighed meat and processed product samples were put in a 1.5 ml Eppendorf tube containing 200 µL of lysis buffer formulation: 0.2 M NaOH, 0.025 M EDTA, 0.1 M NaCl, 0.01 % SDS, 0.01 Tris-Cl, and 0.07 % Tween-20 based on the modified approach (Guan et al., 2019), and incubated (Mettler) at various temperature (85°C and 95°C) and incubation time (5, 10, and 15 minutes) to achieve optimum condition. Each sample was incubated at 800 rpm in a Thermomixer (HLC BioTech/MHR 13) with this treatment, and then 45 µL of Tween 20 was added. The samples were kept in a 4°C refrigerator (Toshiba) for 15 min before being tested for concentration and purity.

DNA Concentration and Purity Assessment

DNA concentration and purity were assessed with a nanodrop spectrophotometer (ND-1000 UV/Vis) at 260 nm and 280 nm. The purity of DNA was determined by the A260/A280 ratio, while the concentration of DNA was expressed in ng/µl.

PCR Amplification

PCR was performed using two sets of species-specific primers for the mitochondrial genes ND4 and Cyt-b. Each 10 µL PCR reaction contained 0.5 µL forward primer (5 pmol/µL), 0.5 µL reverse primer (5 pmol/µL), 3 µL ddH₂O, 5 µL MyTaq™ Red Mix (Bioline), and 1 µL of DNA template. To avoid reagent breakdown, low temperature conditions were maintained. The tube from the PCR mix was gently mixed and spun down into 1.5 mL

tubes. PCR reactions were performed in 0.2 mL thin-walled tubes with a thermal cycler (Applied Biosystem™ PCR System 9700 Thermal Cycler, Thermo Fisher Scientific, USA). PCR condition was set at initial denaturation at 94°C for 5 s, and then 32 cycles with denaturation (94°C for 30 s), annealing (55-60°C for 45 s), extension (72°C for 30 s), followed by final extension at 72°C for 5 min. The results were confirmed by electrophoresis (Guan et al., 2019).

Electrophoresis

PCR amplified results were subjected to horizontal electrophoresis with Mupid 2 Plus System (Takara Bio Inc., Japan). Then it was visualised on a 1.5% agarose gel. Agarose powder was dissolved in 1x Tris-Borate-EDTA (TBE) buffer. 2 µL of ethidium bromide was added to visualise the DNA, and then poured into an electrophoretic gel mould. A comb and electrophoretic plate were inserted into the mould, and the gel was allowed to solidify for 30-40 min at room temperature under closed conditions. After solidification, the gel was submerged in 1× TBE buffer. A total of 5 µL of PCR product (amplified DNA) mixed with 1 µL loading dye was loaded into each well, along with 3 µL of 100 bp DNA ladder as a size reference. Electrophoresis was performed at 50 V for 50-60 minutes. DNA bands were visualised and documented using a gel imaging system (Bio-Rad Chemidoc™/BR-200, Bio-Rad Laboratories Inc., USA)

RESULTS AND DISCUSSION

Optimisation of Lysis Buffer Conditions

The optimisation of DNA extraction was carried out by varying lysis buffer incubation temperatures (85°C and 95°C) and durations (5, 10, and 15 minutes). The results showed DNA purity ranging from 1.70 to 2.25 and DNA concentrations between 66.17 and 469.23 ng/µL (Table 1).

DNA purity is assessed based on the A260/A280 ratio. Regarding Hashemipetroudi et al. (2018), there is typically no protein and RNA contamination when the values are between 1.80 and 2.00. Nucleic acids' absorbance at 260 nm and contamination with protein are at 280 nm (Setiati et al., 2020). The majority of results were outside of the allowed range with slight contamination, possibly from protein (ratio <1.80) or RNA (ratio >2.00), especially in the sausage, 15-minute pork meat, and meatball samples. Most samples exhibited acceptable DNA quality and quantity for PCR amplification, with a few samples exhibiting deviations. These results demonstrated that an efficient method for obtaining amplifiable DNA from processed meat products is direct lysis at 95°C for 5 min.

Table 1
Optimisation results of buffer lysis conditions

Samples	Incubation time	Temperature	DNA concentration	
			(ng/ μ L)	DNA purity
Pork meat	5	85	124.27	1.81
		95	98.07	1.92
	10	85	230.78	1.73
		95	213.79	1.84
	15	85	118.07	1.70
		95	180.01	1.74
Meatball	5	85	121.94	1.94
		95	66.17	1.95
	10	85	102.62	1.81
		95	101.41	1.83
	15	85	85.95	1.73
		95	180.89	1.73
Sausages	5	85	164.23	2.25
		95	469.23	2.11
	10	85	417.02	2.14
		95	205.77	2.14
	15	85	192.15	2.19
		95	407.79	2.14
Corned beef	5	85	191.53	1.81
		95	205.16	1.86
	10	85	79.14	1.89
		95	215.81	1.81
	15	85	193.51	1.83
		95	242.39	1.85

PCR Amplification with ND4 and Cyt-b Primers

The results showed that using multiple temperature and incubation time conditions, successful amplification was observed with the ND4 (120 bp) and Cyt-b (398 bp) (Figure 1). Blanks and negative controls showed no amplification, confirming the absence of contamination.

At 5 minutes of incubation, pork DNA was successfully amplified at both 85°C (A1, A3, A4) and 95°C (B1, B2, B3, B4) using ND4 primers, except in sample A2 (85°C), which yielded no band. Cyt-b amplification at this time point was successful across all samples. At 10 minutes, ND4 amplification was successful in some samples (C1, C3, D3, D4), but failed in others (C2, C4, D1, D2). Cyt-b amplification was generally successful, except in samples C4 (85°C) and D4 (95°C). At 15 minutes, most samples were successfully amplified with ND4, except E2 (85°C), which showed no band. Cyt-b amplification at this time point failed in E2, E4 (85°C), and F4 (95°C).

Possible causes of amplification failure include DNA degradation from high-temperature processing (Yahya et al., 2017), residual contaminants such as proteins, carbohydrates, or

polysaccharides that inhibit Taq polymerase activity (Muflihah et al., 2023; Rezadoost et al., 2016), suboptimal annealing temperature, leading to either non-specific binding (too low) or poor primer-template interaction (too high) (Wijaya et al., 2022), and primer-template mismatch, particularly in degraded DNA (Deekshit et al., 2019). Izadpanah et al. (2018) also observed various band thicknesses. The larger bands suggest that there were more DNA templates (Setiati et al., 2020). Variation in ND4 and Cyt-b primer pairs reflects differences in product size or amplification efficiency. For decayed or processed products, ND4 with a smaller amplicon performed better (Kusnadi et al., 2020) than Cyt-b, which becomes more susceptible to degradation (Matange et al., 2021).

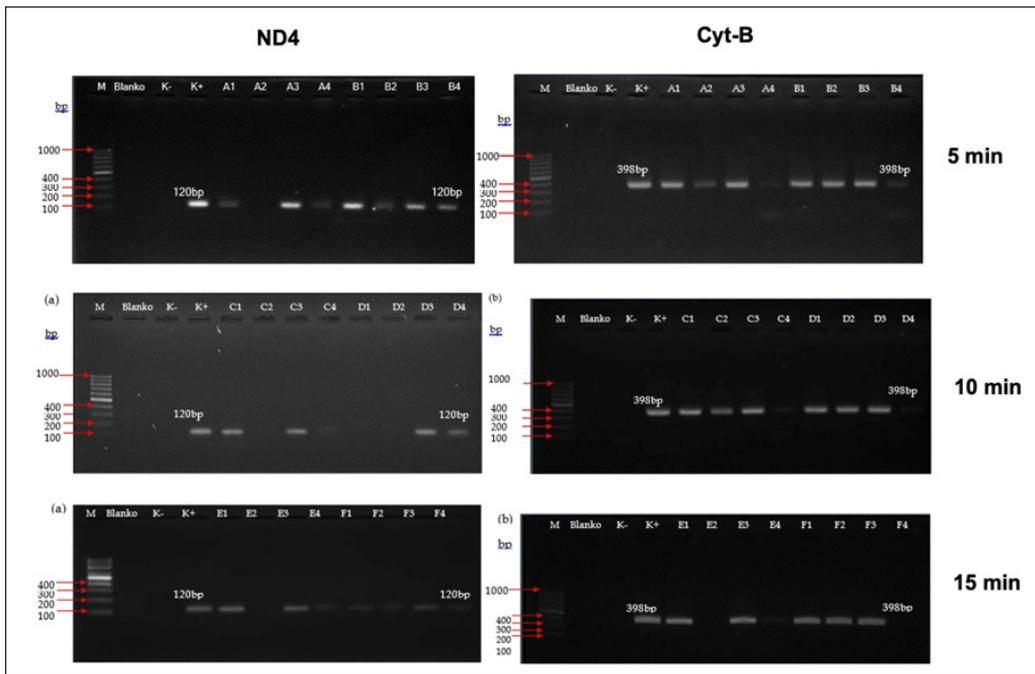


Figure 1. Visualisation of DNA bands on optimum buffer lysis conditions at incubation time of 5, 10, 15 min at 85°C and 95°C for both ND4 and Cyt-b. Note: 5 min treatment: K- = ddH₂O, K+ = Pork meat isolated by Kit, A1 = Meat (5', 85°C), A2 = meatball (5', 85°C), A3 = sausage (5', 85°C), A4 = corned beef (5', 85°C), B1 = meat (5', 95°C), B2 = meatball (5', 95°C), B3 = sausage (5', 95°C), B4 = corned beef (5', 95°C); 10 min treatment: C1 = meat (10', 85°C), C2 = meatball (10', 85°C), C3 = sausage (10', 85°C), C4 = corned beef (10', 85°C), D1 = meat (10', 95°C), D2 = meatball (10', 95°C), D3 = sausage (10', 95°C), D4 = corned beef (10', 95°C); 15 min treatment: E1 = meat (15', 85°C), E2 = meatball (15', 85°C), E3 = sausage (15', 85°C), E4 = Corned beef (15', 85°C), F1 = meat (15', 95°C), F2 = meatball (15', 95°C), F3 = sausage (15', 95°C), F4 = corned beef (15', 95°C).

Parameters such as DNA extraction method, concentration, and purity of DNA, primers, and inhibitors such as proteins, phenols, RNA, and other substances in cells can affect DNA amplification success (Deekshit et al., 2019; Amiteye, 2021; Sophian et al.,

2021). Furthermore, the polysaccharide and other organic content present could inhibit DNA polymerase enzymatic activities (Rezadoost et al., 2016). Primer specificity is crucial for food authenticity and detection of contaminants in small amounts (Arini et al., 2018). Differences in DNA intensity may occur due to DNA degradation throughout the food preparation process. DNA degradation happens when the links between molecules are disrupted due to extreme physical treatment, overheating, and the presence of certain chemicals (Bhoyar et al., 2024). This is consistent with prior research, which indicates that processed product samples are prone to DNA fragment degradation. The processed product has been blended with 38 other substances and heat-treated; therefore, there is a possibility that the DNA present in the sample has been degraded and its concentration has been reduced (Yahya et al., 2017).

Sampling Test Results

The sampling test used optimised conditions (lysis at 95°C for 5 min). DNA purity values ranged from 1.62 to 2.11, while concentrations varied between 126.09 and 321.43 ng/ μ L (Table 2). Some samples fell outside the 1.80-2.00 purity range, indicating minor contamination—likely protein contamination in meatballs and corned beef (A260/A280 <1.80), and RNA contamination in sausages (A260/A280 >2.00) (Hashemipetroudi et al., 2018).

Table 2
Concentration and purity of processed meat products

Samples	Concentration (mg/ μ L)	Purity (A260/A280)
Meatball A*	145.72	1.74
Meatball B	212.59	2.01
Meatball C	126.09	1.80
Sausage D*	321.43	2.11
Sausage E	192.54	1.95
Sausage F	274.63	2.08
Corned beef G*	168.82	1.86
Corned beef H	223.29	1.62
Corned beef I	168.43	1.62

Note. Asterisk (*) indicates good concentration and purity

Figure 2 shows that meatball brand A (A1-A3) was positive for pork DNA with both ND4 and Cyt-b primers, while brands B and C were negative. Sausage brand A also tested positive with both primers; brands B and C were negative. Because the ND4

and Cyt-b primers used in this study are specific for the pig species, it was indicated that commercial meatball brands B and C are not contaminated with pork DNA. For corned beef, all samples showed negative results with Cyt-b primers, while ND4 showed faint or inconsistent amplification. Corned beef brand A might be amplified more efficiently in Cyt-b than ND4 primers.

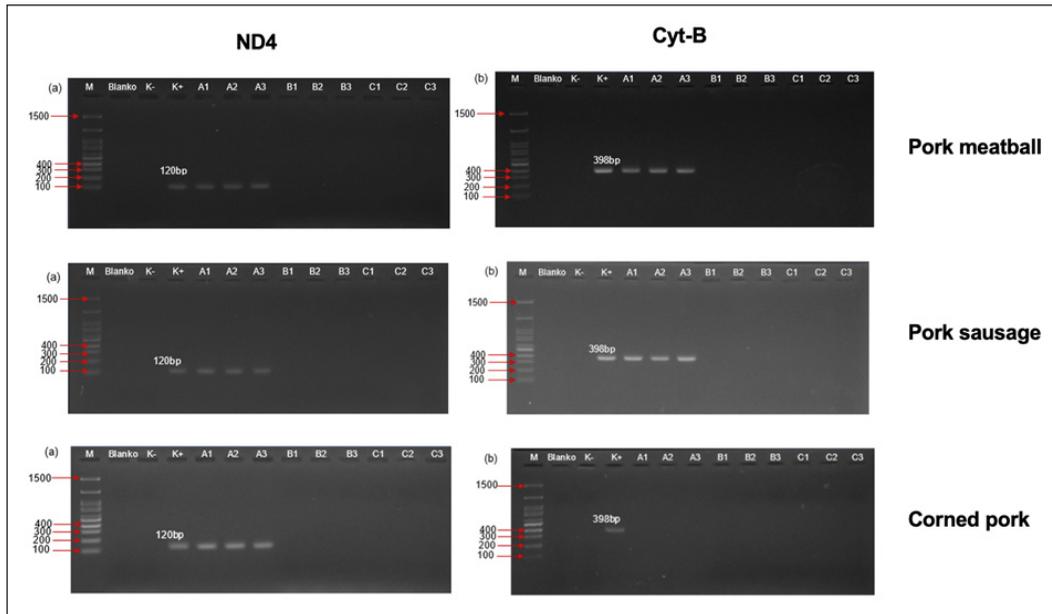


Figure 2. Visualisation of DNA bands on 3 meatballs, 3 sausages, and 3 corned beef brands with three repetitions for both ND4 and Cyt-b. Note: Meatball: K- = ddH₂O, K+ = Pork meat isolated by kit, A1-A3 = meatball brand A (contains pork), B1-B3 = meatball brand B (no pork), C1-C3 = meatball brand C (no pork); Sausage: A1-A3 = sausage brand A (contains pork), B1-B3 = sausage brand B (no pork), C1-C3 = sausage brand C (no pork); Corned beef: A1-A3 = corned beef brand A (contains pork), B1-B3 = corned beef brand B (no pork), C = corned pork brand C (no pork).

The absence of detectable DNA bands in some processed products may result from high-temperature sterilisation (120°C for 15 min), which compromises DNA stability (Matange et al., 2021), ineffective primer binding due to degraded DNA or excessive product length, especially for the longer Cyt-b amplicon (398 bp), and the presence of PCR inhibitors (Yahya et al., 2017). Corned pork is also sterilised at 120°C for 15 min. This treatment can have an impact on the stability of the DNA to be amplified since, while DNA is still present in the sample even when heated to 120°C, the stability of DNA has reduced, resulting in DNA degradation (Matange et al., 2021). Since Cyt-b is longer than the ND4 primer, PCR performance may be affected. According to Kusnadi (2020), overly long primers will make the amplification process less successful, resulting in the degradation of the amplified DNA band. The study findings revealed that the most effective method

for detecting pork DNA in processed meat products is direct PCR using ND4 and Cyt-b primers with optimum lysis conditions established at 95°C for 5 min. When amplifying degraded DNA, ND4 was more suited for the processed product. There was an insufficient sample size and unmeasured sensitivity and specificity of the results. Therefore, validation using real-time PCR is needed to improve the sensitivity and accuracy.

CONCLUSION

The direct PCR approach can amplify DNA from fresh meat and processed meatballs, sausages, and corned pork products, utilising ND4 and Cyt-b primers. Compared to ND4, the Cyt-b primer was more effective at detecting pork DNA in processed products. The optimal temperature and incubation time were established at 95°C for 5, 10, and 15 min (Cyt-b), and 95°C for 5 min (ND4). More study with real-time PCR with primers ND4 and Cyt-b is needed to improve results (sensitivity), raise the accuracy of the smallest detectable DNA concentration, and quantify the amount of contamination in processed products.

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Pure line selection for performance evaluation of Sorghum Breeding Lines for Grain Yield and Its Related Traits

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ABSTRACT

Sorghum is an important cereal crop grown in arid and semi-arid regions for food and feed purposes. Considering its potential use as a carbohydrate source in animal feed, a breeding program was launched at Universiti Putra Malaysia (UPM) to develop local grain sorghum varieties. The research aims to evaluate the performance of selected breeding lines in terms of growth and grain yield traits. The breeding lines include 12 sorghum pure lines, together with two advanced forage sorghum genotypes, Putra SB1 and Putra SB2, as controls. The experiment was conducted in a Randomised Complete Block Design at Field 10 UPM. Traits with high heritability and positive correlation to grain yield were the selection criteria. Results suggest that line V7 (5-3-6) recorded the highest grain yield (1785 kg/ha), while V10 produced the lowest yield (541 kg/ha), and was also the earliest to reach flowering (64 days). The longest height was achieved by V6 (2-36-3), at 294.4 cm, whereas V12 (Upms) recorded the least mean height (166.2 cm). There was moderate intra-population homogeneity observed for growth and yield characteristics, with coefficient of variation

values ranging from 2.3% to 33.7%, because the population was still segregating. The results indicate that V7 demonstrated significant yield potential among the pure lines, highlighting their suitability for developing high-yielding grain sorghum cultivars from the existing population that are adapted to the local climatic conditions of Malaysia. However, further assessment is recommended to purify the evaluated population.

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INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) belongs to the Poaceae family and is cultivated in warm climates worldwide (Xin et al., 2021). It has been the staple and traditional food of the African continent since ancient times (Widowati & Luna, 2022). Sorghum ranks fifth among cereal crops, with a worldwide production of 86.2 million tonnes in 2020 (Bakari et al., 2023). The importance of sorghum lies in its diverse range of utilisation, from human consumption, animal feed, and in industries as well, for the production of bio-composite and biofuel (Silva et al., 2024). The varietal development of grain sorghum has various nutritional benefits, including sufficient fibre content, a low glycemic index, and gluten-free (Bazié et al., 2023). The consumption of sorghum grains includes 55% as food, whereas 33 % of it is consumed as animal feed (Kumar et al., 2013). The main constituents of sorghum grain are starch, proteins, and non-starch polysaccharides. The key features of grain sorghum varieties include a height ranging from three to six feet and a bulky panicle head. These are mainly utilised as a food source for humans or as feed for livestock (Dexuan et al., 2022).

In breeding programs for self-pollinated crops such as rice and beans, the pure line selection strategy has been widely used. (De Oliveira Amaral et al., 2019). Across decades, breeders have opted for a diverse range of techniques for varietal development, but have found pure line selection to be the most suitable for self-pollinated crops. (Rakshit & Bellundagi, 2018). In individual plant selection, the base population is genetically variable but homogenous, and the progeny of selected individual plants are evaluated to identify superior pure lines (Visarada, 2018). Evaluation is usually based on growth and yield-related traits (Vom Brocke et al., 2010). A line is considered a cultivar by its ability to adapt, react, and withstand a particular environment. (Aruna & Cheruku, 2019).

Yield is the most complex among the traits, as it depends upon multiple components. It is a quantitative trait of utmost importance and is immensely influenced by environmental factors. The heritability of yield is very low, and selection entirely based on it is not preferable (Xin et al., 2021). Therefore, parameters contributing to grain yield, such as the number of grains per panicle and 1000-grain weight, are also considered and optimised (Von Pinho et al., 2022). Grains per panicle is a crucial trait in sorghum breeding programs. Plant breeders aim to develop sorghum varieties with higher grain yield potential; therefore, the yield components are also an essential criterion for selecting promising breeding lines. The goal is to breed varieties that produce more grains per plant (Dembele et al., 2021). For practical purposes in agriculture, farmers and growers can use grain per panicle data to select sorghum varieties with potential for higher grain yields (Kovtunov & Kovtunova, 2021). The selection of pure lines also depends on the morphological traits that help determine and differentiate a grain sorghum variety (Bernardeli et al., 2022). Plant height plays a vital role in accepting varieties in a breeding experiment when developing a grain sorghum

variety (Derese et al., 2018). A pure line having a plant height ranging from 3 to 6 feet is considered a grain sorghum variety (Dexuan et al., 2022). A vital quality trait that needs to be addressed while developing a grain sorghum pure line for animal feed is grain colour (Ratnavathi, 2018). The darker-coloured grains in sorghum contain a phenolic compound called tannin, which makes sorghum taste bitter and less desirable (Pan et al., 2019). It negatively affects the overall nutrition and can hinder the digestibility of proteins and carbohydrates. Thus, it will result in the disruption of nutrient metabolism and a decrease in appetite for feed. Therefore, the selection is also based on the colour of the seed coat as well (Farahat et al., 2020).

Heritability is a vital phenomenon for the selection of traits that contribute to and correlate with grain yield. Heritability is a genetic parameter that allows for measuring the genetic influence on traits. It shows the extent to which genetic diversity contributes to phenotypic diversity within a population. The research focuses on heritability estimates to assess the genetic variation in sorghum breeding lines for the proper execution of selection (Herawati et al., 2025).

The current study is part of a breeding program aimed to identify an adapted grain sorghum pure line that is adaptable to the tropical climate of Malaysia. The current breeding program will serve the purpose of germplasm expansion needs, enabling the farmers to cultivate locally adapted sorghum cultivars. It is important to evaluate the genetic diversity in sorghum and the linkages among quantitative traits for the possibility of joint selection of two or more traits. This study will pave the way for considering the inclusion of a new crop, which will be a move towards self-sustainability due to its broad spectrum of utilisation. The deployment of sorghum in Malaysia would primarily focus on grain for feed as an alternative food source with potential energy for animals. (Naharudin et al., 2021).

The current study aims to identify high-yielding grain sorghum pure lines that are adapted to the local climatic conditions of Malaysia. Furthermore, the study aims to assess and purify the existing germplasm to produce locally adapted grain sorghum pure lines that yield adequately to meet local feed requirements. The current findings form part of a comprehensive breeding program that will ultimately lead to the development of a potent grain sorghum variety suitable for local cultivation.

MATERIALS AND METHODS

Planting Materials and Experimental Location

The germplasm was obtained from the Agrogene Bank, Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia (UPM). The plant materials include 14 sorghum lines from grain and forage types (Table 1). Nine lines (V1-V9) had undergone two selection cycles, and three lines (V10-V12) were commercial varieties that have not

been evaluated, and two forage sorghum advanced lines that have undergone 5 cycles (V13 and V14) were included as controls.

Table 1
List of sorghum genotypes and their breeding development status

Label	Accession Number	Type	Number of Selections
V1	1-1-1	Grain	2
V2	1-2-1	Grain	2
V3	1-3-1	Grain	2
V4	1-4-1	Grain	2
V5	1-4-4	Grain	2
V6	2-36-3	Forage	2
V7	5-3-6	Grain	2
V8	6-31-1	Forage	2
V9	6-34-7	Forage	2
V10	White	Grain	0
V11	Sabah	Forage	0
V12	Upms	Grain	0
V13	Putra SB1	Forage	5 (Control)
V14	Putra SB2	Forage	5 (Control)

The study was conducted at Field 10, Universiti Putra Malaysia, Selangor, Malaysia, at coordinates 2.991321088220838° N latitude, 101.71457207742054° E longitude. The soil was sandy loam, with a pH of 5.8. The average temperature was 35 °C, whereas the area received 227.5 mm of average rainfall during the study period.

Experimental Design and Agronomic Practices

Planting began in February, and harvesting was completed by June 2023. The sorghum lines were assigned to rows following a Randomised Complete Block Design (RCBD) with three replications. The inter-plant distance was 25 cm, and the distance between rows was set at 150 cm apart to avoid cross-pollination among the genotypes. A total of 30 plants per genotype was planted and evaluated across replicates. Silver shine was installed to prevent weed growth in this experiment. To ensure uniform growth, sorghum seeds were germinated using Jiffy peat pellets and later were transplanted to the field at 9-10 days after sowing. A basal dose of NPK (15:15:15) was applied 4 days after transplanting, and NPK (12:12:17) was applied 28 days after transplanting, at a rate of 120 kg/ha, to promote vegetative and reproductive growth, respectively. Upon flowering, the panicles were covered with pollination nets to prevent cross-pollination. (Chakravarthi et al., 2018).

Data Collection

The development of sorghum lines was carried out through pure line selection, which continued into the third cycle of selection cycle. Therefore, growth and yield traits were recorded for each individual plant, and the mean performance of each line was used to represent the overall population performance. Plants were harvested at physiological maturity to reduce the effect of biotic factors. This approach helped prevent potential fungal infections caused by rainfall and reduced the risk of bird predation, thus avoiding the depletion of the grains. Each harvested panicle was kept in a separate paper bag and was labelled accordingly. The harvested panicles were kept in a hot air oven for four days at 35°C until the grains reached 8% storage moisture. Data collection was done on phenological, growth, and yield parameters. Phenological parameters, including 50% flowering and 50% physiological maturity, were recorded when the plant reached the desired stage. For the growth parameters, a measuring tape was utilised to determine the plant height, while the number of leaves was counted manually. Yield parameters, including the panicle length and weight, number of grains per panicle, 1000 grain weight, and overall grain yield, were measured post harvest.

Grain yield was determined using the formula:

$$\text{Grain yield (kg/ha)} = \frac{\text{Grain yield (kg) / subplot} \times 1000}{\text{Area/subplot}} \quad [1]$$

(Shahzad et al., 2002)

Data Analysis

Analysis of variance (ANOVA) was used to analyse the mean differences between the populations in RStudio (version 2025.09.2). The mean comparison was done using the least significant difference (LSD) method ($p < 0.05$). Genetic variability analysis was performed using the variability package in RStudio. Correlation between the traits was also measured using the Pearson correlation method ($p < 0.05$). Principal component analysis was performed to observe the percentage-wise contribution of traits to total genetic variation (Bartzialis et al., 2020).

RESULTS AND DISCUSSION

Performance of Morphological Traits and Yield among Sorghum Breeding Lines

The descriptive statistics provided with ANOVA (mean, genetic variance, and coefficient of variation) exemplify all measured traits of 14 sorghum genotypes represented in Table 2.

Table 2
Descriptive statistics and genetic variability parameters for agronomic traits of sorghum genotypes

Genotypes	Trait							1000 grain weight (g)	Grain yield (kg/ha)
	Days to flowering (days)	Days to physiological maturity (days)	Plant height (cm)	Number of leaves (leaves)	Panicle length (cm)	Panicle weight (g)	Number of grains per panicle (grains)		
V1	76.7	105.7	202.1	11.5	20.7	20.6	708.3	23.3	962.3
V2	70.0	103.3	198.4	9.7	21.9	16.2	453.0	26.0	586.4
V3	70.3	103.3	226.9	10.3	18.1	24.6	818.2	22.0	714.3
V4	68.7	108.3	253.1	10.8	20.1	34.8	1129.2	23.6	1452.0
V5	74.3	104.3	228.6	11.8	22.8	13.5	394.2	25.3	596.0
V6	68.7	110.0	294.4	11.6	26.8	30.9	1132.6	22.4	1325.6
V7	68.0	106.0	231.3	9.8	21.1	29.9	786.6	31.8	1785.0
V8	72.0	112.3	261.3	11.3	23.4	33.3	1515.0	18.4	1637.2
V9	72.0	104.3	281.5	11.4	24.5	24.3	968.4	19.4	1058.2
V10	64.3	108.3	238.3	10.5	36.2	18.3	452.9	19.6	541.2
V11	74.3	109.7	228.5	11.1	20.5	24.2	750.6	20.7	849.9
V12	67.0	99.7	166.4	10.1	24.4	36.6	1065.0	29.8	1592.4
V13	80.0	116.0	205.9	12.7	22.1	23.8	922.2	20.7	1066.7
V14	77.0	115.3	253.4	13.1	26.3	15.4	650.0	18.7	799.3
Mean	71.7	107.6	233.6	11.1	23.5	24.7	839.0	23.0	1069.0
Range	15.7	16.3	128.0	3.5	18.2	23.1	1120.9	13.4	1243.8
Genotypic variance	13.1**	20.41***	1034.0***	-0.0045	18.3***	40.8**	70278.6**	14.8***	172525.4***
Heritability	0.4	0.8	0.7	-0.0015	0.9	0.5	0.5	0.7	0.9
CV (%)	5.9	2.3	8.6	15.8	7.4	27.1	33.7	3.8	12.1

Note. CV: Coefficient of variation * significant at p<0.05, ** significant at p<0.01, *** significant at p<0.001, ns non-significant

Table 2 presents significant genetic differences among sorghum genotypes ($p < 0.05$). The overall coefficient of variation (CV) ranged from 2.3-33.7% for the agronomic traits, show substantial variability among lines. The high CV for the number of leaves, panicle weight, and number of grains per panicle indicates adequate environmental impact, which influenced the expression of these traits. Moreover, panicle weight and the number of grains per panicle can be substantially influenced by rust and bird predation (Tsegau & Tegegn, 2020). Furthermore, the ANOVA results also deduced considerable genetic variability for plant height, number of grains per panicle, and grain yield. The genetic variability can provide insight into the genetic improvement of genotypes. It also affirms that these variations have contributed to overall variability. Panicle length, plant height, days to physiological maturity, 1000-grain weight, and grain yield showed high heritability, ranging between 0.7-0.9. Traits with moderate heritability include days to reach 50% flowering, panicle weight, and number of grains per panicle (0.4-0.5) (Table 2). Characters with high heritability also have a lower coefficient of variation, showing less variability, making the selection appropriate. Traits with higher heritability are considered for the selection of genotypes for the next generation (Yaqoob et al., 2015).

Phenological Parameters

A significant difference was observed among the pure lines in the number of days to reach 50% flowering (Figure 1a). The genotype with the earliest flowering was V10 (64.3 days), followed by V4 and V6 (68 days). The line that took the highest days to flower was the control genotypes V13 (80 days) and V14 (77 days). Phenological traits are essential indicators that can help predict crop maturity. The lines that flower early can possibly mature earlier than others; hence, they can be prevented from any biotic and abiotic threats in the field (Mwamahonje & Maseta, 2018).

Significant variations have been observed between genotypes regarding the number of days needed to reach 50% physiological maturity. The genotype that took the fewest days to reach physiological maturity is V12 (99.7 days), followed by V3 and V2 (103.3 days). Control genotypes V13 (116 days) and V14 (115.3 days) took the longest to mature (Figure 1b). A study conducted by Baloch et al. (2023) found that phenological parameters can assist in predicting the optimal timing to prevent pollination and plan the season based on the environmental stresses of a particular location (Baloch et al., 2023). The early maturing lines are the focus of most breeding programs for producing new varieties (Suza & Lamkey, 2024).

Growth Parameters

A significant difference was observed in plant height among the populations. The genotype with the tallest plant height was V6 (294.4 cm), followed by V9 (281.5 cm). However, for

grain sorghum, a dwarf phenotype is preferred. The shortest plant among the evaluated populations was V12, reaching a mean height of 166.2 cm, followed by V2 (198.4 cm) (Figure 2a). Results by Mwamahonje and Maseta (2018) affirmed that to be recognised as a grain sorghum variety, it must be less than 2 meters in height (Mwamahonje & Maseta, 2018). Breeding experiments conducted in tropical regions with high winds and rain have specific challenges. If the plant is taller in size, it will ultimately lodge, leading to significant yield reduction. On the management side, a lower plant height will facilitate the harvesting process (Yan et al., 2023).

Significant and substantial differences were observed between the populations in terms of leaf number. The control genotypes V14 (13.1 leaves) and V13 (12.7 leaves) produced the highest mean number of leaves. The lowest number of leaves was found in V2 (9.7 leaves) (Figure 2b). Kamal and Ahmad (2022) also observed similar variations among the leaves of different sorghum cultivars. They concluded that leaves are the leading site for photosynthesis, which positively influences the grain yield of sorghum. Therefore, an optimum number of leaves will enable the plant to photosynthesise actively by providing more surface area, generating considerable biomass and grain (Kamal & Ahmad, 2022).

Yield Parameters

The longest panicle was produced by genotype V10, with a mean length of 36.2 cm. It was followed by V6 (26.8 cm) and the control genotype, V14 (26.3). V3 produced the smallest panicles with a mean length of 18.1 cm (Figure 3a). A larger panicle can help deduce a good number of grains that may ultimately contribute to increasing grain yield. Studies have also shown a negative correlation between panicle length and grain yield. This is due to the loose and lax nature of the panicle despite having a considerable length (Mamo et al., 2023). The heaviest panicle-producing genotype was V12, with a panicle weighing 36.6 g, followed by V4, with a mean panicle weight of 34.8 g. The value for the lightest panicle was V5, with a mean weight of 13.5 g (Figure 3b). A heavy panicle is a testament to producing more grain yield, but it also depends on factors such as grain size. Panicle weight is an indicator of adaptability and a measure of resource allocation. The weight also depends on the photosynthetic activity of the plant, which eventually facilitates grain filling. (Lin & Guo, 2020).

The genotype producing the highest number of grains per panicle was V8 (1515 grains), while V5 was the least yielding genotype with 394.2 grains (Figure 4a). In a breeding experiment, it is essential to select plants with a desirable seed weight (Surpam et al., 2019). The genotype yielded the highest 1000-grain weight, V7, with a mean weight of 31.8 g. V8 produced the lowest 1000-grain weight with a mean weight of 18.4 g (Figure 4b). Parameters such as panicle length, panicle weight, and 1000-grain weight all contribute to the calculation of grain yield. The grain weight depends on the grain size, and it is the

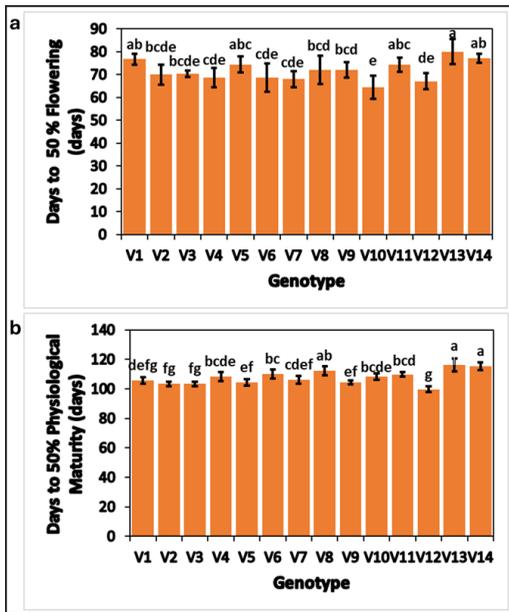


Figure 1. Number of days taken by sorghum pure lines to reach phenological stages: (a) 50% days to flowering and (b) 50% days to physiological maturity. Values are expressed as mean \pm standard deviation (SD), where SD is represented by error bars. Small letters indicate significant differences among genotypes at $p < 0.05$, based on the LSD test

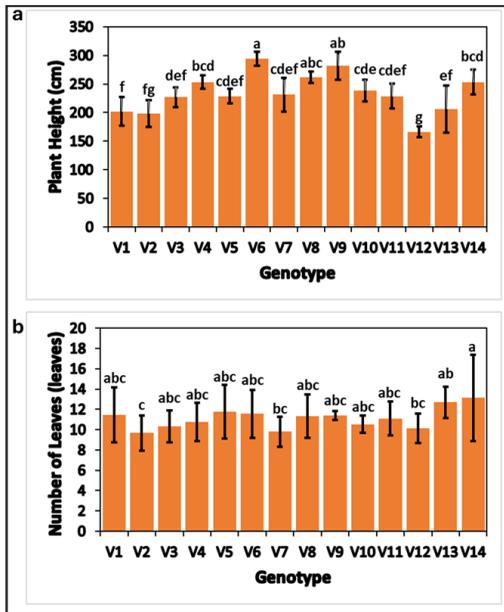


Figure 2. Variation in growth parameters among sorghum pure lines: (a) plant height (cm) and (b) number of leaves. Error bars represent the standard deviation (SD). Small letters indicate significant differences among genotypes at $p < 0.05$, based on LSD

main contributing trait that allows the calculation of grain yield. Grain yield is the ultimate breeding objective for an experiment based on developing a cultivar. (Tao et al., 2018). The genotype producing the highest grain yield was V7 (1785 kg/ha), followed by V8 (1637.15 kg/ha) and V12 (1592.23 kg/ha). The lowest-performing genotype in terms of grain yield was V10, which yielded 541.1 kg/ha (Figure 4c). In a breeding experiment aligned with developing a grain sorghum cultivar, grain yield is the deciding factor. Developing a variety that produces an adequate annual yield is a global necessity to address the alarming issue of food security (Albahri et al., 2023). Improving the crop yield will help address the issue of food security and reduce the environmental impact raised due to the use of chemical fertilisers in such experiments (Dewi et al., 2023).

Correlation Study of Yield and Agronomic Parameters

The analysis of phenotypic correlation measured between the traits on 14 genotypes is presented in Table 3.

Traits related to yield, specifically grain yield and 1000 grain weight, showed a positive correlation ($r = 0.34$; $p < 0.05$). These findings align with those of Tao et al. (2018), who

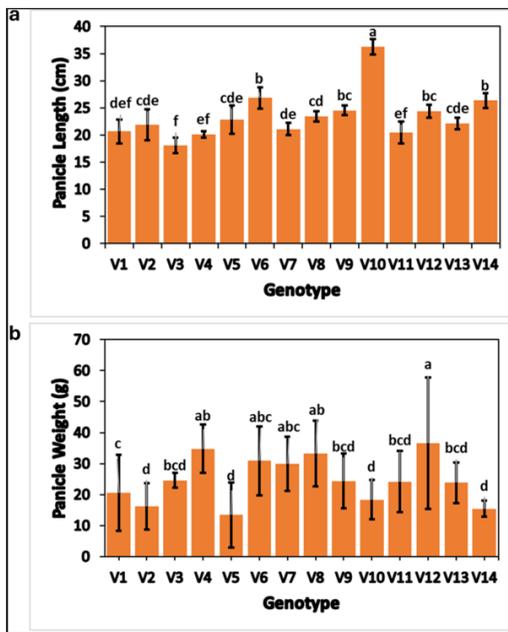


Figure 3. Variation in yield components among sorghum genotypes: (a) panicle length (cm) and (b) panicle weight (g). Error bars represent the standard deviation (SD). Small letters indicate significant differences among genotypes at $p < 0.05$, based on LSD

found that the grain yield of the genotypes showed a high correlation with total grain weight, as 1000-grain weight is indicative of grain size and quality. Therefore, a larger grain size can contribute to greater grain yield (Baye et al., 2022). The number of grains per panicle and panicle weight showed a robust positive correlation ($r = 0.90$; $p < 0.001$). Parida et al. (2022) also reported similar results, where grains per panicle and panicle weight were strongly correlated, as panicle weight is the major contributing factor; hence, a heavier panicle would bear a greater number of grains (Parida et al., 2022). Panicle length had a negative correlation with grain yield and 1000-grain weight ($r = -0.39$; $p < 0.05$), as enormous panicles bear more branches possessing a higher number of grains per panicle, making the grain size smaller and lighter in weight, hence reducing the grain yield. (Asungre et al., 2021).

Among the phenological traits, days to flowering and days to physiological maturity showed a positive correlation ($r = 0.52$; $p < 0.001$). Days to physiological maturity were also positively correlated with the number of leaves ($r = 0.38$; $p < 0.05$). The correlation

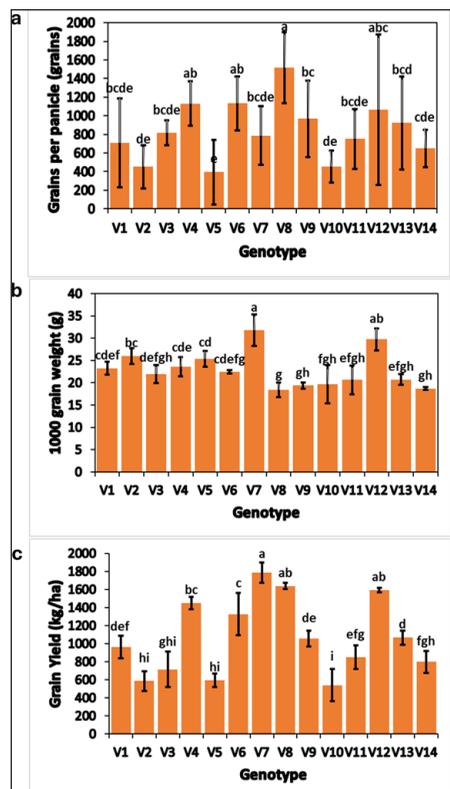


Figure 4. Variation in yield parameters among sorghum genotypes: (a) 1000-grain weight (g), (b) grains per panicle, and (c) grain yield (kg ha^{-1}). Error bars represent the standard deviation (SD). Small letters indicate significant differences among genotypes at $p < 0.05$, based on LSD

in the phenological traits is due to the influence of genetic and environmental factors, combined with plant growth rate mainly influenced by nutrient unavailability (Borrell et al., 2020). Days to physiological maturity were negatively correlated with 1000-grain weight ($r = -0.54$; $p < 0.001$). In a study conducted by Wang et al. (2020), the phenological traits were found to be negatively correlated with grain yield because of the absence of adequate moisture (Wang et al., 2020). The negative correlation can also be due to the presence of early flowering genotypes that lead to a reduction in biomass accumulation, hence provoking a negative impact on grain yield (Ostmeyer et al., 2022).

Table 3
Correlation analysis among agronomic and yield traits

	NOL	PH	PL	DPM	DF	GPP	PW	GY
PH	0.29ns							
PL	0.11ns	0.24 ns						
DPM	0.38 *	0.27 ns	0.16 ns					
DF	0.12 ns	-0.20 ns	-0.34 *	0.52 ***				
GPP	-0.18 ns	-0.29 ns	-0.11 ns	0.08 ns	-0.10 ns			
PW	-0.39 *	0.12 ns	-0.11 ns	-0.08 ns	-0.21ns	0.90 ***		
GY	-0.08ns	0.11 ns	-0.21 ns	0.00 ns	-0.18 ns	0.57 ***	-0.60 ***	
1000GW	-0.27ns	-0.39*	-0.23 ns	-0.54 ***	-0.28 ns	-0.09 ns	0.17 ns	0.34 *

Note. PH: plant height (cm), PL: panicle length (cm), GPP: grains per panicle (grains), PW: panicle weight (g), DPM: days to physiological maturity (days), DF: days to flowering, NOL: number of leaves (leaves), 1000GW: 1000 grain weight (g), GY: grain yield (kg/ha)

*Significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $p < 0.001$, ns not significant.

Principal Component Analysis of Sorghum Genotypes based on Yield and Morphological Traits

The principal component analysis (PCA) is an important tool for extracting significant data from a large data set. The total number of components in PCA is based on the number of variables being analysed (Sinha & Kumaravadeivel, 2016). In this case, the results from the cumulative variance suggest that principal component 1 (PC1) and principal component 2 (PC2) contribute 37.1% and 29.8%, respectively, making it 66.9% of the overall cumulative proportion. The scree plot reflects the variation percentage of each principal component (Figure 4) (Elenen et al., 2019). A biplot is the visual representation of the loading values of the first two principal components. It is constructed based on the loading values for the traits in their respective principal components. Only the higher value from PC1 and PC2 in a biplot is selected because the more considerable loading value indicates a stronger relationship between the variable and its subsequent principal component (Ndiaye et al., 2019) (Figure 5)

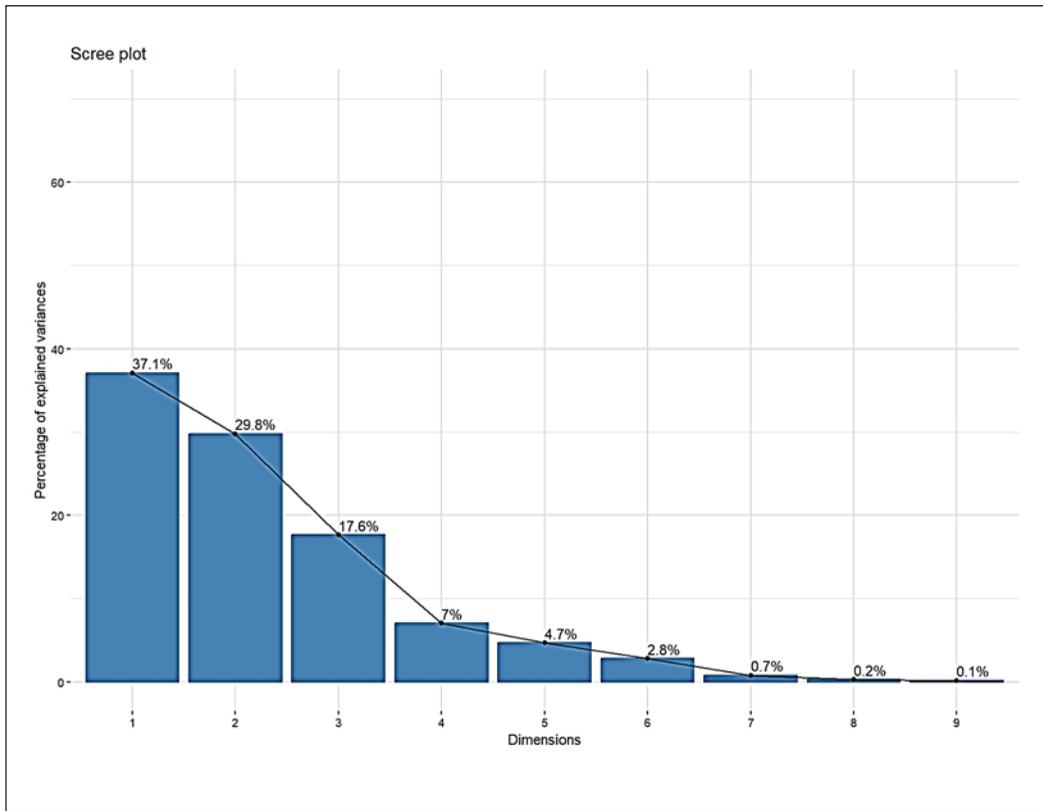


Figure 5. Principal component analysis of agronomic parameters of sorghum genotypes. Scree plot showing the variance percentage of different components

Results from the PCA biplot indicate that V7 and V12 were the most distinctive genotypes, producing the highest grain yield and exhibiting the shortest plant height. Moreover, genotypes V6 and V9 exhibited the highest plant height but yielded minimal grain. The control genotypes took the longest to reach flowering and physiological maturity, resulting in inadequate grain yield (Figure 6).

Similar results have been reported by Kavithamani et al. (2019), where the percentage of cumulative variances of PC1 and PC2 were higher than other components based on agronomic parameters of sorghum cultivars. Usually, the two first components contribute to a large amount of total variance (Kavithamani et al., 2019). Subramanian et al. (2019) presented that data from eight agronomic traits were grouped into three major components accounting for 77 % of total variation, with significant variation confined to the first two components. PCA was able to recognise the key parameters that were responsible for making the population variable (Subramanian et al., 2019)

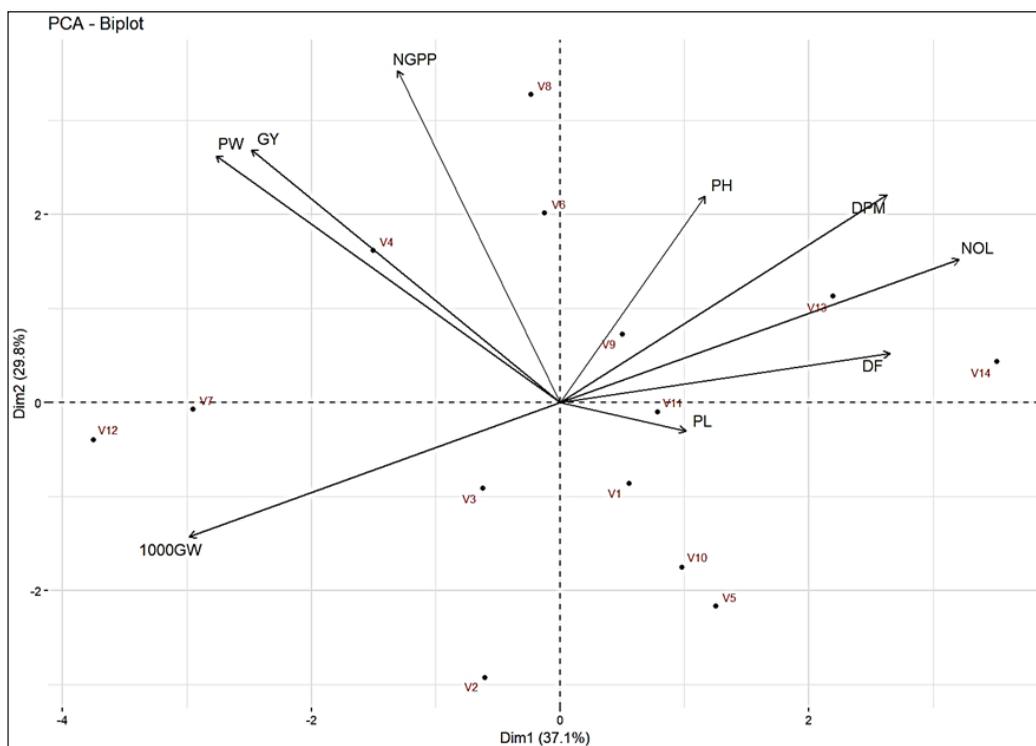


Figure 6. Principal component analysis of agronomic parameters of sorghum pure lines. PCA biplot for the distribution of sorghum genotypes for the first two principal components based on nine quantitative traits. *Note.* PH, plant height (cm); NOL: Number of leaves (leaves); PL: Panicle length (cm); PW: Panicle weight (g); NGPP: Number of grains per panicle (grains); DF: Days to reach 50% flowering (days); DPM: Days to reach 50 % physiological maturity (days); 1000GW: 1000 grain weight (g); GY: Grain yield (kg/ha)

CONCLUSION

The selection study concluded that Genotype 7 produced the highest 1000-grain weight and grain yield. It was also the earliest to reach flowering and physiological maturity, only second to Genotype 12. Genotype 12 recorded the shortest plant, while Genotype 1 produced the highest number of leaves among the evaluated lines. The results based on grain yield as selection criteria decipher adequate potential in the genotypes observed. The decisions made based on heritability estimates suggest that further selection is recommended to purify the existing coterie to develop grain sorghum pure line varieties for animal feed utilisation.

DECLARATION OF INTEREST STATEMENT

The authors do not have any conflict of interest related to the design, data collection, analyses, interpretation or writing of the manuscript.

DECLARATION ON THE USE OF ARTIFICIAL INTELLIGENCE TOOLS

The authors declare that no AI tools were used while preparing the manuscript. The entire work from abstract to conclusion was completed by the authors.

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Short Communication

Do Light Factors Affect Crustacean Larvae Growth?

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ABSTRACT

Light is a key environmental factor that affects every life stage of crustaceans, especially during the larval stage. In early development, after hatching from the egg, crustaceans exist as larvae that float in the water column and are particularly susceptible to light exposure. This short review aims to compile existing findings regarding the effects of light on crustacean larvae. Overall, current findings suggest that a long photoperiod (more than 18 hours of light), higher light intensity, and white light may be particularly beneficial for the survival and growth of crustacean larvae. The collected information may be useful in optimizing rearing protocols in hatchery settings. However, further studies are needed to gain a more comprehensive understanding of how light affects crustacean larvae as a whole.

Keywords: Light, crustacean larvae

INTRODUCTION

Light is a crucial environmental factor that influences every life stage of crustaceans, primarily during the larval stage. Light influences the aquatic environment of crustaceans in terms of spectrum, photoperiod, and intensity (Boëuf and Bail, 1999), which all these factors are affected by season, time of day, depth, water quality, and weather (Bermudes and Ritar, 2008). Light spectrum is filtered by the depth of

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water column of the oceans or lakes, where shorter wavelengths (<390 nm, e.g., violet) and longer wavelengths (>~450 nm, e.g., red and orange) are absorbed by water, leaving the oceans and lakes appear to be blue (Villamizar et al., 2011). Photoperiod refers to the light duration to which crustaceans are exposed within a 24-hour period that regulates biological rhythms, primarily hormonal activity (Nie et al., 2024). Fourteen hours of light and above are considered long, whereas light hours reduced below 12 hours are considered short. Light intensity refers to light brightness, often influenced by the position of the sun. For example, at the ocean surface, light intensity is reduced (0.1-100 lux) during dawn and dusk, and reaches up to 100,000 lux during the daytime (Fouroughifard et al., 2020).

Crustacean is derived from Latin words, referring to chitinous exoskeleton and aquatic crustaceans are categorised into four main groups, including crabs, lobsters, prawns and shrimps (Covich and Thorp, 2001). Life stages of these aquatic crustaceans begin in the form of larvae that float in the water column, which then settle as benthic juveniles. In crabs, the larval stages comprise of Zoea 1 up to Zoea 5 before entering the Megalopa stage. The larval stages could be differentiated based on the setae, maxillipeds (modified appendages) and antennules (sensory antennae) (Li et al., 2022), while the Megalopa stage is a stage resembling juvenile crabs with a flattened body, long eyestalk and the presence of claws (Li et al., 2022). In shrimp (e.g., whiteleg shrimp, *Litopenaeus vannamei*, tiger shrimp, *Penaeus monodon*, and banana shrimp, *Penaeus merguensis*), larval developments consist of six nauplius stages, three zoea stages, three mysis stages, and postlarval stages before entering the juvenile stage (Wei et al., 2014). In lobster (e.g. spiny lobster, *Panulirus* sp.), the larval stage is known as phyllosoma (referring to leaf-shaped body), which floats in the water column for a longer period than other types of aquatic crustacean, with more than 5 months up to 2 years (Briones-Fourzán et al., 2021). In general, all these types of larvae, such as Zoea, mysis, and phyllosoma, had a higher tendency to show positive phototactic behaviour during larval stages, indicating the importance of light for their development, with the eye as the main light-sensing organ that is involved in this role (Boëuf and Bail, 1999; Porter, 2001).

Light factors are paramount for the development of crustacean larvae, and previous evidence suggests that light influences photoreceptors, foraging behaviour, and feeding. However, these effects are still scarce and need to be further reviewed. Hence, the objective of this paper is to review the existing findings related to light and crustacean larvae, focusing on survival, growth, metamorphosis, feeding and the potential mechanism of light response. The information compiled may be beneficial to optimise rearing protocols in aquaculture, overcoming the main issues regarding the reduction of feed supply nowadays. This review compiles information from articles published in scientific journals. We used Web of Science (WOS), Scopus, Google Scholar (GS), and Google as search engines to identify relevant references. The following keywords were used in our searches: "(light) AND (crab OR

lobster OR shrimp OR prawn OR crustacean)." A total of 26 articles were included in this paper, reflecting the limited number of studies available in this field.

Effect of Light on Crustacean Larvae: Photoperiod, Light Intensity and Spectrum

Previous studies have suggested that crustacean larvae grown under long photoperiods (>18 hours of light) exhibit the best survival rates. For instance, larvae of crustaceans such as the blue swimming crab, *Portunus pelagicus*, giant freshwater prawn, *Macrobrachium rosenbergii*, green rock lobster, *Sagmarius verreauxi*, kuruma prawn, *Penaeus japonicus*, red rock lobster, *Jasus edwardsii*, and white-leg shrimp, *L. vannamei* showed the highest survival under these conditions (Figure 1). In crabs, Andrés et al. (2010) found that larvae of the blue swimming crab were able to survive to the megalopa stage under constant darkness (0 hours of light). However, survival was significantly reduced to less than 20%, suggesting that larvae heavily rely on light for feeding. Another study by Ravi and Manisseri (2008) indicated that the Zoea 3 and Zoea 4 stages of blue swimming crab larvae were influenced by light exposure, with longer photoperiods (18 hours of light) often resulting in higher survival rates of up to 85% (Ravi & Manisseri, 2011). Similarly, Ikhwanuddin et al. (2019) observed that blue swimming crab larvae depend on light for more efficient feeding, particularly newly hatched zoea, which were observed feeding more frequently (three to four times) during the daytime compared to nighttime. Bermudes and Ritar (2008) stated that the phyllosoma of the red rock lobster had a significantly higher feeding rate under longer photoperiod (24 hours), which fed up to 20 *Artemia* per individual per day. Similarly, Fitzgibbon and Battaglione (2012) showed phyllosoma of green rock lobster (instar 1- 5) and late-stage phyllosoma (instar 15 - 17) fed higher rate of artemia under 24 hours of light at feeding rates of 0.012 mg and 110 mg *Artemia* per individual per hour, respectively. Sanudin et al. (2014) also demonstrated that larvae of white-leg shrimp had a higher ingestion rate under light conditions (62%) than shorter photoperiod (39.3%), suggesting that shrimp larvae highly depend on their eye for feeding. In contrast, Ikhwanuddin et al. (2019) and Andrés et al. (2010) exhibited continuous darkness caused a decrease in survival of blue swimming crab larvae, with less than 30% and 50% survival, respectively. Likewise, the phyllosoma of red rock lobster had a reduced feeding rate in continuous darkness (Bermudes and Ritar, 2008). Overall, a prolonged photoperiod, longer than 18 hours, improves survival, growth, and feeding rate, whereas continuous darkness had negative effect on survival and feeding rate.

Since most crustacean larvae are visual feeders, their feeding activity increases with light intensity. Higher light intensity enhances visual function, allowing larvae to better recognise and capture prey, up to an optimal brightness level (Lee et al., 2017). Most crustacean larvae species had improved feeding, growth, and survival under higher light intensities (greater than 1000 lux). A study by Ikhwanuddin et al. (2019) found that the

growth of blue swimming crab larvae at stages Zoea 1, Zoea 2, and Zoea 3 correlated with light intensity. A light intensity of 1300 lux resulted in higher growth (specific growth rate: 4% - 4.9% per day) compared to lower light intensity (1.2 lux), which only produced growth of 1.2% - 1.7% per day. This phenomenon has also been observed in fish larvae, for instance, gilt-head bream, *Sparus aurata*, rabbitfish, *Siganus sutor* (Fouroughifard et al., 2020), cod, *Gadus morhua*, chub mackerel, *Scomber japonicus* (Yoon et al., 2010), and walleye pollock, *Theragra chalcogramma* (Porter, 2001), all of which showed similar trends, where higher light intensity (more than 1000 lux) led to higher survival rates.

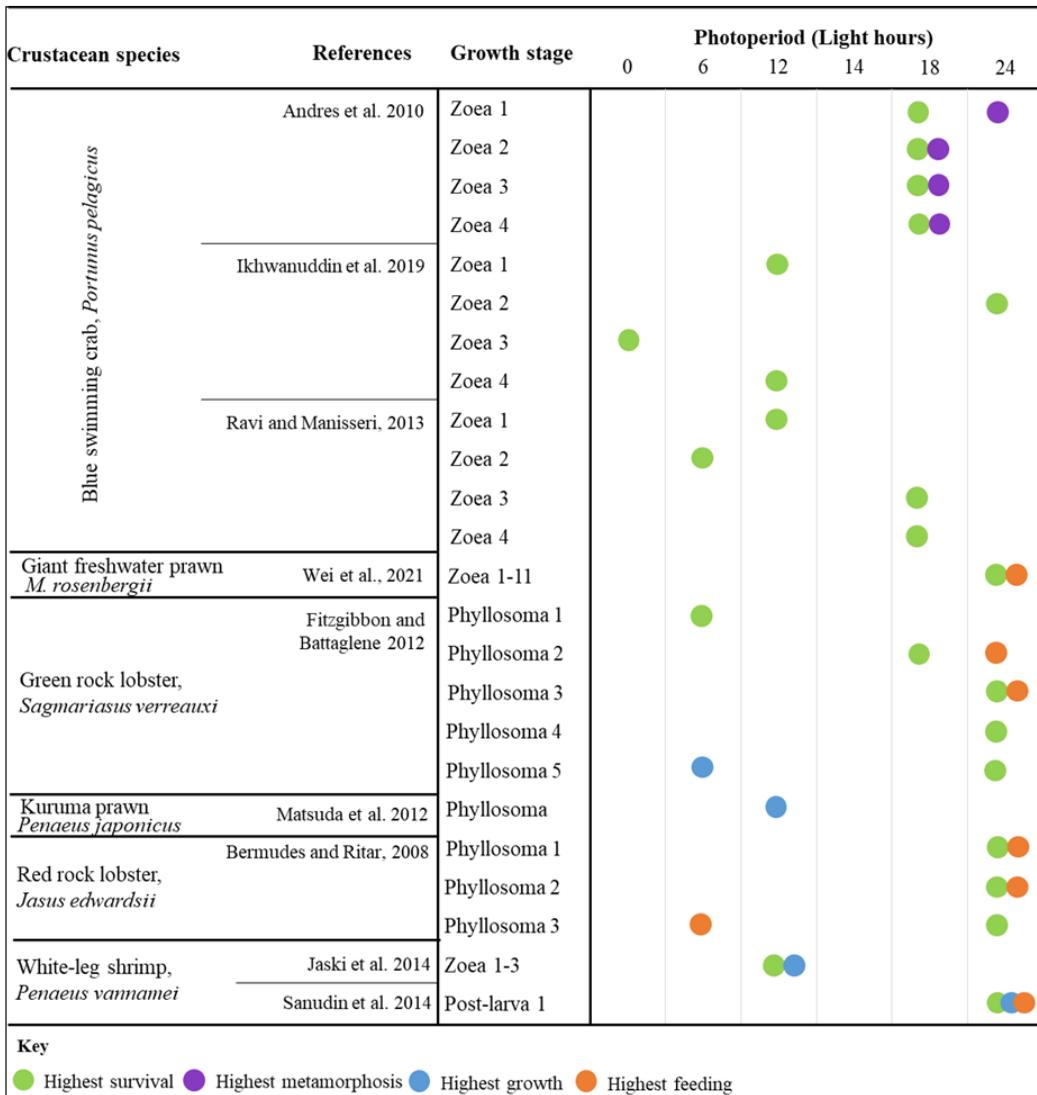


Figure 1. Effect of photoperiod on survival, metamorphosis, growth and feeding of crustacean larvae

Regarding the effect of light spectrum on crustacean larvae, Chen et al. (2022) reported that juvenile crabs reared under blue (460 nm) and cyan (510 nm) spectra exhibited higher moulting rates and lower oxidative stress. The transition of benthic crabs during recruitment from planktonic larvae suggests that juvenile crabs grow optimally under blue light, as blue wavelengths penetrate deeper, reaching the benthic zone (Villamizar et al., 2014). Although studies on the effect of light spectrum on crustacean larvae are limited, it is expected that larvae would perform better under white light, as crustacean larvae typically inhabit the upper water column near the surface. This expectation is supported by Wei et al. (2021) found that larvae of the giant freshwater prawn had higher survival (56%) and metamorphosis rates when reared under white light, whereas larvae exposed to red light showed lower survival (29%) and no metamorphosis. Similar findings have been reported in fish larvae, for example, zebrafish, *Danio rerio* (Villamizar et al., 2011), and goldfish, *Carassius auratus* (Imanpoor et al., 2011), exhibited better growth and feeding activity under white light, while larvae reared under green, yellow, or red light demonstrated reduced feeding activity, slower growth, and higher mortality.

How Light Affects Crustacean Larvae: Compound Eyes, Feeding, and Hormonal Mechanisms

Crustacean larvae are equipped with a larger compound eye as primary visual organs, and this eye consists of clear layers, which seems to be an evolutionary adaptation which is important for camouflage (Mishra et al., 2006; Cronin et al., 2017). The compound eye is comprised of a rounded cluster of small units called ommatidia, and each unit is able to function independently. The number of ommatidia increases with larval stage as a new unit of ommatidia is added along the anterior edge of the eye, hence increasing the larval eye size (Mishra et al., 2006). There is a dense area of light-sensitive cells in an ommatidium, known as the rhabdom, which plays an important role as stimuli in detecting and responding to light, creating phototactic behaviour. Since the ability to observe in dark condition are limited, most of crustacean larvae spend their entire larval stage in the upper water column (Mishra et al., 2006).

Light plays a major role in feeding for most crustacean larvae, as they are visual feeders that rely primarily on sight for foraging behaviour (Yoon et al., 2010). During early developmental stages, other complex sensory organs important for foraging remain underdeveloped (Sanudin et al., 2014). A minimum light intensity is necessary for crustacean larvae to exhibit normal hunting behaviour, and below this threshold, larvae are unable to detect or capture prey (Boeuf and Bail, 1999). This incidence has been demonstrated previously in the larvae of crab, shrimp, prawn and lobster, as summarised in Figure 1. Under extended photoperiod, light stimulates prey-capture activity, increasing feeding rate, and improving larval

growth and survival (Imanpoor et al., 2011). For example, a previous study showed that in laboratory or hatchery settings, both prey (e.g., rotifers and *Artemia*) and crustacean larvae exhibit phototactic behaviour and accumulating in the water surface area, thus increasing the predation success of crustacean larvae (Downing & Litvak, 1999; Villamizar et al., 2011).

One possible hormone involved in light response in crustacean larvae is melatonin (N-acetyl-5-methoxytryptamine). Despite the limited information regarding melatonin in the crustacean larvae, there was evidence indicating the presence of melatonin in the eyestalk of mud crab, *S. paramamosain*, suggesting the role of this hormone in response to light (Chen et al., 2022). Light condition suppresses activity of enzymes such as arylalkylamine N-acetyltransferase (AA-NAT), which play a role in melatonin synthesis. Under darkness condition, AA-NAT is able to synthesise melatonin. In the presence of melatonin, nitric oxide synthase activity is inhibited, stimulating the synthesis of ecdysteroids (Villamizar et al., 2011). Then, ecdysteroids stimulates secretion of methyl farnasoate which plays a role in the growth of crustaceans (moulting) (Chen et al., 2022). Overall process shows that the growth of crustacean larvae occurs during the night or in dark conditions. A shorter dark period (e.g., 4 hours) is likely sufficient for melatonin secretion for promoting moulting in crustacean larvae, but a longer photoperiod (e.g., 18 hours) is necessary to ensure sufficient feeding to provide the energy requirements for larval growth at night. Previous studies indicate that larvae of blue swimming crab, green rock lobster, red rock lobster and white-leg shrimp had higher growth under longer photoperiod with successful metamorphosis reaching up to 80% (Andrés et al., 2010; Bermudes & Ritar, 2008; Fitzgibbon & Battaglione, 2012; Sanudin et al., 2014). Besides, other hormone potentially related to circadian rhythms in crustacean larvae is PDH-II (pigment-dispersing hormone II), since this hormone plays a role in pigment migration in the eye, which affects phototaxis behaviour and vertical migration of larvae. Huang et al. (2014) found that the PDH-II was upregulated under bright light at 0900-1500 but reduced under low light levels at 1800-0600, suggesting the role of this hormone in promoting phototaxis behaviour.

CONCLUSION, GAP STUDIES AND FUTURE PROSPECT

In conclusion, this short review provides insight into the light effect on crustacean larvae. Longer photoperiod up to 18 hours, higher light intensity and white light spectrum could improve growth and survival of crustacean larvae. In the future, further studies on light intensity and light spectrum are necessary to be conducted due to scarce information. Also, the mechanism of light effect on crustacean larvae, primarily in terms of gene expression,

hormones and antioxidant properties, should be investigated. The use of recent technology, such as transcriptomics, may be important to reveal gene and hormonal pathways in crustacean larvae that are affected by light. In aquaculture, one of the main challenges in crustacean aquaculture is to obtain a consistent supply of larvae (seeds). Adjusting light conditions in commercial settings could significantly improve culture efficiency, especially during the prolonged phyllosoma rearing phase. With a better understanding of light's effects on crustacean growth, the duration of the phyllosoma phase, currently lasting up to 12 months, might be reduced to 6 months in laboratory or hatchery conditions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

AUTHOR CONTRIBUTIONS

Ariffin Hidir designed the study, gathered information, analysed data and drafted the paper. Mohd Amran Aaqillah-Amr and Hongyu Ma reviewed the paper. Mhd Ikhwanuddin drafted, reviewed the paper and was involved in funding acquisition, project administration, and supervision.

ETHICAL APPROVAL

Ethical approval is not required as no experiments were conducted in the production of this research paper.

DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are included within the paper. No additional data are available.

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Comprehensive Characterisation of the Robusta Coffee Postharvest Processes: Insight into Physicochemical, Volatile Compounds, and Sensory Preferences

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ABSTRACT

This study investigated how different postharvest processes, specifically wet, semi-wet, and hybrid methods, affect the physicochemical quality and aroma profile of Menoreh Robusta coffee. We mapped the volatile composition of roasted coffee beans using solid-phase microextraction coupled with gas chromatography-mass spectrometry (SPME-GCMS). All samples met the physical standards of the Indonesian National Standard for green beans and the Specialty Coffee Association, but their chemical compositions varied significantly. Principal component analysis (PCA) revealed distinct clustering, confirming that each processing imparts unique chemical signatures to the final product. The hybrid process proved superior in maintaining nutrient density, resulting in higher glucose, fructose, chlorogenic acid, caffeine, fat, protein, and crude fibre contents compared to the other methods. The wet process maximised antioxidant properties, evidenced by the lowest

DPPH IC₅₀ value (7.51 ppm) and the highest ABTS radical scavenging activity (88.32%), as well as the highest total flavonoid (181.22 mg QE/g) and phenolic (139.45 mg GAE/g) levels. Meanwhile, the semi-wet sample had the highest carbohydrate content (65.66%). Analysis of volatile compounds revealed a complex chemical matrix involving ketones, pyrazines, and esters, among others. Sensory assessments showed that although approximately 35% of flavour attributes were similar across treatments, the flavour profile was dominated by distinctive aromas of brown sugar, spices, coconut milk, chocolate, and rubber. All postharvest treatments

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yielded cupping scores exceeding 80, validating the classification of the Robusta Menoreh as a Fine Robusta regardless of the processing strategy used.

Keywords: Flavour characteristics, coffee Robusta, quality, postharvest processing, PCA, PLS-R, SPME-GCMS

INTRODUCTION

Indonesia is the world's fourth-largest coffee producer, with Robusta coffee being the primary source. According to national statistics, over 99% of Indonesian coffee comes from small-scale plantations (Indonesia Ministry of Agriculture, 2022). The Robusta coffee is often associated with a strong, bitter, and earthy flavour, leading to a perception that it is less desirable than Arabica (Liu et al., 2021), although this depends on culture and personal preference. Many consumers tend to prefer Arabica due to its more complex flavour profile (Velásquez & Banchón, 2023), underscoring the importance of improving postharvest handling for the Robusta. Flavour plays a central role in shaping quality perceptions and directly influences price formation and market value (Elhalis et al., 2023). By improving the Robusta's quality, there is clear potential to broaden consumer acceptance and strengthen its position in the coffee market.

The postharvest process produces green beans, which are a crucial foundation for coffee quality. At this stage, green beans carry various flavour precursors that will emerge during roasting (Leblanc, 2021) and determine the coffee's final sensory characteristics (Mulato, 2019). These precursor components include non-volatile compounds related to physicochemical properties and volatile compounds formed during fermentation, which collectively contribute to the overall flavour and sensory perception of coffee (de Oliveira Junqueira et al., 2019; Yusibani et al., 2023).

Physical characteristics of green coffee beans, such as bean colour, water activity, size, and bulk density, influence heat transfer during roasting, thus influencing the development of the Maillard reaction and the formation of complex flavour compounds (Gonzalez-Sanchez et al., 2024; Pereira et al., 2021). In parallel, chemical components, including caffeine, which contributes to bitterness; chlorogenic acid, which influences flavour complexity and potential health benefits; and other phenolic compounds associated with bitterness and astringency, further shape the sensory characteristics of brewed coffee (Mónica Lois et al., 2024; Reza et al., 2023).

The Robusta coffee plays a strategic role in Indonesia, as it is one of the main export priorities for plantation commodities (Syamsuri, 2023). Meanwhile, its flavour characteristics are strongly influenced by diverse local terroirs, yet to date, it remains relatively under-explored scientifically. Variations in climate, soil conditions, and altitude contribute to the complexity and diversity of the sensory characteristics of the Indonesian Robusta (Putri et al., 2019), which holds great potential for developing product quality and differentiation.

Recent advances in postharvest processing, particularly anaerobic fermentation, have opened up new possibilities for enhancing coffee flavour. These techniques can alter key flavour precursors, such as amino acids and sugars, which participate in the Maillard reaction during roasting and play a crucial role in shaping the coffee's final sensory profile (Cao et al., 2023; Leblanc, 2021; Yusibani et al., 2023).

Solid-phase microextraction combined with gas chromatography-mass spectrometry (SPME-GC-MS) provides a solvent-free and efficient approach to profile volatile compounds in roasted coffee, allowing its complex aromas to be captured with minimal sample preparation (Obando & Figueroa, 2024). This technique is suitable for quality assessment and for evaluating how postharvest practices affect aroma development.

In this study, we examined the effect of three postharvest processing methods (wet, semi-wet, and hybrid) on the physicochemical properties and flavour profile of the Robusta coffee. By identifying specific volatile compounds associated with sensory preferences, these findings will provide a scientific basis for optimising postharvest processes, enabling farmers to improve the product consistency and market competitiveness of the Robusta coffee.

MATERIALS AND METHODS

Coffee Postharvest Processes

Ripe red Robusta coffee cherries were collected from smallholder farms in the Samigaluh Subdistrict, Menoreh Hills, Yogyakarta, Indonesia (≈ 900 m a.s.l.), during the July-September 2023 dry season. The area experiences low rainfall (< 50 mm/month), daytime temperatures of 21-30 °C, and is characterised by well-drained volcanic Inceptisol soils. Fermentation treatments were selected to reflect postharvest practices commonly used by local smallholder farmers.

Three postharvest processing methods were applied: wet (removal of skin, pulp, and mucilage followed by 24-hour fermentation and washing), semi-wet (removal of skin, pulp, and mucilage without fermentation), and hybrid (removal of skin, pulp, and mucilage followed by 72-hour anaerobic fermentation in sealed plastic bags). After processing, all beans were sun-dried on raised racks for approximately two weeks. Each treatment was replicated four times, with 5 kg of cherries per replicate. Green beans were evaluated for physical and chemical properties, while roasted beans were analysed for volatile compounds and sensory characteristics.

Physical Characteristics Determination

Green bean defects were classified according to the Indonesian National Standard (SNI 2907:2008). Bean colour (L^* , a^* , b^*) was measured using a chroma meter (Spectra Magic

NX Pro, Konica Minolta). Bulk density and bean volume were determined following the method described by Widyotomo (Widyotomo et al., 2010). Water activity was measured at 25 °C using an Aqualab 4TE, while pH was determined in a 1:20 water-sample slurry using a calibrated LAQUA twin metre to ensure consistent and reliable measurements across treatments (Emanuel et al., 2025).

Chemical Characteristics Determination

Proximate composition was analysed using standard methods. Moisture, ash, and crude fibre contents were determined gravimetrically; crude protein was measured using the Kjeldahl method; crude fat was determined by Soxhlet extraction with *n*-hexane; and total carbohydrate content was calculated by difference (Thiex, 2009). Hot water extraction was applied as a standardised approach for extracting polar coffee constituents. Green beans (5 g) were extracted in ultrapure water at 90 °C for 1 min following a previously reported protocol (Yulianti et al., 2022) with minor modifications. The extract was immediately cooled in an ice bath and vortex-mixed for 2 min to rapidly quench thermal effects and ensure homogenisation, followed by centrifugation. The supernatant was filtered (0.22 µm) for sugar, caffeine, and chlorogenic acid (CGA). Aliquots of the same filtered supernatant were used as stock solutions for the determination of total flavonoid content (TFC), total phenolic content (TPC), and antioxidant activity determined by IC₅₀ of 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) and ABTS radical scavenging assays, following established methods (Priftis et al., 2015; Wołosiak et al., 2022). For the ABTS assay, Trolox was used as a reference antioxidant (TEAC; µmol Trolox equivalents/g sample), and aliquots of the filtered extract were serially diluted (50,000-3,125 ppm) for analysis. Identical extraction conditions were applied across all treatments to enable valid relative comparisons.

Sugars (glucose, fructose, sucrose) were quantified by UHPLC-CAD using an Aminex HPX-87H column with acetonitrile-water (80:20; v/v) as mobile phase. Sugar identification and quantification were based on retention times and external calibration curves over a range of 50-2500 ppm. Limit of detection (LOD) 0.03 ppm for glucose, 0.13 ppm for sucrose, and 0.16 ppm for fructose, respectively, with correlation coefficients (R) = 0.99 for all sugars. To minimise the matrix effects, all samples were analysed under identical conditions within the detector's linear range; therefore, sugar concentrations are interpreted comparatively rather than as absolute values.

Caffeine and CGA were determined by HPLC-UV/Vis using the Zorbax C18 columns. (Yulianti et al., 2022). Caffeine was detected at 254 nm, with concentration determined from a five-point calibration curve (20-100 mg/L) with a LOD of 0.04 ppm and R = 0.99. CGA detection occurred at 320 nm, determining CGA concentration via a five-point calibration curve, with a LOD of 0.01 ppm and R = 0.99. TFC was determined spectrophotometrically at 435 nm using quercetin as the reference standard (Stankovic, 2011), while TPC was

measured at 765 nm with gallic acid as the standard (Wu et al., 2022). Antioxidant activity was evaluated using the DPPH radical scavenging assay, with IC₅₀ values calculated from absorbance at 517 nm (Fadilah & Happyana, 2024), alongside the ABTS assay (Thaipong et al., 2006).

Profiling Volatile Compounds via SPME-GCMS

Coffee samples were roasted according to SCA guidelines for the Robusta coffee using a laboratory-scale roaster with a 250 g batch size. The roaster was preheated to 190-200 °C, and all samples were processed using the same heat input profile. Roasting was carried out to a medium-dark level, with final temperatures of approximately 210-220 °C reached within 9-11 minutes (around ± 3 minutes after first crack). After roasting, all samples were cooled using the same procedure and subsequently analysed for volatile compounds using SPME-GC-MS (Caporaso et al., 2018). Ground roasted (100 mg in 5 mL vial) was equilibrated for 10 min (40°C), followed by fibre exposure (20 min), and injection (5 min) with a 1 cm 50/30 μ m DVB/Carboxen/PDMS fibre. GC analysis was performed using a Zebron ZB-WAX column with helium as the carrier gas at a flow rate of 1 mL/min. The oven temperature was held at 40 °C for 5 minutes, increased to 180 °C at 3 °C/min, and then raised to 250 °C at 10 °C/min with a final hold of 5 minutes. The ion source and interface temperatures were set to 300 °C and 275 °C, respectively. Mass spectra were recorded in electron impact mode (70 eV) over an m/z range of 50-350 with a scan time of 2 seconds (Akiyama et al., 2007; Ribeiro et al., 2018). Volatile compounds were identified using NIST mass spectral library matching, based on the similarity index values, characteristic fragmentation patterns, consistent retention times with known coffee volatiles, and reproducible detection across samples, with relative abundances expressed as peak area percentages.

Sensory Analysis

Sensory evaluation was conducted using the SCA Robusta cupping protocol (SCAA, 2009; UCDA, 2010) by four trained panellists from the Indonesian Coffee and Cocoa Research Institute. Attributes assessed included fragrance, flavour, aftertaste, acidity, sweetness, balance, cleanliness, body, and overall quality. Prior to evaluation, panellists completed a brief calibration session using reference coffees to align sensory descriptors, and all samples were cupped under standardised conditions. The panel size was selected to support descriptive and comparative profiling and an exploratory multivariate framework. To minimise sensory fatigue, sample presentation was randomised, the number of samples per session was limited, and palate cleansing with water was applied between evaluations. Coffee samples receiving a final score of 80 or above were designated as the Fine Robusta.

Statistical Analysis

Data were analysed using one-way ANOVA followed by Duncan's Multiple Range Test (DMRT). Principal Component Analysis (PCA) was used for dimensionality reduction and visualisation of grouping patterns based on standardised green bean chemical variables averaged by treatment and was intended for descriptive rather than inferential interpretation. While Pearson correlation assessed variable relationships. Partial Least Squares Regression (PLS-R) was applied to model the relationship between physicochemical traits and volatile groups, and the influence of volatiles on sensory preferences. It was applied as an exploratory, cross-validated approach with restricted latent components to examine associative covariance among physicochemical traits, volatile groups, and sensory attributes while minimising overfitting. VIP scores were used as relative contribution indicators and not for strict variable selection or inferential conclusions. All analyses were performed using XLSTAT software.

RESULTS AND DISCUSSION

The fermentation method used in this study was selected to reflect prevailing postharvest practices used by smallholder coffee farmers in the Samigaluh (Menoreh Hills) rather than laboratory-optimised conditions. On the Indonesian island of Java, wet fermentation typically lasts 24 hours to help break down the mucilage. Hybrid anaerobic fermentation, on the other hand, typically lasts 72 hours, allowing biochemical changes to occur over a longer period with less oxygen exposure.

Coffee Bean Physical Characterisations

According to SNI 2907:2008, green coffee in the first quality category must have a maximum defect rate of 11%, as defects may lead to bland or bitter flavours. In this study, all postharvest processing methods produced beans within quality I limits, despite the presence of some internal defects (Figure 1b-f).

Agricultural and environmental factors primarily influence coffee's physical characteristics rather than postharvest processing, though they still affect the roasted coffee flavour (Zainuri et al., 2023). Postharvest processings show no significant differences in defects, bulk density, bean volume, water activity, or moisture content (Table 1), which may be attributed to uniformity of raw materials, including origin, variety, and cherry ripeness.

Green beans density classified as low (0.55-0.64), medium (0.65-0.67), or high (0.68-0.70) (Helena Coffee Processing & Export, 2021). In this study, all green beans had a high bulk density of 0.69 to 0.70 g/cm³ (Table 1). High-density beans require more heat and time during roasting, leading to a more complex flavour (Obando & Figueroa, 2024).

Green beans should have 9%-12% moisture content (Indonesian National Standard, 2008), 0.70 maximum water activity (SCA, 2018), which all treatments achieved. The

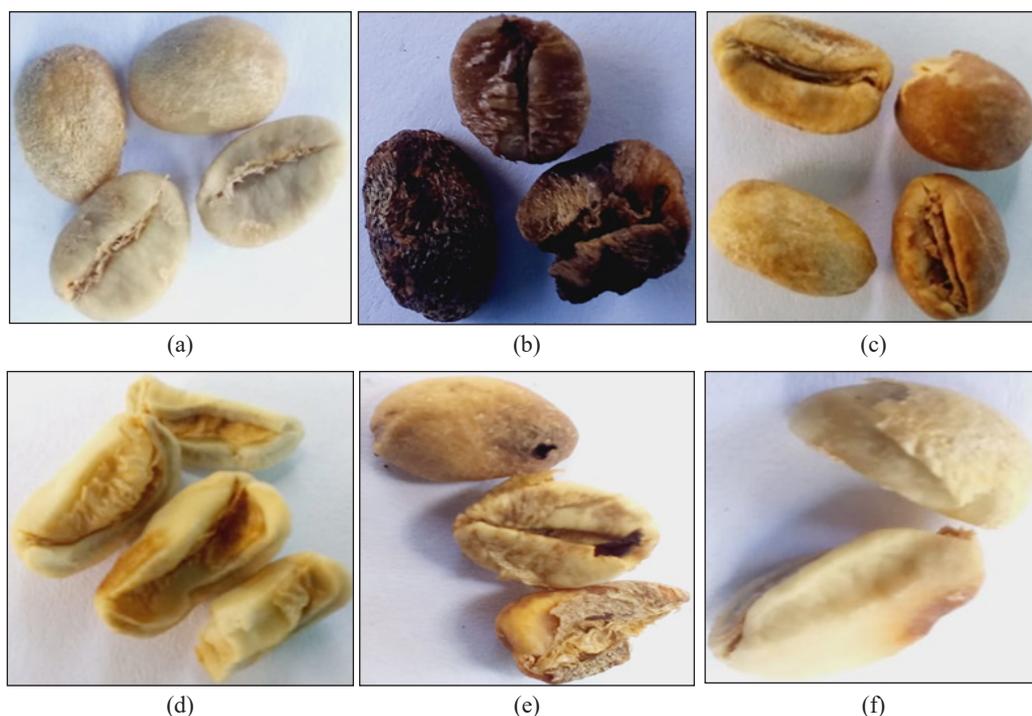


Figure 1. Green coffee beans: (a) Desirable beans vs. internal defects, including (b) Black, (c) Brown, (d) Broken, (e) Hollow, and (f) Shrivelled beans of the Robusta coffee

Table 1
Green bean physical characteristics obtained from three postharvest processes

Physical characteristics	Postharvest processes		
	Wet	Semi-wet	Hybrid
Green bean defect (%)	5.83 ± 1.22 ^a	6.67 ± 1.61 ^a	8.33 ± 1.65 ^a
Bulk density (g/cm ³)	0.70 ± 0.01 ^a	0.69 ± 0.02 ^a	0.69 ± 0.01 ^a
Bean volume (mm ³)	425.37 ± 30.48 ^a	430.73 ± 37.81 ^a	440.70 ± 47.51 ^a
Water activity	0.55 ± 0.01 ^a	0.58 ± 0.00 ^b	0.57 ± 0.00 ^b
Moisture (%)	9.20 ± 0.09 ^a	9.26 ± 0.08 ^a	9.16 ± 0.01 ^a
Colour			
L*	42.65 ± 0.06 ^{b*}	43.55 ± 0.01 ^c	41.48 ± 0.04 ^a
a*	4.97 ± 0.02 ^b	4.56 ± 0.02 ^a	5.61 ± 0.01 ^c
b*	20.53 ± 0.05 ^a	21.10 ± 0.08 ^c	20.76 ± 0.02 ^b

Note. Mean values assigned to the same letter indicate no significant difference at the 5% level based on DMRT ($\alpha = 5\%$)

semi-wet and hybrid processes had higher water activity (0.58% and 0.57%) compared to wet processes (0.55%) due to slower drying with pulp attached (Table 1). Higher water activity reduces air content, enhancing flavour during roasting (Putri et al., 2023)

Postharvest methods influence coffee bean colour and roasting quality (Yulianti et al., 2022), with wet and semi-wet methods producing brighter greens and hybrid methods resulting in darker brown beans (Table 1), indicating longer anaerobic fermentation. Prolonged fermentation increases enzymatic and microbial activity, leading to compound degradation and noticeable changes in bean colour (Obando & Figueroa, 2024).

Coffee Bean Chemical Characteristics

The Robusta coffee beans' chemical characteristics differ greatly depending on the postharvest processing technique used. Key differences were observed in fat, crude fibre, sugar content, caffeine, chlorogenic acid, and antioxidant activity, indicating that processing techniques influence the chemical quality of green beans (Table 2).

Table 2

Green bean chemical characteristics obtained from three postharvest processes

Chemical characteristics	Postharvest processes		
	Wet	Semi-wet	Hybrid
Fat %	6.46 ± 0.06 ^{a*}	6.79 ± 0.06 ^b	7.76 ± 0.06 ^c
Ash %	4.12 ± 0.02 ^a	4.27 ± 0.22 ^a	4.35 ± 0.04 ^a
Crude fibre %	18.19 ± 0.00 ^b	17.90 ± 0.02 ^a	19.96 ± 0.06 ^c
Protein %	14.32 ± 0.05 ^{ab}	14.24 ± 0.08 ^a	14.72 ± 0.36 ^b
Carbohydrate %	65.37 ± 0.44 ^b	65.66 ± 0.10 ^b	63.86 ± 0.10 ^a
Glucose %	0.05±0.00 ^a	0.12±0.00 ^b	0.17±0.00 ^c
Fructose %	0.29±0.00 ^a	0.51±0.02 ^b	0.78±0.03 ^c
Sucrose %	5.18±0.55 ^b	4.96±0.26 ^a	5.47±0.64 ^c
pH of green bean	5.93 ± 0.03 ^a	5.90 ± 0.09 ^a	4.75 ± 2.13 ^a
pH roasted bean	5.17 ± 0.03 ^b	5.17 ± 0.03 ^b	5.10 ± 0.04 ^a
Chlorogenic acid (%)	0.21±0.00 ^{a*}	0.41±0.02 ^b	0.48±0.10 ^b
Caffeine (%)	2.21±0.00 ^a	2.45±0.30 ^{ab}	2.80±0.30 ^b
Antioxidant activity by IC ₅₀ value of DPPH (ppm)	7.51 ± 2.53 ^a	12.88 ± 3.07 ^b	11.58 ± 1.21 ^{ab}
ABTS (%)	88.32 ± 1.40 ^a	85.44 ± 0.54 ^b	84.28 ± 0.47 ^c
TFC (QE mg/g)	139.45 ± 6.08 ^b	96.68 ± 6.05 ^a	109.30 ± 14.80 ^a
TPC (GAE mg/g)	181.22 ± 13.71 ^c	125.37 ± 6.84 ^a	154.84 ± 10.91 ^b

Note. Mean values assigned to the same letter indicate no significant difference at the 5% level based on DMRT. TFC and TPC represent total flavonoid and phenolic contents, respectively

The Robusta green bean fat content has been reported to range from 10% - 16.29% (Speer & Kölling-Speer, 2006; M. Zhu et al., 2021). In this study, lower contents (4.46-7.76%) were observed, which may be attributed to the genetic background, environmental conditions, and cultivation practices specific to the study area (Yani & Novitasari, 2022; Zainuri et al., 2023). The hybrid processes were linked to comparatively higher levels of fat, protein, ash, and sugar among the postharvest treatments (Table 2). The hybrid process produces a higher fat content by retaining mucilage, which promotes lipid synthesis, and involves longer fermentation, which increases microbial activity (Wibowo et al., 2021). Rather than indicating lipid biosynthesis, the higher fat content observed in hybrid-processed beans is discussed in relation to mucilage retention and prolonged fermentation conditions, which may modify the fermentation environment surrounding the bean. As lipid metabolic pathways were not directly assessed, this interpretation is presented as associative rather than as evidence of active lipid synthesis during fermentation (Wang et al., 2025; Zhang et al., 2019). The overall lower fat contents seen in this study compared to widely reported the Robusta reference ranges are probably due to differences in biology and region rather than analytical errors. We used a standard gravimetric Soxhlet extraction method to find the crude fat and reported it as a percentage of the dry weight. This made sure that the analysis was consistent and that the results could be compared between groups.

Ash content in coffee beans affects flavour, condition, and complexity (Janda et al., 2020). The ash content in this study ranged from 4.12% to 4.35%, in accordance with the INS 2907:2008 standard ($\leq 5\%$). Anaerobic fermentation increases the level of free amino acids by partially breaking down proteins. During roasting, proteins interact with phenolic compounds to create melanoidins, which contribute to the brown colour and aroma of coffee (Pereira et al., 2021).

Fermentation contributes to variations in the sugar composition of coffee beans, including glucose, fructose, and sucrose. Longer anaerobic fermentation breaks down mucilage polysaccharides, affecting the coffee's aroma and flavour (Cruz-O'Byrne et al., 2023), with fructose enhancing fruity flavours, glucose offering neutrality, and sucrose providing balanced sweetness (Li et al., 2023). Accordingly, differences in sugar profiles among treatments are interpreted as relative, process-associated variations rather than absolute concentrations.

Fermentation influences the sugar composition of green bean coffee, particularly glucose, fructose, and sucrose. Extended anaerobic fermentation can promote the breakdown of mucilage polysaccharides, which in turn affects aroma and flavour (Cruz-O'Byrne et al., 2023). Fructose is commonly associated with fruity notes, glucose contributes a more neutral sweetness, and sucrose provides balanced sweetness in the cup (Li et al., 2023). Accordingly, differences in sugar profiles among treatments are interpreted as relative, process-related variations rather than absolute sugar concentrations.

Carbohydrate content was calculated by difference and treated as a derived compositional parameter rather than an independently measured variable. Statistical comparisons were therefore applied to each directly measured proximate component before interpreting carbohydrate values, which were discussed in relation to corresponding changes in other proximate fractions rather than as standalone results. This approach helps reduce the risk of overstating treatment effects associated with carbohydrate calculated by difference (McCleary et al., 2020).

During harvest processing, both green and roasted coffee showed pH variations, especially under prolonged fermentation conditions (Pan et al., 2021). Coffee acidity is influenced by these pH changes, which in turn impact perceptions of bitterness, sourness, and scent expression (Bayle, 2019). Accordingly, pH changes among fermentation methods were interpreted as indirect indicators of fermentation-associated biochemical transformations and microbial involvement, rather than as direct evidence of microbial activity (de Oliveira Junqueira et al., 2019).

Postharvest processes also play a crucial role in shaping the chemical composition of coffee, particularly key bioactive compounds such as caffeine and chlorogenic acid (CGA). As identical hot-water extraction conditions (90 °C, 1 min) were applied across all treatments, differences in phenolic and caffeine contents are interpreted as relative process-associated variations rather than as absolute extraction yields. In this study, the hybrid processes, involving longer anaerobic fermentation, resulted in the highest levels of both caffeine and CGA (Table 2). This aligns with earlier research indicating that anaerobic fermentation is associated with reduced compositional changes in caffeine and CGA. As enzymatic and microbial activities were not directly measured, references to enzymatic and microbial pathways are provided solely as a literature-based context, rather than as mechanistic evidence. Observed changes in sugar profiles, phenolic content, antioxidant activity, and fermentation volatiles - related volatiles are therefore interpreted as indirect outcomes commonly associated with the coffee fermentation process (Barney et al., 2023; Swasti et al., 2024). In the hybrid process, fermentation duration and oxygen availability were not experimentally separated, as this treatment reflects an integrated fermentation practice used by farmers. Consequently, the observed physicochemical, volatile, and sensory differences are interpreted as an effect of the combined fermentation environment, rather than as isolated influences of time or oxygen.

Postharvest processes also affect coffee's antioxidant activity, as reflected by DPPH IC₅₀, ABTS assay result, TPC, and TFC, particularly under water-based processing conditions that may enhance the release of antioxidant compounds (Várady et al., 2022). Although extraction temperature can influence the absolute quantification of TPC and TFC values, identical extraction conditions (90 °C, 1 min) were applied across all treatments. Therefore, temperature-related effects are considered systematic rather than treatment-specific.

Accordingly, differences in TPC and TFC are interpreted as relative variations among postharvest processing methods, consistent with previous reports showing higher TPC and CGA yields under hot-water extraction (± 90 °C) compared with lower-temperature conditions (Anh-Dao et al., 2024).

Wet processing exhibits the highest antioxidant activity as indicated by the lowest DPPH IC_{50} value and the highest ABTS assay result, as well as the highest TPC and TFC values (Table 2), due to soaking and washing, which remove inhibitory compounds and enhance β -glucosidase activity (Bressani et al., 2021). This pattern may be related to soaking and washing steps that facilitate the removal of inhibitory compounds and enhance the extractability of phenolic constituents. Similar patterns have been discussed in the literature in relation to enzymatic processes, including β -glucosidase-mediated hydrolysis (Bressani et al., 2021; Várady et al., 2022). However, as enzyme activity was not directly measured in the present study, these references are provided solely as literature-based contextual explanations and not as evidence of increased enzymatic activity under wet processing.

In contrast, hybrid processing, which involves prolonged anaerobic fermentation, may increase microbial enzyme activity, leading to reduced antioxidant potential and increasing IC_{50} values, partly through the hydrolysis of chlorogenic acids into quinic and caffeic acids (Bastian et al., 2021). Consistent with the DPPH IC_{50} results, ABTS radical scavenging activity showed a similar relative pattern across postharvest treatments, supporting a comparative interpretation of antioxidant capacity. Wet processing showed the strongest antioxidant activity, indicated by the lowest DPPH IC_{50} value (7.51 ppm) and the highest ABTS inhibition at 50,000 ppm (88.32%), followed by semi-wet processing (DPPH IC_{50} = 12.88 ppm; ABTS = 85.44%) and hybrid processing (DPPH IC_{50} = 11.58 ppm; ABTS = 82.28%). This trend aligns with the observed patterns in total phenolic and flavonoid contents. The high ABTS inhibition values observed at 50,000 ppm reflect assay saturation at elevated extract concentrations and were therefore interpreted comparatively rather than as indicators of absolute antioxidant capacity.

Pearson correlation analysis result found a strong negative relationship between DPPH IC_{50} value with TFC and TPC, with coefficients of -0.998 and -0.950, respectively. This means higher TFC and TPC levels are linked to stronger antioxidant activity, reflected by lower DPPH IC_{50} values. Additionally, TPC and TFC had a strong positive correlation (0.967), indicating that higher TFC corresponds to higher TPC. A significant relationship ($p < 0.05$) was observed across all correlation analyses.

The PCA biplot (Figure 2) illustrated the differences in the Robusta green coffee beans' chemical characteristics across postharvest processes. The first two principal components (F1 and F2) cumulatively explained 100% of the variation (F1 = 64.46%, F2 = 35.54%), reflecting the use of a selected set of averaged chemical variables for exploratory visualisation rather than inferential statistical analysis. Accordingly, PCA was applied to

illustrate relative separation patterns among processing treatments and not to establish causal relationships.

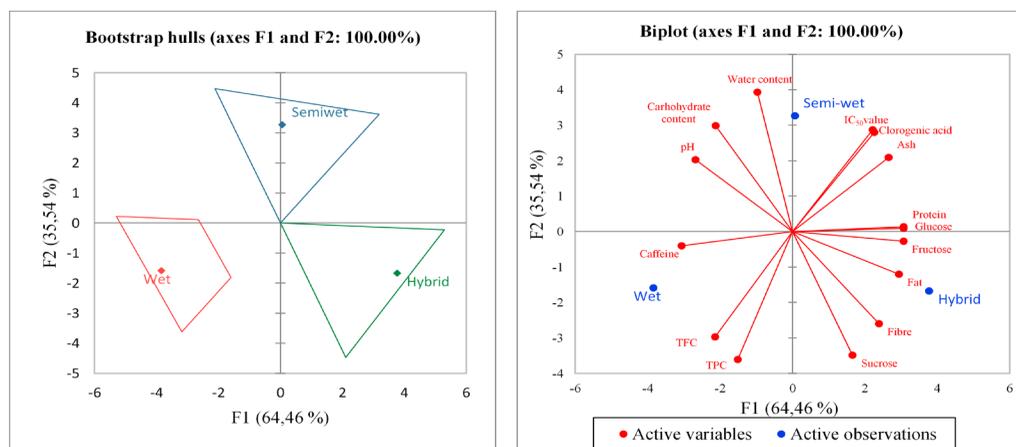


Figure 2. PCA biplot of green bean chemical characteristics across postharvest processing methods.

Note. TFC and TPC represent total flavonoid and phenolic contents, respectively. The first two principal components (F1 and F2) cumulatively explain 100% of the variance and are presented for exploratory visualisation of relative separation among treatments

Based on the squared cosine values of variables and observations, PCA indicated that the hybrid processing method was characterised by elevated levels of glucose, fructose, chlorogenic acid (CGA), fat, ash, fibre, and protein. The wet processing method was associated with the lowest caffeine content and the highest pH value, whereas the semi-wet method showed the highest carbohydrate content.

Roasted Coffee Beans Profiling Compounds by SPME-GCMS

SPME-GCMS analysis identified 36 compounds in roasted coffee from wet, 52 in semi-wet, and 40 in the hybrid method. Among these, only 15 (wet), 21 (semi-wet), and 16 (hybrid) volatile compounds were identified as potential flavour-forming compounds, based on references (Marie et al., 2024; Martinez et al., 2024; Marwani et al., 2024; Obando & Figueroa, 2024). These results reveal variations in flavour potential between postharvest treatments (Table 3).

Consistent dominating compound classes across biological replicates evaluated under similar SPME-GCMS settings supported volatile profile repeatability, with multivariate analysis revealing postharvest processing technique clustering as the primary factor. Because SPME is non-exhaustive and compound-dependent, volatile compounds were classed as flavour-forming or background based on known sensory importance in coffee

Table 3
Compounds, area (%), and retention time (RT) detected in roasted beans from three postharvest methods identified by SPME-GCMS

Compounds	Formula	RT (min)	Postharvest processing (% area)			Odour characteristics
			Wet	Semi-wet	Hybrid	
Keton						
Acetylated hydroxyacetone	C ₅ H ₈ O ₃	5.385	1.63	1.49	1.82	Sweet and fruity Zakariyya et al., 2024)
3-Octanone	C ₈ H ₁₆ O	11.372	nd	0.82	nd	Fruity and floral (Wang et al., 2024)
Pyrazine						
2,5-dimethyl Pyrazine	C ₆ H ₈ N ₂	6.141	1.21	0.78	1.05	Nutty, coffee, spicy, and roasted (Elhalis et al., 2023; Martinez et al., 2022)
Aldehyde						
3-methyl 2-Butenal.	C ₅ H ₈ O	6.204	2.36	2.53	nd	Almond, roasted (Liu et al., 2021)
5-methyl 2-Furancarboxaldehyde	C ₆ H ₆ O ₂	6.948	0.88	0.70	0.87	Nutty, caramelly, roasted, and burned (Yuwono et al., 2019)
Hexanal	C ₆ H ₁₂ O	4.402	nd	7.16	nd	Grassy, green, fatty-green (Caporaso et al., 2018)
E, E-Decadienal	C ₁₀ H ₁₆ O	12.443	nd	0.65	nd	Fruity (Marie et al., 2024)
E-2-Nonenal	C ₉ H ₁₆ O	9.091	nd	0.54	nd	Fatty (Poisson & Schieberle, 2008)
Acetaldehyde	CH ₃ CHO	16.917	nd	0.61	nd	Fruity, pungent, ethereal, fresh (Lester et al., 2021)
Benzaldehyde 2-nitro-diaminomethylidenhydrazone	C ₈ H ₉ N ₅ O ₂		nd	0.58	nd	almond (Mei et al., 2022),
Hydrocarbon						
1,1-dimethyl Cyclohexane	C ₈ H ₁₆	6.709	1.10	1.22	1.60	Spicy (Albak & Tekin, 2016)
Nonadecane	C ₁₉ H ₄₀	18.253; 17.157	0.44	0.67	1.04	-
Hexadecane	C ₁₆ H ₃₄	16.01	nd	1.33	4.96	Fresh, green, fatty, aldehydic (Lester et al., 2021)
Heptadecane	C ₁₇ H ₃₆	17.157	1.68	nd	nd	Waxy (Zhao et al., 2023)
(3-methyl-2-butenyl) Benzene	C ₁₁ H ₁₄	24.756;	0.81	nd	nd	-
Propane	C ₃ H ₈	10.124	nd	0.63	nd	Unpleasant (Nagata, 2003)
Tetradecane	C ₁₄ H ₃₀	16.01; 18.853	0.35	nd	0.24	Waxy (de Oliveira Junqueira et al., 2019)

Table 3 (continued)

Compounds	Formula	RT (min)	Postharvest processing (% area)			Odour characteristics
			Wet	Semi-wet	Hybrid	
Furan						
2-Furanmethanol	C ₈ H ₁₀ O ₃	5.158	6.94	5.25	6.46	Caramel, bready, coffee, spicy (Wu et al., 2023)
2,3-dihydro-4-methyl Furan	C ₅ H ₈ O	2.75	nd	nd	2.75	Caramel, sweet
2-Furanmethanol. acetate	C ₇ H ₈ O ₃	7.414	0.77	1.22	0.83	Fruity, sweet (Afriliana et al., 2018)
Phenol						
2-Methoxy-4-vinylphenol	C ₉ H ₁₀ O ₂	12.342	0.46	nd	nd	Sweet, spicy, cloves, peanut, curry (Wu et al., 2023)(The Metabolomic Innovation Centre, 2024)
Phenol. 4-(2-aminoethyl)	C ₈ H ₁₁ NO	12.355	nd	0.35	nd	-
Ester						
Hexadecanoic acid. methyl ester	C ₁₇ H ₃₄ O ₂	19.551	0.38	0.64	0.97	Fatty oily, waxy (Rodriguez-Campos et al., 2011; Seminde & Chambers, 2020)
2-Butoxy-1-methyl-2-oxoethyl butanoate	C ₁₁ H ₂₀ O ₄	15.884	nd	nd	0.40	Creamy and buttery (G. Zhu et al., 2015)
Sarcosine. N-isobutyryl-. tetradecyl ester	C ₁₁ H ₂₁ NO ₃	24.353	nd	nd	0.30	-
Alcohol						
(S)-3-Ethyl-4-methylpentanol	C ₈ H ₁₈ O	9.128	nd	1.15	nd	Fruity and sweet
Flavonoid						
6,4'-Dimethoxy-3-hydroxyflavon	C ₁₇ H ₁₄ O ₅	25.109	12.80	21.77	11.38	-
Amine						
1-Propanamine. N,2-dimethyl-	C ₅ H ₁₃ N	21.252	nd	nd	0.41	-
Alkaloid						
Caffeine	C ₈ H ₁₀ N ₄ O ₂	18.820	66.44	48.36	61.30	Rubbery, spicy (Seminde & Chambers, 2020)

Note. nd = not detected

literature, and identification was considered preliminary; abundances were given as relative peak area percentages.

Potential fibre saturation effects associated with SPME sampling were reduced by using similar extraction settings across all treatments, including fibre type, extraction duration, temperature, and sample mass. As volatile compounds were evaluated based on relative peak area percentages, any saturation-related bias would be systematic rather than treatment-specific. Accordingly, volatile profiles were interpreted in a comparative context to assess process-related flavour potential rather than as absolute quantitative or causal measures.

Although green bean density can influence optimal roasting conditions, a single roasting profile was applied in this study to prioritise comparability among postharvest treatments rather than roast optimisation. Volatile compound profiles in coffee are notably influenced by postharvest processes, affecting both in terms of their occurrence and relative abundance. Wet processes remove mucilage and water, soluble precursor compounds, while semi-wet and hybrid processes retain these substrates through microbial and enzymatic activities, leading to variations in volatile compounds. SPME-GC-MS analysis shows that the hybrid method closely resembles the wet process (Figure 3a), with the green colour indicating a positive association between postharvest treatments and specific volatile compounds such as hydrocarbons, furans, and esters. Caffeine is not a major volatile in coffee, but it can be detected in SPME-GC-MS analysis due to its semi-volatile nature and heat stability (Marwani et al., 2024).

Although caffeine was detected through SPME-GC-MS in all processing methods, it is not deemed aroma-active due to low volatility and limited odour contribution. Its detection in headspace GC-MS is linked to its semi-volatile nature and thermal stability. Caffeine is reported within the volatile profile for completeness and comparison, but is not considered a contributor to coffee aroma or sensory attributes (Dong et al., 2019).

The wet, semi-wet, and hybrid methods yielded various compound groups (Figure 3b). Variations in these groups are due to postharvest processes (Batali et al., 2022). Flavonoid compounds were found in all methods, with the highest in the semi-wet (Table 3). Flavonoids provide various health benefits (Saud & Salamatullah, 2021) but are odourless (Lin et al., 2022). The high abundance of flavonoid-related compounds observed in SPME-GC-MS analyses is likely due to analytical and matrix-related factors rather than the intrinsic volatility of intact flavonoids. During headspace GC-MS, flavonoids can undergo thermal fragmentation or be detected as semi-volatile derivatives adsorbed by the SPME fibre. Consequently, the representation of relative peak areas may enhance specific compound classes while diminishing others, leading to the interpretation of flavonoid signals as indicative of compositional trends rather than direct evidence of volatile flavonoids (Zhou et al., 2021).

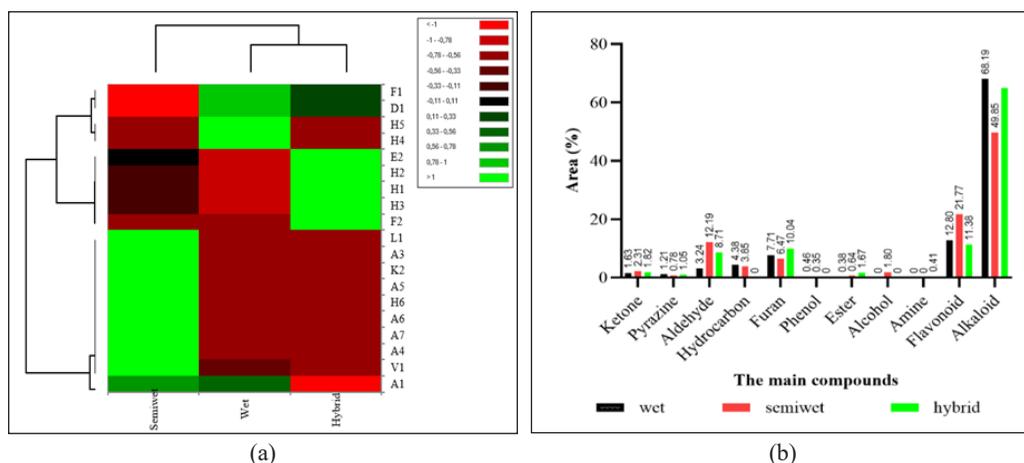


Figure 3. (a) The heatmap and (b) Volatile compounds identified by SPME-GCMS from different postharvest processes

Note. K1= Acetylated hydroxyacetone; K2 = 3-Octanone; PP1 = 2.5-dimethyl Pyrazine; A1 = 3-methyl 2-Butenal; A2 = 5-methyl 2-Furancarboxaldehyde; A3 = Hexanal, A4 = E-2-Nonenal; A5 = E,E-Decadienal; A6 = Acetaldehyde; A7 = Benzaldehyde 2-nitro- Diaminomethylidenhydrazone; H1 = 1.1-dimethyl Cyclohexane; H2 = Nonadecane; H3 = Hexadecane; H4 = Heptadecane; H5 = (3-methyl-2-butenyl) Benzene; H6 = Propane; H7 = Tetradecane; F1 = 2-Furanmethanol; F2 = 2.3-dihydro-4-methyl Furan; F3 = 2-Furanmethanol acetate; P1 = 2-Methoxy-4-vinylphenol; P2 = Phenol 4-(2-aminoethyl); E1 = Hexadecanoic acid; E2 = 2-Butoxy-1-methyl-2-oxoethyl butanoate; E3 = Sarcosine. N-isobutyryl-. tetradecyl ester; L1 = (S)-3-Ethyl-4-methylpentanol; V1 = 6.4'-Dimethoxy-3-hydroxyflavon; D1 = Caffeine

Furans contribute to coffee’s sweetness and aroma through sugar and amino acid interactions. Wet-processed coffee has reduced furans because mucilage is removed, reducing sugar availability (Zakidou et al., 2021). Because coffee chromatogrammes have a lot of complicated areas with lots of furan and aldehyde, co-elution cannot be ruled out completely. To do this, compound identification in these areas was limited to mass spectral deconvolution and repeatable retention behaviour, and conclusions were limited to comparing trends rather than giving exact numbers. In all of these steps, eight volatile compounds are used.

Hydrocarbons detected in the volatile profile are not considered direct contributors to coffee aroma because of their low odour activity and high perception thresholds. Instead, they are better interpreted as process-related markers, reflecting factors such as thermal exposure and lipid degradation during processing. Although they help differentiate samples in sensory models, their contribution to sensory perception is viewed as associative rather than causative.

The absence of certain Maillard-derived sulfur compounds typically found in roasted coffee is probably due to the limitations of the analysis, not because they were completely missing from the study samples. A lot of sulfur-containing aroma compounds are very volatile, reactive, and found in very low amounts, which regular SPME-GC-MS might not

be able to find. In addition, competitive adsorption on the SPME fibre by more abundant compounds and the use of a standardised roasting profile may further limit their detection. Accordingly, volatile profiling in this study emphasises comparative trends among postharvest processing methods, supported by reproducible compositional patterns across biological replicates and multivariate analysis showing that variability among processing methods exceeded intra-method variability.

Odour thresholds and odour activity values were not incorporated in this study, as absolute concentrations of individual volatile compounds were not determined, and odour thresholds are highly compound- and matrix-specific. Given the complexity of the coffee matrix and the interactive effects among volatile compounds, sensory interpretation based solely on odour thresholds may not adequately reflect perceived aroma (Chen et al., 2025).

Sensory Quality

Coffee sensory quality is strongly influenced by postharvest processing (Wibowo et al., 2021). Accordingly, sensory interpretations in this study reflect process-driven descriptive profiles evaluated by trained Indonesian cuppers under standardised protocols rather than culturally generalised consumer preferences. Consumer preference testing was beyond the scope of this study and is identified as a relevant direction for future research linking sensory profiles with market acceptance. All processes achieved cupping scores above 80, classifying them as the Fine Robusta coffee with sensory profiles shown in Figure 4a.

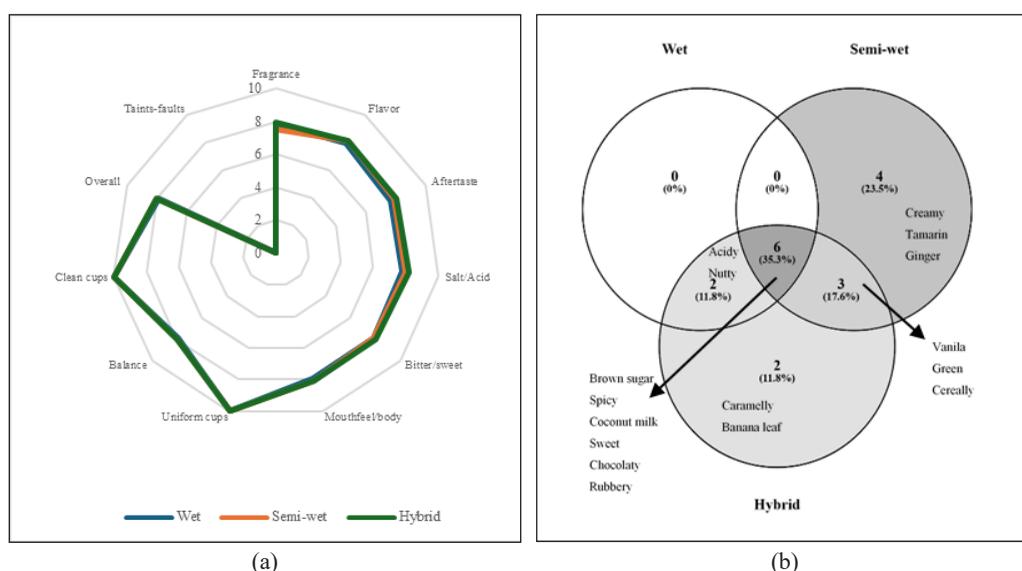


Figure 4. (a) Cupping test results and (b) Flavour in a Venn diagramme from postharvest processes

The hybrid postharvest coffee exhibited the highest sensory score (Figure 4a), characterised by a sweet, clean aftertaste with notes of brown sugar, spice, buttery coconut milk, chocolate, rubber, caramel, and banana leaf. Across postharvest methods, a 35.29% overlap in sensory attributes, including brown sugar, spice, coconut milk, sweet, chocolate, and rubbery notes, which indicates partial sensory similarity rather than a definitive process-driven identity of the Robusta coffee (Figure 4b). Accordingly, sensory differentiation among processes is supported by converging evidence from multivariate separation patterns, statistically significant differences in sensory attributes, and corresponding variations in volatile compound abundances, rather than by the overlap metric alone (Table 3).

Relating Physicochemical Attributes, Volatile Compounds, and Sensory Preferences by PLS-R

Partial Least Squares - Regression (PLS-R) analysis revealed a clear associative relationship between the physicochemical characteristics of green beans and the volatile compounds formed after roasting. Although the explained variance of the volatile predictor block was relatively limited, the PLS-R model explained 62.6% of the variation in the physicochemical properties of green beans (X) and 50.8% of the variation of volatile compounds in roasted beans (Y), highlighting the relevance of precursor-volatile linkages rather than model predictability (Figure 5a).

Green beans from the semi-wet process were associated with ketones, alcohols, and flavonoids, which are commonly linked to fruity and floral flavours, sensory attributes, and are consistent with shorter fermentation and partial sugar degradation (Table 2). These volatile compound groups showed strong associations with moisture content, CGA, and sucrose ($VIP > 1$) (Figure 5b), while TPC and TFC acted as important precursors to volatile phenolics.

Hydrocarbons, phenols, pyrazines, and alkaloids, which correlate to nutty, spicy, earthy, and roasted notes, were linked to the wet process, according to PLS-R analysis. These groups of compounds exhibited strong correlations with bulk density ($VIP > 1$), TPC, and TFC. During fermentation, the hybrid process produces furans, amines, and esters that give off caramel, creamy, and sweet scents. These changes are probably caused by the breakdown of amino acids and microbial activity. Despite having a VIP score below 1, protein content might have an indirect impact through roasting-related non-enzymatic reactions.

In general, PLS-R was used as an exploratory tool to look at how physicochemical characteristics, groups of volatile compounds, and sensory attributes are related to each other, not to make predictive models. The results are analysed in an exploratory context because the volatile predictor block has a low explained variance, and the variable-to-observation ratio is low. Variables exhibiting elevated VIP values are analysed as relative contributors to observed covariance patterns rather than as conclusive determinants of

sensory quality. Consequently, correlations between the physicochemical characteristics of green beans and roasted volatile compounds are regarded as process-driven associations rather than indications of direct causation.

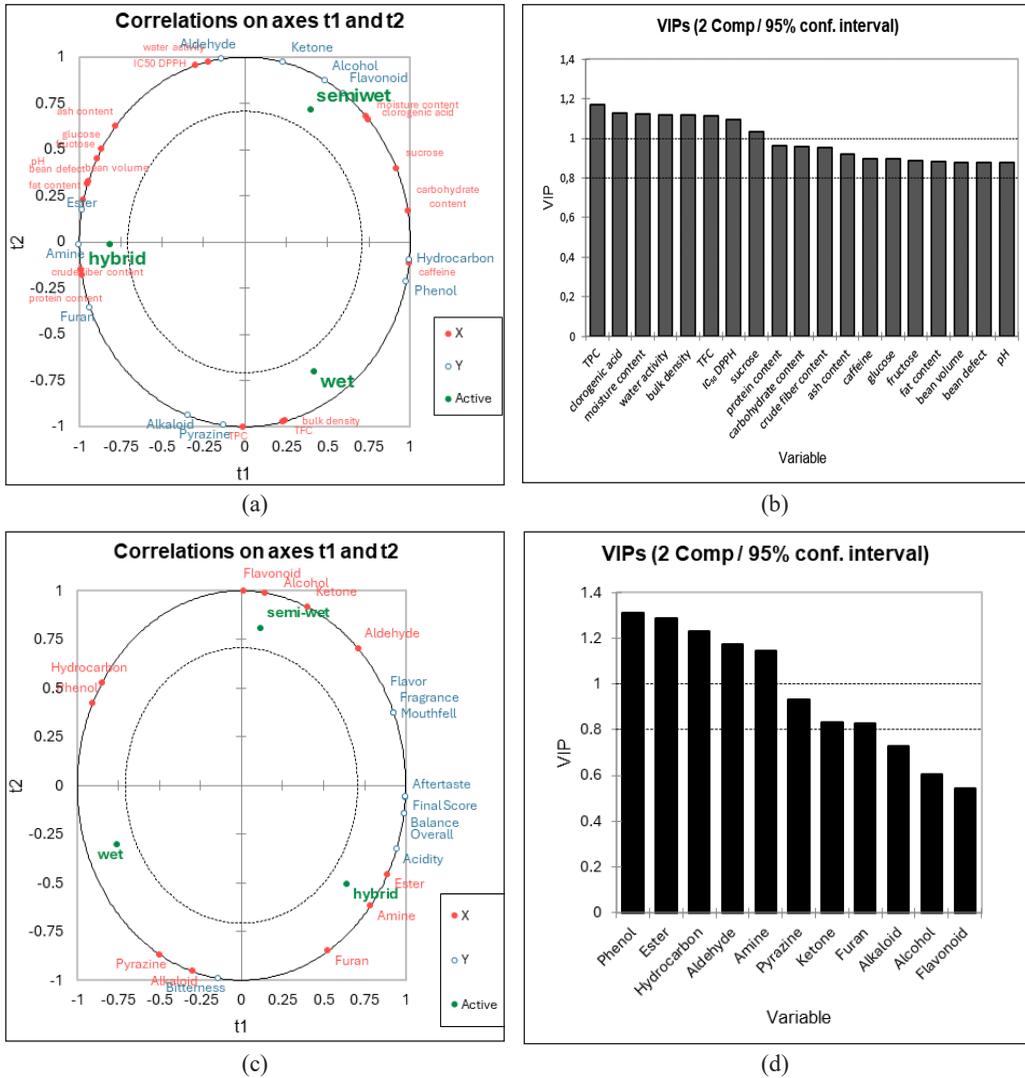


Figure 5. PLS-R biplot correlation and variable importance analysis: (a) Relationship between green bean physicochemical characteristics (X) and volatile compounds of roasted bean (Y), (b) Relationship between volatile compounds (X) and sensory preferences of roasted bean (Y), (c) VIP score of green bean key physicochemical characteristics, and (d) VIP score of roasted bean main compounds contributing to sensory differentiation

The analysis was further extended to examine the relationship between volatile functional groups (X) and sensory preferences (Y) across the three processing methods. In

this model, the PLS-R model explained a high proportion of variance in sensory responses ($R^2Y = 82.8\%$), while the explained variance of volatile predictors remained lower ($R^2X = 38.7\%$) (Fig. 5c). This is acceptable in complex chemical matrices, where volatile data often contain redundant signals and high noise (Genisheva et al., 2018). As shown in Fig. 5d, esters, aldehydes, and amines ($VIP > 1$) contributed positively to sensory quality, particularly in hybrid processing. In contrast, phenols and hydrocarbons ($VIP > 1$) were associated with negative attributes like bitterness, which may reduce consumer acceptance (Genisheva et al., 2018).

The findings suggest that the relationships observed between postharvest processing, physicochemical properties, volatile formation, and sensory expression are relevant to the Robusta coffee, although their expression may vary across regions due to differences in climate and local processing practices. Rather than presenting definitive quantitative benchmarks, the results provide practical insight into how processing choices can be used to steer coffee quality. These outcomes are inherently context-specific and should therefore be interpreted in relation to local environmental conditions and on-farm process management.

Several limitations impact the findings, including the absence of direct measurements of microbial and enzymatic activities, relying instead on literature-based interpretations. Although roasting was standardised by maintaining identical temperature targets and timelines, no instrumental verification was conducted for roast degree, leaving some variability unaccounted for. Furthermore, volatile-sensory relationships were inferred from statistical data rather than validated through aroma experiments. As a result, the differences in volatile profiles are interpreted as primarily due to postharvest processing, with these constraints noted to support flavour potential comparisons, rather than definitive causal conclusions.

CONCLUSION

Postharvest processing was found to influence the physicochemical, volatile compounds, and sensory preferences of the Robusta coffee. The hybrid processing produces a more balanced profile, characterised by relatively higher glucose, fructose, caffeine, and fibre content, while the wet processing is more closely associated with higher antioxidant-related attributes. Semi-wet processing contributed to greater perceived sweetness and body. Sensory evaluation showed a 35.29% overlap in flavour profiles similarity across methods, including brown sugar, spicy, coconut milk, sweet, chocolate, and rubber notes, with all samples classified as the Fine Robusta Coffee. Furthermore, PLS-R analysis indicated that process-related differences in green bean characteristics were associated with the formation of aroma-active volatile compounds and subsequent sensory expression. Together, these results support the use of a process-comparative, decision-oriented approach to guide the Robusta coffee quality development, rather than the selection of a single optimal postharvest processing.

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AI DISCLOSURE STATEMENT

The authors confirm that this manuscript is based on original research data, analysis, and interpretation, and that no generative artificial intelligence tools were used to produce its scientific content.

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Integrated Application of Compost, ZnO Nanoparticles, and NPK Fertiliser in Pot Paddy Cultivation on Inceptisol Enhance Nutrient Use Efficiency and Grain Yield

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ABSTRACT

The integration of technology and innovative practices has been created to address the adverse effects of synthetic fertilisers on agroecosystems, aiming to pinpoint sustainability constraints and enhance agricultural production systems, particularly in rice farming. This current study aimed to determine the impacts of the combined use of compost powder, ZnO nanoparticles (ZnO-NPs), and NPK fertiliser on yield, nutrient use efficiency, and grain yield of paddy. A greenhouse experiment with Randomised Block Design was used to test the effectiveness of nine treatments, namely P0 (no fertiliser), P1 (NPK fertiliser), P2 (300 g compost powder), P3 (P2+ ZnO-NPs 50 mgkg⁻¹ compost), P4 (P2+ZnO-NPs 100 mgkg⁻¹ compost), P5 (P2+ ZnO-NPs 150 mgkg⁻¹ compost), P6 (P3+½NPK), P7 (P4+½NPK), P8 (P5+½NPK). Each treatment had five replications. The treatment P7 (compost + ZnO NPs 100 mg kg⁻¹ + ½ NPK) showed higher N, P, K, and Zn uptake than the NPK treatment. Although the differences were not statistically significant, the results indicate comparable nutrient uptake efficiency. This treatment also had higher dry grain weight per hill and per box (80cm × 60cm × 30cm dimension, with six plant samples), potentially of harvested and milled grain weight, than the NPK treatment. The Relative Agronomy Effectiveness of the P7 increased by 12% compared with the NPK treatment. The agronomic efficiency of P4 increased by 72% compared

with the NPK treatment. This recent study offered new perspectives on the possibilities of utilising nanoparticles, decreasing the chemical Fertilisers used, and enhancing the efficiency of nutrient use and grain yield for lowland rice cultivation. The findings could be confirmed in field trials.

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INTRODUCTION

To date, most of the increase in agricultural productivity throughout Indonesia has been carried out through conventional farming using chemical fertilisers continuously and at high rates to overcome nutrient deficiencies in the soil. The fertiliser needs of farmers in Indonesia should be 13.5 million tons per year. However, Indonesia can produce 3.5 million tons annually, and an additional 7.3 million tons are imported (Central Statistics Agency of Indonesia (CSAI, 2023). Chemical fertilisers supply nutrients to ensure ideal plant growth and yield; however, current production systems struggle to meet rising food demand without substantial fertiliser application (Zhang et al., 2015). However, applying chemical fertilisers negatively impacts soil fertility because these inorganic fertilisers cannot replenish soil humus and, therefore, cannot restore soil fertility in the long term (Purwanto & Alam, 2020). Due to global limitations on available farmland and water resources, optimising mineral fertiliser use is crucial for increasing food production, supporting a growing population, and driving economic progress. Yet, sustainability in agriculture faces obstacles like inefficient nutrient absorption and environmental concerns linked to chemical fertiliser application. However, excessive fertiliser use raises expenses, diminishing farmers' profitability. High rates of conventional fertiliser release that surpass the pace at which plants absorb nutrients and/or transform fertiliser into forms unavailable to plants are the usual causes of low nutrient utilisation efficiency (Chhipa, 2017).

Utilising organic fertilisers and minimising inorganic fertiliser application is an effective strategy for preserving soil health, boosting plant productivity, and improving fertiliser efficiency in farming systems (Lal & Stewart, 2019; Vanlauwe et al., 2015). The use of compost and chemical fertilisers is also the best practice of best practices of an integrated nutrient management strategy that has been extensively studied for its potential to increase agricultural output and sustainably improve Nutrient Use Efficiency (NUE) (Oyetunji et al., 2022). However, using organic fertilisers such as compost derived from organic residues often faces the constraints of slow nutrient release and low nutrient content. Organic fertilisers are not soluble in water, and their nutrients are dispersed into the soil more slowly than conventional water-soluble fertilisers, which are readily dispersed as the fertiliser dissolves (Shaji et al., 2021). Therefore, a growing interest is in developing new fertiliser technologies to maximise efficiency (Van Eerd et al., 2018).

The application of nanotechnology in fertiliser development represents a promising strategy to enhance global food crop production sustainably in the context of climate change (Feregrino-Perez et al., 2018; Pradhan & Mailapalli, 2017; Raliya et al., 2017). Nevertheless, the use of nanomaterials must be carefully managed to prevent adverse effects on plant metabolic processes (Mirbakhsh, 2023; Tarafdar et al., 2015). Nanomaterials such as ZnO, FeO, and ZnFeCu-oxide can significantly enhance seed germination, support plant growth, and elevate crop yield quality in several species, including peanut, soybean, wheat,

onion, spinach, tomato, potato, and mustard greens (El-Saadony et al., 2021; Pradhan et al., 2010; Shojaei et al., 2019). These improvements are attributed to the enhanced nutrient efficiency resulting from nanoparticles' high surface area and active penetration capabilities (Mirbakhsh, 2023). However, research on the application of nanoparticles in rice cultivation remains limited despite rice's strategic and economic importance as a staple food in Indonesia. Therefore, this study investigates the effect of nanoparticle-enhanced compost on the nitrogen use efficiency (NUE) of rice cultivation. The gradual development of nanomaterials improves NUE while lowering N losses and environmental pollution (Panel, 2015; Preetha & Balakrishnan, 2017). Nitrogen use efficiency (NUE) computes the conversion of nitrogen inputs into agricultural products and looks at nitrogen losses in the environment (Norton et al., 2015).

Adding nanoparticles to powdered compost is expected to improve nutrient use efficiency and decrease dependence on chemical fertilisers within rice cultivation systems. In particular, zinc oxide (ZnO) nanoparticles have attracted considerable attention due to their beneficial effects on plant growth and health. Several previous research studies have demonstrated that applying ZnO nanoparticles at optimal doses can significantly stimulate plant development. Zinc is essential for activating various plant enzymes involved in carbohydrate metabolism, maintaining cell membrane stability, facilitating protein synthesis, and regulating auxin synthesis, which collectively promote the growth and development of roots and shoots (Jolli et al., 2020). This research hypothesises that adding ZnO nanoparticles to organic fertilisers can increase the efficiency of inorganic fertiliser use. Therefore, this current research is designed to evaluate the integrated effects of compost powder, ZnO nanoparticles, and the reduction of NPK fertiliser dosage on optimising nutrient use and enhancing grain yield in paddy cultivation. Through this approach, it is expected that innovative solutions can be found to increase rice productivity sustainably and efficiently.

MATERIALS AND METHODS

Experimental Site and Design

A greenhouse experiment was conducted at the field laboratory, the Faculty of Agriculture, Universitas Islam Malang, East Java Province, Indonesia, located at coordinates 7 ° 56 ' 15" S and 112 ° 36 ' 21" E with an elevation of 510 m above sea level. This study tested nine treatments using a randomised block design, consisting of P0 (control, no fertiliser), P1 (NPK fertiliser), P2 (300 g compost powder), P3 (P2 + ZnO-NPs at 50 mg kg⁻¹ compost), P4 (P2+ZnO-NPs at 100 mg kg⁻¹ compost), P5 (P2+ZnO-NPs at 150 mg kg⁻¹ compost), P6 (P3+½ NPK), P7 (P4+½ NPK), and P8 (P5+½ NPK). Each treatment was replicated five times. The planting pot used was a wooden box with dimensions 80 cm × 60 cm × 30 cm. To achieve a soil depth of 25 cm in the box, 100 kg of Inceptisol soil is required. The soil

weight for each pot was calculated using the bulk density method (1.25 g cm^{-3}). Inceptisol soil samples were taken from agricultural land at 0-30 cm depth using a stainless-steel auger (Blake & Hartge, 1986). Six rice seedlings aged 21 days after sowing were planted in a wooden box with a planting distance of $20 \text{ cm} \times 20 \text{ cm}$. The total number of wooden boxes was 45, and the total plant population was 270.

Making of Powder Compost and ZnO Nanoparticles

Composting was carried out in the integrated laboratory of the Faculty of Agriculture, Universitas Islam Malang, where the compost material was first prepared in a composting bin. Cow dung, goat dung, cocopeat, spent mushroom waste, rice husk biochar, and rice bran were the compost materials utilised in a 3:1:3:3:3:1 ratio. The organic matter was first dried and ground to speed up decomposition. Then, it was evenly blended with a hoe to ensure uniformity. Afterwards, a decomposer, activated at a concentration of 2.5 litres

per 500 kg of compost, was added to the composting container. Next, after diluting the decomposer solution with water, it was equally distributed across the organic materials' surface until the materials' moisture content reached 40%. Composting lasted 30 days under aerobic conditions, and maturity was determined by C/N ratio (<20) and stable temperature (Antil et al., 2014; Kumar et al., 2010). Compost production was replicated three times. In addition, to achieve compost powder, the produced compost was dried to 10% moisture and pulverised using a grinder mill. The chemical composition of compost powder is presented in Table 1.

The ZnO-NPs utilised in this recent study were acquired from NanoTech Indonesia Global Co., Ltd., located in Serpong, Indonesia, and obtained using a top-down approach. A bulk ZnO material that has been oven-treated at a temperature of $100 \text{ }^\circ\text{C}$ was reduced in size mechanically using Planetary Ball Milling, which contains four milling jars containing balls. The machine was turned on at 400 rpm for 12 hours until nanometer-scale ZnO was produced. These nanoparticles were identified as spherical, with an average size of roughly 100 nm, based on PSA characterisation (Figure 1) (Ealia & Saravanakumar, 2017). The ZnO-NPs solids were weighed at 15 mg, 30 mg, and 45 mg and added to 300 g of powder compost using a dry mixer. The weighing of ZnO-NPs and powder compost was carried out using several predetermined treatments.

Table 1
Chemical composition of compost powder

Parameter	Results	Methods
pH	7.3	Electrometry
C-Organic (%)	12.96	Walkley and
Organic matter (%)	16.46	Black
N-Total (%)	1.23	Kjeldahl
C/N ratio	10.54	-
P-Total (%)	0.35	
K-Total (%)	0.68	
Ca-Total (%)	1.85	Wet Oxidation
Mg-Total (%)	0.66	with HNO_3 and
Fe-Total (ppm)	7333.70	HClO_4
Zn-Total (ppm)	107.80	
S- SO_4 (ppm)	1859.13	

Soil Properties

The agricultural soil samples obtained from 0-30 cm depth were air-dried, ground, and sieved through a 2 mm mesh, then blended for consistency. The soil type was an Inceptisol. The soil characteristic was Sandy Clay Loam, covering 31.6% clay, 22.8% silt, and 45.6% sand. The soil was very low in organic carbon (0.53%), with pH 5.9 (low), low in total N (0.13%), low in phosphorus (15.37 mg kg⁻¹), and moderate in exchangeable K (0.33 me.100 g⁻¹ soil), high in Capacity Exchangeable Cation (CEC) (25.99 me.100 g⁻¹), very low in Electrical Conductivity (EC) (978 μ S cm⁻¹), moderate in zinc (0.14 mg kg⁻¹), and very high in Basic Saturation (73%).

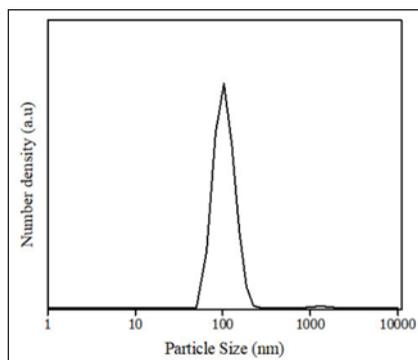


Figure 1. Particle size distribution of ZnO nanoparticles

Agronomic Management System

The 100 kg of air-dried soil samples were flooded and made into mud in the planting box. Application of ZnO-NPs enhanced compost powder was carried out one day before transplanting. This current research involved Inpari 32 variety rice seed, which is frequently cultivated in East Java, Indonesia. This rice variety has a growth cycle spanning 125 days. The seeds were sown on April 18, 2024. The seedlings were transplanted after 25 days of growth into the growing block containing muddy soil with a planting distance of 20 cm \times 20 cm at a density of 6 hills per box, and each hill contained three rice seedlings (Figure 2). One week after planting (WAP), inorganic fertilisers were applied following the recommended dosage for P1 treatment, consisting of urea (100 kg ha⁻¹ \approx 5g per box), superphosphate (100 kg ha⁻¹ \approx 5g per box), and KCl (50 kg ha⁻¹ \approx 2.5g per box). The second fertilisation, conducted at 5 WAP, involved only urea at the same dosage (100 kg ha⁻¹ \approx 5g per box). Treatments P5 and P6 received half the dose of NPK fertiliser, and treatment P7 received a quarter of the dose of NPK fertiliser. The plant nursery included watering into the planting box until the height of the flooding water is 1 cm. Harvesting took place on August 23, 2024.

Sampling and Data Collection

Chlorophyll and Nutrient Content of the Leaf

This study employed the Chlorophyll Meter SPAD-502 to measure chlorophyll content (Ling et al., 2011), and the Kjeldahl method was employed to determine the nitrogen (N)



Figure 2. The growth of paddy plants in a greenhouse from early growth, the maximum vegetative phase and the generative phase

content in the leaves of these crops (Okalebo et al., 2002). The wet combustion method determined the P and K using $\text{HNO}_3 + \text{HClO}_4$ solvent, a Flame photometer for K content, and a UV-spectrophotometer for P content. The Zn content was controlled using an Atomic Absorption Spectrophotometer (Kalra, 1997). Then, leaf nutrient content was assessed 12 weeks after planting (WAP). Nutrient uptake was calculated from the leaf nutrient content multiplied by the plants' total dry weight per hill.

Yield and Yield Components

At maturity, the paddy plant from each box was harvested to determine the paddy plant's total fresh and dry weight. The panicles were manually cut and threshed to determine the grain yield per hill. The number of panicles and the weight of the panicle per hill were determined by calculating the average number of panicles from 6 rice plants per box. Then, the number of grains per panicle was calculated.

Grain Quality

Following harvest, the grain from each box was sun-dried and stored at room temperature for one week to prepare for grain quality analysis. A 100 g grain sample was put into a 90% alcohol solution to determine the weight of the filled and empty grain. Furthermore, the grain was also measured for dry harvest water content by oven-drying at 70 °C for 2 × 24 hours.

Measurement of Nitrogen Use Efficiency

There are some indices of nutrient use efficiency, their calculation using the difference method as follows:

1. RE = Apparent crop recovery efficiency of applied nutrient (kg increase in N uptake per kg N applied)
2. PE = Physiological efficiency of applied N (kg yield increase per kg increase in N uptake from fertiliser)
3. IE = Internal utilisation efficiency of a nutrient (kg yield per kg nutrient uptake)
4. AE = Agronomic efficiency of applied nutrient (kg yield increase per kg nutrient applied) (Dobermann, 2007)

Statistical Analysis

The gathered data were analysed using one-way ANOVA at a 5% significance level in SPSS Statistics version 26.0. If the variance analysis indicated a significant effect, the Tukey test was applied at the same significance level to determine the differences among treatment means.

RESULTS AND DISCUSSION

Chlorophyll Content and Nutrient Uptake

The variance analysis (ANOVA) results showed that the tested treatments significantly affected chlorophyll content and nutrient uptake. The integrated application of compost powder, ZnO-NPs, and NPK fertiliser positively impacted the chlorophyll content compared to the control (P0) and compost alone (P2). The P7 treatment of compost powder + ZnO-NPs of 100 mg kg⁻¹ compost powder + ½ NPK fertiliser in the cultivation of rice increased the chlorophyll content (SPAD value). However, the P7 treatment was not significantly different from the inorganic fertiliser treatment (P1) (Figure 3A).

Increasing the concentration of ZnO-NPs by more than 100 mg kg⁻¹, such as in P5 and P8 treatments, decreased the chlorophyll content (Figure 3A). ZnO-NPs act as a zinc (Zn) source, which is important for various plant physiological processes, such as chlorophyll synthesis and enzyme activation. The results observed in this current study align with those reported by Skiba et al. (2021), who noted that *Pisum sativum* L. showed a decrease in chlorophyll levels, expressed in SPAD units, when cultivated in soil supplemented by ZnO-NPs at concentrations above 100 mg kg⁻¹. Mukherjee et al. (2014) and Küpper et al. (1996) stated that this decrease was attributed to the Mg substitution by Zn. It results from the substitution of the Mg atoms at chlorophyll centers by Zn, which finally hampers the photosynthesis process. Zinc is an essential microelement required in small amounts

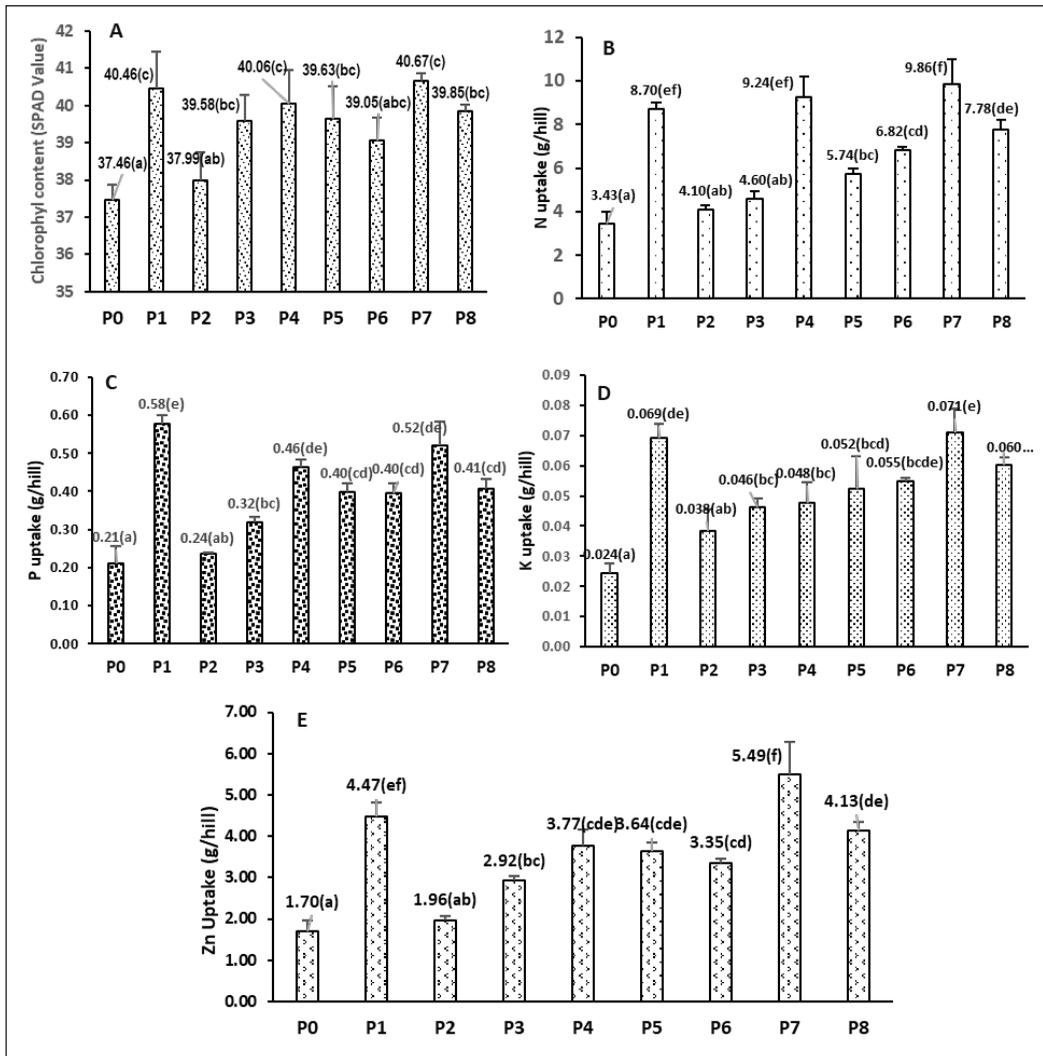


Figure 3. Chlorophyll content (A), N (B), P (C), K (D), and Zn uptake (E) of each treatment compared to control (no fertiliser, P0), NPK fertiliser (P1), and compost powder alone (P2) based on the results of Tukey test ($p < 0.05$). Remark: HSD 5% = 2.05 (A); 1.77 (B); 0.09; (C); 0.017 (D); 1.07 (E)

but significantly affects plant growth (Ayyar & Appavoo, 2017; Subramanian et al., 2014; Suganya et al., 2020).

The P4 treatment of compost powder+ZnO-NPs 100 mg kg⁻¹ and P7 of compost powder+ ZnO-NPs 100 mg kg⁻¹ + ½ NPK had high N and P uptake and was not significantly different from the NPK fertiliser treatment. This treatment of compost powder+ZnO-NPs 50-150 mg kg⁻¹ + ½ NPK (P6-P8) provided similar K uptake as the NPK treatment (Figure 3B-D). The highest Zn uptake was found in the P7 treatment of compost powder+ZnO-

NPs $100 \text{ mg kg}^{-1} + \frac{1}{2}$ NPK, but not significantly different from the NPK fertiliser treatment (Figure 3E). The macro and micro nutrient content of the soil samples used in this study was low to moderate, thus providing a positive response to the application of fertiliser and compost, as well as ZnO-NPs. This result showed that the addition of ZnO-NPs in compost not only functions as a more bioavailable source of zinc but also affects the absorption of other macro and micronutrients in rice plants, so that rice plant growth is better (Yang et al., 2021). ZnO-NPs have a wider specific surface, allowing the release of Zn gradually and efficiently so that plant roots can take it up (Arora et al., 2014). Plant physiology is altered by the nanoparticles in several ways, resulting in modifications at the cellular, organ, and individual plant levels (Dhiman et al., 2021; Marchiol et al., 2019; Marslin et al., 2017; Rizwan et al., 2017). Specifically, nanoparticles may either help or hinder photosynthesis, the vital process that produces bioenergy (Faizan et al., 2020; Kalaji et al., 2017; Tighe-Neira et al., 2018; Wang et al., 2018; Zhao et al., 2014). Increasing the concentration of ZnO-Nanoparticles to more than 100 mg kg^{-1} decreased the nutrient uptake of N, P, K, and Zn in leaves (Figure 3B-E). This is likely due to the effects of excess Zn, but is still not toxic to plants (Kaur & Garg, 2021).

Paddy Yield and Nutrient Utilisation Efficiency

The results of the analysis of variance (ANOVA) established that the tested treatments significantly affected the parameters of rice yield, including the fresh weight and dry weight of plant biomass, number of panicles per hill, number of grains per hill, weight of panicles per hill, weight per individual panicle, dry weight of grains per hill, dry weight of grains per box, and potential production of grains per hectare and Nutrient Utilisation Efficiency. The results of this study showed that all treatments tested gave significant differences based on the 5% Tukey test ($P < 0.05$) in the parameters of rice yield (Table 2 and 3).

The P7 treatment of compost powder+ZnO-NPs $100 \text{ mg kg}^{-1} + \frac{1}{2}$ NPK had a high rice yield component and was equal to the results of the NPK treatment statistically. This treatment had the same fresh and dry weight of biomass, number of panicles per hill as the NPK fertiliser treatment. The treatments of integrated compost powder+ $100\text{-}150 \text{ mg kg}^{-1}$ ZnO-NPs+NPK fertiliser had a higher number of grains per panicle than NPK treatment (P1) and compost powder alone (Table 2), and potential yield per hectare compared to the NPK treatment and other treatments. Therefore, this treatment has the highest Relative Agronomy Effectiveness (RAE value) of 112% (Table 3; Figure 4).

This study showed that combining compost and ZnO nanoparticles can provide better results than a single application of compost. Compost supplies organic material that strengthens soil structure and boosts microbial processes, while ZnO nanoparticles, as a source of microelements, can improve plant mineral nutrition. This is due to increased nutrient uptake and better photosynthetic efficiency, which contribute to greater biomass

Table 2

Fresh and dry weight of biomass, number of panicles per hill, and number of grains per panicle of rice of each treatment compared to control (no fertiliser, P0), NPK treatment (P1), and compost alone (P2)

Treatments	Fresh weight of biomass (g)		Dry weight of biomass (g)		Number of panicles per hill		Number of grains per panicle	
P0	214.28±50.49	a	62.15±9.88	a	24.93±0.81	a	115.87±3.98	a
P1	353.01±14.89	c	98.18±4.79	e	30.30±1.31	bc	119.96±12.33	a
P2	231.94±30.84	ab	65.82±4.76	ab	28.93±0.81	b	116.49±2.44	a
P3	231.54±32.24	ab	70.39±4.37	abc	30.30±0.69	bc	126.49±2.44	ab
P4	260.51±29.71	ab	79.31±2.91	bcd	29.70±0.40	bc	132.98±12.88	ab
P5	271.51±10.09	ab	80.25±6.95	cd	28.93±0.29	b	137.84±12.41	ab
P6	260.47±41.12	ab	78.17±2.85	bcd	29.30±0.79	bc	133.55±6.16	ab
P7	296.54±32.35	bc	85.41±4.32	de	30.63±0.65	c	144.71±10.94	b
P8	271.99±22.56	ab	81.87±7.21	cd	30.20±1.51	bc	146.73±2.58	b
HSD 5%	73.96		13.51		1.66		23.70	

Note. Means followed by different letters in the same column are significantly different at Tukey test, $p < 0.05$

Table 3

Panicle weight per hill, dry weight of grain, and Relative Agronomy Effectiveness of each treatment compared to control (no fertiliser, P0), NPK treatment (P1), and compost alone (P2)

Treatments	Weight of panicles per hill (g)		Weight per individual panicle (g)		Dry weight of grain per hill (g)		Dry weight of grain per box (g)		Relative Agronomy Effectiveness (RAE)
P0	56.31±7.76	a	3.29±0.15	a	37.94±4.12	a	227.67±24.74	a	-
P1	111.06±6.63	c	3.75±0.25	abc	86.51±2.48	bc	519.07±14.29	bc	100.00
P2	67.64±4.46	ab	3.35±0.08	a	47.22±1.80	a	283.34±10.81	a	19.10
P3	66.53±4.76	ab	3.43±0.11	ab	52.82±5.68	a	316.95±34.11	a	30.64
P4	97.06±11.67	c	4.03±0.68	abc	74.75±4.92	b	448.51±29.52	b	75.79
P5	90.17±6.70	bc	4.13±0.27	abc	70.95±3.43	b	425.69±18.49	b	67.95
P6	89.40±4.21	bc	3.93±0.33	abc	72.56±8.41	b	435.35±50.48	b	71.27
P7	117.50±21.22	c	4.58±0.29	c	92.45±10.22	c	554.73±61.34	c	112.24
P8	100.84±4.23	c	4.33±0.26	bc	83.66±2.56	bc	501.96±15.39	bc	94.13
HSD 5%	28.36		0.94		16.77		99.09		

Note. Means followed by different letters in the same column are significantly different at Tukey-test, $p < 0.05$

formation and higher plant productivity. ZnO-NPs may readily release bioavailable Zn^{2+} into the soil, whereupon it may either react with soil organic matter or agglomerate and stick to soil particles, hence influencing soil microorganisms and enzymes (Liu et al., 2015; Santiago-Martín et al., 2016). This condition will greatly assist root development so that more water and nutrients from the soil will ultimately increase rice grain production.

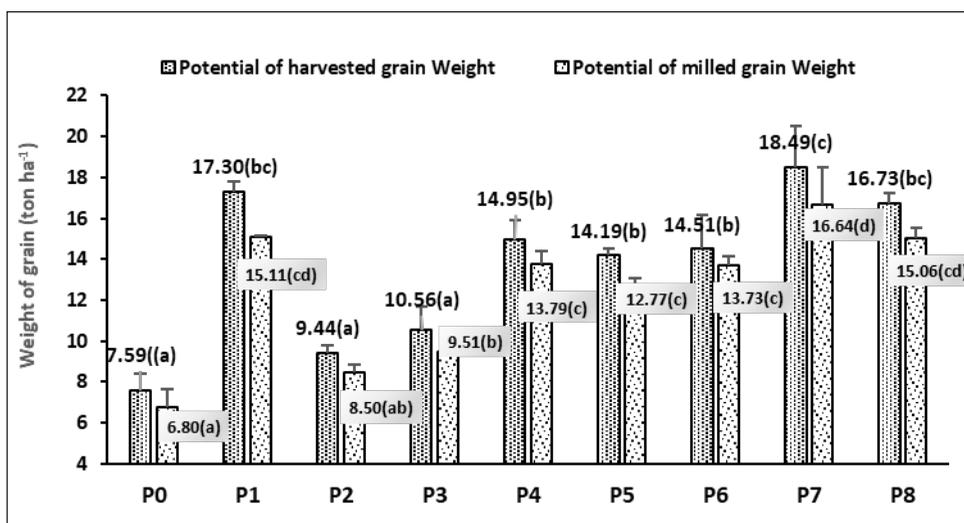


Figure 4. Potential of harvested and milled grain weight of each treatment compared to control (no fertiliser, P0), NPK treatment (P1), and compost alone (P2), based on the results of Tukey test ($p < 0.05$). (Remark: HSD 5% = 3.28; 2.52)

Based on the results of the calculation of nutrient use efficiency using the parameters of the Internal Utilisation Efficiency, the P0 treatment (no fertiliser) and the treatments of powder compost alone (P2) and compost+ZnO NPs 50-150 mg kg⁻¹ (P3 and P5) provided higher efficiency than the NPK treatment (Table 4). However, quantitatively, these treatments gave low yields. Based on the results of the calculation of the Agronomic Efficiency against the control (P0), treatments P4 and P5 (compost powder+ZnO-NPs

Table 4

Internal Utilisation Efficiency (IE), Agronomic Efficiency (AE), Physiologic Efficiency (PE), and Apparent Crop Recovery Efficiency (RE) of each treatment calculated against the control (no fertiliser; P0)

Treatments	IE	AE	PE	RE
P0	34.60±2.01 d			
P1	24.05±1.52 b	187.80±30.73 c	17.66±1.37 bcd	10.62±1.18 c
P2	32.54±0.52 d	62.39±7.18 a	21.64±6.79 cd	2.77±1.19 a
P3	29.80±0.11 c	111.42±17.52 ab	18.83±2.66 bcd	5.95±0.92 ab
P4	19.14±0.90 a	260.07±41.96 d	10.38±2.64 a	24.91±4.09 d
P5	29.70±0.14 c	246.26±44.18 d	23.28±2.04 d	10.55±1.25 c
P6	24.57±0.06 b	77.01±12.85 ab	15.21±3.11 ab	4.92±1.18 ab
P7	20.58±0.32 a	118.28±10.37 b	13.28±2.31 ab	8.73±1.44 bc
P8	23.48±0.26 b	99.52±8.50 ab	15.76±0.93 abc	6.31±0.26 ab
HSD 5%	2.43	51.44	6.17	3.99

Note. Means followed by different letters in the same column are significantly different at Tukey-test, $p < 0.05$

100-150 mg kg⁻¹) provided high-efficiency values in utilising nutrients in the soil and higher than the NPK treatment (P1), while the Physiologic Efficiency and Apparent Crop Recovery Efficiency of P5 treatment (compost powder+ZnO-nanoparticles 150 mg kg⁻¹) had the highest efficiency values.

Grain Quality

The variance analysis (ANOVA) results demonstrated that the tested treatments significantly affected parameters. In addition to increasing quantitative yield, the treatments of compost powder+ZnO-NPs 100-150 mg kg⁻¹ compost (P4 and P5) and the treatment of compost powder+ZnO-NPs 50-100 mg kg⁻¹ compost +½ NPK fertiliser (P6 and P7) improve the grain's value as measured by the percentage of weight of filled and empty grain and grain water content. The treatments had the lowest grain water content (Table 5). The treatments provided better grain quality than the NPK treatment (P2) and control (P0). This study's results align with the research of Zhang et al. (2021), which reported that applying ZnO-NPs increased grain production, dry matter accumulation, and grain quality. Zhao et al. (2014) reported that adding ZnO nanoparticles also improves the quality of cucumber plants.

Zn is an essential element in protein synthesis and formation of pollen grain (Herschfinkel et al., 2007; Mathpal et al., 2015) and enzyme activity since plant enzymes that utilise Zn as a cofactor include SOD, CA, alcohol dehydrogenase and the structural Zn-finger domains mediating DNA-binding of transcription factors and protein-protein interactions (Maret, 2009; Sinclair & Krämer, 2012). Zinc also plays a vital role in seed development, as its deficiency in plants leads to delayed maturity (Hansch & Mendel,

Table 5
Weight of empty and filled grain per 100 g, and water content of grain of each treatment compared to control (no fertiliser, P0), NPK treatment (P1), and compost alone (P2)

Treatments	Weight of empty grain per 100 grammes		Weight of filled grain per 100 grammes		Water content of grain (%)	
P0	5.49±0.22	e	94.51±0.22	a	13.49±0.55	d
P1	4.41±1.01	de	95.59±1.01	ab	15.06±0.33	e
P2	3.83±0.64	cd	96.17±0.64	bc	10.76±0.48	ab
P3	3.14±0.07	abc	96.86±0.07	cd	10.62±0.17	a
P4	2.20±0.22	ab	97.80±0.22	de	12.13±0.16	bc
P5	2.52±0.40	ab	97.48±0.40	de	11.78±0.49	abc
P6	1.23±0.12	a	97.91±0.12	e	12.72±0.61	cd
P7	2.43±0.37	ab	97.57±0.37	de	11.70±0.58	abc
P8	3.41±0.13	bcd	96.59±0.13	bcd	12.99±0.60	cd
HSD 5%	1.23		1.23		1.17	

Note. Means followed by different letters in the same column are significantly different at Tukey-test, $p < 0.05$

2009). Therefore, the addition of ZnO nanoparticles to the compost can affect the quality of rice grain. This most likely indicates that the dry matter content in rice grains increases, which improves the quality of rice as a food source. This is because the ZnO nanoparticles contained in the compost are able to increase the synthesis of certain enzymes involved in protein metabolism, such as the enzyme RNA polymerase. Thus, Zn is a component of protein (Rudani et al., 2018). Overall, the addition of ZnO nanoparticles provides dual benefits, namely increasing the efficiency of nutrient use and increasing the yield and quality of rice plants (El-Saadony et al., 2021).

CONCLUSION

The combination of compost powder+ZnO nanoparticles 100 mg kg⁻¹+½ NPK fertiliser (P7 treatment) increased the N, P, K, and Zn uptake, biomass weight, and number of panicles per hill. This treatment also had a higher harvested and milled grain weight potential than the NPK treatment, with a yield increase of 7% compared to the NPK chemical fertiliser treatment (P1). The Relative Agronomy Effectiveness and Agronomic Efficiency of the P7 treatment increased by 12% and 72% when compared to the NPK treatment. Compost powder+ZnO-NPs 150 mg kg⁻¹ treatments provided higher nutrient use efficiency than the NPK treatment. This research suggests that combining organic fertiliser and nanoparticles should be considered for sustainable rice production in paddy cultivation. However, this study was limited to a pot experiment; thus, field validation under various conditions is recommended to confirm the efficiency of ZnO NP-compost combinations.

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Development of Chrysanthemum Mutants Adapted to Lowland Area using Ethyl Methanesulphonate

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ABSTRACT

Chrysanthemum is a subtropical ornamental plant traditionally cultivated in highland regions. Increasing temperatures caused by climate change are causing a decrease in flower production and quality, thus encouraging the availability of chrysanthemum varieties adapted to warmer lowland environments. This study aimed to induce genetic and phenotypic variations in the Indonesian chrysanthemum variety 'Marina' for potential lowland adaptation using Ethyl Methanesulphonate (EMS) combined with Paclobutrazol (PBZ). EMS concentrations of 0 and 0.77% and PBZ concentrations of 100 and 200 ppm were used as treatment. Morphological parameters, including plant height, number of leaves, number of nodes, diameter of stem, number of flowers, diameter of flowers, and percentage change in flower colour, were evaluated under natural lowland heat stress. Genetic distance was confirmed using RAPD and SSR molecular markers. The results showed that the application of 0.77% EMS and 200 ppm PBZ successfully induced significant phenotypic and genetic variations in the 'Marina' chrysanthemum. Applying 0.77% EMS mutagen has produced several promising putative mutant lines, specifically KM11, KM20, and KM22, which have distinct leaf shapes, bright flower colours, and reduced the time of flowering compared to the parent. Molecular analysis showed significant genetic polymorphisms, with KM11 showing the highest genetic distances (56% similarity) from the parent, while KM22 showed the lowest. These results provide a valuable genetic background for developing lowland-tolerant chrysanthemums, although further analysis of the next generations is required to confirm the stability of these traits for commercial release.

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INTRODUCTION

Chrysanthemum (*Chrysanthemum morifolium*), belonging to the Asteraceae family, is an ornamental plant with a high economic value in domestic and global markets. It is an important Indonesian export commodity, ranked among the top 20 in the world and ranks third in ASEAN (Arumta et al., 2019).

In tropical areas, chrysanthemum is usually grown commercially in highlands between 700 and 1200 m above sea level, due to environmental adaptation to its temperate origins (Kurniati et al., 2013). The plant can also grow optimally at temperatures of 20-26°C in the growth phase and 16-18°C in the flowering phase, with humidity of 70-80% (Sanjaya et al., 2018). However, these conditions greatly limit their production, owing to various factors.

The increasing demand for chrysanthemums and limited land in the highlands have caused farmers to expand chrysanthemum cultivation to the lowlands. The problem in cultivating plants in this area is that the air temperature is relatively higher than in the highlands, especially with global climate change, which impacts the quality and production of chrysanthemum flowers. Temperature above 25°C has been reported to inhibit the flower initiation and delay flower formation in chrysanthemums (Nozaki & Fukai, 2008; Sembiring et al., 2021). Besides that, high temperatures also cause flowers to be dull, pale, and faded. Using chrysanthemum varieties adapted for lowland areas is an effective and economical solution, but until now, we have not obtained Indonesian chrysanthemums with this character. Lowland adaptation is defined not merely as survival, but as the plant's ability to maintain high-value ornamental characteristics, such as stable flower diameter and colour intensity, while completing a normal reproductive cycle under temperatures exceeding the optimal 25°C threshold.

The efficiency of ethyl methanesulphonate (EMS) in inducing mutations in *C. indicum indicum* var. *aromaticum* has been proven in the experiments of Chen et al. (2020) and Nasri et al. (2022). This method has also been used to increase plant tolerance to abiotic stress. Application of 0.5% EMS for 60 to 120 minutes successfully increased the tolerance of *Petunia* to stress (Krupta et al., 2017), drought tolerance in rice plants (Naser et al., 2024), and wheat tolerance in lowlands (Tarigan et al., 2023).

Previous reports indicate that chrysanthemums grown in lowland exhibit etiolation, weakness, and extended flowering. Paclobutrazol is a recommended plant growth retardant and has been widely recommended for suppressing the growth of elongated shoots or internodes. The application of PBZ on chrysanthemum has been proven to be effective in reducing plant height, as well as thickening leaves and stems (Chauhan et al., 2021; Lailaty et al., 2021; Salih & Hussien, 2020). Meng et al. (2022) stated that PBZ could reduce the deleterious effects of high temperature stress on peony growth by reducing lipid membrane peroxidation, activating photosynthesis, and protecting cell structures.

Phenotypic changes in mutants could be easily identified morphologically. However, this method is subjective and is strongly influenced by the environment. Detection of

mutant changes can be done more accurately by using molecular markers, which help to understand the phenotypic effects of mutations (Bhat et al., 2023), as well as the genetic distance between the mutant and the original parent (Kang et al., 2013). Simple Sequence Repeat (SSR) is a molecular marker which commonly used to identify non-coding regions with short repetitive base sequences at specific loci (Weising et al., 2005). SSR markers offer various advantages, such as being easy to use, highly informative, possessing specific loci, reading codominant traits, and applicable for genetic diversity analysis (Choudhary et al., 2023). This study aims to induce genetic and phenotypic variations in the Indonesian chrysanthemum variety 'Marina', which can adapt to lowlands using Ethyl Methanesulfonate (EMS) combined with Paclobutrazol (PBZ).

MATERIALS AND METHODS

Plant Materials

Plant material used for mutation induction was rooted cuttings (8-10 cm long) of the Marina variety. Marina is an Indonesian chrysanthemum variety officially released by the Ministry of Agriculture of the Republic of Indonesia based on decree number 051/Kpts/SR.120/D.2.7/7/2014. Marina is a popular commercial variety with high aesthetic value and yellow flowers, but is traditionally restricted to highland production (1000 m above sea level). The cuttings were transferred to a screen house at the Universitas Pembangunan Nasional Veteran Jawa Timur, at an altitude of \pm 5 m above sea level. The minimum temperature was recorded at 24°C and a maximum of 30°C, with an average temperature of 27.2°C. The humidity level was 75% to 95%.

The material used for molecular analysis included three chrysanthemum mutant genotypes (KM22, KM20, and KM11) and the original chrysanthemum as a control (KN1). Young leaves of EMS-induced mutants and the original chrysanthemum were used for DNA extraction using the CTAB method.

EMS and PBZ Treatment

EMS stock solution 1% (v/v) was used to prepare 0.77% (v/v) solutions using 0.1 M phosphate buffer (pH 7.2), which were then filter-sterilised with an SFCA-PF 0.2 μ m filter (Corning, NY, USA). The concentration of 0.77% EMS was adopted based on its proven efficacy in previous chrysanthemum mutation studies (Latado et al., 2004; Rahmah et al., 2011), ensuring a high probability of viable mutation induction.

The cuttings were immersed in 0.77% EMS solution and sterile distilled water (0% EMS) as a control for 90 min. Explants were washed with sterile distilled water four to five times and transplanted using a mixture of cocopeat, soil, and cow manure in a ratio of 1:1:1. PBZ at 100 and 200 ppm was applied 30, 37, 44, and 52 days after planting, by spraying over the entire surface of the leaves and stems in the morning.

Observations were made on quantitative characteristics (plant height, number of leaves, number of internodes, stem diameter, number of flowers, diameter of flower, percentage change in flower colour) and qualitative characteristics (leaf colour, leaf shape, flower colour, and flower shape). The colour of the leaves and flowers was assessed using the Munsell Colour Charts, and the results were described in terms of hue, chroma, and value.

Data Analysis

The experiment was arranged as a factorial design based on a Completely Randomised Design (CRD). EMS (0, 0.77%) and PBZ (0, 100, and 200 ppm) were considered the first and second factors, respectively. Each treatment was repeated 4 times, resulting in 24 experimental units. Each experimental unit consisted of 10 plants. Data were subjected to an analysis of variance (ANOVA). Significant differences were further analysed using the Fisher's Least Significant Difference (LSD) at the 5% level.

DNA Extraction

Total plant genomic DNA was extracted using the small-scale CTAB method (mini preparation) developed by Doyle (1991) with minor modifications. The chrysanthemum leaf samples were ground from each genotype and mixed with liquid nitrogen in a mortar until they became a powder. The crushed powder was then placed in a 2 ml Eppendorf tube, followed by the addition of 700 μ L of extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 2% (w/v) CTAB (cetyltrimethylammonium bromide), and 2% (w/v) PVP (Polyvinylpyrrolidone) to each sample. Furthermore, 5 μ L of mercaptoethanol was added to each sample, followed by homogenisation of the mixture by carefully inverting the tube, and this was incubated at 65 °C for 15 min in a water bath. The mixture was homogenised again during incubation by inverting the microtube every 5 min.

In the next stage, 700 μ L of chloroform isoamyl alcohol (24:1 v/v) solution was added. The mixture was homogenised using a vortex or centrifuged at 12,000 rpm for 10 min, and the supernatant of each sample was carefully transferred into a new 1.5 mL microtube. Subsequently, 1/10 times the volume of the supernatant was added to each sample, comprising a solution of 3 M sodium acetate (pH 5.2), followed by a cold isopropanol solution, which equalled the volume of the supernatant. The samples were then incubated in a freezer at -20 °C for one hour, and centrifuged at 12,000 rpm for 10 min until a DNA pellet precipitate was formed. The supernatant liquid was discarded, and the DNA pellet was washed with 500 μ L of 70% ethanol solution. The mixture was centrifuged again at 12,000 rpm for 5 min, and the supernatant was discarded. The washed DNA pellet was air-dried until the remaining ethanol was depleted. The dried DNA pellet was dissolved in TE solution (10 mM Tris, pH 8.0, 1 mM EDTA) supplemented with RNase (10 mg/mL). The stock DNA solution was then incubated at 37 °C for 1 h and stored at -200 °C until ready.

PCR Analysis with RAPD Primers

The stock DNA solution, which had been tested for quality and quantity, was then diluted into a working solution at a concentration of 2 ng/μL. The diluted DNA from each sample was then amplified using 7 (seven) RAPD primers: OPA-08 (5'-GTGACGTAGG-3'), OPA-02 (5'-TGCCGAGCTG-3'), OPA-03 (5'-AGTCAGCCAC-3'), OPA-07 (5'-GAAACGGGTG-3'), OPA-04 (5'-AATCGGGCTG-3'), OPA-09 (5'-GGGTAACGCC-3'), and OPA-10 (5'-GTGATCGCAG-3'). DNA amplification was carried out in a total volume of 20 μL consisting of 2 μL of template DNA with a concentration of 2 ng/μL, 10 μL of My Taq HS (Bioline, UK), 1 μL of RAPD primer with a concentration of 5 μM, and sterile ddH₂O.

Amplification was performed on a PCR Thermocycler machine (Biorad, USA) with the following profile: an initial denaturation stage at 94°C for 5 minutes, followed by 45 cycles of denaturation at 94°C for 1 minute, annealing at 37°C for 1 minute, and elongation at 72°C for 1 minute. The PCR reaction was terminated with a final elongation stage at 72°C for 5 minutes. The amplified DNA was then electrophoresed on a 1.2% agarose gel in a tank containing 0.5x TBE buffer at 90 V for 35 minutes. The gel was then immersed in ethidium bromide solution (10 mg/ml) for 10 minutes. The stained gel was then rinsed with distilled water for further visualisation on a UV Transilluminator (Biorad, USA).

PCR Analysis with SSR Primers

DNA was amplified using 5 (five) SSR primers, namely PIF 107 (5'-ATGGTGAAAGAGGAAGCATG-3'), PIF 98 (5'-TGA ACT ATG GTG ACG GCG GT-3'), PIF 7 (5'-TGA GAT CTT TTT TAC CGC GTG TTT-3'), cmGFR 01 (5'-GGTGGAAACGCTCAGCT-3'), and cmGFR 02 (5'-CACGAGCAAGCCATCAG-3').

The Polymerase Chain Reaction (PCR) reaction was carried out in a total reaction volume of 25 μL. The SSR marker-based PCR reaction composition consisted of 10 ng/μl DNA from each test sample, ddH₂O, 2X MyTaq DNA Polymerase, and 0.5 μM primer. The PCR profile used included the following stages, namely initial denaturation at 94 °C for 7 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, attachment of markers at a specific annealing temperature (depending on the annealing temperature of each SSR marker) for 30 seconds, and polymerisation at 72°C for 1 minute. The PCR analysis ended with a final extension stage at 72°C for 7 minutes.

Data Analysis

Data analysis for RAPD and SSR was performed by scoring the DNA bands observed in the electrophoresis results. Each visible band was considered one allele, and DNA bands with the same migration rate were considered at the same locus. Scoring was done in a binary manner: visible bands were scored as 1, absent bands as 0, while unamplified samples

were scored as 9 and considered missing data, resulting in binary scoring data. The binary scoring data were analysed using the UPGMA (Unweighted Pair-Group Method with Arithmetic Mean)-SAHN (Sequential Agglomerative Hierarchical and Nested) programme in NTSYS version 2.1 software. The analysis results were presented as a dendrogram and a genetic similarity matrix.

RESULTS AND DISCUSSION

Morphological Analysis

Quantitative Characteristics

Statistical analysis showed no effect of interaction between EMS and PBZ on plant height, number of leaves, number of internodes, stem diameter, number of flowers, and diameter of flowers. However, a single factor (EMS or PBZ) was influenced at 126 days after planting (Table 1).

Table 1

Plant height, number of leaves, number of nodes, stem diameter, number of flowers, and diameter of flowers of chrysanthemum at 126 days after planting

Treatments	Plant height (cm)	Number of leaves	Number of internodes	Stem diameter (cm)	Number of flowers	Diameter of flowers (cm)
EMS (%)						
0	70.48 ^a	29.03 ^a	23.93 ^b	0.56 ^b	1.04 ^a	10.00 ^a
0.77	69.78 ^a	29.14 ^a	20.26 ^a	0.56 ^b	1.06 ^a	10.79 ^b
PBZ (ppm)						
0	81.38 ^b	33.09 ^b	24.93 ^b	0.50 ^a	1.04 ^a	9.86 ^a
100	67.13 ^{ab}	27.51 ^{ab}	21.84 ^{ab}	0.60 ^{bc}	1.04 ^a	10.57 ^b
200	61.90 ^a	26.66 ^a	19.51 ^a	0.58 ^b	1.08 ^a	10.75 ^b

Note. Means followed by the same letter in the same column are not significantly different at $p < 0.05$

Applying 0.77% EMS produced mutant chrysanthemums with fewer internodes and significantly different from the control. However, the plant height, number of leaves, stem diameter, and number of flowers were not significantly different between the mutant and control (Table 1). The effect of EMS, which reduces the number of internodes, was also observed in the research of Rime et al. (2019), who applied EMS at a concentration of 0.2-1.0% to mango plants.

The application of EMS increased the flower diameter of mutants, which was significantly different from that of the control. EMS causes point mutations; if mutations occur in the genes that regulate cell growth and elongation, or hormone-forming pathways,

they produce phenotypes with larger growth characteristics, such as increased flower diameter and internode length (Subramaniam & Kumar, 2023). Statistical analysis showed a significant interaction between EMS and PBZ on flowering time. Treatment of 0.77% EMS combined with 200 ppm PBZ resulted in the earliest flower bud initiation at 75.55 days, significantly earlier than the control (0% EMS without PBZ), which required 82.25-83.05 days (data not presented). The reduced flowering time (approximately 7 days earlier than the control) without compromising flower quality further validates the adaptive capacity of the mutant. This suggests that the synergistic effect of EMS and PBZ application optimises the plant's ability to cope with high temperature stress.

PBZ was often used to inhibit plant growth and enhance plant vigour, as shown in Table 1, where PBZ treatment (100 and 200 ppm) decreased plant height, leaf number, and internodes. These sizes decreased as the concentration of PBZ increased, due to the inhibition of gibberellin production by hindering the oxidation of kaurene to kaurenoic acid. Consequently, it could further reduce the speed of cell division, reduce vegetative growth, and indirectly redirect assimilates toward reproductive growth for flower formation (Desta & Amare, 2021; Soumya et al., 2021). PBZ also increased stem and flower diameters, enhancing the chrysanthemum's appearance. Numerous studies have shown the effectiveness of PBZ in reducing chrysanthemum plant height (Elhassan et al., 2021; Chauhan et al., 2020) and increasing stem diameter (Salih & Hussein, 2020). These morphological changes induce anatomical alterations, such as increased leaf thickness and stem diameter (Lailaty & Nugroho, 2021).

Qualitative Characters

Leaf Colour and Shape

Plant colour can be described by three factors: hue, saturation, and lightness, or XYZ colour-matching functions (Kasajima, 2019). Hue is a type of colour, such as red, green, and blue. In the Munsell classification system, the degree to which a colour is expressed by 'saturation' or chroma. Lightness represents the brightness of the colours or values. The results showed that EMS and PBZ did not have much effect on leaf colour. All leaves had a green-yellow colour with a hue value of 5GY. However, there was a gradual change in leaf colour (Figure 1). The leaf colour of the wild-type had a lightness value of 7 and a chroma of 10 (bright yellowish green). Meanwhile, EMS or EMS+PBZ chrysanthemum leaves had a slightly darker green leaf colour with the codes "hue", "chroma," and "value," namely 5GY 6/6, 5GY 7/8, 5GY 6/10, and 5GY 6/8, respectively.

The leaves showed variations between mutants, specifically in the sinuses and sinus side between the two lateral lobes (Figure 1). The leaf shape was long, ovate, slender, and elongated, with large serrated leaf edges. The sides of the sinus between the two lateral lobes diverged (opening) and opened wider in EMS without PBZ, and the shape of the

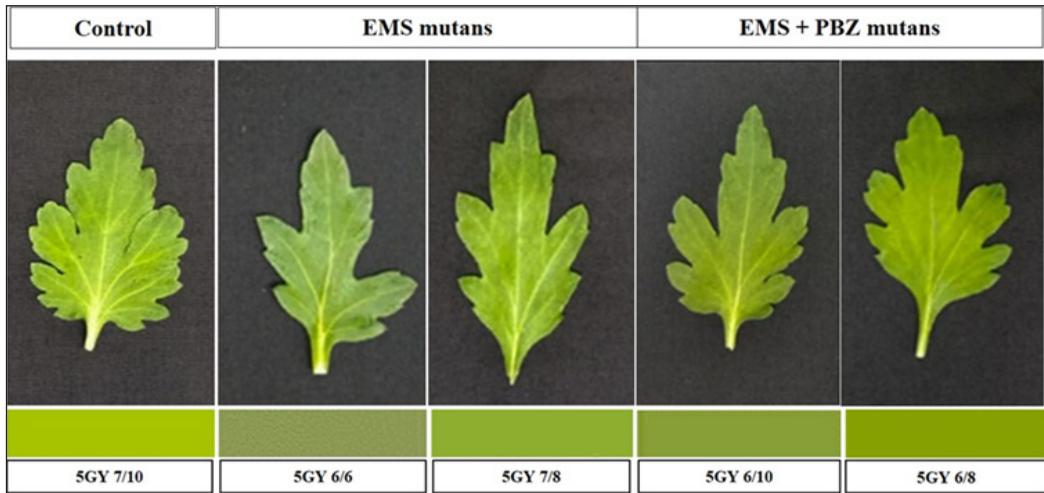


Figure 1. The shape and colour of control and mutant chrysanthemum leaves

sinus between the two lateral lobes was acute (tapered). The control had an ovate leaf shape with converging sides and rounded sinuses (Figure 1). Song et al. (2021) classified chrysanthemum leaf morphology into broad, ovate, and long. This study showed that control chrysanthemum leaves were ovate, whereas leaves of mutants were long ovate.

Flower Colour and Shape

Marina variety in the highlands has yellow flowers, with stacked flower crowns. Cultivation of this variety in the lowlands at an altitude of ± 5 m above sea level causes the colour of the flowers to change from yellow to yellowish white (pale), with thin flower crowns, so that the appearance of the flowers is not attractive. This shows that environmental conditions in the lowlands influence the colour and shape of the flowers.

PBZ treatment without EMS application could not induce flower colour changes (Figure 2). The flower appearance of mutant genotypes in the lowlands showed a wide range of flower colour and shape variations, as presented in Table 2 and Figure 2. The application of 0.77% EMS induced a broad spectrum of flower colour variations, comprising greenish-white (37.5%), white (29.1%), pink (20.8%), dark yellow (12.5%), and yellowish-white (0.1%) (Table 2). In contrast, the control exhibited complete uniformity with 100% yellowish-white flowers. In the greenish-white variants, the green pigmentation was specifically observed on the petal margins and/or the central disc of the flower. Furthermore, EMS treatment resulted in a structural modification of the ray florets, transitioning from an irregular to a regular arrangement.

Table 2
 Percentage of flower colour in EMS chrysanthemum mutants in the lowlands

Concentration of EMS (%)	Flower colour (%)				
	Yellowish white	Greenish white	White	Dark yellow	Pink
Without EMS (0)	100	0	0	0	0
EMS (0.77)	0.1	37.5	29.1	12.5	20.8

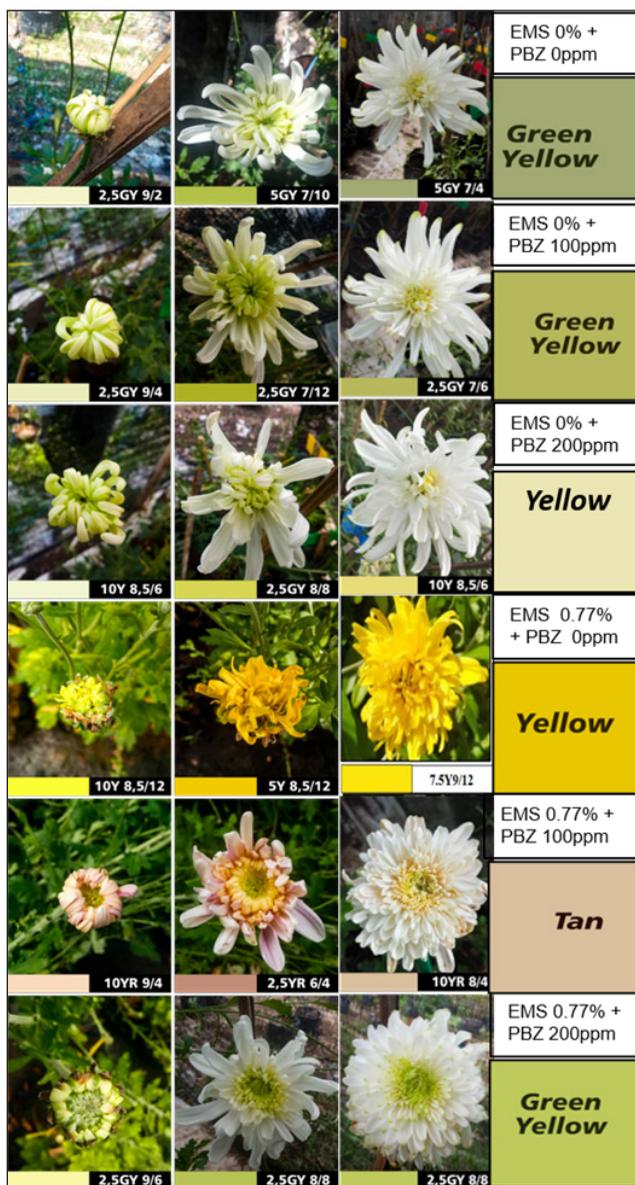


Figure 2. Differences in the shape and colour of flowers between the control and the mutants

EMS and PBZ treatments caused changes in flower shape, flower colour, and flower corolla thickness (Figure 2). EMS application caused a change in the flower colour from yellowish white (7.5 GY 9/4) (wild-type) to various colour variations, such as a dark yellow flower with the colour code 2.5Y 8/12, pink (2.5R 9 /6), greenish-white (2.5 GY 9/2), and pure white (N/9). Changes in flower colour that occurred during EMS+ 100 mg/l PBZ treatment were light yellow (7.5Y 9/12), pink (2.5R 9/2), greenish-white (2.5GY 9/4), and pure white (N/9). The flower colours produced in the EMS + 200 mg/l PBZ treatment were pink (5R 9/2), greenish-white (2.5GY 9/4), and pure white (N/9). Besides causing changes in flower colour, EMS treatment or its combination with PBZ also caused changes in flower shape and corolla thickness. Flowers of the same colour can have different crown thicknesses (Figure 2).

The Marina variety used in this study has a standard type of flower with a decorative shape. The characteristic feature of decorative flowers was a round corolla tightly stacked, and the petals were short in the middle and elongated at the edges. The results showed that chrysanthemum flowers resulting from the EMS mutation had irregular and regular decorative flower shapes (Figure 2). Chrysanthemum flowers in the EMS+ PBZ treatment had a decorative flower shape characterised by long, almost neat, and regular petals, where the length of the innermost petals did not exceed the outermost petals. It appeared that PBZ helped the EMS mutant to form flowers with regular corollas. Chrysanthemum flowers without EMS at all PBZ concentrations had a non-decorative shape with irregular petal lengths from the inside of the flower corolla to the outside of the flower. Although PBZ effectively regulated decorative flowers in EMS mutant plants, it did not occur in control plants or plants without EMS.

EMS treatment significantly affected leaf colour, leaf shape, and flower colour. Lethin et al. (2020) stated that EMS mutagenesis has been applied to various plants to produce morphological diversity and induce the formation of ideal characteristics. Gao et al. (2022) showed that Chinese cabbage plants treated with 0.8% EMS produced changes in leaf colour, shape, and head. Several researchers have reported that the induction of mutations in chrysanthemum plants through the application of EMS produces more flower variations (Ghormade et al., 2020; Din et al., 2023). The use of EMS could increase genetic diversity in plants and make it possible to improve only one desired character, without changing other characters (Purnamaningsih & Hutami, 2016). Nasri et al. (2022) produced mutants of the “Fariba2” cultivar, in which their colour varied from light yellow to orange, pink, and red, while the control plants had yellow-coloured ray florets. Chrysanthemum mutants generated with 0.125% and 0.5% EMS had white and light-yellow floret colours from the cultivar “Homa”, yellow colour. Suryawati et al. (2023) reported that chrysanthemums treated with EMS through an in vitro technique caused the flower colours to change from red-purple (70B) to purple (70A) and red-purple (60A). In this study, the application of

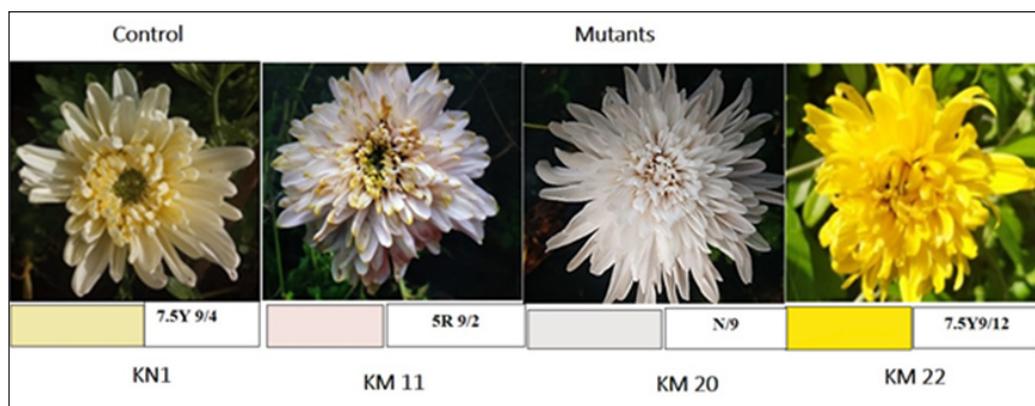


Figure 3. Three candidate chrysanthemum mutant genotypes adapted to the lowlands

EMS and paclobutrazol were proven to have produced mutant plants that could adapt to lowlands and produce flowers of varying colours with normal flower sizes, thick and regular crowns.

Applying 0.77% EMS to the Marina variety (KN1) produced three promising chrysanthemum mutant genotypes in lowlands. These genotypes had good growth performance with attractive flower shapes and colours that were different from those of their parents. Marina variety (control) in lowlands had yellowish-white flowers (7.5Y 9/4), while the mutants had yellow, white, and pink flowers, which included KM22 (yellow 7.5 Y 9/12), KM20 (white N9), and KM11 (pink 2.5R 9/2) (Figure 3).

Distinct phenotypic changes were observed among the selected lines. The flower colour of the KM11 mutant changes from light yellow to magenta-red, characterised by a large diameter and symmetrical ray florets with light green margins. The KM20 mutant exhibited a colour shift to white, featuring regular, symmetrical florets and an increased flower diameter. In contrast, the flower of the KM22 genotype displayed a change to dark yellow, and its florets remained irregular in arrangement. Overall, the synergistic treatment of 0.77% EMS and PBZ (100 and 200 ppm) yielded flowers with enhanced petal thickness and improved structural symmetry. These traits represent significant potential for the commercial development of chrysanthemum varieties tailored for lowland tropical environments (Figure 3). The application of EMS and PBZ represents a strategic cultivation package for lowland production. While EMS induces stable genetic variations for heat tolerance, PBZ acts physiologically to counteract heat-induced etiolation.

RAPD and SSR Molecular Analysis

The genetic similarity matrix using combined RAPD and SSR markers in Table 3 shows the genetic distances between the four chrysanthemum genotypes. Genetic distances ranged from 0.56 to 0.73 across the four genotypes tested. The genetic similarity matrix value

was negatively correlated with the genetic distance, meaning that the lower the value, the higher the genetic distance. The highest genetic distance was observed between the KN1 genotype (control) and the KM11 genotype (pink flowers), and the lowest was the KM22 genotype (yellow flowers). KM11 was the most divergent genotype compared to the control, while KM22 was the most similar.

Based on phylogenetic analysis using the NTSYS tool, the four chrysanthemum genotypes used were separate and had different genetic backgrounds, with a genetic similarity coefficient of 0.73 (Figure 4). KM22 had the closest genetic distance to control (KN1) with a genetic similarity level of 75%, then KM20 had a genetic similarity of 67% to KN1, and KM11 had the furthest genetic distance with a genetic similarity value of 56%. The results obtained showed that the four chrysanthemum genotypes were different individuals. This result was supported by the genetic similarity matrix of the four chrysanthemum genotypes analysed, where the four genotypes had genetic similarities below 73%.

Table 3
Genetic similarity matrix of four chrysanthemum genotypes using UPGMA-SAHN methods

Genotype	KN1	KM22	KM20	KM11
KN1	1.00			
KM22	0.73	1.00		
KM20	0.67	0.67	1.00	
KM11	0.56	0.67	0.65	1.00

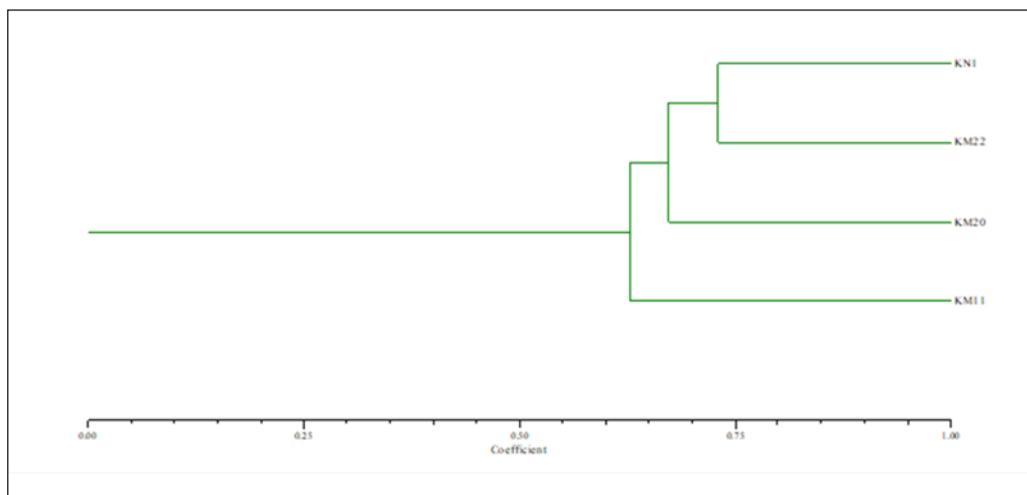


Figure 4. Phylogenetic dendrogram of chrysanthemum mutant genotypes based on the UPGMA-SAHN method

The identification of DNA polymorphisms using RAPD and SSR markers in the first-generation provides significant preliminary evidence of successful genetic induction by EMS. While this study represents the initial phase of selection, the presence of distinct genomic variations suggests that the observed traits, such as increased flower diameter

and reduction in flowering time, are linked to structural changes in the DNA rather than transient phenotypic plasticity, although further verification in subsequent generations is essential to confirm the long-term heritability of these traits.

Some studies on chrysanthemum mutants have shown that RAPD and SSR markers can efficiently identify changes at the genetic level. SSR markers are also potent for studying genetic relationships and could be valuable tools for identifying and classifying chrysanthemum (Chang et al., 2018). Thakur et al. (2022) obtained two prominent genotypes using SSR markers and verified the mutant behaviour of newly evolved chrysanthemum genotypes at the molecular level. These results indicate that the four chrysanthemum genotypes are genetically distinct individuals, confirming that the mutation method successfully produced broad genetic diversity and high potential for developing commercial chrysanthemum varieties in the lowlands.

CONCLUSION

The application of 0.77% EMS and 200 ppm PBZ successfully induced significant phenotypic and genetic variations in the 'Marina' chrysanthemum. Three promising genotype mutants were identified, exhibiting improved floral quality and reducing the time of flowering under lowland conditions. These results proved that chemical mutagenesis is a viable approach to increasing the genetic base of heat-tolerant chrysanthemums. Further evaluation in subsequent generations is required to confirm the stability of these improved traits before they can be officially categorised as stable mutants.

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Site-specific Morphological and Nutritional Variability of *Caulerpa lentillifera* in Malaysia: A Chemometric Approach for Tropical Aquaculture

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ABSTRACT

Caulerpa lentillifera is an important tropical green macroalgae cultivated and harvested across Southeast Asia. Nevertheless, its morphological and biochemical responses towards varying environmental conditions remain insufficiently discussed. This study aimed to characterise the site-specific morphological, nutritional, and mineral variability of *C. lentillifera* collected from three Malaysian populations. Samples were harvested from wild populations in Port Dickson (PD) and Semporna (SS), and from

a cultured pond population from Pulau Langkawi (PL). This study also applied chemometric methods to identify unique compositional patterns relevant to tropical aquaculture. Morphological and proximate analyses found that the PL sample has an assimilator length of up to 94 mm and a moisture content of 96% (wet weight basis), whereas PD samples had higher total nitrogen content (1.36 mg/L), crude fiber (3.45 g/100 g), and rhizoid length (13.8 mm), indicating site-related variability. Principal Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA), and hierarchical heatmaps were used to integrate water-quality, morphometric, nutritional, mineral, and heavy-metal datasets and to visualise site-specific clustering patterns. The PCA explained 62.5% of the total variance, and the PLS-DA model showed clear, yet descriptive, group separation (R^2 and $Q^2 > 0.9$; $p = 0.001$). The fibre, calcium, and carbohydrates were identified as the main discriminant factors. Overall, the analyses revealed distinct site-associated compositional trends rather than definitive classifications. These findings provide baseline insights to guide future site selection and cultivation strategies for sustainable tropical aquaculture.

Keywords: *Caulerpa lentillifera*, chemometric analysis, morphological plasticity, proximate composition, sea grape, site-specific

INTRODUCTION

Seaweed has been an integral part of human diets and health for centuries. To date, most seaweed production is concentrated in Asia, accounting for over 81% of the total worldwide production (Ismail et al., 2024). In 2020, the seaweed aquaculture dominated more than half of the total marine aquaculture, with an annual yield reaching more than 35 million tonnes (wet weight) 2020 (Chopin & Tacon, 2021; Stuthmann et al., 2023). Despite growing interest in seaweed cultivation, most exports focus on brown and red seaweed. Less attention had been given to green macroalgae or *Chlorophyta*. This had created a gap in further understanding of their ecological, nutritional values, and aquaculture potential (Moreira et al., 2021).

Among the green seaweeds, *Caulerpa* spp. is the most prominent genus, with more than 75 species reported (Famà et al., 2002; Preez et al., 2020). Within this, *Caulerpa lentillifera* is the most outstanding species due to its rapid growth, palatability, and potential commercial value. In Southeast Asia, including Malaysia, Thailand, Indonesia, and the Philippines, it is known as 'sea grapes or latok', due to its grape-like features. In coastal regions, *C. lentillifera* is often included in main dishes or eaten fresh due to its palatability and nutritional benefits (Chaiklahan et al., 2020; Khandaker et al., 2021). Due to its rising potential as a functional food, this species has attracted growing economic and cultivation interest.

However, *C. lentillifera* exhibits distinct physiological responses under different cultivation and environmental conditions. This helps to address existing gaps in understanding their adaptation and growth performance (Estrada et al., 2020). To date, many studies have focussed on interspecific variation within the genus *Caulerpa*, but comparatively less attention has been given to intraspecific variation. For example, research in Malaysia primarily focuses on differences among *C. racemosa*, *C. manorensis*, and *C. sertularioides* (Ismail et al., 2020; Yap et al., 2019), while site-specific variability in *C. lentillifera* is relatively underexplored. It is therefore important to understand their site-specific variability within the species and identify the factors that drive intraspecific variation to improve aquaculture cultivation and quality. Although seaweeds exhibit high adaptability, this does not necessarily translate into ecological or cultivation advantages without supporting data.

Thus, this study aims (i) to characterise the morphological, nutritional, and mineral variability across three distinct Malaysian sites; (ii) to identify the key discriminant variables using chemometric approaches; and (iii) to discuss the implications of site-specific variations within the species for tropical aquaculture and resource utilisation. To achieve these objectives, multivariate statistical analyses were employed. Principal Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA), and hierarchical clustering were applied to integrate water-quality, morphological, proximate, and mineral data into a comprehensive analysis. The analysis contributes to identifying the key variables that lead to population differentiation within the species. This study also supports Malaysia's National Agrofood Policy 2021-2030 (NAP 2.0), which aims to promote sustainable aquaculture development, improve cultivation, optimise production, and ensure consistency (Ismail et al., 2024).

MATERIALS AND METHODS

Study Area and Sample Collection

Wild samples were harvested from Port Dickson, Negeri Sembilan (PD, Southern Malaysia; Latitude: 2° 24'58.4 N, 101°51' 24.84"E). It is a semi-exposed coastal area close to the coastal development and active tourism. Another wild sample was collected from Semporna, Sabah (SS, East Malaysia; 4° 28'54.4 "N, 118° 36'40.28 "E). This area is closer to a coral reef ecosystem and is less disturbed by urban development. The cultivated samples were collected from a local aquaculture farm in Pulau Langkawi, Kedah, Malaysia (PL, Northern Peninsular Malaysia) (Figure 1). This site was included based on availability and is not environmentally equivalent to the natural site. While the sampling was conducted between January and April, the dates varied across locations due to logistical and weather constraints.

To avoid bias, samples were harvested around 11:00 a.m. and 3:00 p.m., within a 10-meter radius, and at depths of 0.5 to 1.0 m. This is important to keep preserving site-

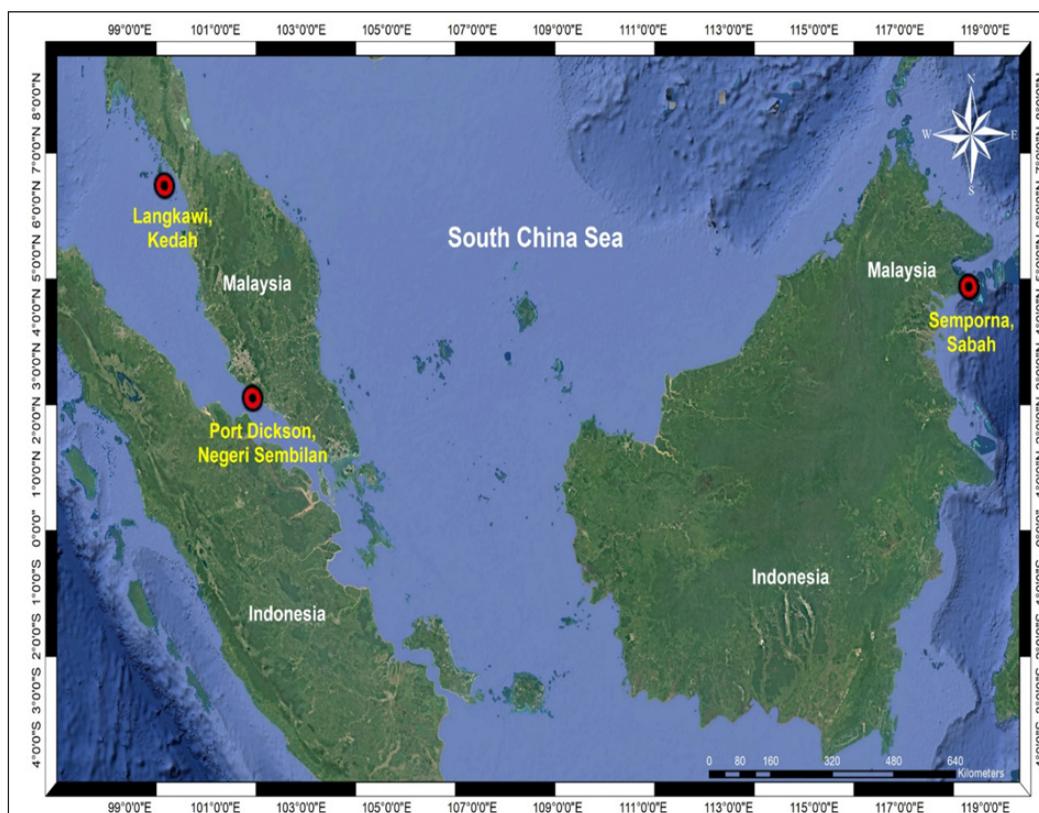


Figure 1. Location of sampling sites: Port Dickson (PD), Semporna (SS), and Pulau Langkawi (PL)

specific differences. At each site, about 10 individual thalli were randomly collected, kept on ice, and transported to the laboratory within 24 hours. Morphometric identification was conducted on individual thalli, and chemical analysis was performed on a composite sample.

Water Quality Assessment

At each site, water temperature ($^{\circ}\text{C}$), salinity (ppt), and pH were measured in situ with a calibrated multiparameter probe (HI98194, Hanna Instruments, USA). Independent water samples were aliquoted within a 5-10 m radius using pre-cleaned amber bottles ($n=10$ per site). Total nitrogen (TN) was determined following the APHA Standard Method (3500 series), while total phosphorus (TP) was quantified after acid digestion using ICP-OES as in USEPA Method 6010D. All results were expressed in mg/L.

Species and Morphometric Identification

Species identification was validated by a phycologist and an aquaculture expert from the International Institute of Aquaculture and Aquatic Sciences (I-AQUAS), Universiti Putra

Malaysia. The identification was recorded based on the seaweed size and structure of the assimilator, branchlet, and rhizoids (Estrada et al., 2020).

Proximate Analysis

Moisture content, ash, crude protein, crude fat, and carbohydrate content of the *C. lentillifera* were determined based on the Association of Official Analytical Chemists (AOAC, 2007) method on the fresh-weight (wet basis). This is to reflect the typical consumption of seaweed (Fisal et al., 2012). The oven-drying method (AOAC 934.01) was used for moisture content, and incineration at 550 °C in a muffle furnace (AOAC 942.05) was used for ash content. The Kjeldahl method with a nitrogen-to-protein conversion factor of 6.25 (AOAC 984.13) was used to determine crude protein, and crude fat was determined by Soxhlet extraction with petroleum ether (AOAC 920.39). Carbohydrate content was determined by difference after subtracting the total percentages of moisture, ash, crude protein, and crude fat, with ten replicates per sample (Abdullah et al., 2021).

Mineral Composition Analysis

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES; Agilent 5100, USA) was used to quantify the mineral composition of the *C. lentillifera*. The seaweed samples were dried in a muffle furnace at 550 °C, as referred to in AOAC (2005). Next, the samples were digested in 7 mL concentrated hydrochloric acid and diluted to 100 mL. The multi-element calibration standards and reagent blanks were also prepared for quality control measurement (Mohd Naeem et al., 2017).

Phytochemical Screening

Dried and fine *C. lentillifera* powder was used in this study to standardise its moisture content (Rasyid, 2017). Aqueous extracts were prepared using a solid-to-solvent ratio of 1:20. Total phenolic content (TPC) was determined using the Folin-Ciocalteu method at 765 nm. Total flavonoid content (TFC) was determined using the calorimetric method at 415 nm. Absorbance was read using a 96-well microplate reader (Model 680, Bio-Rad Laboratories, USA). Calibration curves were prepared using gallic acid (0-250 mg/L) and quercetin (1000-5000 mg/L), with strong linearity (R^2 values = 0.9908 and R^2 = 0.9989, respectively) TPC was expressed as mg gallic acid equivalent per gramme dry weight (mg GAE/ g⁻¹ DW), while TFC was expressed as mg quercetin equivalents per gram dry weight (mg QE/ g⁻¹ DW) (Zheng et al., 2022).

Statistical and Chemometric Analysis

Data are reported as mean ± standard deviation. One-way ANOVA followed by Tukey's post hoc test was calculated using Minitab 21.4.1 software (State College, Pennsylvania,

United States). Chemometric analyses were performed using MetaboAnalyst 6.0. Variables included in Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were selected from complete datasets with no missing values. All variables were mean-centred and auto-scaled to unit variance to account for differences in measurement units. PLS-DA models were validated using k-fold cross-validation, and model performance was assessed using the R^2 and Q^2 values. Variable Importance in Projection (VIP) scores > 1.0 were used to consider important for group discrimination, and clustering patterns were visualised using a heat map.

RESULTS AND DISCUSSION

Water Parameters and Intraspecific Variation

Water temperatures at all sites ranged from 28.67 to 31.5 °C, consistent with standard tropical farming conditions for *Caulerpa* spp. (Zuldin et al., 2019) (Table 1). In the wild environments of PD and SS, elevated temperatures are observed compared to the PL water sample. This difference may reflect local climatic conditions and tidal exposure during in situ sampling, rather than inherent site differences. Importantly, all temperature values remained within the reported tolerance range for *C. lentillifera*, suggesting that temperature variation among sites is unlikely to impose physiological stress during the sampling period (Stuthmann et al., 2023).

There is no significant difference in salinity among sites. This suggests similar patterns of water mixing and tidal influences across sites during sampling (Guo et al., 2015). However, seaweed growth could still be affected by seasonal or short-term freshwater inputs, which can alter salinity. Thus, it is necessary to consider site-specific cultivation practices, especially in an open coastal environment (Mosquera-Murillo, 2016).

Compared to all samples, the highest dissolved oxygen (DO) was observed in PL at 4.31 ± 0.21 mg/L. This range is still comparable to both coastal and semi-aquaculture systems, as reported by Alamsyah, (2016). The variations between samples are likely

Table 1
Comparison of water parameters for *C. lentillifera* harvested from different Malaysian sites

Parameters	PD	SS	PL	p-value
Temperature (°C)	31.47±1.27 ^a	29.72±1.25 ^b	28.67±0.89 ^b	0.000
Salinity (ppt)	31.37±0.55 ^a	31.39±0.71 ^a	31.45±0.72 ^a	ns
Dissolved oxygen (mg/L)	4.14±0.09 ^a	3.77±0.15 ^b	4.31±0.21 ^a	0.000
pH	8.05±0.02 ^b	8.09±0.02 ^a	8.09±0.01 ^a	0.000
Total nitrogen (mg/L)	1.36±0.10 ^a	0.93±0.11 ^b	1.22±0.18 ^a	0.000
Total phosphorus (mg/L)	0.027±0.01 ^b	0.006±0.00 ^c	1.03±0.02 ^a	0.000

Note. Different superscript letters in the row indicate significant differences (Tukey's test, $p \leq .05$). Ns (not significant), PD (Port Dickson), SS (Semporna, Sabah), and PL (Pulau Langkawi)

due to the more stable conditions in the pond system rather than biological activity. In addition, the pH values range from 7.5 to 8.5, indicating a suitable range for growth. It is very important to monitor the pH changes, as they might influence pigment synthesis and secondary metabolism (Ly et al., 2021; Nurkolis et al., 2023).

The PL site also demonstrated higher total phosphorus (TP) (1.03 ± 0.02 mg/L) than other sites. Nutrient accumulation under reduced water-exchange conditions in semi-aquaculture pond systems might also contribute to this (Sulistiawati et al., 2020). While essential for the seaweed growth, excessive accumulation can cause eutrophication. Thus, it is very important to monitor the TP, especially in the cultivation system (Fried et al., 2003).

In contrast, PD showed the highest total nitrogen (TN) content, up to 1.36 ± 0.10 mg/L. This indicates higher nitrogen availability than on the other sites. Yet the TN alone does not reflect bioavailable nitrogen, and elevated concentrations may result from tissue accumulation rather than direct uptake. Coastal activities around sites could also contribute to TN concentration. However, without the specific analysis on the source-specific evidence, this interpretation remains speculative (Chen et al., 2019).

Morphometric Traits across Sampling Sites

Figure 2 shows different parts of the *C. lentillifera*, while Table 2 shows the quantitative measurement of it. A significant difference ($p < 0.05$) was observed in both assimilator

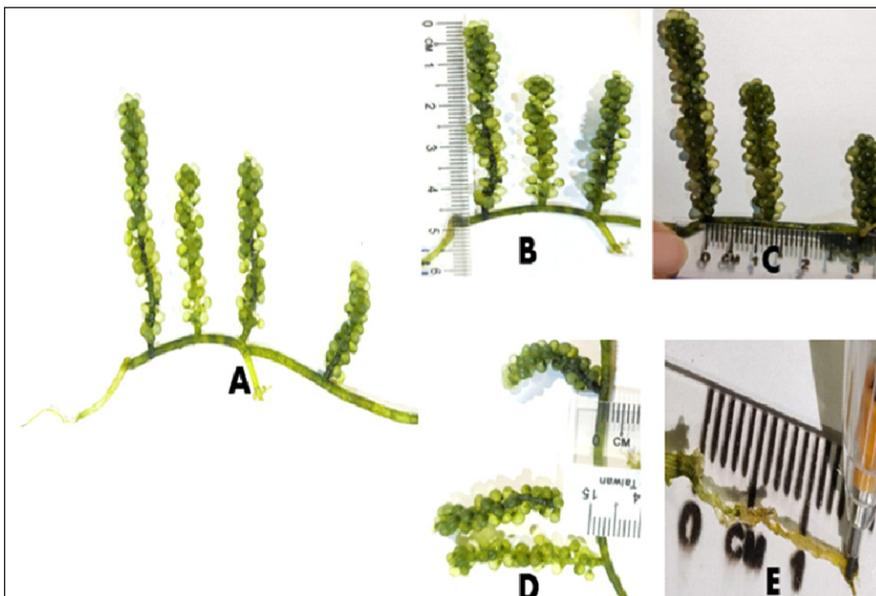


Figure 2. Morphometrics of *Caulerpa lentillifera* based on several parameters. (A) Number of assimilators; (B) Branchlet of the assimilators; (C) Spacing between stolon; (D) Stolon diameter; (E) Rhizoid length

Table 2

Morphometric parameters of C. lentillifera from different locations in Malaysia

Morphometric Parameters/ Location	PD	SS	PL	p-value
Assimilators				
Height (mm)	35.30±6.62 ^b	40.16±3.82 ^b	94.40±7.73 ^a	0.000
Width (mm)	1.71±0.51 ^a	1.53±0.36 ^{ab}	1.26±0.28 ^b	ns
Spacing (mm)	6.00±0.47 ^b	7.55±0.78 ^b	8.18±1.38 ^b	0.000
Number per Stolon	3.15±0.24 ^a	3.0±0.00 ^a	3.20±0.25 ^a	ns
Branchlets				
Ramulus diametre (mm)	1.83±0.23 ^a	1.88±0.20 ^a	2.00±0.23 ^a	ns
Stalk length (mm)	1.81±0.33 ^a	1.70±0.63 ^a	2.00±0.23 ^a	ns
Stalk diametre (mm)	1.00±0.20 ^a	1.17±0.27 ^a	1.07±0.22 ^a	ns
Rhizoids				
Stolon diametre (mm)	1.80±0.24 ^b	2.25±0.16 ^a	1.08±0.14 ^c	0.000
Length (mm)	13.8±3.46 ^a	9.2±2.74 ^b	6.65±1.70 ^b	0.000
Number per rhizoid per stolon	2.80±0.63 ^a	2.60±0.52 ^a	2.30±0.67 ^a	ns

Note. Different superscript letters in the row indicate significant differences (Tukey's test, $p \leq .05$). Ns (not significant), PD (Port Dickson), SS (Semporna, Sabah), and PL (Pulau Langkawi)

and rhizoid length. PL samples exhibited higher double-assimilator heights than SS and PD. These differences suggest the PL site provides more stable, favourable growth conditions, water quality, and nutrient availability for *C. lentillifera* (Estrada et al., 2020). In a cultivated pond like PL, a limited and controlled growth environment could also promote the elongation of assimilators. This is further supported by longer rhizoids in both PD (13.8 ± 3.46 mm) and SS (9.2 ± 2.74 mm) compared to PL (6.65 ± 1.70 mm). In wild environmental conditions, the seaweed needs longer rhizoids as an adaptation to secure stronger anchorage to the substrate.

Some part of the morphometric parameters, like branchlet width, assimilator width, the number of branches per stolon, and the number of rhizoids per stolon, showed no significant differences. These parameters are suggested to be more stable and may be influenced by genetic factors rather than adaptation. In short, the morphological plasticity of *C. lentillifera* is influenced by both genetic and environmental factors. Their adaptation allows them to survive in various hydrodynamic, substrate, and nutrient conditions (Rabia, 2016; Tanduyan et al., 2013).

Proximate Composition on-Site Variation

Table 3 shows the proximate composition of the *C. lentillifera* harvested from different sites. The highest moisture content was detected in the PL sample ($96.60 \pm 0.81\%$), aligned with *C. lentillifera* from Indonesia ($95.01 \pm 0.17\%$) and Taiwan ($94.28 \pm 0.24\%$) (Nofiani

Table 3

The proximate study (wet basis) of C. lentillifera from different locations in Malaysia

Parameter/Site	PD	SS	PL	p-value
Moisture (%)	94.08±0.45	94.94±1.06 ^b	96.60±0.81 ^a	0.000
Ash (%)	1.25±0.21 ^a	0.45±0.06 ^b	0.43±0.06 ^b	0.000
Crude lipid	0.06±0.01 ^b	0.07±0.00 ^a	0.07±0.00 ^{ab}	0.000
Crude protein (g/100 g)	1.0±0.38 ^a	0.43±0.13 ^b	0.56±0.25 ^b	0.000
Carbohydrate	0.16±0.44 ^b	3.97±1.00 ^a	0.86±0.80 ^b	0.000
Crude fibre (g/100 g)	3.45±0.25 ^a	0.14±0.05 ^c	1.48±0.02 ^b	0.000

Note. Different superscript letters in the row indicate significant differences (Tukey's test, $p \leq .05$). Ns (not significant), PD (Port Dickson), SS (Semporna, Sabah), and PL (Pulau Langkawi)

et al., 2018; Ly et al., 2021). This elevated moisture content may be associated with the greater assimilator height observed in PL, as elongated tissues typically retain higher water content to maintain structural integrity and buoyancy.

Higher ash content was detected in PD samples ($1.25 \pm 0.21\%$) than in SS and PL samples. Still, the ash content from all samples lies within the expected range for fresh seaweed (<5%) (Nofiani et al., 2018; Syakilla et al., 2022). This might be due to high calcium and zinc levels in the same samples, suggesting that site-specific minerals influenced ash variation (Zhou et al., 2025). The PD sample also showed higher crude protein (1.0 ± 0.38 g/100 g) than the other sites, indicating accumulation of total nitrogen on the site. While this suggests a possible connection between nitrogen and protein accumulation, further analysis is needed.

Importantly, crude fibre, carbohydrate, and protein were identified as the key variables contributing to separation in subsequent PCA and PLS-DA analyses (see Chemometric Analysis section), thereby linking univariate compositional differences to multivariate clustering patterns.

Mineral Profiles on-site Variation

Table 4 shows that the mineral composition in each sample varies among sites. Samples from PD show the highest calcium (104.00 ± 7.54 mg/100 g) and zinc (0.343 ± 0.26 mg/100 g) levels among the sites. This variation may reflect variation in mineral availability and uptake in an open coastal environment, rather than intrinsic differences of the seaweed (Ismail et al., 2014; Preez et al., 2020).

Higher levels of magnesium, potassium, and sodium are found in PL samples. This pattern is likely due to accumulation under relatively stable pond conditions rather than to direct environmental influences or to the seaweed's intrinsic nutritional superiority. Such accumulation is consistent with reduced water exchange and salt retention commonly

Table 4
Mineral analysis of *C. lentillifera* isolated from different locations in Malaysia

Location/ Mineral (mg/100 g)	PD	SS	PL	p-value	RNI/day (mg/day) limit
Calcium, Ca	104.00±7.54 ^a	32.00±1.97 ^b	26.00±1.63 ^c	0.000	1,000.00
Magnesium, Mg	13.24±2.16 ^b	13.29±0.95 ^b	58.51±1.80 ^a	0.000	310-420.00
Potassium, K	2.69±0.10 ^c	4.93±0.39 ^b	33.60±1.50 ^a	0.000	4.70
Sodium, Na	28.21±2.14 ^c	333.30±24 ^b	550.60±33.8 ^a	0.000	1500
Zinc, Zn	0.343±0.26 ^a	0.165±0.15 ^b	0.116±0.07 ^c	0.000	4.40-6.30
Copper, Cu	1.36±1.20 ^b	1.52±1.18 ^a	1.37±1.31 ^b	0.008	900 µg/day

Note. Different superscript letters in the row indicate significant differences (Tukey's test, $p \leq .05$). Ns (not significant), PD (Port Dickson), SS (Semporna, Sabah), and PL (Pulau Langkawi)

associated with pond-based aquaculture systems, which differ fundamentally from open coastal environments (Greer et al., 2020). While these minerals are nutritionally relevant, the present discussion emphasises their roles as indicators of site and system-specific accumulation rather than dietary contribution. Accordingly, comparison with recommended analyses is descriptive and explanatory in nature and is not intended to imply causality that seaweed consumption quantities vary, and that mineral content is reported on a wet-weight basis.

Heavy Metal Content on-site Variation

In the absence of specific regulations on heavy metals in seaweed in Malaysia, the heavy metal content of the *C. lentillifera* was assessed based on the permissible limits established for leafy vegetables (Table 5). This comparison is intended as a general safety reference rather than a direct regulatory equivalence, as seaweeds differ from terrestrial vegetables in mineral accumulation pathways.

Table 5
The Heavy Metal Comparison of *C. lentillifera* from different locations in Malaysia

Location/ Heavy Metal (mg/kg)	PD	SS	PL	p-value	Malaysia Food Regulation 1985	FAO/ WHO, 2019 limit
Mercury, Hg	ND	ND	ND	-	0.5	0.004
Antimony, Sb	ND	ND	ND	-	0.5	-
Arsenic, As	3.27±0.15 ^b	1.49±0.19 ^c	11.23±0.9 ^a	0.000	1.00	0.5
Lead, Pb	2.08±0.45 ^a	0.95±0.07 ^b	1.11±0.09 ^b	0.000	2.00	2.0
Cadmium, Cd	ND	1.05±0.07 ^a	0.65±0.07 ^b	0.000	1.00	1.0

Note. Different superscript letters in the row indicate significant differences (Tukey's test, $p \leq .05$). Ns (not significant), PD (Port Dickson), SS (Semporna, Sabah), and PL (Pulau Langkawi)

The highest total arsenic was recorded in PL (11.2 ± 0.14 mg/kg) and the lowest in SS (1.58 ± 0.02 mg/kg). These values are consistent with a previous study where seasonal and hydrodynamic factors influence arsenic accumulation in *Caulerpa* spp. (Misheer et al., 2006). The higher arsenic content in PL may result from limited water exchange in the pond systems, allowing dissolved elements to accumulate and be absorbed. However, this remains speculative without identifying the arsenic sources. In fact, the arsenic present in this study was reported based on the total value. A previous study reported that most of the arsenic in seaweed exists as arsenosugars, which are less toxic than the inorganic form and can be reduced through washing, soaking, and boiling (Almela et al., 2005; Yu et al., 2024). Yet, arsenic speciation analysis is required to more accurately assess food safety risks, particularly for on-cultivated *C. lentillifera*, and should be prioritised in future studies.

On the other hand, the PD sample shows a higher lead level (2.08 ± 0.45 mg/kg) than the SS and PL samples. Although higher, all values remained within tolerable intake limits reported for both adults and children (Sharma & Rhyu, 2014; Nag & Cummins, 2022). Mercury (Hg) and antimony (Sb) were detected in any of the samples, further supporting its safety for human consumption. Overall, while the heavy metals were below international and guideline limits, it is very important to continuously monitor these locations to ensure long-term food safety.

Phytochemical Analysis on *C. lentillifera* across Sites

In Table 6, no significant difference ($p > 0.05$) was found in the total phenolic content (TPC) of the aqueous extract from all samples. The uniformly low TPC values are consistent with previous reports, indicating that aqueous extraction (0.15 - 2.04 mg GAE/g DW) of *C. lentillifera* yields relatively low concentrations of polar phenolic compounds (Yap et al., 2019; Syakilla et al., 2022). This suggests that phenolic abundance is not a dominant driver of site-specific variation under the extraction conditions applied in this study.

Nevertheless, a significant difference ($p < 0.05$) in TFC values was observed across all locations, with the values ranging narrowly from 4.57 to 1.63 QE/g DW. The differences might be contributed to by cultivation practices including controlled growth conditions, reduced physical stress, and post-harvest handling. These factors may play a more significant role than environmental exposure alone. In addition, flavonoid content has been shown to vary with extraction parameters, drying method, genetic diversity, and fluctuations due to biological, environmental, seasonal, and yearly variations (Kumar & Roy, 2018; Aryal et al., 2019). Given that PL samples originate from an aquaculture system, these differences should be interpreted with caution, as cultivation practices confound comparisons with wild populations.

Table 6

The total phenolic (TPC) and flavonoid content (TFC) of C. lentillifera were isolated from different locations in Malaysia

Test/Sample	PD	SS	PL	p-value
TPC, mg GAE/g DW	0.44±0.09 ^a	0.42±0.08 ^a	0.51±0.06 ^a	ns
TFC, mg QE/g DW	1.63±0.8 ^b	1.72±1.46 ^b	4.57±3.23 ^a	0.000

Note. Different superscript letters in the row indicate significant differences (Tukey's test, $p \leq .05$). Ns (not significant), PD (Port Dickson), SS (Semporna, Sabah), and PL (Pulau Langkawi)

Chemometric Analysis of Intraspecific Variations in *Caulerpa lentillifera*

Unsupervised Principal Component Analysis (PCA) was adopted to visualise overall patterns of intraspecific variation in *C. lentillifera* variation, followed by supervised Partial Least Squares-Discriminant Analysis (PLS-DA). These analyses are descriptive and explanatory in nature and are not intended to imply causality between environmental parameters and biological traits. This framework is consistent with previous studies that reported that growth-related parameters and biochemical traits may respond independently to environmental conditions rather than exhibiting a direct linear relationship (Wichachucherd, 2019).

The PCA biplot (Figure 3) accounted for 62.5% of the total variance, with PC1 and PC2 accounting for 39.7% and 22.8%, respectively. The positive axis of PC2 is dominated by PD samples and is associated with high calcium (Ca), crude fiber, zinc, and protein content. This clustering indicates that PD samples are enriched in mineral and proximate compositions. However, the PCA alone could not determine whether this is due to the nutrient enrichment or enhanced growth. While Pb had accumulated in the same region, it is not the only factor driving species separation. This is due to the variation in seaweed being driven more by microminerals and nutrient components rather than trace contaminants (Kreissig et al., 2021).

On the other hand, the negative PC1 side is dominated by PL samples. Assimilator height, moisture, phosphate, and total phenolic content were identified as the driving factors. Stable water depth reduced hydrodynamic exposure, and maintenance cultivation could contribute to these. Previously, pond-based studies also suggested that phenolic and antioxidant properties were influenced more by environmental and management factors than by growth (Wichachucherd, 2019). Yet, without direct measurements, this observation is likely descriptive rather than mechanistic.

As for the SS samples, the clustering was found to be distant from both groups. It also shows a strong association between carbohydrate and copper content. This suggests a different metabolic orientation in the samples and reflects energy storage and site-specific

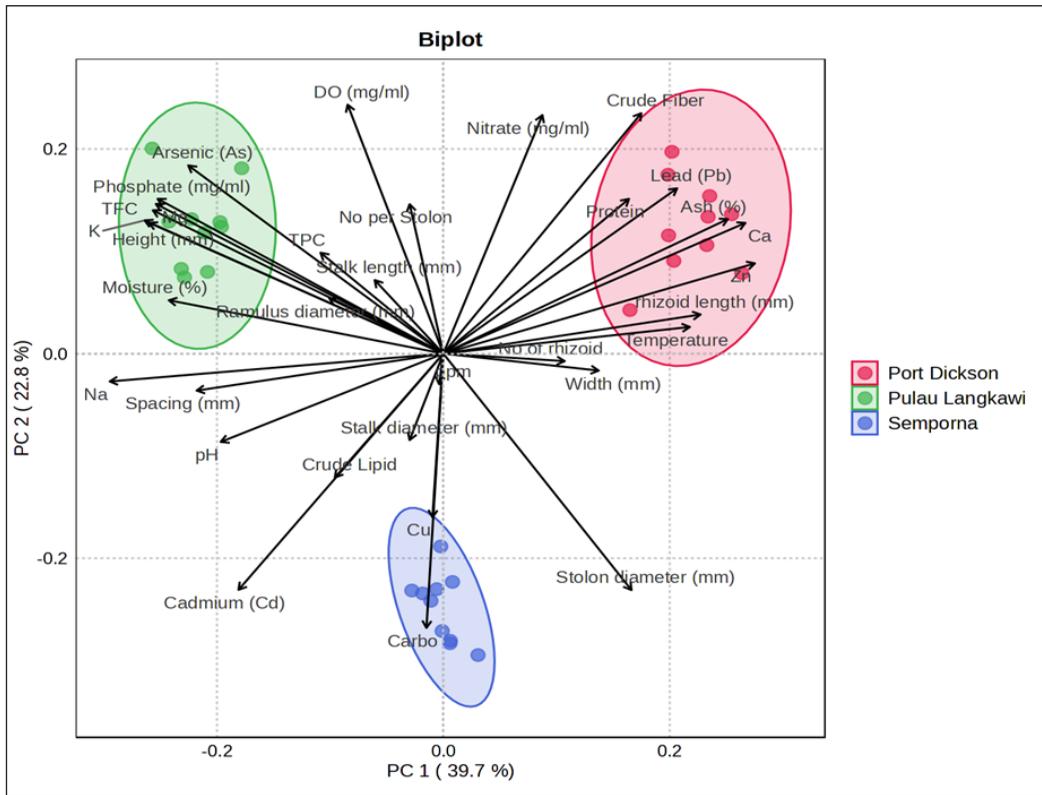


Figure 3. The biplot of *C. lentillifera* harvested from Port Dickson (PD), Pulau Langkawi (PL), and Semporna (SS)

mineral accumulation. This is due to the carbohydrate accumulation and mineral uptake, ate depend on the environmental and growth stage of the seaweed (Kreissig et al., 2021; Syakilla et al., 2022). Nevertheless, without correlation or regression analysis, the observed association should be interpreted with caution.

The PLS-DA models in Figure 4a showed a strong explanatory power with R^2 and Q^2 exceeding 0.90. Permutation testing confirmed the group separation was not due to random chance, as $p = 0.001$ (Figure 4b). Variables with VIP scores greater than 1.3 indicate that crude fibre, calcium, and carbohydrates are key differentiators that support PCA clustering. These variables were consistent with those identified in the univariate proximate and mineral analyses, without implying a causal relationship. Thus, the analytical approaches mirror earlier reports where multivariate separation reflected differential weighting of growth-related biochemical variables rather than a single dominant driver (Ismail et al., 2014; Syakilla et al., 2022).

The hierarchical heatmap was developed to observe trait differences among sites and their clustering within groups (Figure 5). The well-defined clusters on the dendrogram

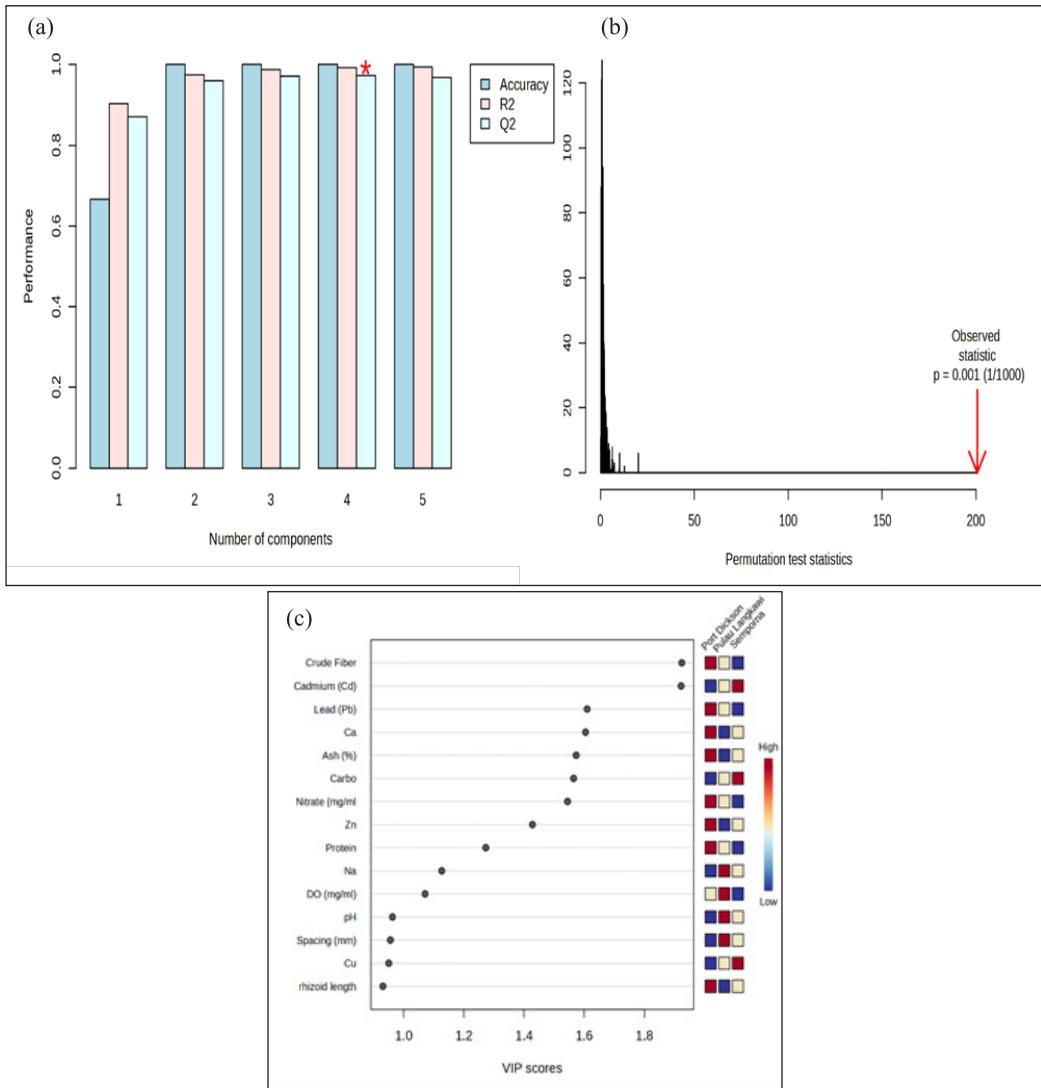


Figure 4. Partial Least Squares Discriminant Analysis (PLS-DA) model performance and validation. (a) PLS-DA scores plot; (b) permutation test; and (c) model evaluation accuracy

strongly support site-level structuring as observed in both PCA and PLS-DA. Furthermore, the colour patterns serve as a signature for each sample and location. For instance, PD samples are high in nitrate, protein, and crude fibre; PL indicates elevated phenolic content; and SS has a high carbohydrate content. Collectively, the chemometric analyses reveal site-specific compositional fingerprints rather than performance rankings, in agreement with previous chemometric studies that emphasise origin-dependent compositional signatures in seaweed (Kreissig et al., 2021). In short, the PCA, PLS-DA, and hierarchical clustering offered complementary insights into intraspecific variability in *C. lentillifera*.

This is important for supporting a basis site selection, origin classification, and cultivation strategies. However, the results do not indicate causal relationships and are influenced by cultivation conditions.

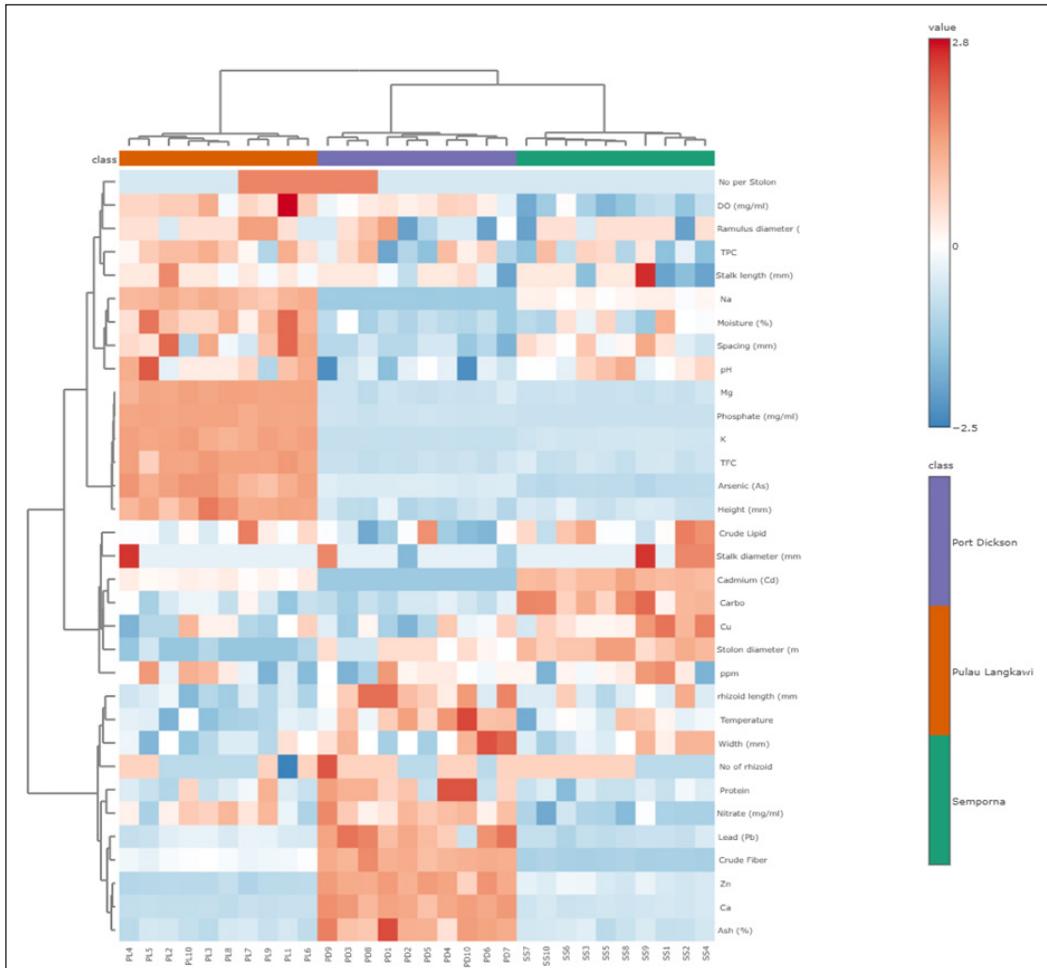


Figure 5. Hierarchical heatmap of nutritional, mineral, and morphometric traits of *C. lentillifera* from three sites in Malaysia

CONCLUSION

These findings showed site-related differences in morphological, mineral, phytochemical, and elemental composition of *C. lentillifera* harvested across Malaysia. These differences are primarily associated with environmental conditions and cultivation practices rather than intraspecific variation. Chemometric analysis identified crude fibre, calcium, carbohydrate, protein, and phenolic content as the key contributors to site differentiation. PD samples generally had higher protein and calcium levels, while SS samples showed stronger

carbohydrate-related traits. PL samples showed higher phenolic content and variation in morphometric characteristics. Although heavy metals are detected at low levels, continuous monitoring is needed.

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Influence of Short-cycle Acacia on Soil Quality

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ABSTRACT

Although short-cycle Acacia plantation has caused negative impacts on the mountainous soil environment, the understanding of these impacts is still unknown in Vietnam. Therefore, this study evaluated the effects of short-cycle Acacia plantation on soil properties under successive seven-year cycles in some areas of Vietnam. A total of 182 soil samples randomly stratified by the Acacia cycles were collected at the topsoil depth of 30 cm to analyse the effects of Acacia cycles

on changes in the soil properties. Non-parametric Kruskal-Wallis & Mann-Whitney tests were used to assess the differences in soil properties under different Acacia cycles and natural forest. Then, the Analytical Hierarchy Process (AHP) was applied to combine the soil properties into the soil quality index for assessing soil quality in the different cycles. The results showed that changes in the soil properties among the Acacia cycles and the natural forest were statistically different ($p < 0.05$). The AHP-based soil quality assessment shows clearly the varying levels of soil deterioration in the successive cycles. It was also found that the indicators of OM, TN, and pH were more closely correlated with the SQI; therefore, soil conservation measures are indispensable for the Acacia soils. The study is a practical contribution to the understanding of

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soil quality change in short-cycle Acacia plantation areas that are common in many parts of the world, especially in the tropics.

Keywords: AHP; Soil Quality Index; Short-Cycle Acacia

INTRODUCTION

Currently, Vietnam's planted forests are about 4.7 million hectares, of which 30% of the total area is Acacia forests. Planted forests have made a great contribution to the Vietnamese economy and created jobs for rural forestry communities. However, planted forests have also caused environmental consequences, especially soil degradation. The effects of planted forests on soil properties reported in previous studies may be negative or positive. On the negative side, the conversion of natural forests to monoculture plantations changes the amount of litterfall and the physicochemical properties of soils (Krashevskaya et al., 2015; Monkai et al., 2018). A decrease in litterfall has resulted in reduced organic matter, biodiversity, and especially soil microorganisms (Cai et al., 2018; Fox et al., 2020; Liao et al., 2012; Wardle et al., 2004). Similarly, other studies also reported that fast-growing tree species have resulted in decreased macronutrients, reduced biodiversity, increased soil erosion, and reduced soil fertility (Bouillet & Bernhard-Reservat, 2001; Liu et al., 2018; Marais et al., 2012; Mensah, 2016). The key causes of soil nutrient depletion and reduced pH are timber logging after short-rotations of monoculture plantation partly due to erosion (Guillaume et al., 2015; Guo et al., 2022). When the wood is harvested, nutrients are also removed from the planting sites. Several case studies reported that plantations resulted in removals of soil macronutrients due to timber logging (Evans, 2022, Jurgensen et al., 1997; Santos et al., 2017; Turner & Lambert, 1986; Weetman & Webber, 1972). Logging also removes the cations of Ca and Mg from the topsoil, thereby increasing soil acidity (Oostra et al., 2006).

On the positive side, several studies have reported supportive effects of planted forests on soil properties. Wall et al., (2005) revealed that soil pH, soil organic matter, and nitrogen contents increase, but soil bulk density decreases in planted forests. In addition, planted forests showed a positive correlation between biomass and soil properties, such as increased soil organic matter, nitrogen, phosphorus, and reduced bulk density (Sang et al., 2012; Sitters et al., 2013; Tchichelle et al., 2016). In particular, the total nitrogen and total organic carbon concentrations were significantly found to be higher when compared with shifting cultivation systems (Hung et al., 2016), and abandoned lands (Dong et al., 2014).

Although the effects of planted forests on soil properties have been investigated in many parts of the world, the effects of short-cycle consecutive Acacia plantations are currently unknown in Vietnam. Therefore, the purpose of the study is to assess the effects of short-cycle Acacia plantation on soil physical and chemical properties. The study began

with an assessment of soil property changes over the seven-year cycles in the study area. The soil properties were then combined into an AHP-based soil quality index to facilitate comparison of the Acacia soil quality among the cycles. The soil quality index not only facilitates comparison of the soil quality over successive Acacia cycles, but it also facilitates spatial comparisons at different scales (Glover et al., 2000; Karlen et al., 2003). The soil quality index can be generated by Boolean logic (Hoseini & Kamrani, 2018), weighted linear combination (WLC) (Silva-Gallegos & Aguirre-Salado, 2017), weighted overlay (WO) (Hassan et al., 2020), multiple linear regression (Khoi, 2024; Leroux et al., 2019), (Akpoti et al., 2019), multi-criteria evaluation (MCE) (Malczewski, 2004, 2006), fuzzy logic (Feng et al., 2017; Zabel et al., 2014), machine learning algorithms (Ebrahim, 2007; Mockshell & Kamanda, 2018; Phillips et al., 2009). In this study, the Analytic Hierarchy Process (AHP) method (Saaty, 1980) is chosen because it is a highly reliable method (Kihoro et al., 2013). It can be used as a consensus mechanism for decision analysis that requires group work (Yalew et al., 2016). AHP considers the evaluation based on the set of criteria and alternative options, from which the optimal decision is made. It also allows estimating the weight of the number of evaluation criteria by the decision maker's pairwise comparison method (Rodcha et al., 2019).

MATERIALS AND METHODS

Location Selection

The study areas were located in the state-owned forestland of Tuyen Quang's Yen Binh & Yen Son forestry companies; Phu Tho's Yen Lap forestry company; Nghe An's Song Hieu forestry company; and Gia Lai's Quy Nhon forestry company (Figure 1). At each study area, the soil sample sites were randomly stratified by the seven-year Acacia cycles, along with nearby natural forest plots on the slope of 8 - 12° and Ferrosols. Acacia tree density in the study areas is from 1,600 to 2,000 trees per hectare. The average annual temperature and rainfall in the northern regions of Vietnam are 22-27 degrees Celsius and 1,500 to 2,000 mm per year, respectively. The average annual temperature and rainfall in Quy Nhon are 27-28 degrees Celsius and 1500-2,500 mm per year, respectively.

Soil Sampling

The management goal is to sustain Acacia timber yield, minimum soil dataset should be chosen in relation to the Acacia timber yield. The study selected organic matter (OM), total nitrogen (TN), total phosphorus (TP), total potassium (TPO), pH, bulk density (BD), and soil porosity (P) because they are related to sustaining Acacia timber productivity. A total of 182 composite soil samples were randomly stratified by the cycles of the Acacia plots of 25m × 25m and nearby natural forest plots. Of which, 34 Acacia's soil samples were collected in the cycle 1; 32 Acacia's soil samples were collected in the cycle 2; 32 Acacia's

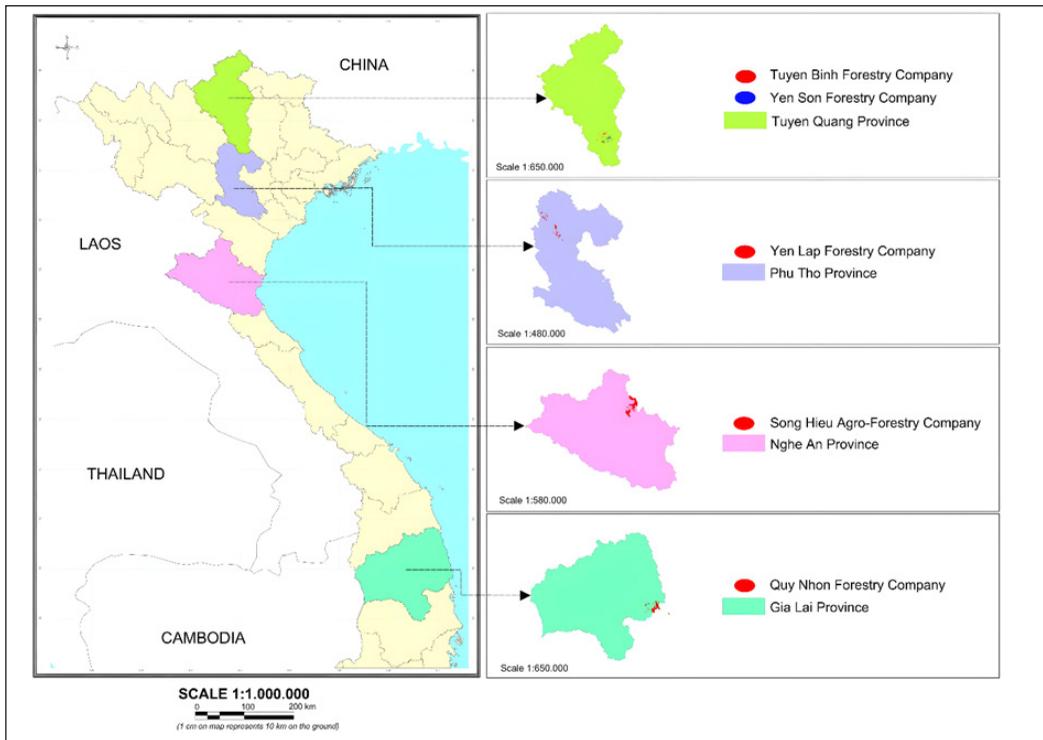


Figure 1. Locations of the study area

soil samples were collected in the cycle 3; 22 Acacia's soil samples were collected in the cycle 4; and 22 Acacia's soil samples were collected in the cycle 5; and 40 natural forest's soil samples were collected in nearby sites of the Acacia plantation areas. At each plot, 18 subsamples were taken along two diagonals of the plot, then mixed into a composite sample. The composite samples were contained in a nylon bag that records the coordinates and other details of the samples, then transported to the laboratory.

Soil Physical Measurement

Soil bulk density and soil porosity were measured by the Vietnamese standard TCVN 11399: 2016 at the soil depth of 30 cm. Soil bulk density was determined from the mass of dry soil per unit volume of a soil sample collected with a cylindrical metal tube with a known volume (100 cm³) in undisturbed soils. Soil porosity was calculated based on the soil bulk density and particle density using the following equation (Mtyobile et al. 2020):

$$\text{Soil porosity \%} = (\text{soil bulk density}/\text{particle density}) * 100$$

Soil Chemical Analysis

The composite samples were air-dried and sieved through a 2 mm sieve, and any fragments were removed. The soil samples were analysed for the soil chemical indicators by the Vietnamese standards (TCVN). Soil pH was measured by the standard TCVN 5979:2007, similar to the ISO 10390:2005. Total organic carbon was determined through wet oxidation with potassium dichromate, as outlined in TCVN 4050:1985. For total nitrogen, the Kjeldahl digestion method was specified in the standard TCVN 6498:1999. Total phosphorus was analysed using the spectrophotometric method after acid digestion described in the TCVN 4052:1985. Total potassium was analysed by flame photometry as described in TCVN 8660:2011. All analyses were conducted in triplicate to ensure data reliability, with appropriate quality control measures, including the use of certified reference materials and blank samples. The analytical procedures were performed under controlled laboratory conditions with calibrated equipment to maintain measurement accuracy and precision throughout the study.

Statistical Analysis

Descriptive statistics were determined for the soil properties, and they were checked for normality by the Shapiro–Wilk test. The study revealed that the soil properties of the Acacia cycle samples did not show a normal distribution; therefore, the non-parametric Kruskal–Wallis and Mann-Whitney test were used to compare the differences under different Acacia cycles and with natural forest at 5% significance level.

Soil Quality Assessment

The soil quality index was aggregated to evaluate soil quality change among the continuous cycles of Acacia. Soil properties were measured in different units, and linear conversion functions were applied to convert the raw values of the soil property to the common scale of 1-100 (Andrews et al., 2004; Karlen et al., 2003).

For OM, TN, TP, TPO and P, the conversion function is given as:

$$\text{Rescaled Score} = \left(\frac{X - X_{\min}}{X_{\max} - X_{\min}} \right) * 100$$

For soil bulk density (BD), the conversion function is given as:

$$\text{Rescaled Score} = \left(\frac{X_{\max} - X}{X_{\max} - X_{\min}} \right) * 100$$

where x = the measured value of a soil property; x_{\max} = the maximum value of a soil property; x_{\min} is the minimum value of a soil property.

For pH, if the pH value < 7, the first formula is used for the conversion, and if pH > 7, the second formula is used for the conversion. Each soil property has a different degree of relative importance on soil quality. This importance was incorporated into the assessment through the weighting of the soil property. The pairwise comparison method in the AHP framework was applied to estimate the weights of soil properties (Saaty, 1980). The soil quality index was calculated as follows:

$$SQI = \sum_{i=1}^n SiWi$$

Si represents the score of a soil property i, and Wi represents the relative weights of a soil property i.

RESULTS AND DISCUSSION

The Soil Properties under the Acacia Cycles

The results of the descriptive statistical analysis are presented in Table 1. Table 1 shows the mean, median and standard deviation (SD) of the soil properties under the Acacia cycles and natural forests in the study areas. Table 1 shows the Kruskal-Wallis test results of successive cycles in the study area. In general, there are statistically significant differences between the Acacia cycles and natural forests ($p < 0.05$) (Table 1). The post-hoc analysis by the Mann-Whitney test showed that all soil properties were clearly different among the Acacia cycles ($p < 0.05$). In addition, the Mann-Whitney test also shows that all soil properties of the Acacia cycles are clearly different from those of natural forests ($p < 0.05$).

The soil analysis results indicate that OM and TN tended to be stable from the first to the fifth cycles of Acacia. Compared with the natural forest, OM and TN decreased slightly over the consecutive cycles. The TP, TPO, and pH showed a clear decreasing trend over the consecutive cycles. The Acacia showed a clear decline compared to the natural forest. The BD showed a clear increase, indicating the hardening of the surface soil in the study areas. In contrast, soil porosity (P) showed a decrease due to the operation of machinery during timber harvesting, planting and tending Acacia forests after each new planting cycle. Comparing our study results with previous studies, some studies have similar results; however, some studies have opposite results. For example, some studies reported that Acacia increased soil pH, TN and TP (Lee et al., 2015), increased fertility (Koutika et al., 2019; Machado et al., 2017; Tchichelle et al., 2016). Similarly, a case study in Central Vietnam (Dong et al., 2014) reported that Acacia had an impact on soil improvement on infertile soils. However, the study by Huong et al. (2015) showed that continuous planting of Acacia from the 2nd cycle caused a decrease in soil nutrients, especially N, P, Ca, and K. Abu Seri and Abd Rahman (2021) reported the results of the investigation on the influence of the age of Acacia on the soil properties of the soil. The results showed that the soil pH, N, P, K, Ca, and soil density also decreased; however, OM and N remained stable.

Table 1
The soil properties under hybrid Acacia cycles in the study sites

Planting cycle	OM (%)	TN (%)	TP (%)	TPO (%)	pH	BD (g/cm ³)	P (%)
Cycle 1 of the Acacia (A1), n = 34							
Mean	2.27	0.140	0.090	0.918	3.87	1.39	41.46
Median	2.27	0.141	0.095	1.180	3.90	1.32	40.33
SD	0.35	0.037	0.028	0.576	0.54	0.27	5.74
Cycle 2 of the Acacia (A2), n = 32							
Mean	2.02	0.128	0.078	0.663	3.58	1.42	40.38
Median	1.95	0.126	0.090	0.770	3.43	1.43	39.10
SD	0.35	0.035	0.029	0.410	0.42	0.23	5.63
Cycle 3 of the Acacia (A3), n = 32							
Mean	1.92	0.128	0.080	0.480	3.49	1.49	37.87
Median	1.94	0.128	0.080	0.240	3.45	1.45	38.15
SD	0.35	0.034	0.034	0.440	0.32	0.23	5.68
Cycle 4 of the Acacia (A4), n = 22							
Mean	1.83	0.109	0.075	0.094	3.25	1.52	40.28
Median	1.77	0.107	0.072	0.093	3.25	1.56	39.81
SD	0.25	0.018	0.023	0.027	0.15	0.24	6.38
Cycle 5 of the Acacia (A5), n = 22							
Mean	1.70	0.099	0.073	0.090	3.10	1.62	36.56
Median	1.68	0.099	0.071	0.093	3.11	1.59	35.90
SD	0.17	0.023	0.014	0.026	0.06	0.09	3.21
Natural Forest (NF), n = 40							
Mean	2.66	0.169	0.117	0.501	3.78	1.29	52.71
Median	2.89	0.184	0.120	0.154	3.71	1.15	52.87
SD	0.60	0.057	0.016	0.530	0.48	0.33	3.19

Note. OM -organic matter, TN -total nitrogen, TP -total phosphorus, TPO -total potassium, pH -soil acidity, BD -bulk density, P -soil porosity. Variation in the soil properties under Acacia cycles and natural forests were tested by Kruskal-Wallis: OM ($\chi^2= 22.94$, $df = 5$, $p < 0.05$), TN ($\chi^2= 27.54$, $df = 5$, $p < 0.05$), TP ($\chi^2= 26.06$, $df = 5$, $p < 0.05$), TPO ($\chi^2= 46.36$, $df = 5$, $p < 0.05$), pH ($\chi^2= 61.33$, $df = 5$, $p < 0.05$), BD ($\chi^2= 27.54$, $df = 5$, $p < 0.05$), P ($\chi^2= 48.99$, $df = 5$, $p < 0.05$). All pair comparisons of the Acacia cycles and natural forest were tested by Mann-Whitney. All soil properties among Acacia cycles are statistically significant ($p < 0.05$)

The results of our soil analysis show that there is a clear distinction between each property among the continuous replanting cycles. The OM and TN indicators at boxplots (a) and (b) of Figure 2 are stable from the second cycle to the third cycle, then show signs of a slight decrease in the fourth and fifth cycles. Comparing the OM and TN of Acacia and natural forest shows differences ($p < 0.05$). The TP content showed signs of decrease in the later cycles compared to the first cycle and natural forests in the boxplot (c) of Figure 2. However, TPO and pH had the strongest decrease, as shown in the boxplots (d) and (e)

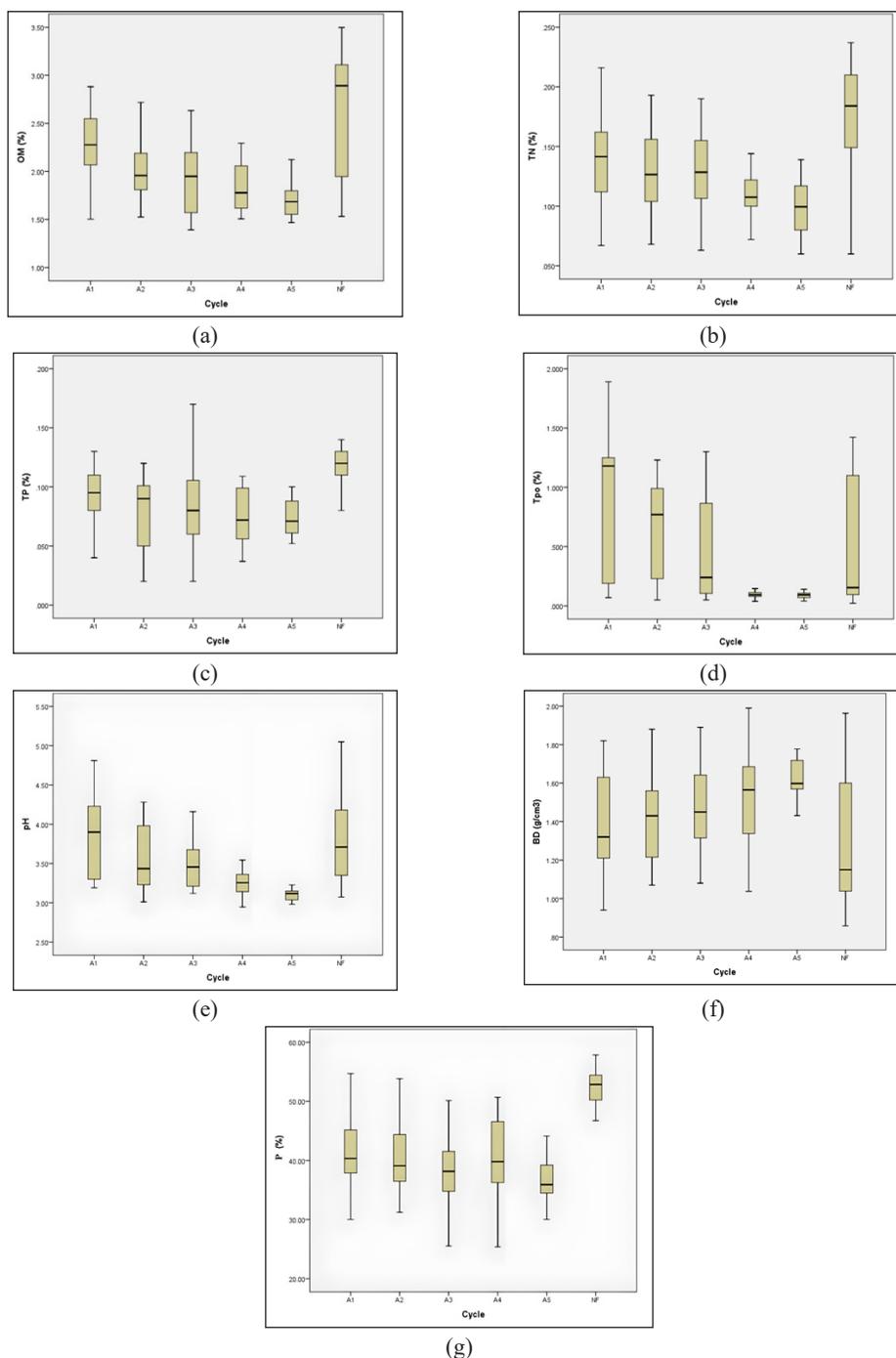


Figure 2. Variation in soil properties under Acacia cycles and natural forest

Note. All pair comparisons of Acacia cycles and natural forest at the boxplots (a), (b), (c), (d), (e), (f), (g) were tested by the Mann-Whitney. All soil properties among the Acacia cycles are statistically significant ($p < 0.05$). A1- Acacia cycle 1, A2 - Acacia cycle 2, A3 - Acacia cycle 3, A4- Acacia cycle 4, A5 - Acacia cycle 5, NF -natural forest

of Figure 2. The soil bulk density at boxplot (f) of Figure 2 shows an increasing trend, indicating that the topsoil shows signs of hardening as an inevitable result of short-cycle Acacia plantation, caused by logging machinery and removal of the surface litterfall. Soil porosity index generally decreased slightly in the first 3 cycles of Acacia rotation but showed a sharp decrease in the 5th cycle due to mechanical logging activities, soil erosion and removal of forest surface litterfall, as showed at the boxplot (g) of Figure 2.

Weighting Soil Properties

From the perspective of agriculture and forestry, farmers as well as land managers often evaluate the relative importance of physical and chemical indicators, which contribute to the overall soil quality. Therefore, the estimation of the relative importance of each physical and chemical soil indicator was essential. The relative role or priority of each soil property can be consulted with soil experts as well as farmers. In this study, soil experts were consulted to build a pairwise comparison matrix of the soil properties in the study based on the AHP method (Karlen et al., 2003; Saaty, 1980). The results of the pairwise comparisons of the soil properties are presented in Table 2.

The weight estimation results show that OM has the highest weight, followed by pH, TN, TP, TPO, soil porosity, and soil bulk density. The computed consistency ratio is at 0.012, which is in the consistent level of less than 0.1. This ensures that the computed weights are logical in expert judgments (Saaty, 1980). From Table 2, OM has the highest weight, 0.326, meaning that OM is the most important factor affecting soil quality. Soil acidity (pH) plays the second most important role in overall soil quality (0,208). Soil acidity is a fundamental chemical parameter because it is related to most of the chemical properties of the soil as well as the activities of soil organisms. Chemical weathering

Table 2
Pairwise matrix of the soil quality parameters using the Analytic Hierarchy Process

Soil Properties	OM	pH	TN	TP	TPO	BD	P	Weights (W)
OM	1.0	2.0	2.0	4.0	4.0	6.0	5.0	0.326
pH	1/2	1.0	1.0	3.0	3.0	5.0	4.0	0.208
TN	1/2	1.0	1.0	3.0	3.0	5.0	4.0	0.208
TP	1/4	1/3	1/3	1.0	1.0	3.0	2.0	0.086
TPO	1/4	1/3	1/3	1.0	1.0	3.0	2.0	0.086
BD	1/6	1/5	1/5	1/3	1/3	1.0	1.0	0.040
P	1/6	1/4	1/4	1/2	1/2	1.0	1.0	0.047

Note. OM -Soil Organic Matter, pH -soil pH, TN -total nitrogen, TP -total phosphorus, TPO -total potassium, BD -soil bulk density, P -soil porosity; maximum eigenvalue (λ_{max}) = 7.083; n = 7; consistency index (CI) = $(\lambda_{max} - n) / (n - 1) = 0.014$; random index (RI) = 1.36; consistency ratio (CR) = CI/RI = 0.012, accepted

in tropical climates often causes natural soil acidity. However, farming activities such as afforestation also add to acidity because farming stimulates pyrite oxidation. Total nitrogen plays an important role in protein biosynthesis and photosynthesis of plants, so the presence of sufficient nitrogen content ensures good plant growth. The weight of total nitrogen estimated from the pairwise comparison matrix is 0.208, which is equal to the weight of the pH. Phosphorus and potassium are essential macronutrients for plant growth. However, their roles in influencing soil quality are considered less important than organic matter and nitrogen, as well as soil pH. The weights of phosphorus and potassium are the same and equal to 0.086. Soil porosity and bulk density are two physical properties that influence soil moisture and soil aeration. However, they are considered the least important because they are usually more stable than soil chemical properties. The weights of bulk density and soil porosity are 0.040 and 0.047, respectively.

Soil Quality Index

The results of assessing the soil quality from seven soil physical and chemical indicators show that, in general, the soil quality in both the natural forest and the Acacia forest at the research sites is at the moderate soil quality level, the total soil quality score of all Acacia and natural forest sites is less than 70 on the scale of 100 (Figure 3 and Table 3). The natural forest sites have a higher soil quality level than Acacia sites. Acacia forest plots in the 3rd to 5th cycles show clear signs of soil quality decline compared to the natural forest sites. It is noted that all the study sites were Acacia forests managed by a state-owned forestry company, and all monitoring plots implemented the same forest management practices without fertiliser addition; therefore, there was no differential influence of fertilisers on soil quality among the selected study sites.

It is noted that soil quality is a concept that is subject to points of view, so the selection of soil quality assessment criteria depends on the preferred concept (Soil Science Society of America, 1995; Anderson & Gregorich, 1984; Larson & Pierce, 1994). In this study, the two groups of soil chemical and soil physical properties were selected; however, the group of soil biological properties was not involved in the assessment because biological property data have not been measured yet. Therefore, the results of the soil quality assessment with this Acacia only reflect an important part of soil quality, but are not completely comprehensive. Therefore, further studies are necessary to supplement soil biological properties. In our view, no chemical fertilisers or pesticides were applied to the Acacia soil and the reference natural forest at the study sites; therefore, the soil ecosystem was not negatively impacted and maintained a relatively stable state over a long period. Under these conditions, the physical and chemical indicators of the Acacia soil reflected the major soil quality properties, helping to maintain the sustainable development of the forest ecosystem. Therefore, although lacking biological indicators, the soil quality index still has significance for comparing soil quality across the selected study sites.

Another issue with the AHP-based SQI assessment is the sensitivity of the soil quality index. Although the AHP method is an effective tool for synthesising the soil properties into a single composite index, allowing for effective comparisons across plots, farms, and even regional or national scales, the determination of the role of each soil property depends on the subjective opinion of the expert. Therefore, in estimating factor weights from the pairwise comparison matrix, it is necessary to check the consistency of the comparison matrix, ensuring that the consistency index $CR < 0.1$. Furthermore, taking the opinions of many experts as well as expert groups helps to minimise biased opinions.

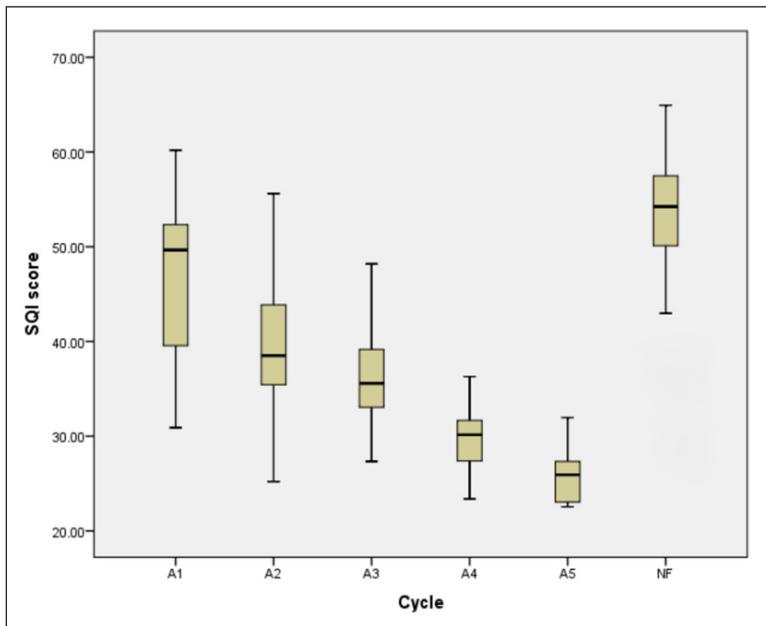


Figure 3. Soil quality index score under different Acacia cycles and natural forest

Table 3
The SQI score under the Acacia cycles and reference natural forest

SQI Statistics	Natural Forest Score	SQI Score under the Acacia Cycles				
		A1	A2	A3	A4	A5
Max	64.94	60.20	55.62	48.19	36.29	31.97
Min	28.73	30.89	25.20	27.33	23.37	22.54
Mean	52.05	46.78	39.20	36.37	29.96	25.87
SD	9.03	8.31	7.15	5.79	3.44	2.76

Pearson’s correlation between the soil quality index and the individual soil properties was analysed to indicate which properties have a major influence on the soil quality. The correlation coefficients between the SQI and the soil properties of OM, TN, TP, TPO, pH,

BD, and P were 0.75, 0.729, 0.42, 0.55, 0.65, -0.46, 0.50, respectively. The two-tailed Pearson's correlation coefficient was statistically significant ($p < 0.05$). The OM and TN had a stronger correlation with the soil quality index, followed by pH ($r = 0.65$), TPO ($r = 0.55$), TP ($r = 0.42$), BD ($r = -0.46$), and P ($r = 0.50$). From the correlation analysis, it implies that the conservation of the soil quality of the Acacia requires maintaining OM, TN, and TPO in the Acacia areas. In particular, it is necessary to implement measures to maintain the soil quality level by returning the biomass of Acacia branches and leaves after harvesting Acacia timber. Forest owners should avoid burning Acacia leaves and branches as an effective measure to return organic matter and mineral nutrients to the soil.

CONCLUSION

The findings of the study indicate that changes in the soil properties among Acacia cycles and natural forests are statistically significant in the selected areas of Vietnam. The results imply that the initial cycle of the Acacia commonly yields a higher SQI score; however, subsequent cycles indicate a decline in soil quality level, owing to changes in individual chemical and physical properties. Apparently, the decline of soil quality is caused by changes in the physical and chemical properties under the Acacia forest. Organic matter, pH, total phosphorus, total potassium, and soil porosity decrease with the consecutive cycles. Only total nitrogen remains quite stable due to the nitrogen fixing of Acacia. Soil bulk density increased in the 3rd to 5th cycles of Acacia, indicating an increase in the ability to harden the topsoil layer. By analysing the Pearson's correlation between the SQI and the individual soil properties, it was found that OM, TN, and pH were more closely correlated with the SQI. This is an indication that soil improvement measures should focus more on protecting and improving these chemical properties. This study is a practical contribution to the understanding of soil quality change in short-cycle Acacia plantation areas that are common in many parts of the world, especially in the tropics.

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***In Vitro* Evaluation of Alpha-asarone Induced Osteogenic Differentiation and Cytotoxicity in MC3T3-E1 Cells**

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ABSTRACT

Osteoporosis affects over 200 million people worldwide, with its prevalence expected to rise due to ageing populations. Side effects associated with long-term use of synthetic treatments have driven interest in natural alternatives. Alpha-asarone, a natural metabolite found in several therapeutic plants, was identified as a major metabolite in *Piper sarmentosum* Roxb. (kaduk), a local plant known for its bone-protective properties. While Alpha-asarone is known for its neuroprotective and anti-inflammatory properties, its osteogenic role remains unexplored. This study aimed to assess the cytotoxicity of Alpha-asarone and its potential to induce osteogenic differentiation *in vitro* using MC3T3-E1 pre-osteoblast cells. Cytotoxicity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay across Alpha-asarone concentrations (0–800 µM). For osteogenic evaluation, cells were treated with 1–20 µM Alpha-asarone over 21 days. Osteogenic differentiation was assessed through ALP activity and von Kossa staining. Untreated cells served as the negative control, while cells treated with ascorbic acid (50 µg/mL) and β-glycerophosphate

(10 mM) served as the positive control. Alpha-asarone exhibited a dose-dependent cytotoxic response in the cytotoxicity assay, with no cytotoxic effects at the concentrations used for osteogenic evaluation. After 21 days, von Kossa staining revealed a significant increase in mineralised matrix formation, indicated by intensified dark brown staining, with a 95.1% increase. ALP activity was also elevated, with the highest increase at 174%. Alpha-asarone at 1 µM produced the most significant enhancement in

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both mineralisation and ALP activity. Alpha-asarone showed its promise as a natural osteogenic agent by effectively and safely inducing MC3T3-E1 cell differentiation.

Keywords: Alpha-asarone, ALP activity, bone mineralisation, MC3T3-E1 cells, natural bone anabolic agent, osteogenic differentiation, osteoporosis prevention

INTRODUCTION

Osteoporosis is a skeletal disorder resulting from an imbalance involving osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells), leading to weakened and fragile bones, prone to fractures (Föger-Samwald et al., 2020). It is often termed a “silent disease” due to its asymptomatic progression (Wei et al., 2024). The global burden includes approximately nine million fractures annually, affecting over 200 million people (Akkawi & Zmerly, 2018; Pisani et al., 2016). In Malaysia, the number of individuals aged 60 or older is projected to grow from 1.4 million in 2000 to 3.3 million in 2050, with 24.1% prevalence in central region postmenopausal women (Lim et al., 2005; Mafauzy, 2000).

The therapeutic goal in bone-related conditions ideally involves slowing down bone loss and promoting new bone formation. However, most current treatment approaches mainly focus on targeting bone-resorption, with limited support to actively promote new bone formation. Consequently, this makes the effective therapies that stimulate new bone formation remain limited (An et al., 2016; Lippuner, 2012). The current established synthetic medicines such as bisphosphonates, estrogen, estrogen receptor antagonists, calcitonin, and teriparatide (Khosla & Hofbauer, 2017; Zhu & March, 2022), although effective in slowing bone loss, present side-effects such as myalgia, osteonecrosis, atypical femur fractures, musculoskeletal aches, adverse gastrointestinal issues, headache, nausea, leg cramps, hot flashes and increased risk of blood strokes (Skjødt et al., 2019; Zhu & March, 2022). Therefore, the therapeutic need remains for agents that actively promote bone formation (An et al., 2016; Lippuner, 2012).

One promising direction in bone-therapy is promoting osteoblast differentiation (Zeng et al., 2020). This multi-stage differentiation process involving proliferation, matrix maturation, and mineralisation is responsible for the development of stem cells into osteogenic progenitors, and, eventually, into mature osteoblasts, essential for bone formation, remodelling, and mineral homeostasis (Carluccio et al., 2020; Yazid et al., 2010). It can be evaluated using several established methods, such as alkaline phosphatase (ALP) activity; an osteoblast marker (Meesuk et al., 2022; Trivedi et al., 2020), and von Kossa staining, which assesses mineralised matrix formation (Matta et al., 2019; Shima et al., 2015).

Natural compounds are increasingly gaining interest and demand in bone-related therapies (Marcucci et al., 2023), due to their reduced incidence of adverse side effects

compared to synthetic drugs, along with their ability to enhance bone health and support long-term use (Qu et al., 2024). Among them, phenylpropanoids such as Alpha-asarone emerge as potential osteogenic agents. It has been reported to exhibit a wide range of promising therapeutic effects, including protection against neurological, liver, and kidney damage, as well as pain relief, antidepressant effects, anticancer properties, antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory activities (Das et al., 2019; Elhoby et al., 2024; Uebel et al., 2021). Alpha-asarone was reported as the major metabolite found in both aqueous and ethanolic extracts of the local plant *Piper sarmentosum* Roxb. “kaduk” (Abidin et al., 2023; Ariffin et al., 2020); traditionally used to treat joint pain, fever, and cough (Sun et al., 2020). Studies have reported its potential in enhancing bone structure and strength, as well as preventing bone loss in osteoporotic rat models (Asri et al., 2016; Asri et al., 2020; Nirwana et al., 2012). Therefore, in addition to the various pharmacological effects of Alpha-asarone, its osteogenic role remains largely unexplored and requires further investigation. This study aimed to evaluate the cytotoxicity of Alpha-asarone, as well as its osteogenic differentiation potential, using the MC3T3-E1 pre-osteoblast cell line.

MATERIALS AND METHODS

MC3T3-E1 Cell Culture

The MC3T3-E1 cell line (ATCC No: CRL-2596™) was used in this study. The cells were cultured in Alpha minimum essential medium (α -MEM; Gibco, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 2% penicillin/streptomycin (Gibco, USA), and 1 mM sodium pyruvate (Gibco, USA). The cell incubation was carried out at 37°C in a humidified environment with 5% CO₂ (Abidin et al., 2021).

Cytotoxicity of Alpha-asarone on MC3T3-E1 Cells

The cytotoxic analysis was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Using 96-well plates, 5×10^3 cells/well were seeded and incubated for 24 hours to allow cell attachment at 37°C in a humidified environment with 5% CO₂. The cells were then treated with Alpha-asarone at concentrations ranging from 0 to 800 μ M for 24, 48, and 72 hours.

Alpha-asarone (Tokyo Chemical Industry Co., Ltd, Japan), with a supplier-certified purity of >98%, was dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Japan) to prepare a stock solution, which was protected from light and stored at -20°C. Working dilutions were freshly prepared prior to each experiment, with the final DMSO concentration maintained at 0.1% (Tian et al., 2022).

On the day of measurement, the old medium was discarded and replaced with fresh medium containing 10 μ L MTT (5 mg/ml; Sigma Aldrich, USA) per well, followed by incubation for 4 hours. The medium was then discarded, and 100 μ L of DMSO was

added to dissolve the formed formazan salts. Absorbance was measured at 570 nm using a microplate reader (Biotek Instruments Inc., CA, USA) (Wang et al., 2015; Yazid et al., 2022). Cell viability of treated cells was normalised to that of untreated cells and reported as a percentage. The half-maximal inhibitory concentration (IC_{50}) was determined using GraphPad Prism software.

Induction of Osteogenic Differentiation using Alpha-asarone

Approximately 3×10^3 cells/well were cultured in 96-well plates, and various concentrations of Alpha-asarone (1–20 μ M) were used to induce osteogenic differentiation. The osteogenic concentration range of Alpha-asarone (1–20 μ M) was selected based on MTT-assay results confirming the absence of cytotoxicity within this range and in accordance with concentrations commonly reported for phenylpropanoid compounds in *in vitro* osteogenic studies; preliminary screening further indicated that these concentrations-maintained cell viability while eliciting measurable osteogenic response. The medium supplemented with 50 μ g/mL ascorbic acid and 10 mM β -glycerophosphate was used as a positive control. Meanwhile, cells cultured in the complete culture medium only were used as a negative control. The cell incubation was carried out in a humidified atmosphere containing 5% CO_2 , at a temperature of 37°C, for a period of 21 days. The medium was changed every 3 days (Abidin et al., 2021).

Alkaline Phosphatase (ALP) Enzymatic Assay

ALP assay was conducted on days 1, 7, 14, and 21. Cells were washed with phosphate buffer saline (PBS; Gibco, USA) and lysed using 0.1% Triton X-100 (Sigma Aldrich, USA). The amount of total protein was determined by adding the cells with Bradford reagent for 5 minutes at room temperature, followed by measuring absorbance at 595 nm. ALP enzyme activity was measured by incubating the cells for 30 mins at 37°C in alkaline conditions (pH 10) with 0.1 M sodium bicarbonate–sodium carbonate buffer, 2 mM $MgSO_4$, and 6 mM p-nitrophenyl phosphate (pNPP), i.e., the enzyme substrate. The reaction was stopped with the addition of 1.5 M NaOH, and absorbance was measured at 405nm using a microplate reader (Biotek Instruments Inc., CA, USA). ALP activity was measured as specific activity, with unit activity per total protein (mg). The activity was normalised to untreated cells and expressed as a percentage (Abidin et al., 2023; Hadzir et al., 2014; Yazid et al., 2010).

Mineralisation Analysis using Von Kossa Staining

On the day of analysis, cells were washed using PBS (Gibco, USA) and fixed using 10% (v/v) formalin (Sigma Aldrich, USA) prepared in PBS for 30 min. Following washing with

deionised water thrice and staining the cells using freshly prepared 5% (v/v) silver nitrate solution (Sigma Aldrich, USA) for 30 min under ultraviolet light, followed by washing with deionised water thrice. After which addition of fresh 5% (v/v) sodium carbonate in 25% (v/v) formalin took place for 5 min, followed by 5% (v/v) sodium thiosulfate (Sigma Aldrich, USA) for 2 min to remove any unreacted silver nitrate. The cells were washed thrice and air-dried, to be viewed under the light microscopy (Olympus Scientific Solutions, Japan), in which dark brown or black-stained cells represent mineralisation. ImageJ software was used for mineralisation quantification (Abidin et al., 2021; Abidin et al., 2023; Hadzir et al., 2014). Mineralisation quantification was performed using ImageJ software with identical threshold settings applied to all images within each experiment set. Images were acquired under consistent magnification and lighting conditions, and threshold parameters were defined based on control samples and uniformly applied across all treatment groups to minimise observer bias.

Statistical Analysis

All the experiments were conducted in triplicate ($n=3$) and represented as the mean \pm standard deviation. Statistical analysis was conducted using GraphPad Prism software using a two-way analysis of variance (ANOVA) test followed by Dunnett's post hoc test. The result was considered statistically significant at a p -value of <0.05 . No statistical outliers were excluded from the analysis. Given the exploratory nature of this *in vitro* study, formal a priori power calculations were not performed; however, reproducibility was supported through independent experimental repeats with consistent effect sizes observed across multiple time points.

RESULTS

The cytotoxicity analysis of Alpha-asarone on MC3T3-E1 cells showed a dose-dependent manner, represented by reduced cell viability percentages as the concentration increases (Figure 1). The percentage of cell viability exhibited a significant difference ($p<0.05$) compared to the negative control (0 μM), whereby Alpha-asarone showed the lowest viability percentage after 72 hours of treatment at 48.1% (600 μM) and 19.5% (800 μM). The IC_{50} values of Alpha-asarone on MC3T3-E1 cells were only determined after 48 and 72 hours of treatment with 688.7 μM (143.43 $\mu\text{g/mL}$) and 447.8 μM (93.26 $\mu\text{g/mL}$), respectively.

During differentiation, ALP enzymatic activity was measured, as depicted in Figure 2, showing the ALP activity normalised to the negative control (untreated cells), set as 100% vs the differentiation days. ALP activity exhibited a significant increment ($p<0.05$) in the treated cells compared to the control group throughout the 21 days of incubation with Alpha-asarone. The highest ALP activity was observed on day 14 at 1 μM (174%),

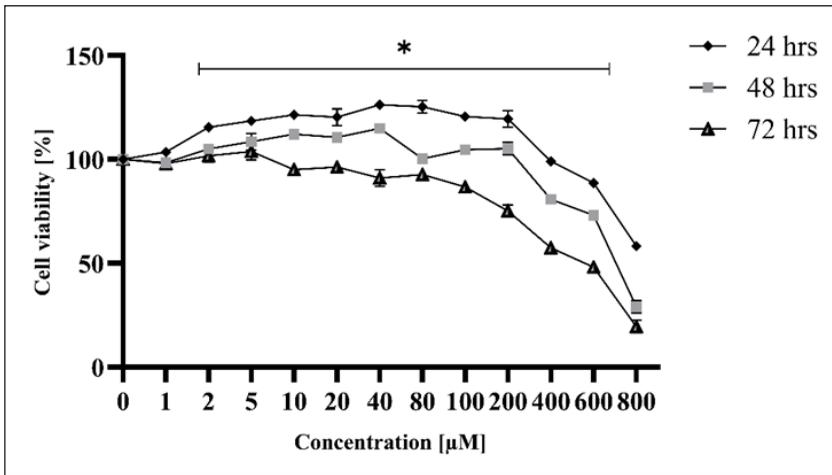


Figure 1. The MC3T3-E1 cell viability (%) during the treatment with Alpha-asarone in a range of concentrations (0–800 μM) for 24, 48 and 72 hours, normalised to untreated cells. The (*) denotes the significant difference compared to untreated cells

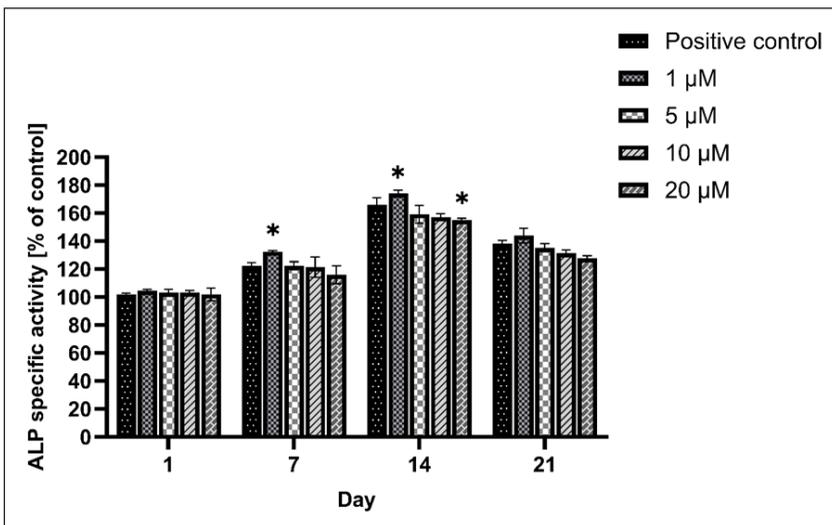
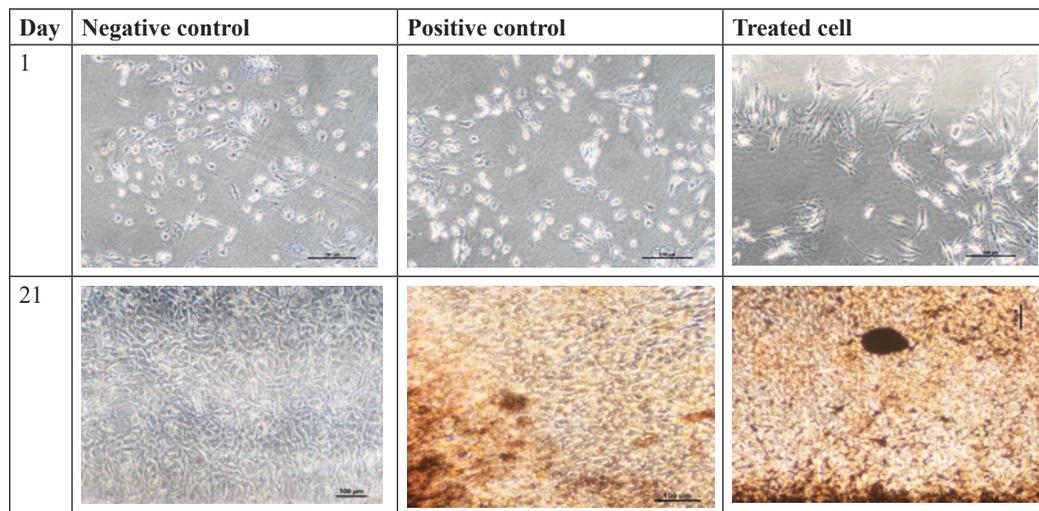


Figure 2. The ALP specific activity (%) of MC3T3-E1 cells treated with Alpha-asarone for 21 days, normalised to untreated cells. The (*) denotes the significant difference compared to the positive control

followed by 5 μM (159%), 10 μM (157%), and 20 μM (154.9%), indicating a concentration-dependent enhancement of early osteogenic differentiation, with maximal effects at lower concentrations.

Von Kossa staining was used to evaluate the effect of Alpha-asarone on the mineralised matrix formation (late stage of differentiation) during the 21-day treatment period, as depicted in Figure 3A and Figure 3B. Figure 3A shows the representative microscopic

A.



B.

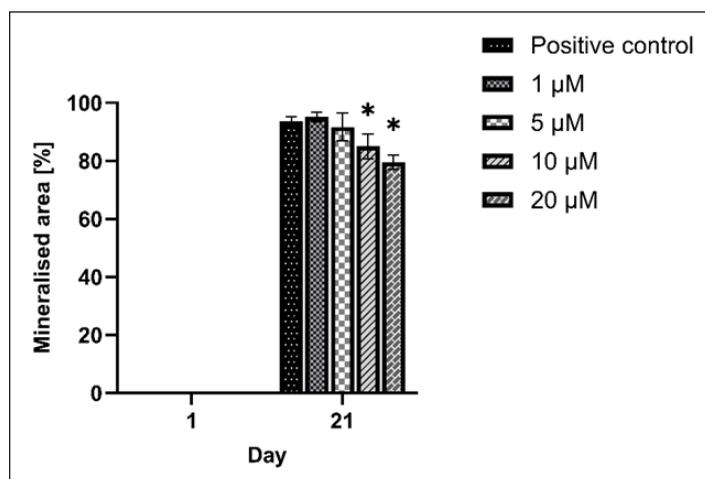


Figure 3. Mineralisation analysis of MC3T3-E1 cells treated with Alpha-asarone for 21 days. Figure (3A) shows the representative microscopic visualisation of stained MC3T3-E1 cells following von Kossa staining. Figure 3B shows the percentage of quantified mineralisation. The (*) denotes the significant difference compared to the positive control

visualisation of stained MC3T3-E1 cells, and Figure 3B shows the percentage of quantified mineralisation. The treatment with 1 µM Alpha-asarone exhibited the highest mineralisation after 21 days (95.1%), followed by 5 µM (91.7%), 10 µM (85%), and 20 µM (79.6%). Meanwhile, 5 µM showed comparable effects ($p > 0.05$), 10 µM and 20 µM exhibited lower mineralisation on day 21, compared to the positive control. Meanwhile, the negative control showed no mineralised matrix formation up to day 21.

Morphological assessment further supported these findings, as Alpha-asarone -treated MC3T3-E1 cells exhibited increased cell density, enhanced extracellular matrix deposition, and the formation of mineralised nodules, particularly at lower concentrations. These morphological changes were consistent with the observed increase in ALP activity and von Kossa staining intensity.

DISCUSSION

Prior to differentiation analysis, the cytotoxicity of Alpha-asarone on MC3T3-E1 cells was assessed (Figure 1). MC3T3-E1 cells were employed due to their well-characterised and highly producible osteogenic differentiation, making them an accepted and reliable model for initial mechanistic and safety screening before any subsequent validation in human primary osteoblast and *in vivo* systems. The cytotoxicity analysis findings suggested that Alpha-asarone exhibited a weak and moderate cytotoxicity on the cells at 48 and 72 hours, respectively (Fithrotunnisa et al., 2020). The findings also showed that Alpha-asarone is deemed non-cytotoxic for the cells at the various concentrations below the reported IC₅₀. The IC₅₀ values obtained (447.8–688.7 µM) are substantially higher than the concentrations used for osteogenic differentiation (1–20 µM), indicating a wide therapeutic safety margin *in vitro*. While direct extrapolation to *in vivo* plasma concentrations is limited, these findings demonstrate that osteogenic effects occur well below cytotoxic levels. The study is positioned as a proof-of-concept, with pharmacokinetic and bioavailability studies required for *in vivo* relevance. Accordingly, the present work should be interpreted as an exploratory study, providing foundational evidence to guide subsequent statistically powered investigations in human-relevant and *in vivo* models. The wide concentration range (0–800 µM) was selected to comprehensively characterise the cytotoxic profile of Alpha-asarone, exhibiting low to moderate cytotoxicity across different cell types at relatively high concentrations. Therefore, extending the range ensured capture of both non-toxic and cytotoxic thresholds, allowing a clear distinction between safe osteogenic concentrations and cytotoxic levels.

Alkaline phosphatase (ALP) enzyme is one of the most well-known markers of osteoblastic differentiation. When osteoblast cells undergo the differentiation process to perform their specialised function of bone formation, ALP enzyme activity is elevated, indicating an ongoing and active bone formation taking place, given the fact that it is secreted by active osteoblast cells (Trivedi et al., 2020). In this study, Alpha-asarone at all concentrations, and the positive control induced a significant time-dependent increase in ALP activity of MC3T3-E1 cells ($p < 0.05$), shown from day 7 to 14, compared to the untreated cells, which indicates osteogenic differentiation induction (Figure 2).

Alpha-asarone at 1 µM consistently resulted in the highest ALP activity throughout the differentiation period, with the highest observed after 14 days compared to the negative

control. In addition, ALP activity for 1 μM Alpha-asarone was significantly higher ($p < 0.05$) than the positive control on days 7 and 14, indicating its notable potential to efficiently enhance ALP activity, crucial for bone differentiation and formation. Meanwhile, 5 μM and 10 μM showed comparable ALP activity enhancement with no significant difference ($p > 0.05$), while 20 μM exhibited lower ALP activity on day 14 compared to the positive control. This signifies that at low concentrations, particularly 1 μM , Alpha-asarone was effective in regulating ALP activity, demonstrating its potency and potential to show the highest biological effect using small doses.

ALP enzyme activity of all concentrations of Alpha-asarone showed a decline after day 14, as well as the positive control. This decline is linked to the mineralisation of MC3T3-E1 cells; as besides ALP being an indicator of osteoblastic differentiation, it is also crucial for the last stage of differentiation, i.e., mineralisation, due to its function in providing inorganic phosphate (Yazid et al., 2018; Vimalraj, 2020), which together with calcium ions form hydroxyapatite crystals; the hard and strong tissue leading to bone formation (Boonrungsiman et al., 2012). This finding is consistent with other previous findings, in which MC3T3-E1 cells exhibited a decreasing trend in ALP activity, corresponding with mineralisation (Yazid et al., 2022). Another study has also confirmed these findings on differentiated MC3T3-E1 cells, whereby ALP activity decreased after 21 days (Kwon et al., 2014).

The evaluation of mineralisation is important as it represents the last stage of differentiation, when osteoblast cells lay down the calcified mineralised matrix, crucial for bone strength, structure, and function (Bourne et al., 2021). Hence, evaluating the effect of Alpha-asarone on MC3T3-E1 cell mineralisation provides valuable insights into its osteogenic potential. Upon staining with silver nitrate, silver is deposited in place of calcium ions reduced by light, resulting in the formation of metallic silver mineralised nodules, noted by osteoblasts stained in dark brown or black (Yazid et al., 2010). As observed in microscopic visualisation (Figure 3A) and quantification of mineralisation percentage (Figure 3B), no mineralisation was observed at day 1 in both the treatment and control groups. The formation of mineralised matrix in all Alpha-asarone-treated cells and the positive control was evident by day 21, signifying osteogenic differentiation induction. While von Kossa was employed as a well-established method to visualise phosphate-containing mineralised nodules and was supported by complementary ALP activity data, future studies will incorporate calcium-specific assays such as Alizarin Red S staining to further validate and extend the assessment of osteogenic mineralisation.

These findings suggest that Alpha-asarone enhances osteogenic mineralisation marked by the dark-brown stained cells, with 1 μM being the most effective, further supporting the findings of ALP enzymatic activity. Meanwhile, negative control (untreated cells) showed no staining of MC3T3-E1 cells, due to the absence of necessary differentiation factors.

Quantitative analysis of the stained areas further confirmed these findings and indicated that Alpha-asarone enhances bone matrix formation, reflecting its osteogenic potential.

The findings in this study showed the interconnected relationship between ALP activity and mineralisation in osteogenic differentiation. Both ALP activity and mineralisation analyses demonstrated that 1 μ M Alpha-asarone optimally enhanced osteogenic differentiation, as evidenced by the highest ALP levels and subsequent mineralisation. As previously reported, a study on MC3T3-E1 cells treated with *Piper sarmentosum* extract demonstrated that the concentration exhibiting the highest ALP activity also resulted in the highest mineralisation (Abidin et al., 2021).

The ability of Alpha-asarone to induce the differentiation of MC3T3-E1 cells could be attributed to its pharmacological and therapeutic effects. In a previous study, Alpha-asarone exhibited potent antioxidant activity, including 2,2-Diphenyl-1-picrylhydrazyl radical scavenging, metal chelation, and lipid peroxidation inhibition, suggesting its potential as a powerful agent for combating oxidative stress. The presence of reactive oxygen species has been found to promote apoptosis of bone formation-associated cells, such as osteocytes and osteoblasts, growth and differentiation (Lu et al., 2017), favouring bone resorption and ultimately causing bone loss (Marcucci et al., 2023). Hence, the possession of strong antioxidant activity provides a favourable environment for bone formation and differentiation. Overall, the present study demonstrates that Alpha-asarone promotes osteogenic differentiation of MC3T3-E1 cells, as evidenced by increased ALP activity and mineralised matrix formation. While these findings provide robust preliminary evidence of osteogenic activity, their generalisation beyond the MC3T3-E1 murine pre-osteoblast model should be interpreted with caution, as this cell line primarily serves as a highly reproducible and well-characterised platform for early-stage screening. Consequently, future studies employing human primary osteoblast and relevant *in vivo* bone models will be essential to confirm biological relevance and translational potential.

Alpha-asarone is known to exhibit neuroactive effects at higher systemic doses, which warrants consideration when evaluating its translational potential. Importantly, the concentrations employed in this study (1–20 μ M) were substantially lower than levels reported to obtain neurotoxic or behavioural effects *in vivo*. Furthermore, the present work focusses on cellular osteogenic responses under controlled *in vitro* conditions rather than systemic exposure. Nevertheless, comprehensive safety profiling, including neurotoxicity and organ-specific assessments, will be important in future *in vivo* studies prior to clinical translation.

To our knowledge, this is the first study to evaluate the effect of Alpha-asarone on ALP activity and mineralisation. Moreover, this study explored a natural compound, addressing the growing interest in alternative therapies for bone-related conditions. It also evaluated the compound's potential to promote osteogenic differentiation and bone formation,

representing an important therapeutic avenue. This finding is derived from a murine pre-osteoblast *in vitro* model and cannot be directly extrapolated to human therapy. Accordingly, the present study is a proof-of-concept investigation aimed at identifying the osteogenic activity of Alpha-asarone under controlled experimental conditions. While MC3T3-E1 cells provide a highly reproducible and well-characterised platform for early-stage screening, interspecies differences in cellular responses, metabolism, and pharmacokinetics necessitate cautious interpretation. Therefore, future studies incorporating human primary osteoblasts, pharmacokinetic and bioavailability profiling, and validation in relevant *in vivo* osteoporosis models will be essential to bridge the translational gap and establish clinical relevance.

Specific experimental refinements that would strengthen this work include validation in human primary osteoblasts, incorporation of calcium-specific mineralisation assays, molecular pathway analyses, and *in vivo* efficacy and safety studies. These refinements are planned for future investigations to build upon the current findings. The potential ethical and regulatory considerations must also be acknowledged when proposing Alpha-asarone as a therapeutic agent. These include comprehensive safety profiling, neurotoxicity evaluation, and assessment of long-term exposure risks. Despite being a naturally occurring compound, Alpha-asarone would still require full preclinical toxicology studies and adherence to regulatory compliance frameworks before any therapeutic application can be considered.

However, as preliminary research, these findings serve as a foundation for future studies. Therefore, as a future recommendation, these findings may be implied and used in other models, such as human primary cells and further *in vivo* models, for clinical validity. In addition, elucidation of the molecular mechanisms underlying its osteogenic effects is important. To minimise variability, Alpha-asarone was sourced from a single commercial supplier with certified purity, and fresh stock solutions were prepared using standardised protocols. Nevertheless, batch-to-batch variation can act as a potential limitation. Future studies should incorporate analytical verification methods such as high-performance liquid chromatography (HPLC) to confirm compound consistency and strengthen reproducibility.

FUTURE RECOMMENDATIONS

Future studies should incorporate additional assays to strengthen the claims of bone anabolic potential of Alpha-asarone. Specifically, a calcium-specific mineralisation assay such as Alizarin Red S staining would provide complementary validation to von Kossa staining by directly quantifying calcium deposition. Furthermore, osteogenic gene expression profiling of key markers, including *RUNX2*, *COL1A1*, and osteocalcin (*OCN*), would demonstrate the transcriptional regulation underlying the observed differentiation. Protein-level validation through Western blotting or immunocytochemistry, together with mechanistic pathway analyses, would further clarify the molecular basis of Alpha-asarone's osteogenic effects. Beyond *in vitro* assays, *in vivo* bone formation studies coupled with

micro-computed tomography (micro-CT) analyses are recommended to substantiate the anabolic potential and provide translational relevance. These approaches will collectively strengthen the evidence base and support the clinical development of Alpha-asarone as a natural bone anabolic agent.

CONCLUSION

In conclusion, Alpha-asarone demonstrated promising potential to promote osteoblastic differentiation of MC3T3-E1 cells, as evidenced by significant increases in ALP activity and mineralised matrix formation compared with the untreated and positive control groups. Alpha-asarone at a concentration of 1 μ M significantly exhibited the highest osteogenic effect without inducing cytotoxicity. These findings suggest Alpha-asarone may serve as a safe and effective natural agent for enhancing osteogenic differentiation.

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Review Article

Innovative Applications of Mycogenic Metal Oxide Nanoparticles for Eco-friendly and Sustainable Aquaculture Practices: A Review

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ABSTRACT

Nanotechnology has emerged as a transformative field across various industries, with agriculture being a key area of impact. Metal oxide nanoparticles (MONPs) are particularly valuable due to their versatile applications in enhancing agricultural productivity and sustainability. However, the traditional chemical synthesis of MONPs has raised environmental concerns, prompting researchers to seek alternative methods. As a result, green synthesis of MONPs using eco-friendly methods and biological materials has become a popular and sustainable alternative for industrial and agricultural applications. Fungi are highly versatile microorganisms that have the ability to produce

a diverse range of secondary metabolites that can be utilised as reducing agents for the production of MONPs. Despite this potential, there is a lack of comprehensive review articles that specifically examine the role of fungi from different genera as nanofactories for MONPs, along with their biological capabilities in promoting sustainable aquaculture practices. Therefore, this review seeks to bridge this gap by providing a comprehensive overview of fungal species capable of synthesising MONPs. In addition, this review highlights the recent advancements in mycogenic MONPs, with a focus on their potential applications, particularly in aquaculture.

Keywords: Aquatic health management, aquaculture innovation, eco-aquaculture solutions, environmental sustainability, nanotechnology in aquaculture

INTRODUCTION

Agriculture is vital to the economy of both developed and developing nations, and rising global populations will significantly increase food demand in the coming decade. However, climate change and global warming are already impacting the efficiency and quality of food production worldwide (Food & Agriculture Organisation, 2019). Moreover, global warming drives shifts in disease epidemiology by altering ecosystems, increasing vulnerability, and enhancing pathogen exposure (Ghazali et al., 2018). A sustainable food system must reduce climate impact, protect ecosystems, restore biodiversity, and ensure a safe, nutritious food production.

Nanotechnology offers innovative solutions in agriculture and environmental management through nanovaccines and nanosupplements for enhanced disease control. It involves manipulating materials at the nanoscale (1–100 nm), where increased surface area enhances applications in biosensors, nanomedicine, and bionanotechnology. Metal oxide nanoparticles (MONPs) offer high stability, easy synthesis, tunable properties, and resistance to swelling. Their charge allows integration into diverse systems and functionalisation for various applications (Nikolovo & Chavali, 2020).

MONPs can be synthesised through green, chemical, and physical methods, with the agricultural sector favouring the eco-friendly approach. The capacity of different biological systems to produce metallic nanoparticles varies significantly. This variation arises from differences in enzymatic activity and intrinsic metabolic processes, leading to the fact that not all living organisms are capable of synthesising nanoparticles (Ojha, 2022). Nonetheless, fungi have emerged as the preferred organisms for nanoparticle production.

Compared to bacteria, fungi are more advantageous in nanoparticle biosynthesis due to their rich repertoire of bioactive metabolites, superior aggregation abilities, and higher overall efficiency (Ghosh et al., 2021). Numerous studies have explored mycogenic MONP synthesis, but their application in aquaculture remains limited despite showing significant

potential for system improvement. They are particularly effective in controlling bacterial diseases, mitigating environmental pollutants, and functioning as vaccine carriers or immunostimulants (Danaraj et al., 2022).

By integrating MONPs into aquaculture practices, reliance on antibiotics can be minimised, thereby contributing to sustainable aquaculture and addressing the critical issue of antimicrobial resistance. This review highlights the diversity of fungi in MONP production, their current application in aquaculture, and discusses challenges and future directions, including scalability and commercialisation, environmental and safety considerations, and future research areas.

BIOGENIC METALLIC OXIDE NANOPARTICLES (MONPS)

MONPs are usually produced by hydrolysing metal salts at room temperature or below 100°C. Their remarkable physical and chemical properties stem from their small size and the high density of corner and edge surface sites. In contrast, biosynthesis involves the generation of compounds from simple precursors by living organisms, their metabolites, or biomolecules. This method is recognised as the most efficient, biocompatible, and safe approach for nanoparticle synthesis, making it the preferred choice for many applications (Jadoun et al., 2021).

Both chemical and biological processes use reducing agents for synthesis, but green chemistry approaches are gaining popularity to minimise harmful by-products. Biogenic MONP synthesis employs microbes, plants, or animal-derived substances to generate nanoscale materials via biomolecule production in a bottom-up approach (Figure 1). This widely accepted method enables atoms to self-assemble into nuclei, forming nano-sized particles. Conversely, the microbial synthesis of MONPs is regarded as an eco-friendly

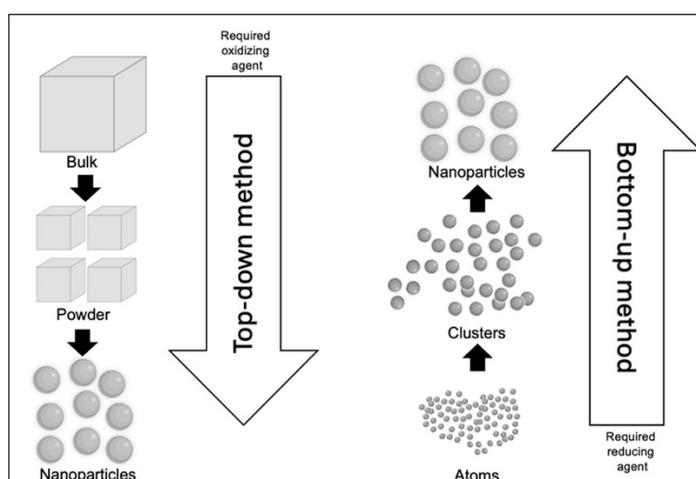


Figure 1. The synthesis of metallic oxide nanoparticles involves both top-down and bottom-up approaches

approach, as it typically takes place in aqueous media, under ambient temperature and pressure conditions, and near-neutral pH levels.

Various parameters, such as pH, pressure, temperature, and solvents, influence MONP synthesis using biogenic precursors. For instance, Wijesinghe et al. (2021) used *Tephrosia purpurea* extract for ZnO nanoparticle synthesis, highlighting pH's crucial role in optimization. A key advantage of biologically synthesised MONPs is the natural protein layer on their surfaces, which enhances suspension stability and prevents agglomeration (Durán & Seabra, 2012).

FUNGI AS NANOFACTORIES FOR MONPS

Traditional MONP synthesis relies on hazardous, costly methods with high energy demands and environmental risks. In contrast, green synthesis is sustainable, reducing waste, pollution, and toxicity while using renewable materials. Microbial-mediated green synthesis offers a sustainable alternative, utilising algae, bacteria, and fungi to produce MONPs via extracellular and intracellular mechanisms (Figure 2).

Fungi are promising sources of bioactive compounds, with ascomycetes and other species producing about 6,400 such molecules (Guilger-Casagrande & Lima, 2019). Their diverse intracellular enzymes, surface proteins, and reducing agents make them efficient biological nanoparticle synthesisers (Narayanan & Sakthivel, 2011). Furthermore, fungi uniquely tolerate and accumulate heavy metals, potentially enhancing nanoparticle production from bulk materials (Guilger-Casagrande & Lima, 2019).

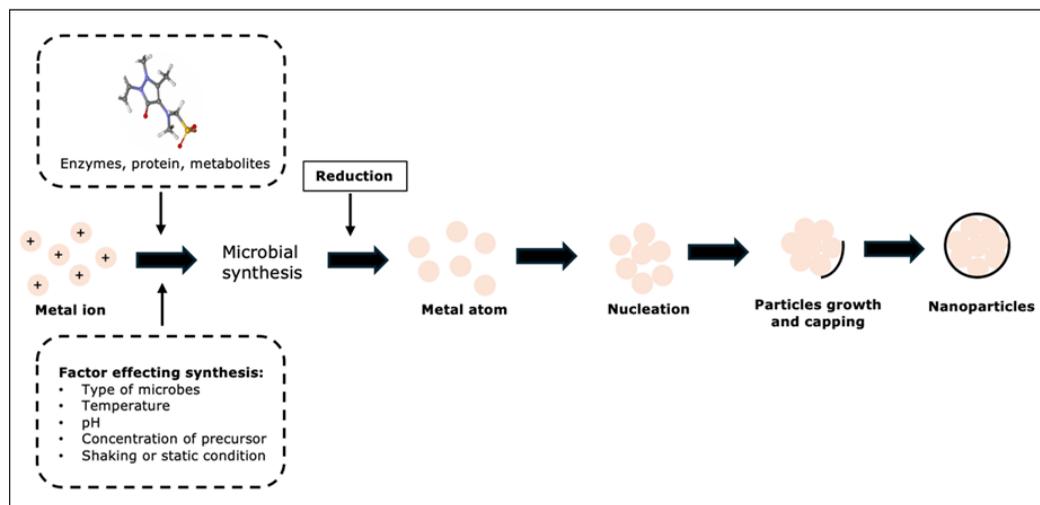


Figure 2. Microbial-mediated synthesis of nanoparticles involves the capture of metals, their enzymatic reduction, and capping, all facilitated by biomolecules such as proteins, enzymes, and metabolites from microbial extracts, which play a crucial role in maintaining nanoparticle stability. Adapted with Modification from Ghosh et al. (2021)

Selecting fungi for nanoparticle biosynthesis offers several notable advantages. As previously stated, fungi demonstrate exceptional tolerance to high concentrations of metal ions and exhibit highly efficient bioaccumulation capabilities, making them ideal candidates for this process. Fungi produce significantly more nanoparticles than bacteria due to their enhanced secretion of extracellular proteins, which aid nanoparticle formation. Additionally, fungal-derived nanoparticles typically exhibit low toxicity, making them ideal for various applications (Karunakaran et al., 2023).

Various genera of fungi, including certain metropolitan and rare fungal species, have been reported to possess the ability to synthesise MONPs. Metropolitan fungi such as *Trichoderma*, *Penicillium*, *Fusarium* and *Aspergillus* have been extensively explored as nanofactories for biosynthesis of MONPs. Beyond these well-known groups, other genera with similar potential are highlighted in the section below:

Trichoderma Spp

Trichoderma is a genus within the Fungi Kingdom, specifically classified under the Ascomycota Division, Pezizomycotina Subdivision, Sordariomycetes Class, Hypocreales Order, and Hypocreaceae Family. This genus comprises various species that are economically significant due to their capacity to produce industrial enzymes, antibiotics, and bioactive compounds (Consolo et al., 2020). To date, a total of 375 species of *Trichoderma* have been identified and confirmed worldwide (Cai & Druzhinina, 2021). These organisms are gaining prominence in nanotechnology, especially in the synthesis of MONPs.

Recent studies indicate that they can resist various nanocompounds; however, we still do not fully understand how this tolerance impacts their ability to synthesise MONPs (Ramírez-Valdespino & Orrantia-Borunda, 2021). The synthesis of nanoparticles driven by *Trichoderma* may involve enzymes such as reductases, which act as bioreductive agents in nanoparticle biofabrication. Previous research has shown that *Trichoderma* can accumulate metals through a mechanism involving reductase enzymes like NADPH-dependent nitrate reductase (Simões et al., 2020). This sequential process facilitates the reduction of metal salts, ultimately leading to the formation of nanoparticles.

Previous studies have highlighted the presence of phenolics, proteins, amino acids, aldehydes, ketones, and other functional groups on *Trichoderma*-mediated ZnO nanoparticles, which contribute to the reduction, capping, and stabilisation of the nanoparticles (Zaki et al., 2021). This indicates that compounds found in *Trichoderma Spp* can serve as both bioreducers and biostabilisers in the synthesis of MONPs. The resulting ZnO nanoparticles were found to be between 8 and 23 nm in size, exhibiting fungicidal activity against various soil pathogens.

Similarly, Singh et al. (2024) identified hydroxyl, carboxyl, flavonoid, amine, carbonyl sulfide, and carbonyl groups on ZnO nanoparticles mediated by *T. harzianum*. Their study

demonstrated that *T. harzianum* can produce ZnO nanoparticles ranging from 617 to 195 nm, with an average size of 314 nm, which also display biological activity. Despite the beneficial compounds associated with *Trichoderma* Spp, the biogenic synthesis of MONPs using these fungi and the exploration of their biological properties remain relatively underexplored.

Penicillium Spp

The genus *Penicillium* is among the most widespread and commonly encountered groups of soil fungi in nature. It is classified into four subgenera: *Aspergilloides*, *Penicillium*, *Furcatum*, and *Biverticillium*. Recently, the first three subgenera have been reclassified under the *Penicillium* genus, while *Biverticillium* has been reassigned to the *Talaromyces* genus (Perrone and Susca, 2017). It encompasses species with significant applications across medicine, pharmaceuticals, agriculture, and biotechnology. Notably, the discovery of penicillin from *Penicillium* fungi revolutionised the treatment of bacterial infections, profoundly impacting human health.

Additionally, this genus includes diverse species that play vital roles in decomposing organic materials and are recognised for their ability to produce extracellular proteins, particularly enzymes (Otero et al., 2020). Beyond their medicinal importance, *Penicillium* species are also crucial in areas such as food spoilage, biotechnology, and plant pathology, with a current total of 483 accepted species (Houbraken et al., 2020).

Penicillium fungi play a crucial role in the green synthesis of MONPs. Zakariya et al. (2022) demonstrated iron oxide (FeO) nanoparticle production using a cell-free aqueous extract of *Penicillium* Spp, highlighting the role of proteins as reducing and capping agents. Fungal enzymes and compounds like phenolic and amine groups further aid in nanoparticle formation and stabilisation. Their study reported FeO nanoparticles (3.31–10.69 nm) with strong antibacterial activity. Similarly, Ali et al. (2021) found that *P. roqueforti* biosynthesises FeO nanoparticles (5–16 nm) with antibacterial and antifungal properties.

El-Batal et al. (2020) found that the culture filtrate of *Penicillium chrysogenum* can generate copper oxide (CuO) nanoparticles with an average diameter of 9.70 nm, exhibiting notable antifungal and antibacterial properties. Their study revealed the presence of N–H bonds, suggesting that this functional group plays a crucial role in the synthesis and stabilisation of CuO nanoparticles. Apart from that, Honary et al. (2012) demonstrated that *P. aurantiogriseum*, *P. citrinum*, and *P. waksmanii* are capable of synthesising CuO nanoparticles. This research highlights the ability of various *Penicillium* species to produce monodispersed particles and emphasises the role of proteins in fungal culture filtrates as bioreducing and biostabilising agents.

Fusarium Spp

Fusarium is a genus of filamentous fungi that includes many species which produce mycotoxins, are notorious plant pathogens in agriculture and making infections in humans from time to time. These fungi are ubiquitous and can exist in various habitat such as water, soil and plant-associated media (Hernández-Ochoa et al., 2020). The genus *Fusarium*, with approximately 70 species, shows great genetic and biological/ecological diversity that is reflected in the various production of secondary metabolites (Toghueo et al., 2020). *Fusarium* is one of the most exploited fungal genera for nanoparticle fabrication, utilised by many researchers.

Some species can produce a diverse array of metabolites, showcasing impressive chemical variety and notable bioactivities. For example, *F. oxysporum* is highly valuable in industry and biotechnology due to its wide range of enzymes. These enzymes have various applications, including the production of cutinases, nitrilases, glycoside hydrolases, fructosyl amino acid oxidase, laccases, lipoxygenase, nitric oxide reductase, decarboxylases, keratinase, phospholipase B, and triosephosphate isomerase (Ibrahim et al., 2021), which may potentially be employed to produce MONPs.

Previously, it was reported that a cell-free filtrate of *F. oxysporum* successfully synthesised vanadium oxide (V_2O_5) nanoparticles, with sizes ranging from 10 to 20 nm (Gholami-Shabani et al., 2021). The study revealed the presence of carbonyl groups from proteins and amino acids in the fungal cell-free filtrate, which acted as bioreducing and capping agents for the mycosynthesis of V_2O_5 nanoparticles. Interestingly, the V_2O_5 nanoparticles synthesised by *F. oxysporum* demonstrated significant biological activity, particularly antimicrobial activity.

Similarly, a study by Gupta and Chundawat (2020) demonstrated that *F. oxysporum* can produce ZnO nanoparticles ranging in size from 18 to 25 nm. This study also identified extracellular proteins in the ZnO nanoparticles, which likely facilitate the reduction of zinc ions. Additionally, phenols, including polyphenols such as terpenoids and flavonoids, were found to act as bioreducing agents in the synthesis process. These nanoparticles were also found to possess biocatalytic activity to produce bioethanol from rice straw.

Apart from that, *Fusarium Spp* has been shown to produce MONPs. Kavitha et al. (2020) synthesised ZrO_2 nanoparticles using *F. solani*, revealing hydroxyl and carbonyl groups on their surface. Similarly, Mohamed et al. (2019) synthesised ZnO nanoparticles using *F. keratoplasticum*, observing two shapes with sizes ranging from 10–42 nm and 8–38 nm. They suggested that proteins or fungal bio-filtrate extracts bind to ZnO nanoparticles via free amino or carboxyl groups, influencing nanoparticle shape.

Aspergillus Spp

The genus *Aspergillus* is one of the most prevalent fungal groups, comprising several hundred species (Samson et al., 2014). These saprophytic filamentous fungi are commonly found in soil or organic matter. They are highly adaptive and resilient to changing environmental conditions, capable of growing across a broad temperature range and at relatively low humidity levels. Most *Aspergillus* species produce a wide array of secondary metabolites and fermentative enzymes, establishing a significant connection with human health, daily life, and industrial production (Yang et al., 2022).

Among fungal sources, *Aspergillus* stands out as a particularly promising candidate for MONPs production. This is due to its over 350 species, which exhibit significant biochemical versatility and a high capacity for protein secretion (Rai et al., 2022). Additionally, the compounds produced by *Aspergillus* are classified as generally regarded as safe (GRAS), making them suitable for industrial use.

Previously, a study by Singh et al. (2020) demonstrated that *A. flavus* can biosynthesise ZnO nanoparticles with an average size of 90.05 nm, exhibiting notable antifungal activity against *Alternaria solani*. The research identified various compounds involved in the biosynthesis process, including hydroxide, alkene, alkane, nitric oxide, aliphatic chains, sulphonic acid, alkyl halides, and silicon oxycarbide. This highlights the versatility of *Aspergillus* Spp in nanoparticle biosynthesis, attributed to its rich and diverse metabolite profile.

Furthermore, El-Saadony et al. (2021) reported that *Aspergillus* species, particularly *A. niger*, can synthesise ZnO nanoparticles (~45 nm). Functional groups like alcohols, amines, amides, and phenols contributed to biosynthesis, while proteins in the fungal supernatant stabilised the nanoparticles. These ZnO-NPs also showed strong bactericidal activity against fish pathogens.

Apart from ZnO nanoparticles, *Aspergillus* species have also been reported to synthesise CuO nanoparticles with sizes below 100 nm (Mani et al., 2021). CuO nanoparticles synthesised using *A. terreus* are formed due to the presence of proteins and enzymes in the endophytic fungus, which transform copper nanoparticles into their oxide form, CuO. These biogenic CuO nanoparticles have also been reported to possess several biological activities, including antimicrobial activity.

Other Fungal Genera

Cordyceps Militaris

Medicinal fungi have long been valued in human civilisation, with *Cordyceps* being prominent in traditional Chinese medicine. *Cordyceps militaris*, from the Clavicipitaceae family, is known for its nutritional and medicinal benefits, particularly its bioactive

metabolite, cordycepin, which has antibacterial, antitumor, antiviral, and antifungal properties (Choi et al., 2011).

The ability of *C. militaris* to produce MONPs can be seen through research by Dias et al. (2022). This study observes the ability of this fungal species to produce ZnO nanoparticles with an average particle size of 1.83 nm. The FTIR spectral analysis confirmed that various components of the aqueous mushroom extract of *C. militaris* played a role in capping and stabilising the nanoparticles. For example, proteins, alcohols, polyphenols, aromatic acid esters, phenolics, and β -glycosidic bonds of β -glucans were present.

Similarly, a study conducted by Li et al. (2019) synthesised zinc oxide nanoparticles via co-precipitation methods from *C. militaris*. This study revealed the ability of *C. militaris* to produce ZnO nanoparticles with a size of about 10.15 nm. A further characterisation revealed the presence of alcoholic OH groups, CH₂ asymmetric stretching, carbonyl group, carboxylic acid group, CN stretching vibration for phenol and amine group, and alkene group. Despite this, the capacity of *C. militaris* to produce MONPs beyond ZnO is notably limited.

Xylaria Spp

The genus *Xylaria* (Xylariaceae) is a widely distributed fungus in marine and terrestrial environments, known for producing diverse bioactive metabolites with antibacterial, antioxidant, and cytotoxic properties. (Chen et al., 2024). Additionally, this fungal genus has been reported to synthesise ZnO nanoparticles, with particle sizes ranging from 30 to 50 nm (Sumanth et al., 2020). Various compounds, such as phenolic OH groups, hydroxyl groups, polyphenols, keto-enol structures, alkenes, alkanes, and aromatic functional groups, have been implicated in the formation of these nanoparticles, which also demonstrate antimicrobial activity against pathogenic fungi and bacteria.

A study by Nehru et al. (2023) showed that *Xylaria arbuscula* synthesised ZnO nanoparticles (116 nm) with antioxidant, antimicrobial, antidiabetic, and anti-inflammatory properties. Characterisation of the biomolecules associated with the ZnO nanoparticles revealed the presence of compounds such as alcohols, polyphenols, aliphatic hydrocarbons, amides, and aromatic amines, which may serve as capping and reducing agents in the conversion of zinc ions to ZnO nanoparticles. This research highlights the potential of *Xylaria* species for the mycosynthesis of metal oxide nanoparticles (MONPs). However, literature on the synthesis of MONPs other than ZnO by *Xylaria* species remains limited.

Pleurotus Djamor

The genus *Pleurotus* includes several nutritionally and medicinally valuable species, though not all are commonly cultivated (Vega et al., 2022). *Pleurotus djamor* (Rumph.

ex Fr.) Boedijn, found in tropical and subtropical regions, thrives at temperatures up to 30°C and produces fruiting bodies within 1–2 weeks, making it ideal for tropical cultivation (Salmones & Mata, 2015). Studies show its crude extracts possess analgesic, anti-inflammatory, antipyretic, antimicrobial, and antiplatelet properties, along with strong free radical scavenging activity (Susem et al., 2013), highlighting its potential for MONP synthesis.

A study by Manimaran et al. (2021a) demonstrated that *P. djamor* aqueous extract synthesised ZnO nanoparticles (70–80 nm). FTIR analysis confirmed the presence of aromatic rings and carboxylic acid groups, facilitating nanoparticle formation. These mycosynthesised ZnO nanoparticles exhibited strong larvicidal, antibacterial, anticancer, and antioxidant properties, highlighting their potential for biomedical and environmental applications.

Similarly, Manimaran et al. (2021b) reported that *P. djamor* aqueous extract synthesised TiO₂ nanoparticles (31 nm). FTIR analysis identified functional groups like hydroxyl, amine, and carboxylic acid. These TiO₂ nanoparticles showed potent larvicidal, antibacterial, and anticancer activities, making them promising for pest control, antimicrobial therapies, and cancer treatment.

APPLICATIONS OF MYCOGENIC MONPs IN AQUACULTURE

Aquaculture is shifting from traditional to intensive farming, enabling higher stocking densities in limited water spaces. This shift significantly raises the risk of transmissible and infectious diseases (Santos & Ramos, 2018). The overuse and misuse of artificial feeds in aquaculture, driven by poor management and limited farmer training, often degrade the environment and increase disease prevalence (Hossain et al., 2022). Global aquatic products, encompassing aquatic plants, fish, crustaceans, molluscs, and other species such as bullfrogs and jellyfish, constitute the third largest source of food protein for humans, after cereals and milk, and account for 16.4% of the total animal protein supply (Du et al., 2022). Environmental contamination and disease prevalence pose major challenges in aquaculture, driving recent technological advancements to address these issues.

Mycogenic MONPs, acknowledged as an innovative and transformative method, offer a wide range of applications and significant potential in the field of aquaculture (Figure 3). It enables new methods for drug management and vaccine development, protecting fishery resources from pathogens. The rapid rise of nanotechnology presents promising solutions for advancing aquaculture. MONPs have gained interest in agriculture for their high surface area, stability, and tunable properties. However, despite their eco-friendly and biocompatible nature, mycogenic MONPs remain underexplored compared to other synthesis methods.

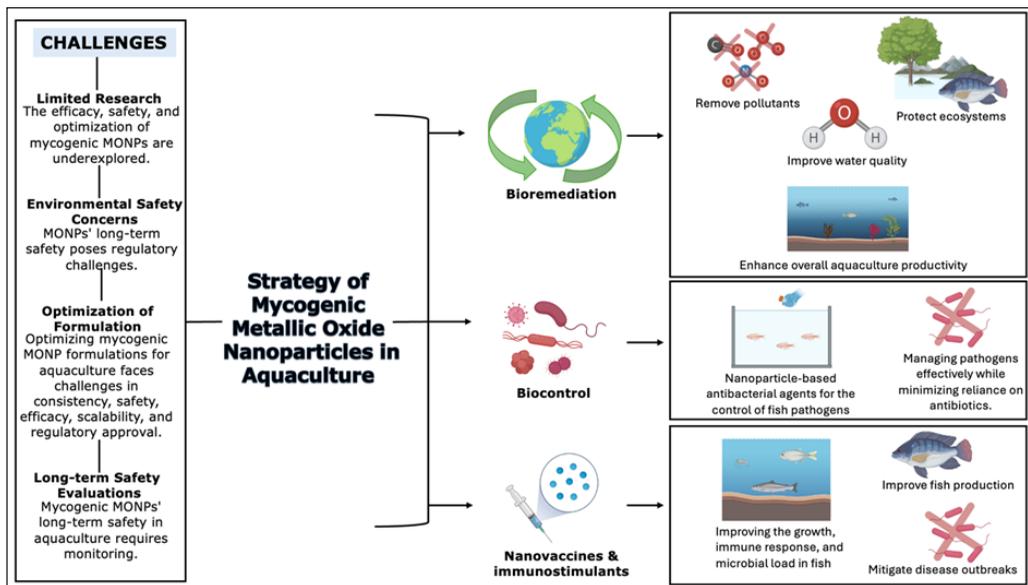


Figure 3. Strategy of mycogenic metallic oxide nanoparticles (MONPs) in aquaculture with applications in bioremediation, biocontrol, nanovaccines, immunostimulants, and associated challenges

Nanovaccines and Immunostimulants

The rise of infectious diseases in aquaculture demands advanced therapeutic strategies. MONPs offer superior biocompatibility, targeted delivery, and immune enhancement (Singh et al., 2021), making them ideal for nanovaccines and immunostimulants. Mycogenic MONPs stand out for their eco-friendly production, scalability, and unique biological functionalities. Their synthesis relies on enzymatic and metabolic secretions that control particle size, morphology, and function, ensuring suitability for aquaculture applications.

In this context, El-Saadony et al. (2021) found that mycogenic ZnO nanoparticles from *Aspergillus niger* improved *Oreochromis niloticus* performance, feed efficiency, and behaviour. Treated fish showed enhanced liver function, higher LYZ and NBT activity, lower cortisol, and increased testosterone and growth hormone levels, indicating better stress management and growth regulation.

Additionally, mycogenic ZnO nanoparticles exhibited strong antibacterial properties, achieving the lowest bacterial loads in water and fish tissues while effectively targeting key pathogens, including *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Aeromonas hydrophila*. These findings highlight their potential as a powerful therapeutic in aquaculture.

The integration of nanotechnology, particularly MONPs, is revolutionising therapeutic approaches in aquaculture. As nanovaccines and immunostimulants, MONPs enhance immune activation and provide sustained protection against infections. However, research

on mycogenic MONPs in these roles remains scarce, highlighting the need for further studies. Future research should compare the efficacy of different mycogenic MONPs, optimise formulations, assess long-term safety, and expand applications across aquaculture systems to maximise their potential as sustainable disease control tools.

Biocontrol Potential

Aquaculture plays a vital role in global food security and economic sustainability. However, the industry is continually challenged by the prevalence of infectious diseases, which cause significant losses in fish populations. In recent years, MONPs have emerged as a promising tool in combating fish diseases due to their unique physicochemical properties, including antimicrobial activity, biocompatibility, and stability, offering innovative solutions to safeguard aquatic health.

Al-Shammari et al. (2022) demonstrated the efficacy of mycogenic ZnO nanoparticles synthesised using *Fusarium* sp. against *Saprolegnia parasitica*. Species from the Saprolegniaceae family, including *Achlya* and *Saprolegnia*, infect fish eggs in both aquaculture and natural environments, posing challenges during incubation. At 100 ppm, mycogenic ZnO nanoparticles achieved 79% inhibition of *Saprolegnia* Spp, comparable to malachite green. This highlights their potential for controlling saprolegniasis in fish hatcheries.

Moreover, the potential of mycogenic MONPs as antibacterial agents against aquatic pathogens is evident in a study by Hassan et al. (2021). Mycogenic magnesium oxide (MgO) nanoparticles synthesised using *Rhizopus oryzae* exhibited antibacterial properties against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which cause significant health issues in fish and fisheries products (Sivaraman et al., 2022; Shahrokhi et al., 2022). Their presence in fish products also poses a risk of foodborne illnesses, emphasising the need for nanoparticle-based pathogen control.

Similarly, a study by Flores-Rábago et al. (2023) demonstrated the efficacy of mycogenic CuO nanoparticles synthesised using *Ganoderma sessile* extract as antibacterial agents against *S. aureus* and *P. aeruginosa*. These mycogenic nanoparticles effectively inhibited both bacteria at low concentrations, with *P. aeruginosa* being the most susceptible. Despite the potential of mycogenic MONPs in aquaculture, research in this area, particularly for combating aquatic diseases, remains limited. Further studies are needed to explore their application in aquaculture systems, ensuring environmental safety, and evaluating their impact on aquatic organisms and overall productivity.

Bioremediation in Aquaculture

Water pollution occurs when unnatural substances enter water bodies, disrupting the natural cycle. Aquatic systems process waste, but excessive pollutants and overuse from

agriculture, industry, and development reduce their capacity to assimilate waste (Malik et al., 2020). Fish species are crucial to human-related aquatic communities. Furthermore, Environmental pollution is rapidly increasing due to urbanisation, industrialisation, and population growth. The textile and dyeing industries are major contributors, releasing synthetic dyes and heavy metals that harm aquatic life, degrade water quality, and threaten human health.

Previously, MONPs have been reported to show a promising use in wastewater purification, removal of heavy metals and dyes, and microbial contaminants (Nizamuddin et al., 2019). Due to the growing demand for oxide nanoparticles and nanomaterials, research into their synthesis using fungi has been rapidly advancing in recent years (Loshchinina et al., 2023). A study by Kumar et al. (2024) reported the application of mycogenic FeO nanoparticles synthesised by *Myceliophthora thermophila* as a catalytic reducer of *p*-nitrophenol (PNP) into *p*-aminophenol, a toxic organic compound commonly found in industrial wastewater. Additionally, these nanoparticles demonstrated the ability to decolourise bromophenol blue and malachite green.

PNP is highly soluble in water, resistant to biodegradation, and threatens aquatic ecosystems by disrupting enzymatic processes, bioaccumulating in the food chain, and harming biodiversity. Malachite green, widely used in aquaculture, is highly toxic and poses long-term ecological and human health risks (He et al., 2023). Similarly, improper disposal of bromophenol blue degrades water quality and disrupts aquatic ecosystems (Saad et al., 2024). These findings highlight the need for effective waste management to reduce environmental impact.

A study by Darwesh et al. (2023) reported the ability of CuO nanoparticles synthesised by *Fusarium oxysporum* OSF18 as a microbial, heavy metals, and textile dye remover from industrial wastewater. The mycosynthesised CuO nanoparticles embedded in alginate beads demonstrated exceptional performance, achieving microbial disinfection efficiency of 99.995%, removal of heavy metals (93% for Pb, 55% for Cr, and 30% for Ni), and dye decolourisation efficiency of 90%. These findings mark a significant advancement toward developing an eco-friendly, cost-effective, and user-friendly solution for the bioremediation of wastewater from the textile industry, ultimately contributing to the preservation of aquatic ecosystems.

Next, a study by Mahanty et al. (2023) reported the ability of FeO nanoparticles synthesised by *Corynespora cassiicola* as a bioremediation to adsorb heavy metals, viz. Pb, Ni, Cu, Zn, Cd, and Cr from water. The comparative evaluation of mycogenic FeO nanoparticles against other green adsorbents clearly demonstrated their superior effectiveness as heavy metal adsorbents. Among these heavy metals, chromium (Cr) stands out as one of the most hazardous, posing severe risks to both health and the environment. This study highlights the potential of mycogenic FeO nanoparticles for efficient heavy metal removal in treating industrial effluents and aquaculture water.

Although previous studies have highlighted the potential of mycogenic MONPs as a photocatalytic and bioremediation agent, it is important to note that future efforts should focus on advancing their application, understanding their mechanisms, and ensuring environmental and economic sustainability. For example, conducting field trials and pilot studies by implementing MONP-based water remediation in small-scale aquaculture farms can help validate lab-scale results in real-world conditions.

CHALLENGES AND FUTURE DIRECTIONS

The successful use of mycogenic MONPs in aquaculture requires overcoming key challenges and exploring future advancements. This includes scaling up production for commercial viability, ensuring environmental safety, and enhancing efficacy. Key aspects discussed include scalability, commercialisation, regulatory compliance, and future research to drive innovation. Tackling these challenges will help transition mycogenic MONPs from lab innovations to sustainable aquaculture solutions.

Scalability and Commercialisation

Scaling up mycogenic MONP synthesis is crucial for commercial aquaculture applications. While lab-scale production shows promise, industrial-scale synthesis requires optimising fungal cultivation, standardising protocols, and improving yield efficiency. This section explores key aspects of scalability, including optimising fungal cultivation processes, standardising synthesis protocols, and improving yield efficiency to ensure consistent nanoparticle quality. Additionally, it highlights the need to address economic feasibility, regulatory compliance, and environmental sustainability. Overcoming these challenges can make mycogenic MONPs a viable and eco-friendly solution for improving aquaculture.

Optimising Fungal Cultivation and Improving Yield and Efficiency

Large-scale mycogenic MONPs synthesis faces challenges in maintaining consistency and Optimising reaction conditions. It also requires cost-effective fungal cultivation with robust strains for high-yield production (Javed et al., 2024). Apart from that, maximising nanoparticle yield without compromising quality depends on enhancing the reaction efficiency between fungal metabolites and precursor materials. Production efficiency can be further improved by Optimising reaction conditions such as pH, temperature, and reaction time using advanced models like Response Surface Methodology (RSM). Available in software like MINITAB, RSM combines statistical and mathematical methods to optimise processes effectively (Lamidi et al., 2022). It helps determine the optimal factors for the highest nanoparticle yield.

Genetically engineered fungal strains could enhance metabolic capabilities, improving nanoparticle yield and consistency. Genome mining has recently identified genes encoding

enzymes for peptide assembly, regulation, resistance, and secondary metabolite synthesis (Leal et al., 2024). These genes form biosynthetic gene clusters (BGCs), allowing coordinated pathway expression. By introducing or modifying specific genes, fungi can be engineered to overproduce metabolites that act as reducing and stabilising agents in nanoparticle synthesis. Several transformation systems have been developed, including chromosomal engineering, *Agrobacterium*-mediated transformation, and CRISPR/Cas9-based genome editing (Jin et al., 2021). Genetic modifications can also enhance fungal tolerance to temperature and pH fluctuations, improving their robustness in large-scale cultivation.

Standardising Production Processes and Ensuring Economic Feasibility

A major challenge in applying mycogenic MONPs is optimising their synthesis for consistency, scalability, and cost-efficiency. Standardising the process ensures uniform nanoparticle size, shape, and properties, which are crucial for efficacy. Integrating automation and bioreactor technologies can significantly enhance process efficiency and reproducibility, making large-scale production both feasible and reliable (Wainaina & Taherzadeh, 2023). Additionally, it is essential that the production process remains cost-competitive compared to conventional nanoparticle synthesis methods, which can be evaluated through comprehensive cost-benefit analyses (Hassaan et al., 2020). Scaled-up production must also comply with environmental and safety regulations, focusing on effective waste management and reducing the ecological footprint.

Demonstrating Efficacy in Real-world Conditions

A major challenge in promoting mycogenic MONPs in aquaculture is proving their effectiveness and addressing adoption barriers. Field trials and pilot studies on farms are crucial to demonstrating their benefits in water quality, pathogen control, and fish health, as mentioned earlier. These studies will also offer insights into environmental impact, economic feasibility, and long-term sustainability.

Additionally, collaboration with aquaculture practitioners and industries is crucial for integrating this innovation into existing systems. Engaging stakeholders through workshops, knowledge exchange, and partnerships will enhance adoption. Overcoming challenges through collaboration will help mycosynthesised MONPs evolve from lab innovation to a scalable, sustainable solution for aquaculture.

Environmental and Safety Considerations

As the use of mycogenic MONPs in aquaculture grows, it is essential to address their potential environmental and safety impacts (Liu et al., 2024). This includes evaluating

their effects on aquatic ecosystems, ensuring sustainable practices, and minimising risks to both target and non-target organisms.

Comprehensive Risk Assessment

Although research on the toxicity, bioaccumulation, and environmental effects of mycogenic MONPs in aquaculture is limited, studies on other nanoparticles reviewed by Nam et al. (2014) provide valuable insights. This underscores the need to assess their interactions with environmental substances. Toxicological studies should examine their effects on target and non-target aquatic species, including plants, invertebrates, and microbial communities. Bioaccumulation studies are also essential to determine their accumulation in aquatic organisms and potential transfer through the food chain. Additionally, environmental impact assessments should focus on their degradation, persistence, and behaviour in aquatic ecosystems.

Develop Safer Nanoparticle Formulations

Nanoparticles function differently from their bulk counterparts due to their small size and large surface area (Tirumala et al., 2021). This enhances interactions with biological systems, making surface modifications essential to reduce the reactivity and toxicity of MONPs. Mycogenic MONPs may naturally acquire protein or biomolecule coatings from fungal byproducts, improving stability, reducing toxicity, and enhancing biocompatibility (Campaña et al., 2023). Thorough testing of these eco-friendly MONPs is crucial to assess toxicity and ensure compliance with environmental and aquatic health regulations.

Establish Regulatory Frameworks

Currently, no standardised regulatory frameworks govern nanotechnology in food and agriculture (Fajardo et al., 2022). Therefore, developing robust policies is crucial for the safe use of nanoparticles in these industries. Collaboration with regulatory agencies ensures mycogenic MONPs comply with environmental and aquatic health regulations, promoting responsible innovation while minimising risks. Clear guidelines also support the adoption of mycogenic MONPs in aquaculture, fostering sustainable advancements while maintaining public and environmental safety.

Regular Monitoring and Surveillance

A key challenge in applying mycogenic MONPs in aquaculture is understanding their long-term effects on aquatic ecosystems, especially their influence on antimicrobial resistance. Biomonitoring programmes are crucial for assessing aquatic health and tracking resistance patterns where mycogenic MONPs are used. These programmes provide insights into

their impact on microbial populations (Parada et al., 2019) and their role in resistance development. Regular monitoring helps detect adverse effects early, allowing timely interventions to reduce risks and support the sustainable use of mycogenic MONPs in aquaculture.

Future Research Areas

The green synthesis of MONPs is rapidly growing, yet fungal-based MONP synthesis and its aquaculture applications remain underexplored. Hence, this section highlights future research directions in this area.

Vaccine Development and Delivery System

The potential of mycogenic MONPs as a platform for vaccine development and delivery represents an exciting and promising area for future research. These nanoparticles offer distinct advantages, including biocompatibility, stability, and the ability for surface functionalization, which are crucial for effective vaccine delivery. Additionally, the mycosynthesis of MONPs enhances their safety and eco-friendliness, further establishing their suitability for this purpose.

One of the key areas of focus is the ability of MONPs to serve as carriers for antigen delivery through surface modification. These nanoparticles can be functionalized to carry specific antigens, enabling targeted immune responses (Wang et al., 2024). Future research can explore ways to optimise the functionalisation process, ensuring that antigen integrity is preserved and immunogenicity is enhanced.

Another key aspect is the adjuvant potential of MONPs, which enhance vaccine effectiveness through immunostimulatory properties (Wang et al., 2024). They prolong antigen exposure, target immune cells, and boost responses in aquaculture. Research can explore their synergy with traditional adjuvants and their role in both innate and adaptive immunity. Integrating mycogenic MONPs into aquatic vaccines offers a novel strategy against bacterial infections. Studies may assess their ability to elicit strong immune responses while maintaining fish health and growth. Additionally, research could examine their role in protecting antigens from degradation for safe delivery to target tissues.

The biodegradability and safety of mycogenic MONPs are also critical considerations for their application in vaccine delivery. Future investigations should include long-term safety assessments, examining cytotoxicity, immunogenicity, and the potential for accumulation in biological systems. The fungal-based synthesis pathway provides a unique advantage in ensuring eco-friendly production, but thorough studies are needed to confirm these benefits.

Advancing research in these areas could pave the way for mycogenic MONPs to revolutionise vaccine delivery systems. Their scalable, cost-effective, and efficient

properties hold significant promise for enhancing aquatic animal health and addressing the challenges of infectious diseases in aquaculture.

Biosensor Application

Aquatic ecosystems face growing threats from pollutants, including heavy metals, pesticides, and harmful microorganisms, which highlight the urgent need for efficient monitoring tools. Mycogenic MONPs offer significant promise for developing sensitive and eco-friendly biosensors tailored for aquatic environments, owing to their distinctive physicochemical properties. One critical area of focus is the detection of heavy metals. Mycogenic MONPs, such as ZnO nanoparticles synthesised through fungal methods, exhibit remarkable conductivity and photocatalytic properties, making them ideal for detecting trace levels of metals like mercury (Hg), lead (Pb), and cadmium (Cd). Despite the broad synthesis of metal oxide nanoparticles from transition metals, the application of mycogenic MONPs in heavy metal detection remains relatively limited.

Another key application is monitoring aquatic pathogens. Biosensors with MONPs detect harmful microorganisms like *Vibrio* Spp, *Aeromonas* Spp, and *Streptococcus* Spp. Electrochemical biosensors use biologically relevant materials such as proteins, antibodies, or DNA to interact with target analytes, generating measurable electrical signals (Channabasavana Hundi Puttaningaiah, 2024). Functionalised MONPs, when combined with DNA or antibodies, enable rapid and specific detection of pathogens (Zimina et al., 2022). Additionally, MONPs are instrumental in assessing pesticides and organic pollutants in wastewater. Their photocatalytic properties, particularly in FeO, ZnO, or TiO₂ nanoparticles, can be harnessed for sensing and degrading organic pollutants in diverse environments (Ashour et al., 2023). Fungal-derived MONPs offer enhanced specificity and a reduced environmental footprint compared to chemically synthesised alternatives. By capitalising on the unique capabilities of mycogenic MONPs, researchers can drive innovations in aquatic biosensing technologies, fostering environmental sustainability and advancing aquaculture management practices.

Aquatic Feed Supplement

The use of mycogenic MONPs in aquatic feed supplementation offers great potential for improving aquaculture productivity. Synthesised through eco-friendly fungal-based methods, these nanoparticles have high surface area, bioavailability, and biocompatibility, making them ideal for feed enhancement. ZnO nanoparticles, in particular, serve as effective mineral supplements, providing essential micronutrients for growth, skeletal development, and metabolism (Yusof et al., 2023). Their nanoscale size ensures efficient absorption, addressing nutritional deficiencies in aquaculture diets.

Mycogenic MONPs enhance immunity, improving disease resistance and reducing antibiotic reliance in aquaculture. They also interact with gut microbiota (Xia et al., 2017), promoting gut health and feed efficiency, leading to better growth and overall fish health. Additionally, their antimicrobial properties suppress harmful pathogens, lowering disease incidence and improving survival rates. By leveraging these multifunctional benefits, integrating MONPs into aquaculture feed can drive sustainability, productivity, and environmental stewardship.

CONCLUSION

Numerous studies have highlighted the potential of various fungal species to synthesise MONPs and their diverse biological activities. Fungal genera such as *Trichoderma*, *Penicillium*, *Fusarium*, *Aspergillus*, and others have demonstrated considerable promise as natural nanofactories for MONP production due to the presence of various secondary metabolites and proteins. In addition, nanoparticles synthesised by these fungi have demonstrated a range of biological activities. Harnessing these fungi for MONP synthesis could significantly contribute to sustainable aquaculture practices. Nevertheless, additional research is necessary to thoroughly investigate their maximum potential. This review also discusses the challenges while highlighting future directions focused on mycogenic MONPs and assessing the long-term environmental impact of using MONPs in aquaculture. In conclusion, fungal-synthesised MONPs offer a promising alternative for sustainable aquaculture, though further research is needed to overcome challenges and enhance application.

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AI USAGE STATEMENT

The authors confirm that no generative artificial intelligence (AI) tools were used in the writing or preparation of this manuscript. The manuscript was prepared entirely by the authors. We believe the high AI similarity score may represent a false positive result due to conventional scientific writing structure and terminology.

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Preliminary Study on the Safety and Efficacy of a Newly Manufactured STVac7 Vaccine against Pneumonic Mannheimiosis of Goats

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ABSTRACT

Pneumonic manheimiosis is a respiratory disease of small ruminants caused by *Mannheimia haemolytica*. It leads to deaths and great economic loss, and the most effective control measure is through vaccination. This study evaluates the safety and efficacy of a newly manufactured STVac7 vaccine in goats against *M. haemolytica*. Five groups of goats with 3 goats per group received either freshly prepared vaccines (Group 1), 21-month-old formulations (Group 2), high dose vaccination (Group 3), vaccine containing a stabiliser and preservative (Group 4) and control, no vaccination (Group 5). Key clinical signs, including respiratory rates, body temperatures and adverse events, particularly sneezing and coughing, were monitored throughout the 12-week study period. All goats were challenged with 10⁹ CFU/mL of live *M. haemolytica* in week 10. Following vaccination, mild sneezing and coughing were occasionally observed. Post-challenge, clinical signs were most severe in the control Group 5, while *M. haemolytica* was isolated from all groups, with a higher frequency in the control Group 5. This was consistent with the more severe gross and histopathological lung lesions in control Group 5, while bronchus-associated lymphoid tissue (BALT) was mostly observed in Group 1 and least observed in control Group 5. Subsequently, high levels of IgG were detected in

vaccinated goats, particularly Group 4. Fibrinous bronchopneumonia, a key lesion of pneumonic manheimiosis was observed only in the control Group 5. In summary, the STVac7 vaccine is safe and effective in protecting goats from severe pneumonic manheimiosis.

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INTRODUCTION

Mannheimiosis, a significant respiratory disease affecting goats and sheep, is caused by the bacterium *Mannheimia haemolytica* (Shiferaw et al., 2006), a Gram-negative bacterium belonging to the Pasteurellaceae family. This pathogen is renowned for its role in causing acute and chronic pneumonia, particularly in ruminants (Taunde et al., 2019). *Mannheimia haemolytica* thrives in the respiratory tract of goats, causing damage to the lung tissues that impair the respiratory function. The bacterium's potent toxins and virulence factors contribute to the severity of the disease (Zecchinon et al., 2005).

Mannheimiosis is globally distributed and can affect small ruminant populations in various countries. It is more prevalent in regions with intensive livestock production and can lead to considerable economic losses due to reduced productivity, increased mortality rates, and treatment costs (Wang et al., 2018; Girma et al., 2023). Goats exposed to stressors such as inclement weather, transportation, parasitism and malnourishment are highly susceptible to the disease (Abera & Mossie, 2022; Taunde et al., 2019). The widespread occurrence of the disease underscores its importance and highlights the need for effective control and prevention strategies.

Pneumonic mannheimiosis typically presents with dyspnoea, coughing, nasal discharge, and increased respiratory rate (Rawat et al., 2019). Affected goats may exhibit decreased feed intake and lethargy. Severe cases can lead to high morbidity and mortality rates, particularly in young and immune-compromised animals. Gross pathological findings often include lung consolidation, pleuritis and fibrinous adhesions, particularly in the cranioventral aspect of the lungs (Emikpe & Akpavie, 2012; Rawat et al., 2019). On histopathological examination, lung lesions are characterised by bronchopneumonia, interstitial pneumonia, emphysema and haemorrhage. These lesions collectively contribute to impaired lung function and compromised respiratory efficiency.

Efforts to manage pneumonic mannheimiosis primarily involve disease prevention through vaccination and biosecurity measures (Shahudin et al., 2018). Intranasal vaccines, such as those containing live attenuated *M. haemolytica* antigens, have proven effective in inducing local immunity in the respiratory tract (Tenuche & Emikpe, 2015). These vaccines can help reduce the severity of clinical signs and lessen the economic impact of the disease. Intranasal vaccines offer the advantage of stimulating a rapid mucosal immune response at the site of entry, enhancing the animal's ability to fend off the bacterium upon exposure, while also ensuring ease of delivery and better vaccine coverage (Annas & Zamri-Saad, 2021). Proper vaccination protocols, including timing and booster doses, are critical for optimal protection.

STVac7 is an intranasal spray vaccine that has been developed by Universiti Putra Malaysia. It contains inactivated whole cells of *M. haemolytica* and was found to cross-protect against serotypes A2, A7, and A9 (Sabri et al., 2000). While studies on the efficacy

of laboratory-scaled STVac7 spray vaccine have been carried out, the safety and shelf-life efficacy of this vaccine following manufacturing under a GMP facility have yet to be determined. This paper reports the impact of administering a newly manufactured STVac7 vaccine and a 21-month-old STVac7 vaccine in goats. At the same time, the study also assesses the toxicity or adverse effects of administering a high dose of a freshly prepared STVac7 and the STVac7 containing polysorbate 80 and benzyl alcohol as a stabiliser.

MATERIALS AND METHODS

Ethical Approval

All use of animals and experimental protocols was granted approval by the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia, under the reference number UPM/IACUC/AUP-R004/2023.

Animal Selection and Management

A group of 15 adult Boer breed goats between 25 and 30 kg bodyweight, comprising both male and female specimens, was carefully chosen for the study. These selected goats were unvaccinated and exhibited apparent good health. Before the final selection, serum samples were collected from each goat and subjected to ELISA to determine the antibody levels against *M. haemolytica*. Those with low antibody levels against *M. haemolytica* were subsequently chosen. These selected goats underwent a 7-day acclimatisation period and were housed at the Animal Experimental House within the Faculty of Veterinary Medicine at Universiti Putra Malaysia (UPM) for the duration of the study.

During acclimatisation, all goats received a subcutaneous administration of ivermectin at 1 ml per 50 kg bodyweight, as a component of a routine deworming protocol. Throughout the course of the study, the goats' diet consisted of freshly harvested Napier grass and a commercial goat pellet, both supplied at a rate equivalent to 1.0% of their body weight. Additionally, they were provided unrestricted access to drinking water.

Experimental Design

At the start of the experiment, selected goats were randomly divided into five groups with 3 goats in each group. Groupings were done by a member of the research team based on animal ID. Each group was kept in a separate enclosure. Subsequently, different types of STVac7 vaccine were administered intranasally into each group, respectively. The vaccines were manufactured in a GMP facility at BioAngle Vacs Sdn. Bhd., based on the protocol described by Sabri et al. (2013) and were administered to the respective group as outlined in Table 1.

Vaccinations were performed on two separate occasions, on days 1 and 14 of the study. Subsequently, on day 70, all goats across the groups were challenged with an inoculum containing 10^9 CFU/ml of live *M. haemolytica* A7 through an intratracheal administration method to ensure disease development (Smith et al., 2020). On day 91 of the study, or 21 days after challenge, all goats were euthanised by means of slaughtering and were immediately followed by necropsy procedures.

Table 1

Summary of grouping and the various types of vaccine preparation administered

Group	Treatment
Group 1	Freshly manufactured STVac7 vaccine (< 1 month; 10^6 cells/mL)
Group 2	STVac7 that has been kept for 21 months. The vaccine was stored at room temperature and was checked for stability and presence of intact antigen at 3-month interval
Group 3	High-dose freshly prepared STVac7 vaccine (< 1 month; 10^8 cells/mL)
Group 4	Freshly manufactured STVac7 vaccine containing 0.01% Polysorbate 80 and 2% Benzyl Alcohol as vaccine stabilisers (Corcoran & Ray, 2014; Ieven et al., 2021)
Group 5	PBS placebo vaccine

Clinical Observations and Samplings

Following administrations of the vaccine, all goats were closely monitored for adverse effects of vaccination, which included coughing, sneezing, lethargy, loss of appetite, oronasal discharge and mortality. The monitoring was conducted at 24- and 48-hours post-vaccination.

After the challenge with the live *M. haemolytica* A7, daily observations were carried out for three consecutive weeks to monitor for clinical signs of pneumonic manheimiosis. These signs included oronasal discharge, pyrexia (rectal temperature $\geq 40^\circ\text{C}$), coughing, sneezing, dullness or lethargy, abnormal respiration, loss of appetite, depression, and death.

Throughout the study, all animals were consistently observed for health status. Any abnormal clinical signs or adverse effects were meticulously recorded by a veterinarian. The severity of these events was assessed, with particular attention to the frequency and duration of coughing and sneezing, which were classified as mild or severe. Mild reactions were defined by occasional or intermittent symptoms, while severe reactions were characterised by continuous or frequent occurrence.

Prior to and at weekly intervals following vaccination, serum samples were collected from all goats throughout the 12-week study period. The sera were subjected to ELISA according to Poonsuk et al. (2023) to determine the antibody levels. The serum was diluted to 1:1,200 while the secondary antibody was diluted to 1:10,000.

For dead or euthanised animals, a thorough examination of all organs was carried out by a veterinary pathologist, with particular emphasis on the lungs, while liver and kidney

lesions were observed for goats in Groups 3 and 4. Gross lesions that were present within the lungs were documented and described. Subsequently, pneumonic lesions were scored based on the percentage of areas affected (Table 2) according to Bkiri et al. (2023). Samples of lung, liver, and kidney were collected into sterile plastic bags for the isolation of *M. haemolytica* (Abate & Fentie Kassa, 2023). Similarly, samples were collected for histopathology analysis, where lungs were examined for pneumonic lesions, while liver and kidneys were examined for toxicity lesions, particularly for Group 3 and 4. The lungs encompassed the right apical lobe and the cranial part of the left apical lobe, preserved by immersion in a solution of 10% neutral buffered formalin, allowing for fixation to occur over a period of no less than 28 h.

The formalin-fixed lung samples were subjected to routine processing for histopathology examination involving paraffin embedding, sectioning at 4 μ m and stained with haematoxylin and eosin. Each slide was blindly examined under light microscopy by a veterinary pathologist, involving five microscopic fields at magnification 200 \times , before the severity of the histopathology lesions was scored. Score 0 represented a normal tissue, signifying unaffected tissue; score 1 represented mild severity, involving less than 25% of the tissue; score 2 denoted moderate severity, affecting between 25% and 50% of the tissue; and score 3 represented severe lesions, involving more than 50% of the tissue. At the same time, all bronchi and bronchioles were examined at 100 \times and 200 \times magnifications and their numbers, along with the number of bronchus-associated lymphoid tissue (BALT), were counted and noted before the numbers of BALT were then normalised by dividing the number of BALT by the number of bronchi.

Statistical Analysis

All data were methodically arranged into tabular formats before comparative analysis among the different groups was performed using the One-way Analysis of Variance (ANOVA) statistical method. This was followed by the implementation of Tukey's post hoc test to identify specific group differences. Additionally, cumulative severity scores for each organ and respective group were calculated, facilitating a comprehensive assessment of the overall impact. Similar comparative evaluations were conducted to analyse the cumulative severity scores across the groups. All statistical analyses were done using SPSS version 20.

Table 2
Summary of gross lung lesion scoring

Extent of the Lesion	Score
0% (normal)	0
1–24%	2
25–49%	4
\geq 50%	6

RESULTS

Adverse Effects

Between 24 and 48h post-initial vaccination, one goat from each of Groups 1, 2, 4, and 5 showed mild, transient sneezing ($p>0.05$). Similarly, a goat from Group 1 showed mild sneezing, while a goat from each of Group 2 and Group 3 showed mild, transient coughing following a booster dose on week 2 ($p>0.05$). Table 3 summarises the adverse events among goats following challenge with live *M. haemolytica*. Analyses revealed no significant ($p>0.05$) differences between all treated groups.

Respiratory Rate and Body Temperature

Table 4 summarises post-challenge respiratory rate and body temperature for the five groups of goats. Both respiratory rate and body temperature were within the normal range, although there were significant ($p<0.05$) differences in the respiratory rates of treated goats compared to control but not the body temperatures ($p>0.05$). Groups 1 and 2 showed significantly ($p<0.05$) higher, while Group 3 showed significantly ($p<0.05$) lower respiratory rates than control Group 5. Groups 1, 3, and 4 showed significantly ($p<0.05$) lower body temperature than that of control Group 5.

Table 3

Adverse events among goats following challenge with live Mannheimia haemolytica

Adverse Events	Group 1 (n=3)	Group 2 (n=3)	Group 3 (n=3)	Group 4 (n=3)	Group 5 (n=3)
Mild sneezing	2	2	1	2	2
Severe sneezing	0	1	0	0	0
Mild coughing	2	1	1	0	2
Severe coughing	0	0	0	1	0
Dullness/lethargy	1	2	1	0	0
Oronasal discharge	1	1	2	2	1
Loss of appetite	0	0	0	1	0
Depression	0	0	0	0	0
Death	0	0	0	0	0

Note. All data showed no significant differences between groups

Table 4

Average respiratory rate and body temperature following challenge of goats subjected to different treatments

Parameter	Group 1 (n=3)	Group 2 (n=3)	Group 3 (n=3)	Group 4 (n=3)	Group 5 (n=3)
Respiratory rate (breaths/min)	36.8 ± 8.4 ^c	36.2 ± 13.6 ^c	31.1 ± 5.3 ^b	34.8 ± 9.5 ^a	33.9 ± 9.3 ^a
Body temperature (°C)	38.6 ± 0.3 ^a	38.9 ± 0.3 ^b	38.6 ± 0.4 ^a	38.6 ± 0.2 ^a	38.8 ± 0.4 ^b

Note. ^{a,b} Different superscripts in the same row indicate significant differences ($p<0.05$)

Gross Pathology

The lungs of all animals in Group 1 showed consolidated and pneumonic lesions in the right apical and accessory lobes, with areas of petechiations. Cut surface revealed the presence of frothy oedema fluid. One goat showed pneumonia and mild petechiations with meaty lung consistency involving the left side of the lung. Group 2 showed acute pneumonia with a reduction in sponginess, involving the entire lungs or some parts of the lungs. A low amount of oedema fluid was observed on the cut surface. One goat of this group showed mild congestion and haemorrhage, involving the three left lung lobes. Similarly, Group 3 showed acute pneumonia with a reduction of sponginess involving the entire lungs or some parts of the lungs. The lung parenchyma of all goats showed congestion in the left cranial and caudal parts of the apical lobes, and the right apical lobe. No consolidation was noted. A low amount of oedema fluid was observed from the cut surface of all lungs, particularly in the pneumonic and congested areas.

In Group 4, only one goat showed mild oedema and acute pneumonia involving the right apical lobe. No other lesion was seen. On the other hand, all goats of Group 5 showed lesions consistent with fibrinous pneumonia. In two goats, fibrin tags were seen on the visceral pleural surface of the left cranial and caudal parts of the apical lobes, as well as the right apical lobe. The parenchyma of the left cranial and caudal parts of the apical lobes, the right apical lobe, the accessory lobe, and some parts of both caudal lobes were consolidated. The other goat showed fibrinous deposition leads to adhesion of the left cranial part of the apical lobe to the pericardium, and the right goat showed moderate consolidation and pneumonia in the middle lung lobe. These lobes were consolidated, along with the left and right caudal lobes. Congestion and oedema were observed in the lungs of all goats, involving most of the lung lobes.

Total lung lesion scoring of all treated groups was mild (range between 0.22-0.15 and 1.56 ± 0.47) with no significant difference ($p > 0.05$) between the treated groups but were significantly ($P < 0.05$) lower than the control Group 5 that showed moderate lung lesions of 3.67 ± 0.46 . Similarly, consolidation and presence of fibrin, which are among the major gross lung lesions of pneumonic mannheimiosis were significantly ($p < 0.05$) more severe in control Group 5 and were insignificant ($p > 0.05$) among the four treated groups. Acute pneumonia is another important lung lesion, which was significantly ($p < 0.05$) less severe in Groups 1, 2 and 4 than the control Group 5 (Table 5). Pulmonary congestions were extremely low in treated groups but was moderately high in control Group 5 ($p < 0.05$). Pulmonary haemorrhages (petechiation) are not a consistent lesion in pneumonic mannheimiosis, and this study revealed insignificant ($p > 0.05$) differences between all groups.

Table 5

Gross lesion scoring in the lungs of goats experimentally infected by Mannheimia haemolytica

Lesions	Group 1	Group 2	Group 3	Group 4	Group 5
Pneumonia	2.00 ± 0.00 ^b	4.00 ± 1.15 ^{a,b}	5.33 ± 0.67 ^a	0.67 ± 0.67 ^b	5.33 ± 0.67 ^a
Consolidation	1.33 ± 0.67 ^b	1.33 ± 0.67 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	4.67 ± 0.67 ^a
Congestion	0.00 ± 0.00 ^b	1.33 ± 1.76 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	4.00 ± 1.15 ^a
Fibrin	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	2.67 ± 0.67 ^a
Oedema	1.33 ± 0.67 ^b	2.00 ± 0.00 ^b	2.00 ± 0.00 ^b	0.67 ± 0.67 ^b	4.67 ± 0.67 ^a
Haemorrhage	1.33 ± 0.67 ^a	0.67 ± 0.67 ^a	0.67 ± 0.67 ^a	0.00 ± 0.00 ^a	0.67 ± 0.67 ^a
Total score	1.00 ± 0.24 ^b	1.56 ± 0.41 ^b	1.56 ± 0.47 ^b	0.22 ± 0.15 ^b	3.67 ± 0.46 ^a

Note. ^{a,b} Different superscripts in the same row indicate significant differences (p<0.05)

Histopathology Description

In this study, the histological examinations of the lungs of goats infected with *M. haemolytica* revealed several distinct lesions. Many of the bronchi of goats in Group 1 showed large-sized and activated BALT (Figure 1a). Mild to moderate bronchopneumonia was noted, characterised by a low to moderate number of lymphocytes surrounding several bronchi and bronchioles (Figure 1b), but rarely involving the neighbouring lung parenchyma. However, inflammatory reactions in the lungs were characterised by interstitial pneumonia involving alveolar macrophages, which were seen in all goats of Group 1 (Figure 1c) but affected only certain areas of the lung, causing mild to moderate thickening of the alveolar septa. Most areas of the lung parenchyma appeared normal with occasional mild atelectasis, while mild to moderate emphysema was occasionally seen in some areas of the lung sections. Pulmonary haemorrhage was mild and was seen only in one lung lobe of one goat. On the other hand, the number of lymphocytes was especially high in the wall of bronchi and bronchioles that showed the presence of BALT.

Lungs of Group 2 revealed a very low number of respiratory airways with the presence of BALT. When BALT was present, they appeared small to moderate in size (Figure 2a). Nevertheless, moderate to very severe bronchopneumonia was noted (Figure 2b), characterised by moderate to high numbers of lymphocytes found surrounding many bronchi and bronchioles, which sometimes involved the neighbouring lung parenchyma. Inflammations of the lungs were characterised only by interstitial pneumonia, causing moderate to severe thickening of the alveolar septa. Therefore, areas of normal lung parenchyma were rarely identified, with the exception of one lung lobe of one animal that showed extensive normal lung parenchyma. Atelectasis was mild, while emphysema was mild to moderate, seen in many areas of the sections. Severe pulmonary haemorrhage was seen only in one lung lobe of one goat. Pulmonary oedema (Figure 2c) and interlobular oedema were none to moderate.

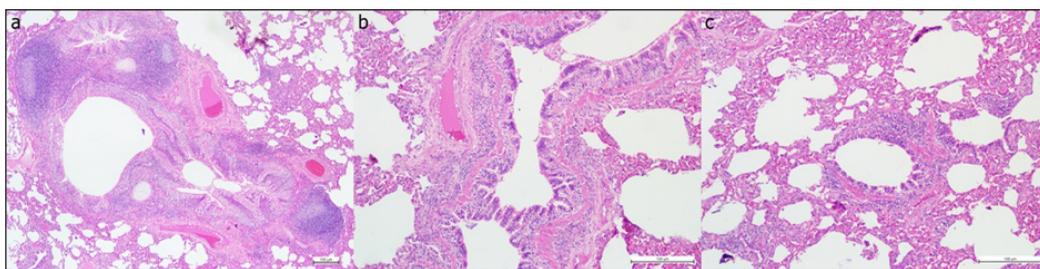


Figure 1. Examples of histopathological findings in the lungs of Group 1. a: Large BALT surrounding the respiratory airways (bar=100 μ m); b: Mild bronchopneumonia characterised by a low infiltration of lymphocytes (bar=100 μ m); c: Moderate bronchopneumonia and interstitial pneumonia (bar=100 μ m)

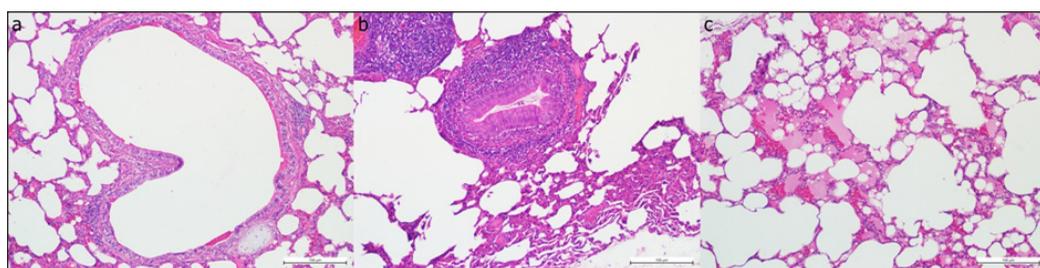


Figure 2. Examples of histopathological findings in the lungs of Group 2. a: Rare observation of an extremely small BALT (bar=100 μ m); b: Severe bronchopneumonia with emphysema in the surrounding parenchyma (bar=100 μ m); c: Mild pulmonary oedema and haemorrhage (bar=100 μ m)

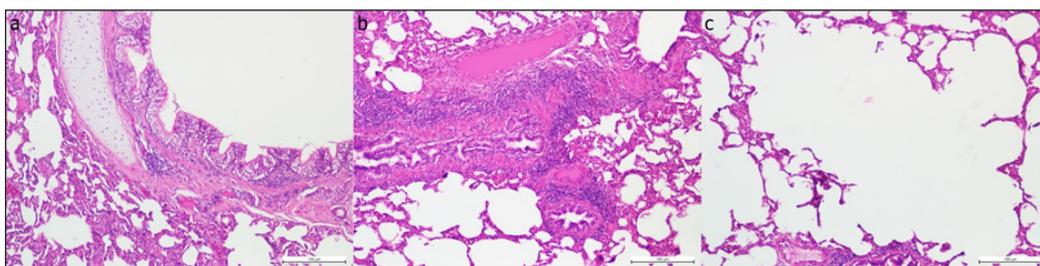


Figure 3. Examples of histopathological findings in the lungs of Group 3. a: Rare observation of a small BALT (bar=100 μ m); b: Severe bronchopneumonia (bar=100 μ m); c: An area of pulmonary emphysema (bar=100 μ m)

Lungs of Group 3 showed a low number of respiratory airways with the presence of BALT. When BALT was present, they appeared small in size (Figure 3a). Moderate to severe bronchopneumonia was apparent (Figure 3b), featuring a moderate to high influx of lymphocytes surrounding many bronchi and bronchioles, and their neighbouring lung parenchyma. However, inflammations of the lungs were characterised only by interstitial pneumonia, which was extensive, leading to moderate to severe thickening of the alveolar septa. Areas of normal lung parenchyma were occasionally identified, and emphysema was

mild to moderate (Figure 3c), seen at many areas of the sections, while atelectasis was mild. Perivascular oedema and pulmonary oedema were not observed.

The lungs of Groups 4 showed some bronchi with medium- to large-sized, activated BALT (Figure 4a). Mild to severe bronchopneumonia was noted, characterised by a low to high number of lymphocytes surrounding several bronchi and bronchioles that occasionally involved the neighbouring lung parenchyma. As Group 1, the number of lymphocytes tends to be high, especially in the wall of bronchi and bronchioles that show the presence of BALT. Inflammations of the lungs were characterised by interstitial pneumonia, which was seen in all goats of Group 4, but affected only certain areas of the lung, causing mild to moderate thickening of the alveolar septa (Figure 4b). Areas of normal lung parenchyma were frequently identified (Figure 4c), occasionally accompanied by limited regions of mild atelectasis and mild to moderate emphysema.

The lungs of Group 5 showed an extremely low number of respiratory airways, which showed the presence of BALT. When present, they were extremely small. However, broncho-pneumonia was generally severe to very severe, characterised by a high to very high number of lymphocytes surrounding several bronchi and bronchioles, and tends to involve the surrounding lung parenchyma (Figure 5a). Inflammations of the lungs were

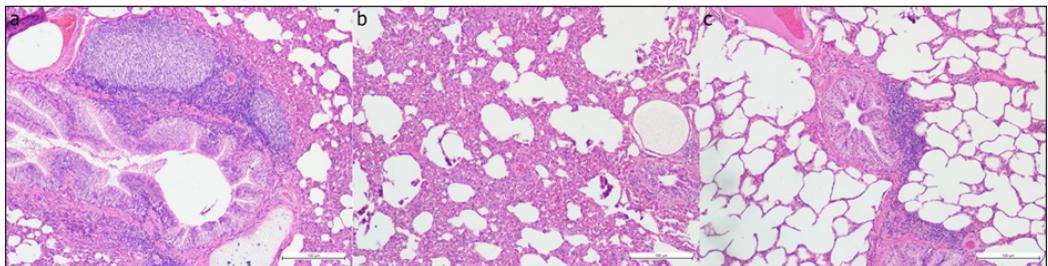


Figure 4. Examples of histopathological findings in the lungs of Group 4. a: Large-sized BALT surrounding a respiratory airway (bar=100 μ m); b: Moderately severe interstitial pneumonia (bar=100 μ m); c: Moderate bronchopneumonia, note that the surrounding parenchyma is not affected (bar=100 μ m)

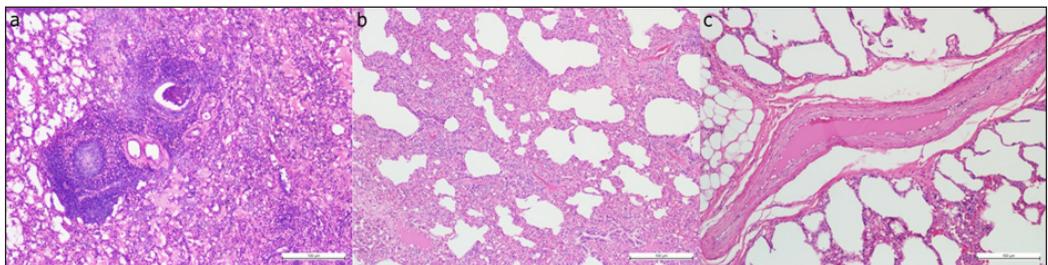


Figure 5. Examples of histopathological findings in the lungs of Group 5. a: Severe bronchopneumonia and pneumonia, accompanied by pulmonary oedema (bar=100 μ m); b: Severe interstitial pneumonia (bar=100 μ m); c: Perivascular oedema as evidenced by separation and lifting of the connective tissue surrounding the blood vessel (bar=100 μ m)

characterised by interstitial pneumonia, which was seen in all goats and affected extensive areas of the lung sections, leading to severe to very severe thickening of the alveolar septa (Figure 5b). Areas of normal lung parenchyma were only occasionally identified; emphysema was moderate to severe, while atelectasis was mild. Mild to severe pulmonary haemorrhage was observed in two goats, and mild presence of fibrin in the alveolar space and pleural surface was seen in all goats of this group. Mild pulmonary oedema was observed in one goat; pleural oedema was moderate to severe (Figure 5c), while interlobular oedema was severe. None of the groups showed significant gross and histopathology lesions in the livers and kidneys.

Histopathology Evaluation

Table 6 summarises the mean scores of lung lesions along with the standard errors of mean (SEM) for each lesion in each group. Among the lesions, bronchopneumonia, emphysema, presence of fibrin, interlobular oedema, perivascular oedema, and total lesion score displayed significant ($p < 0.05$) variations across groups. Scores for bronchopneumonia were notably high in Group 5 compared to the other groups ($p < 0.05$). Pneumonia was primarily observed as interstitial pneumonia with thickening of the alveolar septa. Despite the severity of pneumonia in Group 5, no significant difference ($p > 0.05$) was observed among all groups.

Emphysema was observed in all groups, with the most severe in Group 4 and the least severe in Group 1. In fact, emphysema was significantly less severe ($p < 0.05$) in Group 1 compared to Groups 4 and 5, but not significant ($p > 0.05$) compared to Groups 2 and 3. On the other hand, the presence of fibrin was only noted in Group 5, a significant ($p < 0.05$)

Table 6

Histopathological lung lesion scores in goats following an experimental infection by Mannheimia haemolytica

Lesions	Group 1	Group 2	Group 3	Group 4	Group 5
Bronchopneumonia	1.17 ± 0.20 ^{a,b}	1.33 ± 0.15 ^{a,b}	1.10 ± 0.16 ^{a,b}	0.97 ± 0.18 ^a	1.67 ± 0.18 ^b
Pneumonia	1.50 ± 0.22 ^a	1.53 ± 0.21 ^a	1.23 ± 0.21 ^a	1.37 ± 0.21 ^a	2.00 ± 0.21 ^a
Emphysema	0.37 ± 0.10 ^a	0.77 ± 0.14 ^{a,b}	0.70 ± 0.13 ^{a,b}	0.90 ± 0.11 ^b	0.87 ± 0.13 ^b
Atelectasis	0.20 ± 0.07 ^a	0.17 ± 0.07 ^a	0.37 ± 0.13 ^a	0.17 ± 0.10 ^a	0.57 ± 0.15 ^a
Haemorrhage	0.53 ± 0.09 ^a	0.67 ± 0.15 ^a	0.30 ± 0.10 ^a	0.33 ± 0.10 ^a	0.63 ± 0.14 ^a
Fibrin	0.00 ± 0.00 ^a	0.33 ± 0.10 ^b			
Pleural oedema	1.07 ± 0.14 ^a	1.70 ± 0.20 ^a	1.43 ± 0.19 ^a	1.50 ± 0.18 ^a	1.70 ± 0.16 ^a
Interlobular oedema	0.37 ± 0.12 ^a	0.53 ± 0.13 ^a	1.63 ± 0.19 ^b	0.63 ± 0.17 ^a	0.93 ± 0.20 ^a
Perivascular oedema	0.00 ± 0.00 ^a	0.33 ± 0.13 ^b	0.33 ± 0.12 ^b	0.20 ± 0.09 ^a	0.77 ± 0.19 ^b
Pulmonary oedema	0.00 ± 0.00 ^a	0.07 ± 0.05 ^a	0.27 ± 0.14 ^a	0.00 ± 0.00 ^a	0.30 ± 0.13 ^a
Total score	0.52 ± 0.05 ^a	0.71 ± 0.05 ^{a,b}	0.74 ± 0.06 ^b	0.61 ± 0.05 ^{a,b}	0.98 ± 0.06 ^c

Note. ^{a,b} Different superscripts in the same row indicate significant differences ($p < 0.05$)

observation compared to other groups. However, interlobular oedema was significantly more severe ($p < 0.05$) in Group 3 compared to the remaining groups, while perivascular oedema was most severe in Group 5, and was significant ($p < 0.05$) compared to Groups 1 and 4.

Ultimately, the mean total lung lesion score indicated that Group 5 exhibited the highest lesion severity score (0.98 ± 0.06), which was significant ($p < 0.05$) compared to the other groups. In contrast, Group 1 showed the lowest total lung lesion score of 0.52 ± 0.05 , significantly ($p < 0.05$) lower compared to Groups 3 and 5, but not significantly ($p > 0.05$) compared to Groups 2 and 4.

BALT Evaluation

Among the five groups, Group 1 exhibited the highest number of BALT (17.67 ± 5.55), followed by Group 4 (12.17 ± 3.74), while Group 2 showed the lowest number of BALT (5.50 ± 1.57). However, no significant difference ($p > 0.05$) was observed between all five groups. For the number of bronchi and bronchioles observed, Group 5 recorded the highest count of these respiratory airways at 50.83 ± 3.74 , while the lowest count was observed in Group 1 (34.00 ± 3.62). Similarly, no significant difference ($p > 0.05$) was noted for the number of respiratory airways evaluated. The BALT count was normalised and compared between the groups, revealing that Group 1 displayed the highest normalised BALT count (52.86 ± 13.92), followed by Group 4 (25.51 ± 8.34). No significant difference ($p < 0.05$) was observed between Groups 1 and 4. The remaining Groups 2, 4, and 5 generally exhibited low normalised BALT counts ranging between 13.92 ± 3.87 and 16.96 ± 4.46 . The normalised BALT count of Group 1 was significantly higher ($p < 0.05$) compared to Groups 2, 4, and 5 (Table 7).

Table 7
Number of BALT in the lungs of goats following an experimental infection by *Mannheimia haemolytica*

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5
No. of BALT	17.67 ± 5.55	5.50 ± 1.57	7.33 ± 2.80	12.17 ± 3.74	8.00 ± 1.41
No. of bronchi and bronchioles	34.00 ± 3.62	38.00 ± 3.93	35.33 ± 6.60	48.50 ± 4.28	50.83 ± 3.74
Normalised BALT count	52.86 ± 13.92^b	13.92 ± 3.87^a	16.96 ± 4.46^a	$25.51 \pm 8.34^{a,b}$	16.61 ± 4.01^a

Note. ^{a,b} Different superscripts in the same row indicate significant differences ($p < 0.05$)
No superscripts in the same row indicate no significant difference ($p < 0.05$) between all groups

Bacterial Isolation

Table 8 summarises the rate of bacterial isolation from the lung, liver, and kidney of goats from different groups following vaccination and subsequent challenge with live *M.*

haemolytica. The bacterium was successfully isolated from all five groups, with Group 5 exhibiting the highest percentage of isolation at 89.0%, which was significantly ($p < 0.05$) higher than Group 3 at 44.0%. Groups 1, 2 and 4 displayed similar bacterial isolation rates of 67.0%, which was not significant ($p > 0.05$) compared to Group 5.

Table 8
Rate of isolation of *Mannheimia haemolytica* from lungs, liver, and kidneys of goats of different groups

Organ	Group 1 (n = 3)	Group 2 (n = 3)	Group 3 (n = 3)	Group 4 (n = 3)	Group 5 (n = 3)
Lungs	2 (66.7%)	2 (66.7%)	2 (66.7%)	3 (100%)	2 (66.7%)
Liver	2 (66.7%)	2 (66.7%)	0 (0.0%)	1 (33.3%)	3 (100%)
Kidneys	2 (66.7%)	2 (66.7%)	1 (33.3%)	2 (66.7%)	3 (100%)
Average %	67% ^{a,b}	67% ^{a,b}	44% ^b	67% ^{a,b}	89% ^a

Note. ^{a,b} Different superscripts in the same row indicate a significant difference ($p < 0.05$)

Serum Antibody Response

Figure 6 summarises the level and pattern of antibody response by goats of different groups. Following administration of STVac7 at Week 0, there was a significant ($p < 0.05$) elevation of antibody against *M. haemolytica* in Groups 1, 3, and 4 compared to control Group 5 at Week 1. Group 2 displayed lower antibody levels and remained similar level ($p > 0.05$) as the control Group 5. At Week 2 post-vaccination, all vaccinated groups showed

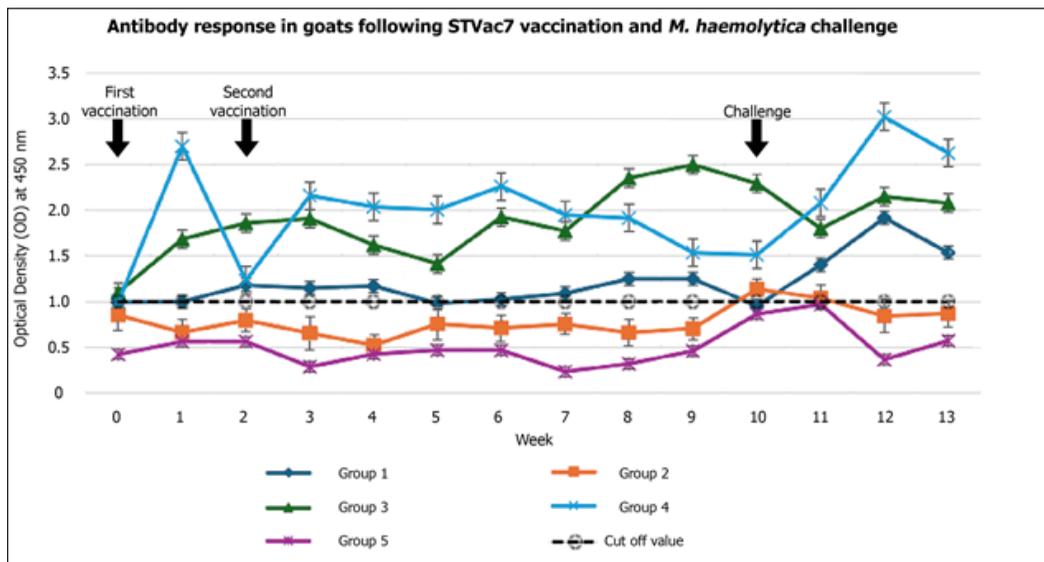


Figure 6. Antibody levels and pattern of different groups of goats following vaccination with STVac7 at weeks 0 and 2 followed by challenge with live *M. haemolytica* at week 10

significantly ($p < 0.05$) higher antibody levels than the control group. Following the booster dose at Week 2, antibody levels of all vaccinated groups remained significantly ($p < 0.05$) higher than the control Group 5 for 5 weeks post-second vaccination.

At the challenge in Week 10, all vaccinated groups showed higher antibody levels than the control Group 5. Following the challenge, antibody levels increased further in Group 3 and 4, with Group 4 showing ($p < 0.05$) higher antibody levels than all other groups for 2 weeks post-challenge. Group 3 experienced a decline in IgG levels at Week 11 post-challenge but exhibited a gradual increase in the subsequent week.

In general, all vaccinated groups showed higher antibody levels than the control group throughout the 12-week study period. Upon challenge at week 10, the antibody levels of all vaccinated groups were high, indicating protection against infection except Group 5 (Figure 6). Nevertheless, it seemed that vaccination with STVac7 containing stabilisers and preservatives was most effective in inducing a robust humoral immune response against *M. haemolytica*.

DISCUSSION

Findings from this study suggest that GMP-manufactured STVac7 is safe and effective. All groups did not show significant clinical signs following vaccination, either with freshly prepared vaccine, with 21-month-old vaccine, with a higher dose or with vaccine that contains a stabiliser and preservative. The few animals that showed mild sneezing and coughing were regarded as normal (Stockler et al., 2020), possibly due to inhaling the powder-formed concentrate supplementation given to them each morning. Furthermore, all vaccinated groups showed high antibody levels, except those vaccinated with the 21-month-old vaccine, but remained higher than the control unvaccinated throughout the study period. Therefore, at the time of challenge, the antibody levels of all four vaccinated groups were high, indicating the efficacy of these vaccine preparations. In fact, pathology findings revealed that all vaccinated groups developed various types of microscopic and macroscopic lesions, but were extremely mild compared to the control unvaccinated goats of Group 5, which showed fibrinous pneumonia involving the antero-ventral aspect of the lungs. Fibrinous pneumonia is recognised as a classical pulmonary lesion in cases of pneumonic manheimiosis (Sharma et al., 2011). Although pneumonia was noted in all vaccinated groups, it was generally mild and lacked the fibrinous component. Furthermore, pulmonary consolidation and oedema were most severe in Control Group 5. In fact, gross and histopathology could not conclude that a certain type of vaccine preparation can confer the best protection against pneumonic manheimiosis due to insignificant differences between vaccinated Groups 1, 2, 3, and 4.

Nevertheless, based on the severity scores of bronchopneumonia, emphysema, interlobular oedema and the total lung lesions, it is suggested that the freshly prepared

STVac7 vaccine containing preservative and stabiliser is the best vaccine preparation. In fact, adding 0.01% Polysorbate 80 and 2% Benzyl alcohol into the vaccine was found to produce good effects (Wahlgren et al., 2025). Polysorbate 80 is a non-ionic surfactant known for its role in stabilising protein-based biologics (Doost et al., 2018), potentially preventing antigen degradation or aggregation over time. Its inclusion may help maintain antigenic integrity and improve delivery by enhancing solubility and dispersion of the antigen particles, particularly in powder-formed vaccines. Benzyl alcohol, a commonly used antimicrobial preservative in injectable and mucosal formulations, likely contributes to the vaccine's long-term stability and sterility without compromising the immunogenicity (Ruiz et al., 2003). In this case, both freshly prepared and preservative-added vaccines resulted in high normalised BAL count and high antibody levels (Effendy et al., 1998) compared to the challenged non-vaccinated goats throughout the study period. These minimise the development of lung lesions following challenge by live *M. haemolytica*. Although a higher concentration of antigen in the vaccine resulted in a slightly higher lung lesion score and a low normalised BAL count, the antibody pattern was high and impressive.

A newly manufactured STVac7 has shown early indication of an effective vaccine following this preliminary study. However, further studies should focus on large-scale field trials to validate STVac7's efficacy under diverse farm conditions and disease challenges. Exploring the duration of immunity and need for boosters is also important for long-term protection. Optimising the formulation with stabilisers and preservatives to improve shelf-life without reducing efficacy will help advance STVac7 as a practical, scalable solution for controlling pneumonic manheimiosis in small ruminants. Therefore, STVac7 can be an alternative vaccine against pneumonic manheimiosis, especially when reports have suggested low efficacy of the currently available pneumonic manheimia vaccine in some parts of the world (Zamri et al., 2025). Furthermore, this intranasal vaccine is easy to use and user-friendly, especially for the many smallholders.

CONCLUSION

Vaccinating goats with STVac7 vaccine against pneumonic manheimiosis either the freshly prepared, the 21-month-old, the higher dose or those with stabiliser and preservative, resulted in high antibody levels and subsequent mild pathology lesions. However, the freshly prepared STVac7 vaccine containing stabiliser and preservative is the best vaccine preparation to be used against pneumonic manheimiosis. Administration of the freshly prepared vaccine resulted in the least severe macroscopic and microscopic lesions, as well as the highest normalised BAL count.

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Effect of Black Soldier Fly Larvae Frass on the Growth of Pak Choy and Selected Soil Chemical Properties

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ABSTRACT

Black soldier fly (*Hermetia illucens*) larvae frass has gained increasing attention as a potential biofertiliser due to its nutrient content and role in organic waste recycling. This study evaluated the effects of black soldier fly larvae (BSFL) frass on the growth of Pak Choy (*Brassica rapa* L.) and selected soil chemical properties under rain-shelter conditions. Five fertiliser treatments were arranged in a completely randomised design with six replicates: NPK fertiliser (15:15:15), single frass application (BF1), double frass application (BF2), combined NPK with single frass application (NBF1), and combined NPK with double frass application (NBF2). Plant growth was assessed using shoot and root fresh and dry weights, while soil pH, total nitrogen, and available phosphorus were measured after a 30-day growth period. Data were analysed using analysis of variance followed by least significant difference tests. Fertiliser treatments significantly influenced plant biomass,

soil pH, and available phosphorus, whereas soil total nitrogen did not differ significantly among treatments. The combined NPK and double frass treatment (NBF2) produced the highest shoot fresh weight, representing a 56.6% increase compared with NPK alone. Frass-only treatments resulted in biomass comparable to NPK fertiliser, indicating that BSFL frass was able to support Pak Choy growth under controlled conditions. Frass application also resulted in moderately higher soil pH, while available phosphorus declined in combined fertiliser treatments. Overall, BSFL frass shows potential as a supplementary organic fertiliser

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for short-cycle leafy vegetables; however, field-scale validation and longer-term nutrient dynamics require further investigation.

Keywords: Black soldier fly farming, circular economy, organic waste composting, sustainable agriculture, waste to wealth

INTRODUCTION

In traditional agricultural practices for reducing agricultural waste, most farmers follow the decomposition procedures of burying waste back into the soil. This procedure can be called composting, as it reduces waste into nutrient-rich composts with the help of soil microbes or other decomposing agents. One of the most popular composting methods is through vermicomposting, with the help of earthworms (*Eisenia fetida*). This process of converting food waste to organic fertiliser through decomposers is one of the most practical cultures used in agriculture.

Another type of composting that has emerged in agriculture recently is BSFL compost. The larvae are from the black soldier fly (*Hermetia illucens*) (Diptera: Stratiomyidae), a decomposing agent alternative to traditional decomposition processes. Black Soldier Fly Larvae (BSFL) is one of the best insects for bioconversion processes. The larvae have an extremely voracious appetite and can consume a wide range of organic waste (Alattar et al., 2016), including food waste, crop straw (Gao et al., 2019) and animal manure (T. Liu et al., 2019; Z. Liu et al., 2018). The BSFL have received extensive attention due to their high levels of lipids and proteins.

The production of frass from BSFL also contributes much more to crop production improvements in yield and growth rates. Black Soldier Fly Larvae frass is rich in organic matter, which can help increase soil fertility and encourage microbial proliferation in soil (Zahn, 2017). Microorganisms break down organic matter to enrich the surrounding soil with nutrients the plants can use. With BSFL frass, fertiliser usage such as NPK fertiliser can be reduced (Schmitt & de Vries, 2020). Therefore, this helps to save costs and reduce the negative environmental impact.

Many research studies focus on pointing out the bioconversion performances of BSFL. However, few studies or articles have evaluated the effects of the BSFL frass in terms of nutrient values and the growth performance of plants. Synthetic fertilisers such as NPK fertiliser or organic fertilisers such as livestock waste products, can be utilised easily to improve soil quality and plant growth performance. However, there are some drawbacks to using these types of fertilisers. If synthetic fertiliser is used excessively, farmers will risk the excessive loss of nutrients when applying it to their crops (Sabry, 2015). Besides that, using livestock waste products as fertilisers could also potentially pose problems to crops when applied more than usual. For example, the high nitrogen content in the manure can cause nutrient toxicity to crops if not properly composted. Moreover, using BSFL frass as

a fertiliser is relatively new in the agriculture industry, and not many research institutes and universities focus particularly on this product. Therefore, this study was initiated to evaluate the fertilising performance of BSFL frass on the yield of Pak Choy and selected chemical properties in the soil compared with synthetic fertiliser NPK (15:15:15) application.

METHODOLOGY

Experimental Sites

The project was initiated and performed in three areas within the research field: the establishment of the Black Soldier Fly and production of frass inside the poultry facility (Figure 1), the establishment of Pak Choy plants was done for one planting season under the rain shelter, and the laboratory was used for ascertaining the plant growth performance and soil chemical properties.

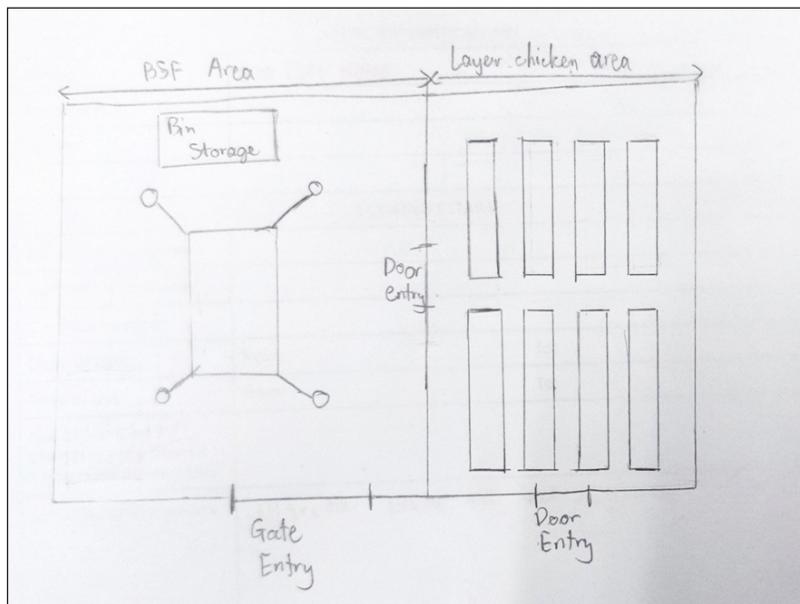


Figure 1. Illustration of BSFL establishment

Frass Production and Characterisation

The BSFL cage was built, and the adult flies were introduced into the cage. The modified egg trap for the flies to lay eggs was also constructed to collect and reproduce the larvae efficiently. There were also two plastic container boxes prepared for the larvae that functioned as the feed supplement area and the collection of frass, which was separated after the pupation stage of the flies and then transferred to another container and left to dry for a week in a dry and cool area to remove the moisture. The feeds supplied were food waste

from the cafeteria, fresh poultry manure, and crop waste (e.g., Kangkung). The rate of feed given was on a consistent organic feedstock mixture throughout the production period.

During the larvae stage, monitoring of the cage for prevention of infestation of other fly species laying eggs onto the feed besides adequate resupplying of feed, monitoring of escaped prepupae from the feed box and control of moisture and temperature by spraying mists of water into the cage were done daily, although moisture control was less required since the climate of Malaysia is hot and humid (Temperature range: 24-32°C; Relative humidity: 80-90%) (Orangutan Appeal UK, n.d), which provides optimal conditions for survivability and reproduction of larvae of BSF.

Fresh BSFL frass contained high initial moisture content typical of insect-derived residues. Prior to application, frass was air-dried under shaded, well-ventilated conditions for seven days until a stable, friable texture was obtained. The moisture content of the dried frass was approximately 25-30%, as estimated gravimetrically based on weight loss during the drying period. This drying step was conducted to reduce variability associated with excess moisture, improve handling consistency, and standardise application across treatments. All frass used in the experiment originated from a single production batch and was applied on a fresh weight basis.

Soil Preparation and Baseline Characterisation

Topsoil and Silabukan soils were collected from the university research farm, air-dried, and sieved through a 2-mm mesh to remove debris and ensure uniform particle size. The soils were thoroughly mixed in bulk prior to filling polybags to ensure homogeneity. Baseline soil chemical properties, including pH, total nitrogen, and available phosphorus, were analysed before treatment application and are presented in Table 2. Soil texture was determined using the Munsell Colour System and classified as sandy clay loam.

Planting and Crop Management

Curly Dwarf Pak Choy (*Brassica rapa* L.) seedlings were transplanted into polybags and grown for 30 days (FAO, 2024). All treatments received irrigation from the same water source at equal volumes throughout the experimental period to avoid differential nutrient inputs. Environmental conditions within the rain shelter, including temperature and relative humidity, were monitored regularly. Polybags were spaced 5cm apart to prevent nutrient cross-contamination through drainage.

Experimental Design and Treatments

Polybags (16" diameter × 16" height) were arranged on a uniform concrete surface to reduce spatial variability in soil moisture and nutrient movement. Based on this uniformity, a completely randomised design (CRD) was adopted. Five fertiliser treatments were

evaluated with six replicates per treatment. The treatments consist of NPK= NPK (15:15:15) fertiliser (NPK); BF1= one time BSFL Frass application (BF1); BF2= two times BSFL Frass application (BF2); NBF1= NPK (15:15:15) fertiliser + one time BSFL frass (NBF1); and NBF2= NPK (15:15:15) fertiliser + two time BSFL frass (NBF2). Frass application rates were selected based on ranges reported in previous BSFL frass studies and were intended to evaluate agronomically relevant responses rather than strict nutrient equivalence with NPK fertiliser, in addition of relevant information from Jabatan Pertanian Semanjung Malaysia (1998). The descriptions of each treatment are shown in Table 1.

Table 1
Description of the treatments used in the experiment

Treatment ID	Description	Rate (t ha ⁻¹)	Amount Per Polybag (g)
NPK (Control)	Conventional application of NPK (15:15:15) fertiliser	0.6	3
BF1 (Frass)	One Time (1x) BSFL Frass application	2.5	12.5
BF2 (Frass)	Two Times (2x) BSFL Frass application	5	25
NBF1 (NPK + Frass)	NPK (15:15:15) fertiliser + 1x BSFL frass	0.6 (NPK) + 2.5 (frass)	3 + 12.5
NBF2 (NPK + Frass)	NPK (15:15:15) fertiliser + 2x BSFL frass	0.6 (NPK) + 5 (frass)	3 + 25

*Source: Jabatan Pertanian Semanjung Malaysia (1998)

Frass Characterisation, Plant Growth, and Soil Analysis

Three types of parameters are measured in this experiment: (i) frass characterisation on total N and available P values, (ii) growth of Pak Choy, and (iii) selected soil chemical properties. Frass was collected after larval pupation, air-dried under shaded conditions for seven days to stabilise moisture content, and stored in sealed containers prior to application. All frass applied in the experiment originated from a single production batch to minimise variability in nutrient composition. Frass characterisation focussed on total nitrogen and available phosphorus, and potassium analysis was not conducted due to analytical constraints.

The parameters of plant growth measured are shoot fresh weight and dry weight, root fresh weight and dry weight and plant height. Plant height was measured every 10 days after the transplant. Fresh weight and dry weight of Pak Choy shoots and roots were measured with an electronic balance during 30 DAP, and the samples were transferred to the lab after being harvested. The results were recorded for dry weight after drying in the oven at 80 °C for 24 hours.

The soils were sampled before planting and during the harvesting of the Pak Choy. The soils were air-dried, ground with a mortar and pestle and sieved through a 2 mm sized

sieve. The ground samples were prepared for chemical analyses. Soil pH was analysed by two methods: (i) 1:2.5 (soil: distilled water) and (ii) 1:2.5 (soil: 0.01 M CaCl_2), then shaken with a rotary shaker at 180 rpm for 15 minutes, and then left for the suspension to settle for 1 day. Total nitrogen content was analysed by putting 2 g of soil in a CHN analyser. Soil available P was determined using the colourimetric method (Murphy & Riley, 1962). The P extraction solution was prepared by mixing 4.3 mL HCl and 0.7 mL H_2SO_4 in a 1 L volumetric flask and adding distilled water to the final volume; the reagent A was prepared by mixing 6 g ammonium molybdate and 74 mL H_2SO_4 solution in 500 mL and left cooled before added with 0.1454 g potassium antimony tartrate dissolved in 200 mL water, transferred to 1L volumetric flask and brought to volume with distilled water; and Reagent B was prepared by mixing 1.32 g of ascorbic acid with 250 mL reagent A. Calibration procedure was done by preparing potassium dihydrogen phosphate (KH_2PO_4) as P standard. A small amount of KH_2PO_4 was dried in an oven at 105 °C for 1 hour and left to cool in a desiccator before being prepared in 4.3935 g and mixed with distilled water in a 1 L volumetric flask at 1000 ppm concentration. Next, a 5 ppm KH_2PO_4 solution was prepared by diluting 5 mL of 1000 ppm KH_2PO_4 solution in another 1L volumetric flask. Finally, 5 different concentrations of solutions (0, 0.1, 0.2, 0.3, 0.4, and 0.5 ppm) in 50 mL volumetric flasks were prepared from a 5 ppm KH_2PO_4 solution and added with 8 mL reagent B before adding distilled water to the final volume. They were left for 15 minutes and read in a spectrophotometer at 882 nm for a standard curve. The extraction procedure was done by adding a 5 g soil sample and 20 mL extraction reagent in a plastic vial and shaking with 180 rpm in a rotary shaker for 10 minutes, then filtering in another vial to obtain supernatant. 2.5 mL supernatant was transferred to a 25 mL volumetric flask and mixed with 8 mL reagent B before adding distilled water to the final volume. The final solution was shaken and left for 15 minutes. The solution was read in a spectrophotometer at 882 nm for a standard curve, and the data was recorded.

Data Collection and Statistical Analysis

Plant height and shoot fresh weight were recorded at harvest. Soil samples were collected after harvest for analysis of pH, total nitrogen, and available phosphorus. Data were analysed using one-way analysis of variance (ANOVA). Normality and homogeneity of variance were verified using Shapiro-Wilk and Levene's tests, respectively. Mean separation was performed using the least significant difference (LSD) test following significant ANOVA results. Plants that were completely destroyed by pest damage were excluded from analysis using listwise deletion (< 5%), and degrees of freedom were adjusted accordingly. Statistical Analysis Software (SAS) version 9.4 was used to analyse the data collected.

RESULTS

Total N and Available P of Frass

Table 2 shows the nutrient content of BSFL Frass produced. Total nitrogen was 3.16%, and available phosphorus was 124 mg L⁻¹.

Table 2

Black soldier fly larvae frass nitrogen and phosphorus composition

Total Nitrogen (%)	Available Phosphorus (mg L ⁻¹)
3.16	124

Plant Growth

Shoot Fresh Weight and Dry Weight

Shoot fresh weight ($p = 0.020$) (Figure 2) and dry weight ($p = 0.045$) (Figure 3) of Pak Choy were significantly affected by fertiliser treatment. The combined application of NPK fertiliser with double-rate BSFL frass (NBF2) produced the highest shoot fresh weight among all treatments, at 19.44 g for shoot fresh weight and 1.69 g for shoot dry weight. Frass-only treatments (BF1 and BF2) did not differ significantly from the NPK-only treatment, indicating comparable biomass production under these treatments. Single-rate combined application (NBF1) resulted in intermediate shoot fresh weight values.

Root Fresh Weight and Dry Weight

The root samples also showed a similar trend as the shoot samples in both fresh weight ($p = 0.01$) (Figure 4) and dry weight ($p = 0.038$) (Figure 5). The data distribution's highest and lowest mean value was by NBF2 and BF2, at 1.024 g and 0.44 g for root fresh weight and 0.16 g and 0.088 g for root dry weight, respectively. Frass-only treatments resulted in comparable to NPK fertiliser, which shows there is no significance for interaction between NPK and all treatments.

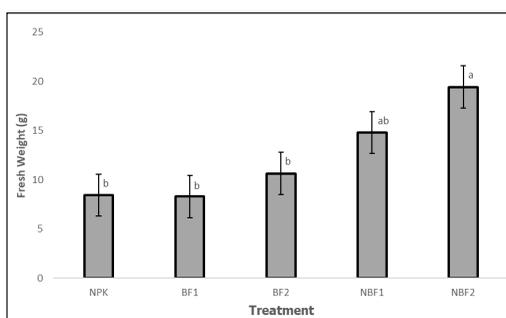


Figure 2. Effect of treatments on shoot fresh weight. Bars represent mean \pm standard error (SE)

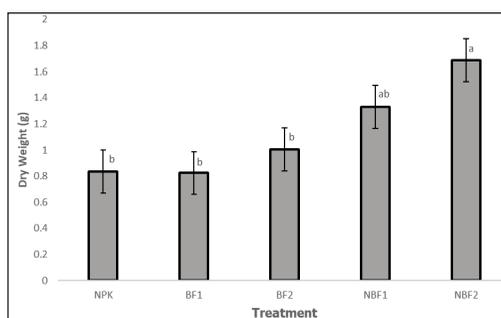


Figure 3. Effect of treatments on shoot dry weight. Bars represent mean \pm standard error (SE)

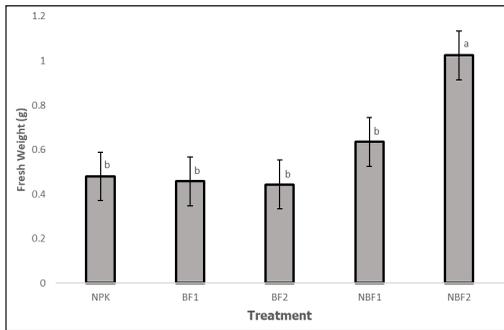


Figure 4. Effect of treatments on root fresh weight. Bars represent mean \pm standard error (SE)

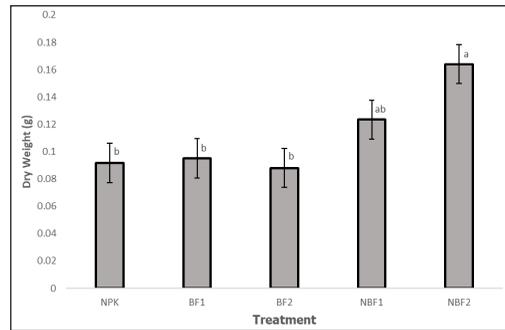


Figure 5. Effect of treatments on root dry weight. Bars represent mean \pm standard error (SE)

Plant Height

Plant height recorded throughout the growth stages (10, 20, 30 DAP) showed an increasing effect for treatments BF1, BF2, NBF1 and NBF2, compared to the decreasing trend from NPK treatment (Figure 6). In comparison, with the effects of 1 missing data value from BF2 and NBF2 each, they showed higher growth values than the other treatments in each treatment category (frass treatment and mixture treatment, respectively). Besides that, only BF2 displayed the highest value at 30 DAP at 9.04 cm with the effect of one missing value, while the lowest value at 30 DAP comes from NBF1, at 7.82 cm. Overall, the treatments BF1, BF2, NBF1 and NBF2 showed better growth results than the control treatments, and the doubled application rate of treatments BF2 and NBF2 showed an increase with a significant effect on plant height than BF1 and NBF1 treatments.

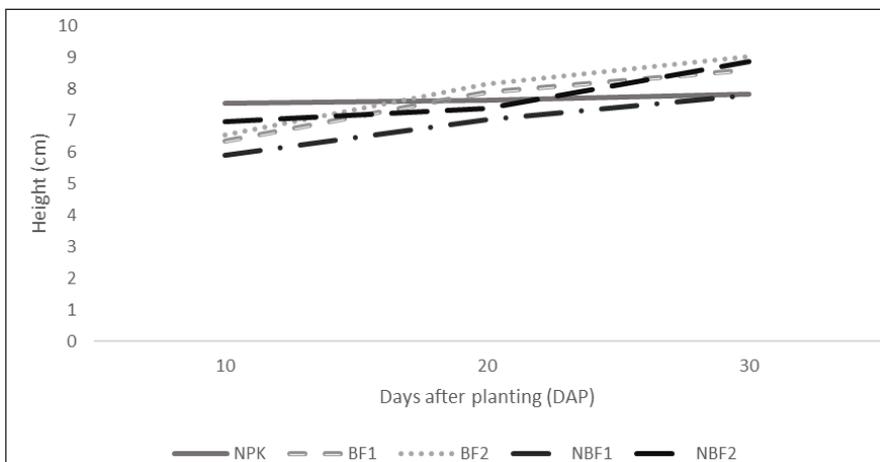


Figure 6. Effect of treatments on plant height

Pretreated Soil Chemical Properties

The recorded nutrient values of pretreated soil are shown in Table 3. pH values on water and the calcium chloride method were at 6.47 and 6.35, respectively. Total N content in soil was at 0.98%, and P available in soil was recorded at 1.88 mg L⁻¹.

Table 3

Nutrient values of pretreated soil

Soil parameter	Value
pH H ₂ O	6.47
pH CaCl ₂	6.35
Total N	0.98%
Available P	1.88 mg L ⁻¹

Soil pH

The pH readings showed that the soil from treatments BF1 to NBF2 was slightly alkaline at pH 8.00 for the H₂O method (Figure 7) and almost neutral (pH 7.00) for the CaCl₂ method (Figure 8), generally. Significant differences were shown in mean values among the four treatments in relation to the control NPK for both methods ($p < 0.0001$). Besides that, results from both methods also showed some similarities in comparison. Firstly, both results showed that the mean pH values from the control treatment NPK had the lowest values compared to the other four treatments. Secondly, the mixture of NPK fertiliser and BSFL frass from NBF2 showed the highest mean pH value among the treatments. However, the level of significance between BF1, BF2, NBF1 and NBF2 is very low, indicating pH of the soil level has little influence on these types of treatments.

There were some differences in the results shown by both methods. First, BF2 showed the second-highest mean value using the H₂O method, at 8.13. Meanwhile, NBF1 showed the second-highest mean pH value when measured by CaCl₂ at 7.23, while the second-lowest mean value was shown at 8.05 when measured by H₂O.

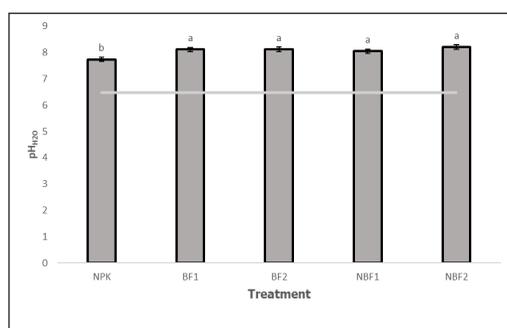


Figure 7. Effect of treatments on soil pH using the H₂O method. Bars represent mean \pm standard error (SE)

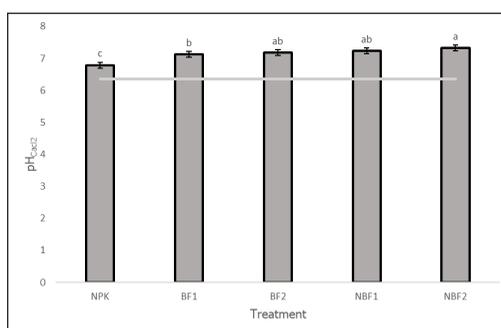


Figure 8. Effect of treatments on soil pH using the CaCl₂ method. Bars represent mean \pm standard error (SE)

Soil Total N and Available P

The mean values of total nitrogen content for the five different treatments were generally at approximately 1.00%, with the controlled treatment of frass fertiliser at BF1, while the mixture of NPK fertiliser and BSFL Frass (NBF2) resulted in very low mean values of 1.02% and 1.03%, respectively (Figure 9). The results showed no significant differences ($p = 0.566$) in total nitrogen content in the soil for the different treatments. Overall, doubled treatment of BSFL Frass at NBF1 showed the highest mean value of total N at 1.06%, and single treatment of BSFL Frass at BF1 showed the lowest total N content at 1.02%.

Phosphorus content available in soil generally decreases gradually from treatments NPK to NBF2 (Figure 10). There were significant differences in the data distribution among the 4 treatments in relation to the control treatment ($p < 0.0001$). Phosphorus available content from the control treatment NPK provided the highest mean value amongst the treatments at 11.06 mg L^{-1} . On the other hand, both frass treatments, BF1 and BF2, provided higher mean values than both mixtures of NPK fertiliser and frass treatments, NBF1 and NBF2, with BF1 being higher than BF2. In comparison, frass treatment BF1 showed a higher mean value than BF2, with a difference of 1.29 mg L^{-1} , while mixture treatment NBF2 showing higher value than NBF1, with a difference of 0.28 mg L^{-1} . The interaction within frass-only and mixed-fertiliser treatment groups shows no significance.

Additional Observations

There were some observations made during the growth stage of the Pak Choy as well. During the second week of the growth stage, the Pak Choy samples were growing variably for each treatment. Some samples were significantly larger in size, notably from treatments NBF1 and NBF2, which were the largest amongst all samples. Despite that, all samples from the five treatments showed growth during the growing period. Comparatively, treatments BF1 to NBF2 showed more significant growth in relation to the control treatment NPK in terms of growing size and plant damage scale that was caused by pest attacks and scorching.

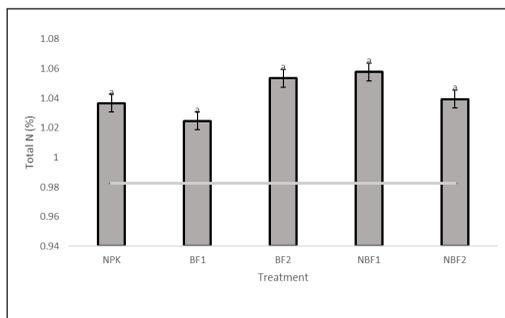


Figure 9. Effect of treatments on soil total nitrogen. Bars represent mean \pm standard error (SE)

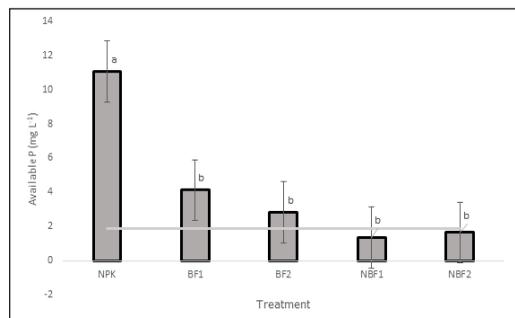


Figure 10. Effect of treatments on soil available phosphorus. Bars represent mean \pm standard error (SE)

There were visible types of pests inflicting damage. At the earlier stage, the Pak Choy samples were damaged variably from less to more by leafminers and Giant African snails (natively known as 'siput babi'), with Giant African snails appearing the most. With application of the homemade natural pest repellent and chitin obtained from carcasses of the adult BSF and pupa skin, the occurrence of leafminers was reduced to none the following week and the appearance of Giant African snails on the leaf surface of the samples. During the pest attack period, the samples from treatments BF1 to NBF2 had fewer pest attack occurrences compared to the control treatment NPK. From all 6 Pak Choy samples, 5 of the samples from Treatment 1 were damaged by pest attack, notably the worst from replicates 3 and 4, but were still intact with leaves remaining on the plants. Treatment 2 and 4 samples had less damage scale overall, with only partial damage caused by pests on BF1-R3 and NBF1-R4, while no visible damage was observed on the other samples. For treatment 3 and 5, 1 of 6 samples was damaged severely and was completely devoured by the pests, which explains the existence of missing data values for plant fresh weight, dry weight and final plant height, as well as damage intensity caused by the snails.

DISCUSSION

Frass Nutrient Properties

The nitrogen and phosphorus content of BSFL frass was shown to be a total N of 3.16% and an available P content of 124 mg L⁻¹. These nutrients provide a minimum supply for plant nutrient uptake to achieve positive growth. However, in reference to the article from Temple et al. (2013), the total N value of the frass was recorded at 4.54%, which is much different from the results shown, and no information on available phosphorus was found. The feasible explanation for these resulting values is that these products depend on the feeding value of the larvae, which is affected more by the protein content in the feed due to the larvae's requirement for protein to grow and enter the pupate stage for the metamorphosis process into adult flies.

Most insect substrates contain high soluble nitrogen content that can alter plant nutrient uptake, soil N availability, and influence growth positively, and BSFL frass is no exception. A study by Kagata and Oghushi (2012) stated that applying the insect N-rich frass on potted *B. rapa* samples resulted in a higher plant intake of inorganic N than that of insect-poor N frass. Besides that, the soil amendment performance of BSFL frass has proven similar to that of chicken manure. Therefore, it has been proven that BSFL frass has the potential for smallholders to replace livestock manure as a fertiliser treatment for crops since the frass can improve overall farm productivity (Quilliam et al., 2020).

Despite the comparison, various NPK values of frass from various production facilities in different countries exist. This can be due to the effects of the quality of feeding materials, some possible external factors such as temperature, humidity, and light intensity on the

reproduction rate of the adult flies, and management methods. Nevertheless, the nutrient values of the frass proved to be good for the fertilisation of crops, similar to the application of organic fertilisers in addition to the provision of inducing defensive mechanism triggers in the plants, whereby most of the organic fertilisers used by farmers do not contain such beneficial properties.

Besides that, BSFL frass was also observed to provide defensive properties for the plants to combat pest and pathogen attack, based on the additional observations made for treatments NPK to NBF2 that had included application of frass, which were observed to have a significant decrease in pest attack occurrence during the second week of growth stage. This is because the BSFL frass contains chitin, the component of insect cuticles that triggers the defensive response in the plants when applied, preventing the plants from being damaged by pest and disease attacks. Chitin content in the frass can be produced by harvesting a composting substrate from the BSF breeding area that contains moults of the larvae and the pupae cases (Quilliam et al., 2020). This provides more improvements in promoting plant growth without transmission of pathogens and pest attack with the BSFL frass (Choi & Hassanzadeh, 2019).

Plant Growth

Overall, Pak Choy's growth performance had an interesting outcome. Despite having lower NPK values of frass content and mixture content in the soil, the samples with frass and mixture fertiliser treatments showed more positive growth than the control treatment of NPK fertiliser application under controlled rainshelter conditions. NBF2 provided a better outcome due to the abundant nutrient supply for plant growth from the combination of frass and NPK fertiliser that increased the plant biomass of the Pak Choy. This may be due to the increase in N content in the leaf of Pak Choy in response to the increasing amount of frass, as soluble organic N from the application of frass was readily available for the plants (Kagata & Oghushi, 2012). Similar outcomes were also shown for the root fresh weight and dry weight of the Pak Choy samples, and explanations of the differences in the root fresh weight and dry weight.

Next, on plant height, the Pak Choy samples from BF2 had the highest values amongst the other samples, which provided a good assumption that the growth is affected positively more by the frass than the NPK fertiliser application. This means that BSF frass has the ability to provide more soluble N, P, and K in soil for plants' inorganic nutrient uptake to support growth compared to the NPK fertiliser. Comparatively, the mixture of frass and NPK fertiliser in treatments from NBF1 and NBF2 had a higher effect on the plant height than the frass treatments in BF1 and BF2 due to the combination of both fertilisers. Some studies agreed that frass can affect crops' growth more significantly than commercial synthetic fertilisers, such as NPK (15:15:15) fertiliser (Beesigamukama et al., 2020; Quilliam et al., 2020).

The results demonstrate that BSFL frass was able to support Pak Choy growth comparable to conventional NPK fertiliser under controlled rain-shelter conditions. Similar biomass production between frass-only and NPK treatments suggests that frass provided sufficient nutrients to sustain short-cycle leafy vegetable growth. The enhanced biomass observed under combined frass and NPK treatments indicates a complementary effect between organic and inorganic nutrient sources.

Soil Selected Chemical Properties

The pH values of the soil samples from treatments BF1 to NBF2 lean towards slightly more alkaline for the H₂O method and neutral for the CaCl₂ method, relative to the control treatment, which had slightly lower pH values, despite leaning towards neutral for both methods. The only feasible explanation for the occurrence is that BSFL frass has the ability to regulate soil pH to reach a neutral level, enabling more positive growth for the Pak Choy plants than the NPK fertiliser. The pH is one of the important factors influencing the provision of essential nutrients, which is the amount of essential nutrients available in the soluble soil. It also affects the root's ability for nutrient uptake. If the pH is too high (alkaline) or too low (acidic), there will be adverse effects on the nutrient absorption by the plants, and the soil will not be compatible for the plants to survive.

Nitrogen content in soil from treatments BF1 to NBF2 was shown to have more variability in total nitrogen content. For treatments BF1 and BF2, there is a notable increase in total nitrogen content with increasing application rates, which means that doubled application provided a better nitrogen supply for plant growth, including inorganic N absorption by roots. NBF1 was shown to have the highest total nitrogen content, which explains that the mixture provided the best nitrogen supply outcome for the soil in comparison with the other 4 treatments. Since there were no significant differences between the 5 treatments ($p > 0.05$), soil nitrogen supply is not generally affected by any fertiliser application, either NPK fertiliser, frass or a mixture of frass and NPK fertiliser. The pretreated soil total nitrogen (TN) value of 0.98% appears relatively high compared with mineral agricultural soils; however, this value reflects total nitrogen associated with a topsoil-based potting medium rather than plant-available nitrogen. The soil used in this study was collected from surface horizons enriched with organic matter, where nitrogen is predominantly present in organic forms bound within soil organic matter and microbial biomass. In pot experiments, soil is confined within a limited volume and is not subject to leaching losses, which can further concentrate organic nitrogen pools relative to field conditions. In addition, the reported TN value represents bulk nitrogen content and does not directly indicate nitrogen availability to plants, which depends on mineralisation dynamics rather than total concentration. The absence of significant differences in soil total nitrogen among treatments after harvest supports the interpretation that plant growth responses were

driven by nutrient availability dynamics and soil chemical conditions rather than initial bulk nitrogen levels.

Available phosphorus content in soil for treatments from BF1 and BF2 had higher values than NBF1 and NBF2. NBF1 and NBF2 recorded lower values due to the inclusion of frass that controls the P content in the soil, and the NPK fertiliser has a higher P_2O_5 value, which is at 15%. Despite such comparisons, the frass treatment was proven to have phosphorus content in the soil. Besides that, the recorded values of lower P content for BF1 to NBF2 compared to the control treatment NPK were shown to be irrelevant in comparison to studies by Temple et al. (2013) that displayed a positive response to an increase in soil P levels by increasing frass treatments for pak choy plants. The results could be correlated to the differences in soil status in comparison to this study, where in the field site from the research of Temple et al. (2013), the soil was classified as Humic Gleysol, which was clayey and had a high waterlogging status, while the soil from this study had high organic matter content due to topsoil included, therefore influencing the P content in the soil. Other explanations could be related to the influence of pH caused by frass input from BF1 and BF2, and mixture fertiliser input from NBF1 and NBF2 that altered the P content in the soil. With the regulation effect on pH in soil by the frass treatment and mixture treatment, the soil developed a nutrient supply for plants in high amounts, which has potential for the moderation of available P in soil. Since no other studies provided a sufficient and clear explanation of the soil P effects, both seemed most feasible concerning the results obtained.

Generally, the mixture of frass and NPK fertiliser was shown to perform better in nutrient availability in the soil than in the control and frass treatments. Despite such results, the frass treatment alone performed well regarding nitrogen and phosphorus availability in the soil due to its ability to regulate nutrient availability. The explanation above correlates with the discussion by researchers from Klammsteiner et al. (2020), Beesigamukama et al. (2020) and Temple et al. (2013).

Frass Application Rate Recommendations and Other Possible Mixture Combinations

The doubled application rate of treatment of frass from BF2 provided the most significant total nitrogen content compared to the single application rate of treatment of frass from BF1 based on the results combined, and therefore, theoretically, the crops should be applied frass at doubled rates to achieve better growth and yield. The combined fertiliser application also improved plant growth and yield outcomes, with increasing application rates providing better plant growth positivity. With reference to findings on plant growth performance by Beesigamukama et al. (2020), Zahn (2017) and Temple et al. (2013), it is accepted that increasing the application rate of BSFL frass can promote higher growth performance and yield.

The mixture treatment of frass and NPK fertiliser was also shown to have very positive results on soil nutrients and plant growth performance. Similar to the frass treatment group,

the doubled application rate of NBF2 showed better overall performance than NBF1 based on the overall results, which provides a concrete assumption that crops should be fertilised at twice the application rate for the mixture type. With the increasing application rate only from frass and the addition of inorganic fertiliser at a constant rate, both types of fertiliser tend to provide a higher improvement boost of soil nutrient supply and plant growth compared to solely frass application because of the addition of nutrient supply from the NPK fertiliser that plays a supporting role in the improvement of soil nutrient supply and plant growth while frass has the additional function of improving plant defence properties against invasion of pests and diseases.

However, for farmers who emphasise consideration of cost effectiveness and optimum frass application rates relative to income, application rates as low as 2.5 t ha⁻¹ could provide viable plant growth and yield for many farmers, especially for smallholders. The compilation of results above from pak choy yield value and soil nutrient aspects proved that 2.5 t ha⁻¹ of frass application rate offer similar outcome to NPK fertiliser application, considering the cost of buying NPK fertilisers and targeted nutrient improvement in soil that are the main concerns for many farmers. Therefore, frass can be utilised as an alternative to synthetic fertilisers due to its fertilisation value and accessibility.

Role of Microbial and Non-nutrient Effects

Microbial-mediated growth promotion and chitin-induced plant responses were not directly measured in this study. Although previous studies have reported such effects for insect frass, the present findings cannot distinguish nutrient effects from microbial or biochemical contributions. Therefore, these mechanisms are discussed as potential explanations supported by literature rather than experimentally verified outcomes.

Pest-related Observations

Reduced pest damage was observed in some frass-amended treatments; however, pest incidence was not quantified statistically and organic pest repellents were applied during the experiment. Consequently, pest-related observations are descriptive and should not be interpreted as causal effects of frass application.

Agronomic, Ecological, and Policy Context

From an agronomic perspective, the findings suggest that BSFL Frass can be used as an alternative nutrient source for leafy vegetable production under controlled conditions.

Research Limitations and Future Research

Although the overall results displayed positive feedback on the use of BSFL frass, the results are still lacking in terms of information on the usage rate and nutrient properties

of frass due to limitations, such as time constraints, unavailability of selected laboratory equipment and chemicals required for analysis of frass and soil, and lack of references to global research articles related to this field. This project was initiated based on recent information from recent journal articles, and there was no background information or any base studies regarding Black Soldier Fly at our university. Hence, the research findings were limited to pH, total N, and available P in frass and soil; plant growth, shoot and root dry weight, fresh weight, and plant height. Research constraints and lack of analytical resources are also the factors that prevented potassium dynamics, microbial properties, leaf nutrient concentrations, and biosafety parameters were not assessed.

There were also some unprecedented events and mistakes during the project period. The project was also done over a lengthy duration, which took approximately 8 months from the beginning to the end, as the frass obtained from the breeding area took 3 months to produce, and the planting process took 5 months due to some mistakes made, such as usage of soil for plants, invalidated planting pots for the planting process, and crops being damaged intensively by pests during the growing period. The first mistake was using soil, where Silabukan soil was applied first, without knowing that the soil texture was not suitable for the plants used in this project. The solution for the soil problem was to mix topsoil with silabukan soil at a ratio of 3:1, as recommended by my supervisor. The second problem was that the planting pots were not suitable, as the previous pots used were plastic bottles, which could cause problems with the overall root and shoot growth of Pak Choy samples, resulting in the use of polybags as a solution. The last mistake was not caring for the plant samples well enough, resulting in damage to leaves caused by pests and pathogens. The replanting process was done two times: the first time planting on a soil mixture and without the application of homemade natural pest repellent and application of chitin, and the second time with over-application of pest repellent, letting the plants be exposed to the sun, which caused scorching damage to the plants. These discrepancies must be addressed to obtain more accurate results and provide reliable outcomes by other researchers. Future studies should incorporate multi-cycle field trials, comprehensive nutrient profiling, microbial analyses, and economic evaluation to better assess the fertiliser substitution potential of BSFL frass.

CONCLUSION

This study demonstrated that BSFL frass influenced Pak Choy growth and selected soil chemical properties under rainshelter conditions. Mixed NPK fertiliser and BSFL frass treatment (NBF2) resulted in the highest plant biomass, while frass-only treatments produced growth responses comparable to conventional NPK fertiliser. These results indicate that BSFL frass can contribute to nutrient supply for short-cycle leafy vegetables without hindering plant growth.

Soil pH was moderately increased following frass application, whereas no significant difference among treatments was found in soil total nitrogen, suggesting that growth responses may be influenced by factors beyond bulk soil nutrient concentrations. Changes in available phosphorus highlight the complexity of nutrient interactions when organic and inorganic fertilisers are combined.

Although BSFL frass shows potential as a supplementary organic fertiliser, conclusions regarding fertiliser substitution, pest suppression, and nutrient regulation should be interpreted cautiously due to the pot-based experimental design, single cropping cycle, and limited soil chemical measurements. Further research incorporating field trials, multiple cropping cycles, and comprehensive nutrient and microbial analyses is necessary before broader agronomic recommendations can be made.

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Clinicopathological Profiling of Chronic Kidney Disease in Cats Presented at University Veterinary Hospital, Universiti Putra Malaysia from 2016-2021

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ABSTRACT

Chronic kidney disease (CKD) is common in cats, typically showing increased prevalence in geriatric patients. This study profiled clinicopathological changes across CKD stages in 136 cats -without concurrent diseases- selected from the Veterinary Laboratory Services Unit database. Cases were classified into stages II, III, and IV based on the International Renal Interest Society (IRIS) creatinine guidelines. Signalment and clinicopathological data including hematology, serum biochemistry and urinalysis were analysed. Domestic Shorthair (77.2%) was the most common breed affected, followed by Persian (5.9%) and mixed breed cats (5.9%). Males (60.1%) were more frequently diagnosed than females (37.5%). Notably, cats aged 5 to 8 years (n=48) were overrepresented with CKD regardless of stage. Erythrocyte counts and haemoglobin levels were significantly higher ($p<0.05$) in stage II than in stage IV. Similarly, packed cell volume, reticulocyte counts, and urine specific gravity were significantly higher ($p<0.05$) in stage II than in stages III and IV. Levels of band and segmented neutrophils, as well as monocytes increased as stages advance, reaching the highest in stage IV cats ($p<0.05$). Phosphate level was significantly higher ($p<0.05$) in stages IV than in stage II and III. Urea and creatinine concentrations in stage IV were approximately four times higher than those in stage II. High-normal sodium and albumin, low-normal chloride and normal potassium were common across all stages. These findings suggest that non-regenerative anaemia and tubular dysfunction characterise later CKD stages. Understanding these patterns is vital for monitoring disease progression and optimising therapeutic interventions.

Keywords: Chronic kidney disease (CKD), CKD stage, clinicopathological profile, feline

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INTRODUCTION

Chronic kidney disease (CKD) is a metabolic disease that is frequently seen in cats, in which the prevalence increases with age (Bartges, 2012; Brown et al., 2016). The

prevalence of CKD in geriatric cats has climbed from 35% (Krawiec & Gelberg, 1989) to as high as 80% (Chen et al., 2020). Although geriatric cats have a higher prevalence, all cats of all ages are likely to be affected with CKD (Bartges, 2012; Chen et al., 2020).

Chronic kidney disease occurs due to kidney structure and function damage over a long period, which subsequently become more severe and irreversible (Bartges, 2012). Chronic kidney disease can be contributed by various underlying diseases such as amyloidosis, toxication, chronic pyelonephritis or glomerulonephritis, feline infectious peritonitis, lymphoma, polycystic kidney disease, feline leukaemia and hypoxia (Brown et al., 2016; Sparkes et al., 2016). Progressive damage to the kidney causes a reduction of large amounts of functional nephron units and gradually impairs glomerular filtration rate (GFR) for excretion of metabolites, leading to build up of by-products such as blood urea nitrogen (BUN) and creatinine in the body (McLeland et al., 2015).

Chronic kidney disease can be diagnosed using a combination of thorough physical examination, patients' history, complete blood count (CBC), serum biochemistry, urinalysis, abdominal radiograph or ultrasonography, and renal scintigraphy or renal biopsy. According to the International Renal Interest Society (IRIS) staging system, CKD can be classified into four stages using serum creatinine level in fasted patients as a measure of GFR (IRIS, 2023). Symmetric dimethylarginine (SDMA), an amino acid that is the by-product of intranuclear protein methylation, was introduced as a renal biomarker primarily excreted through renal filtration and is considered to be more sensitive than serum creatinine as the former is less influenced by muscle mass (Hall et al., 2014; Sargent et al., 2021). Serum SDMA detects as little as 25% loss of kidney function, while creatinine typically does not rise until 75% of function is lost. Unlike creatinine, SDMA is not influenced by confounding conditions such as muscle mass, making it more reliable for assessing kidney function (Sargent et al., 2021).

At the terminal stage of oliguric feline CKD patients, the classical clinicopathological findings include azotaemia (elevated serum creatinine and BUN), hyponatremia, hypochloremia, hyperkalemia, hyperphosphatemia, isosthenuria (S.G. 1.008-1.012), non-regenerative anaemia, and proteinuria (Elliott & Barber, 1998; Polzin, 2013; Reynolds & Lefebvre, 2013). Histologically, it was found that kidney lesions are more destructive and irreversible in stage III and IV than stages I and II CKD (McLeland et al., 2015). It is hypothesised that the severity of lesions between CKD stages may also reflect in the clinicopathological parameters. Nevertheless, CKD in feline patients may not usually be presented with the typical clinicopathological findings in clinical practice, as it may be influenced by various risk factors of CKD, such as age, comorbidities and environmental factors (Brown et al., 2016). Given that Malaysia's tropical climate predisposes feline populations to dehydration that may induce hemoconcentration, there is a risk that the underlying abnormalities may be masked that can compromise the accuracy of assessment

of CKD based on clinicopathological parameters. Despite these challenges, comprehensive clinicopathological profile of feline CKD remain sparse within the Malaysian context. Hence, the main objective of this study is to profile clinicopathological parameters (haematology, serum biochemistry and urinalysis) in cats presented with various stages of CKD at University Veterinary Hospital (UVH), Universiti Putra Malaysia (UPM).

MATERIALS AND METHODS

Case Inclusion

In this retrospective study, the digital clinicopathological data of all cases presented at UVH, UPM and submitted to the Clinical Pathology Laboratory, Veterinary Laboratory Services Unit (VLSU), UPM between the year 2016 and 2021 was obtained with permission. Data was screened and narrowed to feline cases that have all three results of CBC, serum biochemistry and urinalysis. Patients' history and disease diagnosis confirmatory from UVH clinicians were not attainable in this study due to limited access during the COVID-19 pandemic.

The clinicopathological data was retrospectively refined to feline cases that were suspected to have CKD based on a few criteria. Firstly, the cases must have serum creatinine ≥ 140 $\mu\text{mol/L}$. Secondly, the cases have no evidence of feline lower urinary tract disease from the urinalysis results. The evidence includes pale reddish or reddish coloured urine, or presence of numerous erythrocytes or leukocytes per high power field under microscopic examination. Thirdly, cases should not have other concurrent diseases such as cardiovascular, liver disease and diabetes mellitus. A total number of 136 cases fulfilled all three criteria and were selected for this study.

Clinicopathological Data

Complete clinicopathological profiles from each case were compiled, including signalment, haematological, serum biochemistry and urinalysis parameters. Using serum creatinine concentration results, the cases were categorised into stages II (140-250 $\mu\text{mol/L}$), III (251-440 $\mu\text{mol/L}$) and IV (>440 $\mu\text{mol/L}$) following the IRIS staging system of CKD for cats (IRIS, 2023). Stage I serum creatinine value (<140 $\mu\text{mol/L}$) was excluded from the inclusion criteria. It is acknowledged that the IRIS staging used in this study is based on the serum creatinine concentration only, although creatinine together with SDMA concentrations are preferred. Due to limitations of obtaining other parameters that includes SDMA from UVH database, the staging of CKD in these patients were categorised descriptively using serum creatinine concentration.

Statistical Analysis

Statistical analysis was performed using IBM SPSS statistical software, version 23 with CKD stages (II-IV) as the independent variable, and the categorical variables (breed, age, sex, and urine protein) and clinicopathological parameters as dependent variables. Descriptive analysis was employed for the categorical variables. Chi-square and Fisher's exact analyses were then performed to determine the association between the signalment and CKD stages. Data for clinicopathological parameters that were normally distributed which are the packed cell volume (PCV), mean corpuscular volume (MCV), lymphocytes count, albumin to globulin ratio (A:G), and the concentrations of hemoglobin, albumin, globulin and mean corpuscular hemoglobin (MCHC) were analysed using one-way ANOVA; data that were not normally distributed including the total counts of red (RBC) and white (WBC) blood cells, band and segmented neutrophils, monocytes, eosinophils and reticulocytes, the concentrations of serum sodium (Na), potassium (K), chloride (Cl), calcium (Ca), BUN, creatinine, total protein, and the urine specific gravity (USG) were analysed using Kruskal-Wallis Test. The tests were done to compare the mean or median of clinicopathological parameters with the CKD stages, respectively. When there is statistical significance, corresponding post hoc Fisher's LSD and pairwise comparison were then performed. Results with a p value of less than 0.05 were considered statistically significant.

RESULTS

Breed, Sex, and Age Distribution

There was no significant association found ($p > 0.05$) between CKD stages and breed, sex, and age.

Breed

Among 136 cats that were suspected with CKD, Domestic Shorthair represented the majority of the study population, followed by Persian and Cross/Mixed as shown in Table 1.

Table 1
Breeds of cats that were suspected with chronic kidney disease

Breed	CKD Stage (n)			Frequency (n)	Prevalence (%)	95 % CI	
	II (%)	III (%)	IV (%)			Lower limit	Upper limit
Domestic Shorthair	47 (34.6%)	22 (16.2%)	36 (26.5%)	105	77.2	69.6	83.6
Persian	2 (1.5%)	1 (0.7%)	5 (3.7%)	8	5.9	2.8	10.8
Cross/Mixed	2 (1.5%)	2 (1.5%)	4 (2.9%)	8	5.9	2.8	10.8

Table 1 (continued)

Breed	CKD Stage (n)			Frequency (n)	Prevalence (%)	95 % CI	
	II (%)	III (%)	IV (%)			Lower limit	Upper limit
Domestic Longhair	4 (2.9%)	1 (0.7%)	2 (1.5%)	7	5.1	2.3	9.8
Siamese	2 (1.5%)	1 (0.7%)	1 (0.7%)	4	2.9	1.0	6.8
Maine Coon	0 (0.0%)	1 (0.7%)	0 (0.0%)	1	0.7	0.1	3.4
Norwegian Forest	1 (0.7%)	0 (0.0%)	0 (0.0%)	1	0.7	0.1	3.4
Unknown	1 (0.7%)	1 (0.7%)	0 (0.0%)	2	1.5	0.3	4.6
Total	59 (43.4%)	29 (21.3%)	48 (35.3%)	136	100.0		

Sex

Male cats have a higher proportion of CKD than female cats. Among toms, 44 were castrated, while among queens, 39 were spayed as shown in Table 2.

Table 2

Sex distribution of cats that were suspected with chronic kidney disease

Sex	CKD Stage (n)			Frequency (n)	Prevalence (%)	95 % CI	
	II (%)	III (%)	IV (%)			Lower limit	Upper limit
Intact Male	14 (10.3%)	7 (5.1%)	17 (12/5%)	38	27.9	20.9	35.9
Castrated Male	18 (13.2%)	9 (6.6%)	17 (12.5%)	44	32.4	24.9	40.5
Intact Female	6 (4.4%)	4 (2.9%)	2 (1.5%)	12	8.8	4.9	14.5
Spayed Female	20 (14.7%)	8 (5.9%)	11 (8.1%)	39	28.7	21.6	36.7
Unknown	1 (0.7%)	1 (0.7%)	1 (0/7%)	3	2.2	0.6	5.8
Total	59 (43.4%)	29 (21.3%)	48 (35.3%)	136	100.0		

Age

The age of cats that were suspected with CKD were categorised into several age groups (Table 3). Age group with the highest frequency was 5-8 years, followed by 9-12 years and 13-16 years. Cats aged 5-8 years also represented the most prevalent age group across all CKD stages.

Table 3
Age distribution of cats that were suspected with chronic kidney disease

Age (Year-old)	CKD Stage (n)			Total	Prevalence (%)	95% CI	
	II (%)	III (%)	IV (%)			Lower limit	Upper limit
0 - 4	10 (7.4%)	2 (1.5%)	6 (4.4%)	18	13.2	8.3	19.7
5 - 8	18 (13.2%)	10 (7.4%)	20 (14.7%)	48	35.3	27.6	43.6
9 - 12	12 (8.8%)	6 (4.4%)	13 (9.6%)	31	22.8	16.4	30.4
13 - 16	11 (8.1%)	7 (5.1%)	5 (3.7%)	23	16.9	11.3	23.9
17 - 20	4 (2.9%)	1 (0.7%)	2 (1.5%)	7	5.1	2.3	9.8
Uncertain	4 (2.9%)	3 (2.2%)	2 (1.5%)	9	6.6	3.3	11.7
Total	59 (43.4%)	29 (21.3%)	48 (35.3%)	136	100.0		

CKD = Chronic kidney disease

Clinicopathological Parameters

Haemogram

Overall, the RBC, haemoglobin and PCV decreased from stage II to IV in CKD cats (Table 4). The stage II cats had RBC and haemoglobin concentration significantly higher ($p < 0.05$) than stage IV cats. Meanwhile, the PCV of stage II cats was found significantly higher ($p < 0.05$) than stages III and IV CKD cats.

The reticulocyte count in stage II cats was significantly higher ($p < 0.05$) than cats with CKD stages III and IV. Nevertheless, the reticulocyte count median at all stages were below the normal range. There was no significant difference of MCV and MCHC between CKD stages.

Leukogram

The WBC, band and segmented neutrophils, and monocytes increased with stage, in which values at stage IV were significantly higher ($p < 0.05$) than stage II (Table 5). The eosinophil count was significantly lower ($p < 0.05$) at stage IV than stages II and III.

Table 4

Haemogram of cats with chronic kidney disease according to stages

Parameter	CKD Stage	n	Mean	Median \pm SD
RBC ($5-10 \times 10^{12}/L$)	II	57	7.15	$7.19^a \pm 1.54$
	III	28	6.59	$6.51^{ab} \pm 1.92$
	IV	47	6.19	$5.93^b \pm 2.15$
Haemoglobin (80-150 g/L)	II	57	116.16 ^a	119.00 ± 25.73
	III	28	103.86 ^{ab}	98.55 ± 32.48
	IV	47	93.89 ^b	89.60 ± 28.86
PCV (0.24-0.45 L/L)	II	59	0.31 ^a	0.31 ± 0.06
	III	29	0.28 ^b	0.26 ± 0.07
	IV	28	0.26 ^b	0.25 ± 0.07
MCV (39-55 fL)	II	57	43.96	44.00 ± 4.20
	III	28	42.32	43.00 ± 4.73
	IV	47	43.06	44.00 ± 4.67
MCHC (300-360 g/L);	II	57	371.88	367.00 ± 35.40
	III	28	375.57	375.00 ± 34.56
	IV	47	366.30	360.00 ± 35.33
Reticulocytes (0.5-1.5/100RBC)	II	44	1.06	$0.20^a \pm 1.61$
	III	26	0.28	$0.10^b \pm 0.68$
	IV	43	0.52	$0.10^b \pm 1.17$

CKD = Chronic kidney disease; RBC = Red blood cells; PCV = Packed cell volume;

MCV = Mean corpuscular volume; MCHC = Mean corpuscular haemoglobin concentration

^{ab} = Mean or median within column with different superscripts are significantly different ($p < 0.05$)

Table 5

Leukogram of cats with chronic kidney disease according to stages

Parameter	CKD Stage	n	Mean	Median \pm SD
WBC ($5.5-19.5 \times 10^9/L$)	II	57	12.41	$10.30^a \pm 7.26$
	III	28	14.39	$12.70^{ab} \pm 8.49$
	IV	47	17.80	$14.10^b \pm 11.29$
Band Neutrophils ($<0.3 \times 10^9/L$)	II	57	0.20	$0.11^a \pm 0.18$
	III	27	0.27	$0.13^{ab} \pm 0.27$
	IV	47	0.32	$0.21^b \pm 0.28$
Neutrophils ($2.5-12.5 \times 10^9/L$)	II	57	8.74	$7.21^a \pm 5.43$
	III	27	10.97	$9.73^{ab} \pm 6.08$
	IV	47	14.09	$11.18^b \pm 9.20$
Lymphocytes ($1.5-7.0 \times 10^9/L$)	II	57	2.08	1.81 ± 1.07
	III	27	2.35	2.02 ± 1.68
	IV	47	2.09	1.75 ± 1.57
Monocytes ($0.2-0.8 \times 10^9/L$)	II	57	0.55	$0.37^a \pm 0.50$
	III	27	0.74	$0.51^{ab} \pm 0.55$
	IV	47	0.99	$0.66^b \pm 0.99$
Eosinophils ($0.1-1.5 \times 10^9/L$)	II	57	0.80	$0.61^a \pm 0.71$
	III	27	0.72	$0.66^a \pm 0.54$
	IV	47	0.36	$0.23^b \pm 0.54$

CKD = Chronic kidney disease; WBC = White blood cells

^{ab} = medians within column with different superscripts are significantly different ($p < 0.05$)

Although WBC were within normal range in all stages, the mean of band and segmented neutrophils and monocytes in stage IV were above normal. Meanwhile the lymphocyte count was at low normal in all CKD cats.

Biochemistry

The median of phosphate concentration in stage IV was above normal range and significantly higher ($p < 0.05$) than stages II and III CKD cats (Table 6). The sodium concentration was at high normal while chloride was at low normal in all stages. The potassium level was within normal range across all stages of CKD.

The concentration of BUN and creatinine increased with stages in which stage IV values were approximately four times higher when compared with stage II.

The median of serum protein concentration was 22 to 26% above normal range in all stages of CKD. The albumin and globulin concentrations across all stages were within and above normal range respectively. The median of A:G was at low normal in stages II and IV, while stage III was lower than normal. There were no significant differences in the median of serum protein, albumin and globulin concentration between all stages.

Table 6

Serum biochemistry parameters of cats with chronic kidney disease according to stages

Parameter	CKD Stage	n	Mean	Median \pm SD
Sodium (146-156 mmol/L)	II	54	151.52	152.00 \pm 6.42
	III	28	152.64	152.50 \pm 6.22
	IV	46	152.73	151.55 \pm 10.52
Potassium (3.9-5.5 mmol/L)	II	54	4.74	4.70 \pm 0.78
	III	28	4.65	4.65 \pm 0.97
	IV	46	5.05	4.50 \pm 1.43
Chloride (110-132 mmol/L)	II	54	115.15	114.50 \pm 6.00
	III	28	115.74	115.50 \pm 7.90
	IV	46	112.46	112.50 \pm 11.20
Calcium (2.2-2.9 mmol/L)	II	17	2.56	2.60 \pm 0.21
	III	6	2.67	2.73 \pm 0.13
	IV	5	2.33	2.57 \pm 0.77
Phosphate (1.1-2.8 mmol/L)	II	43	1.61	1.60 ^a \pm 0.44
	III	26	2.38	1.95 ^a \pm 1.75
	IV	35	5.06	5.40 ^b \pm 2.48
Urea (3.0-10.0 mmol/L)	II	59	16.92	15.10 ^a \pm 13.89
	III	29	27.27	24.60 ^b \pm 12.99
	IV	48	56.33	50.50 ^c \pm 22.97

Table 6 (continued)

Parameter	CKD Stage	n	Mean	Median \pm SD
Creatinine (60-193 μ mol/L)	II	59	186.73	186.00 ^a \pm 31.90
	III	29	328.31	319.00 ^b \pm 54.25
	IV	48	782.27	653.50 ^c \pm 348.84
Serum Protein (55-75 g/L)	II	48	94.04	94.05 \pm 11.02
	III	26	92.32	91.80 \pm 9.61
	IV	45	94.89	93.00 \pm 16.39
Albumin g/L (25-40 g/L)	II	48	30.28	30.75 \pm 4.45
	III	26	28.40	28.20 \pm 4.93
	IV	45	30.48	28.60 \pm 7.41
Globulin (25-45 g/L)	II	48	63.76	63.70 \pm 13.04
	III	26	63.92	63.10 \pm 11.47
	IV	45	64.40	64.50 \pm 15.14
A:G (0.5-1.4)	II	48	0.49	0.50 \pm 0.15
	III	26	0.47	0.45 \pm 0.12
	IV	45	0.50	0.50 \pm 0.16

CKD = Chronic kidney disease; A:G = Albumin to globulin ratio

^{ab} = medians within column with different superscripts are significantly different ($p < 0.05$)

Urinalysis

The median of USG of stage II CKD cats was significantly higher ($p < 0.05$) than stages III and IV cats (Table 7).

Table 7

Urine specific gravity of cats with chronic kidney disease according to stages

Parameter	CKD Stage	n	Mean	Median \pm SD
USG	II	59	1.026	1.022 ^a \pm 0.013
	III	29	1.015	1.015 ^b \pm 0.004
	IV	48	1.015	1.015 ^b \pm 0.006

CKD = Chronic kidney disease; USG = Urine specific gravity

^{ab} = median within column with different superscripts are significantly different ($p < 0.05$)

There was significant association ($p < 0.05$) between CKD stages and urine protein concentration (Table 8). No evidence of proteinuria (negative) was the most common finding in stages III (15/29; 52%) and IV cats (22/48; 46%). Stage II cats had proteinuria of 1+ concentration as the highest frequency (21/59; 36%), followed by no proteinuria (16/59; 27%).

Table 8
Protein concentration in urine of cats with chronic kidney disease according to stages

Protein	CKD Stage			Total	Percentage
	II	III	IV		
Negative	16	15	22	53	39.0
1+	21	7	7	36	25.7
2+	10	1	8	19	14.0
3+	11	2	11	24	17.6
4+	1	4	0	5	3.7
Total	59	29	48	136	100.0

CKD = Chronic kidney disease

DISCUSSION

This study provides a comprehensive profile of clinicopathological changes across various stages of feline CKD. A higher proportion of male than female cats suspected with CKD found in the current study is consistent with other studies (Greene et al, 2014; Piyarungsri & Pusoonthornthum, 2016). The prevalence of CKD was notably higher in neutered cats than in intact cats. This finding could be supported by another study whereby absence of testosterone or oestrogen in neutered cats was reported to have smaller kidney size than intact cats, which could be related to higher risk of getting CKD (Shiroma et al., 1999).

The prevalence of CKD cats that were younger than 16 years old were higher than geriatrics, in contrast with Lulich et al. (1992). Young cats below five years of age with CKD could be a progression of acute kidney injury (AKI) into CKD (Schmiedt et al., 2016). However, due to limited access to confirmatory reports such as diagnostic images by the clinicians, AKI cases may have been included in the analysis.

Hemogram results revealed that the RBC, haemoglobin and PCV declined from stage II to stage IV, a consistent observation with a previous study by King et al. (2007). In addition, the reticulocyte count, MCV and MCHC values were also suggestive of non-regenerative normocytic normochromic anaemia. These results suggest there is a positive correlation between the progression of CKD stages and the prevalence of non-regenerative anaemia in feline patients. A previous study reported that anaemia was more often seen in patients with terminal stage of CKD due to decreased or total absence of erythropoietin (Lawson & Jepson, 2021). However, the high MCHC values can be due to haemolysis during blood sampling.

Leukogram analysis revealed that cats with stage IV CKD frequently exhibit systemic inflammation or concurrent infection. Cats with CKD tend to have a leukocyte profile characterised by neutrophilia, lymphopaenia and eosinopaenia when compared with healthy cats, more pronounced in the end-stage of the disease (Kralova et al., 2016). This

leukogram pattern was also seen in CKD dogs (Kralova et al., 2009). It was suggested that decreased lymphocytes particularly T cells, is common in CKD cats especially at the end-stage, compromising patients' immunity, which then results in increased susceptibility to infection or inflammation (Kralova et al., 2016).

Inflammation or infection of the renal system such as tubulointerstitial nephritis and lower urinary tract bacterial infection have been reported as common features in CKD (Brown et al., 2016; Reynolds & Lefebvre, 2013). Cats with CKD have a lower antioxidant capacity due to oxidative stress, which increases the risk of infection and inflammation (Keegan & Webb, 2010). Oxidative stress can accelerate apoptosis of neutrophils and decrease neutrophil functions as seen in dogs (Silva et al., 2013). In humans, it was reported that oxidative stress can cause scarring of the kidneys and systemic inflammation in later stages of CKD (Vaziri, 2004). Leukocyte counts provide critical insights into CKD staging and may serve as a prognostic predictor of patients' survival time (King et al., 2007). This supports the findings of the current study where there was an ascending trend of leukocytosis observed as the CKD stages advances.

Typical electrolyte imbalances in serum biochemistry of CKD cats were not observed in the present study. Instead of hyponatraemia, the sodium concentration was found to be at high normal. This finding is likely attributable to reduced fluid intake, profuse polyuria, or a combination thereof that results in dehydration and subsequently haemoconcentration (Elliott & Barber, 1998). Vomiting, polyuria and anorexia were more likely seen in CKD cats at the time of diagnosis, which can lead to haemoconcentration (Greene et al., 2014). However, in depth clinical assessment of hydration status like mucous membrane and skin turgor elasticity were not obtained due to limited access to clinical information, hence other causes of high normal sodium including hemoconcentration cannot be ruled out.

The normokalaemia observed is contrary to previous studies that reported hyperkalaemia at end-stage CKD (Elliot & Barber, 1998; King et al., 2007). Hyperkalaemia is typically seen in CKD due to reduced GFR and the presence of tubular injury, which causes impaired compensatory mechanism of potassium excretion by the tubules (DiBartola, 2016). Nevertheless, it was reported by Reynolds and Lefebvre (2013) that hyperkalaemia is rather uncommon in CKD cats (prevalence 5.8%) compared with hypokalaemia (prevalence 18-30%), although the latter mostly applies to CKD IRIS stages II-III. While the exact mechanisms of hypokalaemia in CKD cats are not fully understood, it is likely multifactorial, involving insufficient dietary potassium intake, excessive urinary excretion, and stimulation of renin-angiotensin-aldosterone system resulting from sodium-restricted renal diets (Adams et al., 1993; Polzin, 2013). Episodes of vomiting in CKD that leads to metabolic alkalosis may cause exchange of hydrogen ions with potassium, which leads to hypokalaemia (DiBartola, 2016). Conversely, hyperphosphataemia was observed in stage IV patients, a finding that corroborates previous research that reported seven out of eight

cats developed hyperphosphataemia with the greatest severity noted at terminal stages of the disease (Schaefer et al., 2021). This observation might be due to poorer glomerular excretion and tubular reabsorption as the disease worsens (Kidder & Chew, 2009).

The progressive elevation of BUN and creatinine from stage II through stage IV reflects the declination of GFR as the disease severity advances. The increased prevalence of infection and inflammation in the CKD patients may likely to cause hyperglobulinaemia and concomitant rise of serum protein. In CKD, affected glomerular filtration barrier will cause leakage of albumin into the urine (Kovarikova, 2015) and consequently develop hypoalbuminemia (Rayhel et al., 2020). The normal value of albumin observed could be explained with the possibility of dehydration leading to false elevation of albumin concentration whilst the actual concentration may be lower. However, as mentioned earlier, the clinical presentation of hydration status was not confirmed due to limited data to be included in this study.

The observed progressive decline in USG from stage II to stage IV indicates that the renal tubular concentration capacity undergoes significant deterioration as feline CKD advances. The USG in stages III and IV were also approaching isosthenuria (USG 1.008-1.012) suggesting that the urine osmolality was almost isotonic with plasma, which aligns with observations of Elliott and Barber (1998). Another study also reported that the mean of USG in CKD cats was 1.009 whereas in normal cats was 1.050 which was sufficiently concentrated (Deguchi & Akuzawa, 1997). Consequently, failure of the renal concentrating mechanism drives a polyuric state that necessitates a corresponding increase in water intake. Without this sufficient compensatory intake, dehydration ensues (Greene et al., 2014).

Since serum albumin concentration were found to be within normal range, it is expected to find that most of the cases presented showed no signs of proteinuria. Cases that had low concentration of protein in the urine could be due to insensitivity of the urine dipstick to detect protein (Grauer, 2011). Although these findings may also suggest that glomerular functions were not as compromised as the tubules in CKD cats presented to UVH (Hokamp & Nabity, 2016). Nevertheless, it is widely recognised that urine dipstick analysis lacks the precision required for a definitive diagnosis of proteinuria. Assessment of the urine protein to creatinine ratio (UP:C) and detection of microalbuminuria would represent more robust diagnostic modalities for evaluating kidney disease patients. Other forms of diagnosis such as histopathology can further identify the affected and damaged kidney structures and their level of severity.

CONCLUSION

This study demonstrates that the clinicopathological hallmarks of feline CKD significantly influenced by age and hydration status, often leading to non-classical biochemical presentations. The prevalence of high-normal sodium and normal albumin concentrations likely reflects masked hemoconcentration, underscoring the necessity of standardised

hydration status in tropical climates. Nevertheless, the clinicopathological profile of the cats involved in this study suggests that the severity of CKD worsens from stages II to IV. The emergence of non-regenerative anaemia and impaired tubular concentrating ability are pathognomonic of advanced stages. Furthermore, increased inflammatory markers identified suggest that CKD cats were more susceptible to inflammation or infection.

It is acknowledged that this study largely focused on stage II to stage IV CKD as the bias is inherently linked to the reliance of serum creatinine as a primary diagnostic marker. Stage I cases is attributed to the subclinical nature of early-stage of renal insufficiency. Azotaemia becomes detectable only after 50 to 75% of the functional renal mass becomes compromised. Consequently, Stage I patients often evade clinical detection. This also highlights inclusion of SDMA testing could have mitigated this limitation.

Another major limitation in this study is that the confirmatory diagnosis of CKD by the clinicians was not obtained, which may lead to inclusion of AKI cases. It is highly recommended that further studies such as prospective designs with more comprehensive information including confirmatory diagnosis and staging of CKD inclusive of SDMA concentration and UP:C, clinical assessment of hydration status and diet, to better delineate the clinicopathological changes in each stage of CKD in feline patients.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding this study.

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