Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

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Pertanika Journal of Tropical Agricultural Science
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About the Journal

Overview

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

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

Pertanika Journal of Tropical Agricultural Science 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.

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

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

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

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

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

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

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

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

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

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

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

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

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

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Foreword

Welcome to the second issue of 2025 for the Pertanika Journal of Tropical Agricultural Science (PJTAS)!

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

This issue contains 18 articles: four review articles; one short communication; and the rest are regular articles. The authors of these articles come from different countries namely Brunei, India, Indonesia and Malaysia.

A selected article entitled "Plant Growth Regulators Application to Enhance Flowering and Fruit Production in Gac (*Momordica cochinchinensis*)" assessed the effects of four plant growth regulators —indole-3-acetic acid (IAA), gibberellic acid (GA), benzyl adenine (BA), and maleic hydrazide (MH)—at varying concentrations (0, 40, 80, and 120 ppm) using a randomized complete block design (RCBD) with five replications. Results showed that MH at 40 and 80 ppm significantly improved flower development, ovary diameter, early anthesis, and fruit yield, highlighting its potential in gac cultivation. The detailed information of this article is available on the page 339.

A study by Fatin Nabila and team entitled "Evaluating AedesTech Mosquito Home System (AMHS) Effectiveness on Aedes Mosquitoes" analyzed the efficacy of the AedesTech Mosquito Home System (AMHS), an autodissemination ovitrap with pyriproxyfen, through laboratory trials on *Aedes albopictus* and *Aedes aegypti*. The trials examined the impact of an attractant, trap positioning, and oviposition site selection. The laboratory results indicated that the Mosquito Home Aqua (MHAQ) solution with attractant consistently attracted *Ae. aegypti* effectively (Welch's Analysis of Variance) F (2,68.66) =5.22, p=0.01). However, its efficacy with *Ae. albopictus* was suboptimal compared to other treatments (Two-way ANOVA, F=0.16, df=2, p>0.05), highlighting the need for considering additional attractants. Full information on this study is presented on the page 451.

A review article entitled "Food Wastes for Enhancing Soil and Crop Productivity in Tropical Acid Soils" determines excessive use of inorganic fertilizers degrades soil quality, whereas organic bio-fertilizers, enriched with beneficial microbes,

offer a sustainable alternative. This review examines that food wastes such as eggshell wastes, washed rice water, fruits, vegetables, and animal wastes have positive effects on improving soil and crop productivity. Bio-fertilizers offer environmental, socio-economic, and agricultural benefits, including improved soil fertility, enhanced crop yields, and disease resistance. Further details of this study are found on the page 511.

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
Mohamed Thariq Hameed Sultan

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Plant Growth Regulators Application to Enhance Flowering and Fruit Production in Gac (*Momordica cochinchinensis*)

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ABSTRACT

Gac fruit (*Momordica cochinchinensis*) has garnered substantial interest due to its potential as a rich source of lycopene and β-carotene, prompting higher demand for large-scale production. However, the development of its female flowers is hindered by the dioecious nature of the gac plant, demanding manual pollination to enhance fruit yield. In addition, the female flower of the gac plant starts late, depending on environmental variables such as temperature, moisture, and photoperiod. Accelerating flowering onset and augmenting pollination could substantially amplify gac fruit production, provided a comprehensive comprehension of exogenous plant growth regulators is attained. Accordingly, the current study investigates the role of plant growth regulators at various concentrations in developing female flowers and fruit production in gac plants. A field planting experiment was conducted using a five-replication factorial randomized complete block design (RCBD). The combination treatments comprised two factors of interest, which were four different types of plant growth regulators (indole-3-acetic acid [IAA], gibberellic acid [GA], benzyl adenine [BA], and maleic hydrazide [MH]) at four different concentration rates (0, 40, 80, and 120 ppm). Notably, Gac plants treated with MH at concentrations of 40 and 80 ppm exhibited significant performance in node and pistillate count, ovary diameter, early onset of first flower anthesis, and fruit yield. These findings underscore the potential of MH as a potent growth regulator for enhancing gac fruit production. Nonetheless, more research on

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agronomic practices, control environment modifications, and postharvest handling is required for the profitable production of the gac fruit.

Keywords: Benzyl adenine, gac fruit, gibberellic acid, indole-3-acetic acid, maleic hydrazide, pollination

INTRODUCTION

Momordica cochinchinensis, commonly known as gac, is a distinguished Cucurbitaceae family member. Thrive is a perennial vine that flourishes in home gardens, intertwining effortlessly between lattices and branches, indicating its adaptability and ease of cultivation (Aoki et al., 2002; Vuong, 2000). Revered as the "fruit from heaven," Gac fruit possesses a variety of nutritional benefits, including its ability to promote well-being, longevity, and vitality. Studies in recent years have found this fruit to contain high levels of carotenoid antioxidants, particularly lycopene and beta-carotene (Bhumsaidon & Chamchong, 2016; Burke et al., 2005; Müller-Maatsch et al., 2017; Tran et al., 2016; Vuong et al., 2006;;). There are eight times more lycopene in the aril than in tomatoes and five times more beta-carotene in carrots (Aoki et al., 2002; Chuyen et al., 2015; Singh et al., 2001). Gac fruit has demonstrated significant commercial potential through various value-added products, including juice, oil, and powder (Do et al., 2019). These products enhance culinary dishes and serve as dietary supplements, promoting health benefits linked to the fruit's phytochemical composition, particularly its carotenoids such as lycopene and β-carotene (Vuong et al., 2006). The increasing availability of gac fruit products in global markets reflects a growing consumer interest in health-oriented foods. A study by Pham et al. (2022) projected that the global market for gac fruit products will grow at a compound annual growth rate (CAGR) of 8.5% from 2020 to 2025, driven by rising demand for natural and functional foods. Key markets for gac products include China, India, and the United States, highlighting its significant export potential (Pham et al., 2022).

Additionally, gac fruit is promoted as a highly nutritious fruit due to its carotenoids, which present natural antioxidants that help prevent certain cancers. Therefore, improving production to meet the increasing demand for gac fruit as a health product is essential. However, the dioecious nature of gac plants, where male and female flowers are found on separate plants, poses significant challenges for fruit production (Bharathi & John, 2013; Parks et al., 2013; Tran et al., 2020). Insect-mediated pollination in gac fruit faces several challenges, including a short blooming period, minimal nectar, and the need to visit multiple plants for successful pollination. This necessitates hand pollination to achieve optimal fruit yield and quality, outperforming insect-mediated and natural pollination in terms of fruit size and sensory attributes (Parks et al., 2013; Pessarakli, 2016). Current horticultural practices in gac fruit plantations often result in suboptimal male-to-female plant ratios and delays in flowering observed in seed-propagated plants, further complicating efficient pollination and fruit production.

Although gac's horticultural potential is compelling, inadequate cultivation guidelines leave growers grappling with a variety of uncertainties, including the male-to-female plant ratio and delays in flowering observed in seed-propagated plants. Nevertheless, augmented gac fruit production will only be achieved through careful attention to flower initiation, pistillate flower quantity and quality, and fruit setting rates. Research on the modulatory effects of growth regulators has revealed the intriguing possibility of inducing bisexual flowers, thereby signaling a potential paradigm shift in gac cultivation (Puzari, 1999; Sanwal et al., 2011). It has been demonstrated that using growth regulators in the leaves of Cucurbitaceae family crops can regulate various physiological processes, especially flowering and sex expression alteration (Rajbhar, 2023).

Plant growth regulators (PGRs) are chemical substances transported by vascular tissues, enhancing the source-sink relationship and stimulating photo-assimilates' translocation to endorse fruit development and eventually increase productivity (Nayak, 2022). The critical role of exogenous and endogenous growth regulators, such as cytokinins, gibberellins, ethylene, and auxins, in plant sex determination has been investigated, especially in the Cucurbitaceae family (Thomas, 2008; Yamasaki et al., 2005). Endogenous plant hormones can be influenced or induced by exogenous plant hormones (Hikosaka & Sugiyama, 2015), ensuring the accuracy of the flowering period through a complex network of genes that integrate endogenous and environmental signals (Campos-Rivero et al., 2017). Once pollination occurs during the anthesis period, cell division is induced by the synthesis and action of endogenous growth regulators, especially auxin (Dorcey et al., 2009); therefore, it would trigger fruit development (Kumar & Kumar, 2016).

Sex ratio and sequence of flowering are determined by environmental factors, including auxins, gibberellic acid, ethylene, and ascorbic acid levels (Bharathi & John, 2013). Sex alteration in plants can be attained by modifying mineral nutrition, temperature, photoperiod, and phytohormones (Baset et al., 2014; Ha, 2014; Megharaj et al., 2017). It is where hormones play a significant role in sex alteration (Thomas, 2008; Grumet & Taft, 2012). The substantial effect on sex expression was discovered when the foliar application of naphthalene acetic acid (NAA), GA, maleic hydrazide (MH), indole acetic acid (IAA), silver nitrate, ethrel, boron, kinetin and morphactin was applied to bitter gourd plants at the two to four leaf stage (Prakash, 1976). Furthermore, Gosai et al. (2020) reported major increases in early pistillate flower appearance on cucumber treated with 100 ppm MH and ethephon. Conversely, although exogenous application of GA at 20 to 40 mg/l was discovered to increase pistillate and staminate flower numbers, comparatively high concentrations of 60 mg/l GA increased only pistillate flowers (Ghosh & Basu, 1983). However, spraying ethephon onto male spiny gourd plants at any concentration did not impact the plants, while applying 400 ppm silver nitrate, $AgNO_3$, led to the greatest number of bisexual flowers (Naik et al., 2018). Sanwal et al. (2011) also stated that female gac

plants treated with 500 mg/l $AgNO_3$ could induce the highest hermaphrodite flowers with pollen viability comparable to typical male plants.

In recent years, the hybridization of several Mormordica species, including the gac fruit plant, has been conducted (Mohanty et al., 1994), and studies on plant growth regulator effects on the gac fruit plant have indicated the possibility of developing new varieties with bisexual flowers (Puzari, 1999; Sanwal et al., 2011). The intricate interplay between hormones, environmental factors, and mineral nutrition determines the phenotypic expression of sex in plants, fostering a transformative journey toward unlocking the plants' genetic potential. Nevertheless, minimizing the risks associated with plant growth regulator residues depends on the specific regulator used, the application rate, and the crop type, requiring agricultural practitioners and farmers to follow proper application practices, adhere to recommended waiting periods before harvest, and regularly monitor applications. Understanding the maturity stages of gac fruit is crucial in optimizing harvest timing and ensuring maximum yield and quality. Gac fruit undergo several maturity stages, each characterized by distinct physical attributes. Initially, the fruit exhibits a green skin color, gradually turning orange as it matures.

By maturity index 4, the skin becomes a vibrant orange to reddish-orange, with the pulp inside transforming from pale yellow to a deep orange-red. The firmness of the fruit at this stage is firm but slightly yielding, making it ideal for harvest. However, there is currently insufficient information regarding the effects of the application of plant growth regulators on sex expression modification and fruit production in gac plants. Using growth regulators and meticulous scientific inquiry, this study attempts to overcome some of these difficulties faced in gac fruit production to meet both small- and large-scale demand. Thus, the current study aims to determine the application of different plant growth regulators and their concentrations in promoting female flower development and fruit production of gac plants.

MATERIALS AND METHODS

Field Plot Preparation and Agronomic Practices

Field planting started in February 2020 at the Bukit Kor, Universiti Malaysia Terengganu, Marang, where a plot measuring 24.5 m × 28 m was plowed and rotavated. The planting area comprises Nami series soil and is located at latitude 5° 21’ North and longitude 103° 2’ East, with an altitude of about 32 m above sea level (asl). The site is in a tropical rainforest with an average annual rainfall of 2552.5 mm, average temperature of 27.8°C (min 23.4°C and max 30.5°C), and 81.7% relative humidity. This particular crop requires a sturdy trellis to support the climbing vine, which must be regularly trained. For this study, the related trellis set comprised Balau plane woods of 5.08 × 5.08 cm and 2 m tall with a 2.5 m horizontal wood attached and netting between two vertical pillars, where

one replication was equal to one plant with one trellis unit. There was approximately 2 m distance between the trellis sets in a single row and column (Figure 1). Female and male gac cuttings as planting material were propagated before planting, and each successfully propagated plant was transplanted to the field after five weeks.

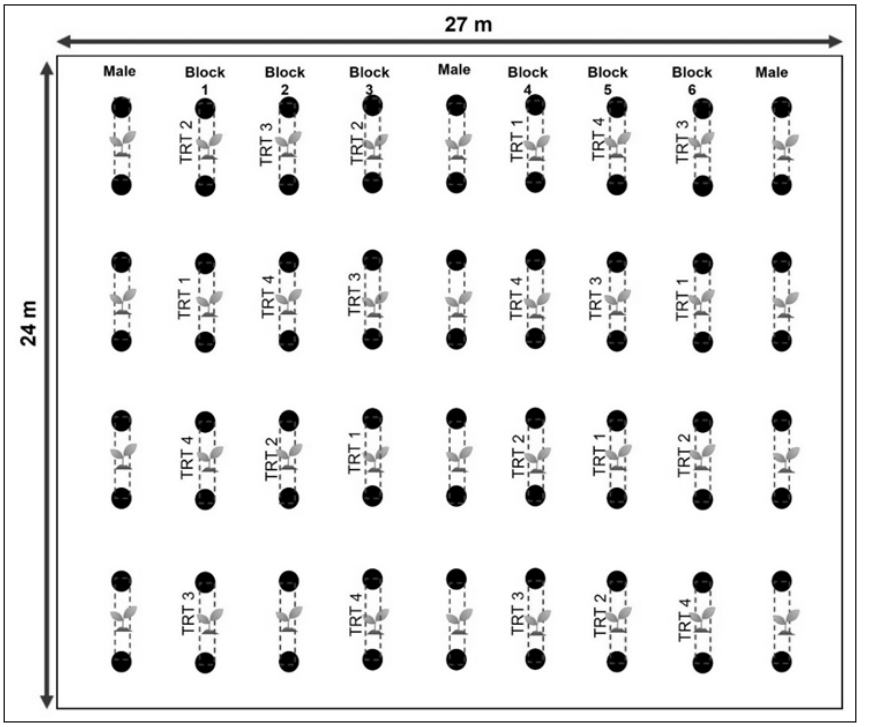

Figure 1. Experimental layout of gac cultivation plot

The planting preparations were completed before transplanting, such as applying 10 t/ha compost manure and the setup of weed mats and irrigation systems. Fertilizers were applied at the rates of 100 kg/ha urea: 20 kg/ha triple super phosphate (TSP): 72 kg/ha muriate of potash (MOP) during planting until flowering, and 48 kg/ha urea: 20 kg/ha TSP: 72 kg/ha MOP, from flowering until harvesting. Weeding was performed every month before fertilizer application. This research applied a holistic approach to pest and disease control, integrating physical, biological, and cultural practices to manage pests and diseases effectively. Physical controls included removing infected plants and using yellow sticky traps to capture flying insects. Biological controls involved the application of neem oil, a natural pesticide known for its efficacy against a wide range of pests and its minimal impact on non-target organisms. By employing these methods, we aimed to reduce the reliance on chemical pesticides, promoting a sustainable and environmentally friendly approach to pest and disease management.

After completing the necessary preparations, including the application of compost manure, the setup of weed mats, and the installation of irrigation systems, the gac cuttings were transplanted to the field after five weeks of propagation. The subsequent planting process, from February 2020 onwards, marked the commencement of the study. Throughout the cultivation period, spanning over one and half years, the crop's growth and development were meticulously monitored and managed, adhering to the established protocols for fertilization, pest and disease control, and trellising techniques.

Plant Growth Regulators Application

The female gac plants were sprayed with IAA, GA, BA, and MH at four different concentration levels (0, 400, 800, and 1200 ppm) during the pre-flowering stage, which was about 30 days after shoot sprouting. Morphological differentiation between male and female gac plants was conducted before the experimental treatment by observing flower structures. Larger flower buds identified female plants with ovary structures, while male plants exhibited smaller flower buds without ovary structures. There were 65 female plants that comprised the study's experimental units, consisting of 13 combination treatments with five replications and 12 male plants that served as pollen producers. The PGRs were sourced from reputable suppliers: IAA and MH were obtained from Sigma-Aldrich (USA), GA, and BA from Merck (Germany). Stock solutions of each PGR were prepared by dissolving the powdered compound in a small volume of ethanol, followed by dilution with distilled water to achieve the desired concentration. Spraying was conducted in the early morning to ensure optimal absorption until the solution thoroughly covered the entire plant. For the control group, the plants were not sprayed with any plant growth regulators.

Data Collection

The number of nodes, number of pistillates, ovary diameter, days to first flower, days from pollination to harvest, and number of fruits per plant were collected during the planting period. Other than that, the number of nodes at the first flower initiation of every plant was counted manually and recorded. During flowering until the end of the harvesting period, the number of pistillate was counted using a visual count daily for each plant. The ovary diameter was measured for the first five flowers of each plant using a digital caliper, and the data was recorded during flower anthesis. Days to the first flower were recorded for each plant during flower anthesis and pollination. The days from pollination to harvest for each fruit set were recorded upon pollination until harvest at maturity stage four. Data was recorded for only the first five flowers that appeared and were successfully pollinated until harvesting. The flowers were tagged, and the pistillate flower anthesis and harvesting dates were recorded. Gac fruit undergoes several maturity stages, each marked by specific changes in color, texture, and biochemical composition, with five distinct stages identified by Tran et al. (2016) (Figure 2).

Figure 2. Gac fruits with different maturity index

The gac fruits, manually harvested at maturity index 4, had bright and vivid orange-red peel colors, with vibrant orange to reddish-orange skin, bright red or deep orange pulp, and a firm but slightly yielding texture. The fruits are harvested at maturity stage four because this stage represents the optimal point for several important physicochemical properties (Tran et al., 2016). At this stage, the gac fruit exhibits the highest levels of beneficial compounds, such as lycopene and beta-carotene, which are important for their antioxidant properties. The texture is also ideal for processing and consumption. Harvesting at this stage ensures the best nutritional content, flavor, and texture quality, making it suitable for fresh consumption and further processing. Additionally, data on the number of fruits per plant were recorded daily and summed up during the harvesting period.

Experimental Design

The study was designed using a factorial randomized complete block design (RCBD) with five replications (one plant of each replication) to investigate the impact of two factors on the gac vine 30 days after shoot sprouting. The factors included four types of plant growth regulators (IAA, GA, BA, and MH) and four concentration rates of the plant growth regulators (0, 40, 80, and 120 ppm). The data obtained from the experiment were analyzed using a two-way analysis of variance (ANOVA), and significant treatment means were separated using Duncan's multiple range test (DMRT) at $p < 0.05$ (SAS, version 9.3). The effects of the concentration of each plant growth regulator type on the dependent parameters were evaluated and compared using a pooled Least Significant Difference (LSD) test at $p \leq 0.05$. For plant growth regulator types and concentrations that displayed significant interaction, the effects differed by concentration. To understand the nature of the interaction between the different plant growth regulators and their concentrations on the dependent variables, additional partitioning of the interaction sum of the square was performed, followed by regression analysis. Pearson's correlation coefficients were calculated using

the Procedure Correlation (PROC CORR) procedure, and the interpretation of correlation coefficient values was based on the study by Schober et al., (2018). The correlation coefficients were used to determine the strength and direction of the relationship between the independent and dependent variables.

RESULTS AND DISCUSSION

The current study demonstrated that four selected plant growth regulators at different concentrations resulted in various growth and development performances during the planting period. Significant interaction was observed between the different plant growth regulators and concentrations on the number of nodes, number of pistillates, ovary diameter, days to first flower, days from pollination to harvest, and number of fruits per gac plant except the total fruit weight per plant (Table 1). However, no significant regression effects were seen between the number of nodes, number of pistillates, ovary diameter, days to first flower, days from pollination to harvest and number of fruits per gac fruit plant, with the concentration rates of the plant hormones. Thus, the effects of the different concentrations on the number of nodes, number of pistillate, ovary diameter, days to first flower, days from pollination to harvest and number of fruits per plant for each plant hormone type were compared using a pooled least LSD test at $p \leq 0.05$ (Figure 3).

Table 1
The main interaction effects of plant growth regulator types (IAA, GA, BA, MH) and concentrations (0, 40, 80, 120 ppm) on growth and yield parameters of gac plants

Factor	Number of nodes	Number of pistillate	Ovary diameter (mm)	Days to the first flower	Days of pollination to harvest	Number of fruits/ plant	Total fruit weight/ plant (kg)
Plant growth regulators (PGR)							
IAA	36.0 a^z	19.9 b	10.94 b	63.7 a^z	57.2 a	12.83 ab^z	9.90 a
GA	33.3 a	18.3 b	10.80 b	59.5 ab	57.7 a	11.42 b	9.37 a
BA	35.9 a	25.5 a	11.04 b	58.9 ab	57.6 a	13.17 ab	10.38 a
MH	35.8 a	24.8 a	11.48 a	56.3 b	57.2 a	14.17 a	11.69 a
Concentrations (C) (ppm)							
0 (Control)	30.7 b	18.7 b	10.62 b	70.7 a	59.3 a	9.33 b	6.87 b
40	41.8 a	24.9 a	11.25 a	54.7 b	57.2 b	14.67 a	12.55 a
80	34.8 b	21.6 ab	11.32 a	56.3 b	56.8 b	12.58 a	10.03 a
120	33.8 b	23.3 a	11.05 a	56.8 b	56.4 b	15.00 a	11.90 a
PGR x C	*	*	*	*	*	*	ns

Note. Significant at $p \leq 0.05$ and not significant at $p > 0.05$
z = Means with the same letter within a column, and the factor is not significantly different at $p=0.05$ using Duncan's Multiple Range Test (DMRT). *, ns=Significant and not significant at $p \leq 0.05$, respectively

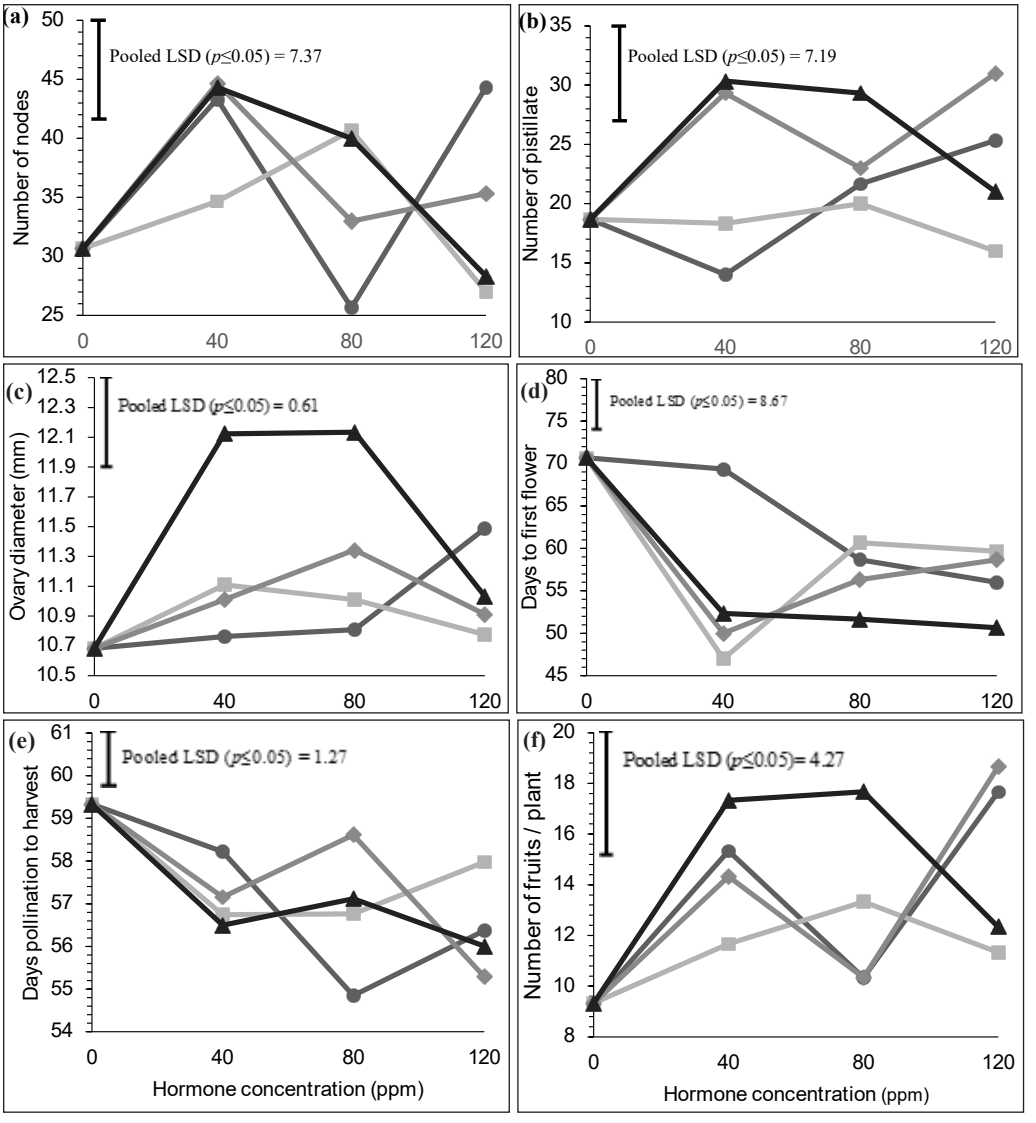

Figure 3. Number of nodes (a); number of pistillates (b); ovary diameter (c); days to first flower (d); days from pollination to harvest (e); and number of fruits/ plant (f) on gac plants with different types of plant growth regulators IAA (●), GA (■), BA (♦) and MH (▲) and plant growth regulators concentrations at 0, 40, 80 and 120 ppm concentrations. n = 5

Generally, it was observed that the higher node numbers were for the plants treated with IAA at 40 and 120 ppm, BA at 40 ppm, and MH at 40 ppm concentrations (Figure 3[a]). However, there were no differences in node numbers for plants treated with GA at 80 ppm and MH at 80 ppm, with optimal node numbers around 40 to 44.3. It has been reported that bottle gourd plants treated with 10 ppm IAA exhibited an increase in the number of nodes

and leaves (Rahman, 1992). Additionally, 60 ppm GA application on muskmelon resulted in a higher number of nodes than other plant growth regulators (Chaurasiya et al., 2016). At the same time, Gosai et al. (2020) reported that MH at 100 ppm increased the number of nodes and primary branches in cucumber. Application of BA at 10 ppm or more on peas showed that BA influences the growth and development of peas, particularly in relation to the number of nodes and flowering (Sprent, 1968). There are a number of mechanisms by which these plant growth regulators affect node formation, such as IAA promoting cell elongation and division, GA stimulating stem elongation, MH stimulating branching, and BA promoting cell division and differentiation. These interactions demonstrate the complex regulation of plant hormones and their impact on plant morphology.

As displayed in Figure 3(b), gac plants treated with BA at 40 ppm and 120 ppm and MH at 40 ppm and 80 ppm exhibited 44.3% and 49.7% higher number of pistillate, respectively, than the control. However, the pistillate numbers of the gac plants with control treatment demonstrated no significant differences with those treated with IAA and GA at 40, 80, and 120 ppm, BA at 80 ppm, and MH at 120 ppm concentrations. These findings are comparable with the study by Hikosaka and Sugiyama (2015), where the application of BA at 2000 ppm increased pistillate numbers in cucumber. Similarly, Hidayatullah et al. (2009) also reported that MH at 450 µmol/L increased the number of pistillates by three-fold compared to the control. The application of MH at 450 µmol/L or 100 ppm was stated to help enhance endogenous auxin hormones, signaling intersects with other hormonal pathways to promote pistillate flower development (Gosai et al., 2020). Our results align with these studies, indicating that lower concentrations of BA and MH can also positively affect pistillate numbers in gac plants.

Additionally, a number of nodes showed significant, positive, and strong correlations (R= 0.745) with a number of pistillate on gac fruit plants treated with four plant hormones at different concentrations (Table 2). This finding followed studies reported by Kumari et al. (2018) and Manisha and Pal (2014) in cucumber, where there was a positive and substantial relationship between the number of nodes and the number of pistillates of the first flower. Plant growth, including the number of nodes and pistillate, was influenced by photosynthetic activity and assimilation. It would translocate to multiple sinks, enhancing growth and overall yield (Eifediyi & Remison, 2010). The information on the correlation between plant growth variables is crucial for enhancing yield production, especially the number of nodes and pistillate (Manisha & Pal, 2014). These findings suggest that the application of IAA, GA, BA, and MH at varying concentration levels can effectively stimulate cell elongation and division, thereby influencing plant growth dynamics (Gosai et al., 2020; Rademacher, 2000). Understanding the relationship among variables through correlation studies may provide insight into strategies for optimizing fruit quality and productivity (Prabhakar & Kushwah, 2017).

Table 2
Correlation coefficients (r) for each pair of parameters in gac fruit plant treated with indole-3-acetic acid (IAA), gibberellic acid (GA), benzyl adenine (BA), and maleic hydrazide (MH) at 0, 40, 80 and 120 ppm concentration

	Number of pistillate	Ovary diameter (mm)	Days to the first flower	Days of pollination to harvest	Number of fruits/ plants	Total fruit weight/ plant (kg)
Number of nodes	0.75**	0.38^{ns}	-0.34^{ns}	-0.14^{ns}	0.68*	0.72**
Number of pistillate	--	0.45*	-0.39^{ns}	-0.19^{ns}	0.73**	0.78**
Ovary diameter (mm)	--	--	-0.37^{ns}	-0.20^{ns}	0.38^{ns}	0.46*
Days to the first flower	--	--	--	0.53*	-0.51*	-0.53*
Days of pollination to harvest	--	--	--	--	-0.34^{ns}	-0.35^{ns}
Number of fruits/plants	--	--	--	--	--	0.91***

Note. For the correlation coefficient, n = 24. *,**, ***, ns = moderate, strong, very strong correlation and not significant at $p \leq 0.05$

Meanwhile, the ovary diameter of gac fruit treated with MH at 40 and 80 ppm concentrations was larger than that of the other plant growth regulator combination treatments and 12% higher than the control (Figure 3[c]). In addition, the gac fruit treated with IAA at 120 ppm, BA at 80 ppm, and MH at 40 and 80 ppm concentrations had higher ovary diameters by 6% to 12.7%, respectively, compared to the control. The application of MH contributes to alterations in the auxin level, resulting in enhanced fruit size (Gosai et al., 2020) since auxin is responsible for cell division, elongation, and differentiation. During the anthesis period, ovary growth and cell division experienced a temporary slowdown until pollination and fertilization occurred (Gillaspy et al., 1993). Fertilized ovules typically reinitiate cell division, initiating fruit development. However, ovary diameter would increase throughout flower anthesis until fruit maturation and ripening by the effect of plant growth regulators (Tantasawat et al., 2015). These findings were upheld by the correlation coefficient results showing a significant, positive and moderate correlation (R=0.446) between ovary diameter and a pistillate number of gac fruit plants treated with four types of plant hormones at different rates (Table 2). It also indicated that a higher ovary diameter would affect a higher pistillate number when treated with IAA, GA, BA, and MH at 0, 40, 80, and 120 ppm. Pistillate number and ovary diameter might be related to photosynthetic activity and assimilation, contributing to plant growth and development (Eifediyi & Remison, 2010).

The gac plant with the control treatment experienced significantly longer days to first flower than all the other plant growth regulators with combination treatments of IAA, GA, BA, and MH, at 40, 80, and 120 ppm, except for the gac plant with IAA at 40 ppm treatment (Figure 3[d]). Gac plants treated with GA at 40 ppm took significantly less

time to flower anthesis than those treated with other treatments yet were similar to those treated with BA at 40 ppm and MH at 40, 80, and 120 ppm. The plant's first flower treated with GA at 40 ppm emerged 23 to 24 days earlier than the control. Comparable to the findings reported in Ahmad et al.'s (2019) study, bitter gourd sprayed with GA at 100 ppm demonstrated the shortest duration for first flower emergence, occurring four to five days earlier than the control. There was also a shorter period for the first flower emergence of cucumber treated with MH at 200 ppm (Kaur et al., 2016). Generally, the treatments with plant growth regulators demonstrated shorter days to first flowering, which would benefit fruit production (Gosai et al., 2020).

The gac plant treated with IAA at 80 ppm experienced a 4.5 to 1.5 days shorter duration from pollination to harvest compared with the other combination treatments. However, it is similar to the BA at 120 ppm and MH at 120 ppm treatments, as illustrated in Figure 3(e). Conversely, the day's pollination to harvest of the gac fruit plant with controls was longer than the gac fruit plant treated with IAA at 80 and 120 ppm, GA at 40, 80 and 120 ppm, BA at 40 120 ppm, and MH at 40, 80 and 120 ppm concentration. However, the gac fruit plant with control had similar days of pollination to harvest, with IAA 40 ppm and BA 80 ppm. After pollination, fruit growth and development commonly involve plant hormone synthesis and regulation, especially GA and auxin (Obroucheva, 2014; Ozga & Reinecke, 2003). Note that IAA promotes fruit cell elongation and, thus, the rapid increase and exponential growth of cell size in cucumbers (Liu et al., 2020). Some studies have revealed that the auxin concentration peak coincides with the cell elongation growth rate (Pattison et al., 2014).

In addition, Rylott and Smith (1990) discovered that cytokinin stimulated active cell division within the embryo, attracting assimilates from other plant parts in the developing pods. Accordingly, the assistance of plant growth regulators might be one factor that accelerates fruit growth development after pollination. On the other hand, days to the first flower have a significant, positive and moderate correlation with days pollination to harvest (R=0.531) of gac fruit plant treated with four plant hormones at different concentrations (Table 2). These findings indicate that the extended period of the first flower's appearance influenced the longer period of pollination needed to harvest gac fruit. A similar result was reported by Kumari et al. (2018) in cucumber, where there was a significant and positive relationship between days to first pistillate flower and days of pollination to harvest.

According to the results of the current study, the number of fruits on the gac fruit plant treated with BA at 120 ppm was significantly higher than a plant with control, IAA at 80 ppm, GA at 40, 80 and 120 ppm, BA at 40 and 80 ppm, and MH at 120 ppm concentration (Figure 3[f]). However, the gac fruit plant treated with BA 120 ppm had a similar number of fruits to plants of IAA at 40 and 120 ppm and MH at 40 and 80 ppm (Figure 3). The weight of the fruit and the quantity of fruits produced per plant would affect the crop yield, thus influencing the primary economic output (Ghani et al., 2013).

The current study indicated that the gac plants treated with IAA at 40 and 120 ppm, BA at 120 ppm, and MH at 40 and 80 ppm had the highest number of fruits produced per plant. This concurs with the results of Ahmad et al. (2019) and Akter and Rahman (2013), where cucumbers treated with IAA at 200 ppm and 10 ppm had more fruits than the control. It is also reported that the application of MH at 200 ppm may influence auxin levels, which enhances the fruit set and development of cucumbers (Pattison et al., 2014; Gosai et al., 2020). The utilization of plant growth regulators such as auxin, GA, and cytokinin may be involved in the photosynthesis activities and thus contribute to improving plant growth and development, with higher fruit quality and production.

According to the main and interaction result shown in Table 1, there was no significant interaction effect between types of plant hormone and concentration on total weight per plant and the average gac fruit weight of each plant. There were also no significant differences between gac fruit plants treated with IAA, GA, BA, and MH plant hormones in terms of total weight per plant and average gac fruit weight per plant. The average weight of each plant and the average gac fruit weight per plant were approximately 10.33 kg and 788.5 g of each gac fruit weight, respectively. However, the total weight per plant with control was significantly lower than the gac fruit plant with 40, 80 and 120 ppm concentration plant hormone. In addition, the average gac fruit weight per plant of the control treatment was significantly lower than the average gac fruit weight of 40 and 80 ppm concentration plant hormone. However, it was similar to the average weight of 120 ppm plant hormone concentration. The total weight per plant and average weight per fruit upon the application of exogenous plant hormone was proven to increase compared to control in various reports (Ahmad et al., 2019; Baset et al., 2014; Hidayatullah et al., 2009; Mahala et al., 2014; Nagamani et al., 2015; Tantasawat et al., 2015).

Based on the current study, the fruit number of gac fruits plants treated with different types of plant hormone and concentration showed significant, positive and moderate to strong correlation with the number of nodes (R=0.683) and number of pistillate (R=0.729) and days to first flower (Table 2). However, there was also a notable, negative, and moderate correlation (R=-0.511) between the number of gac fruits per plant and the days until the first flowering when the plants were treated with various plant hormone types and concentrations. Similar results were found on total weight per plant, where there was a significant and positive relationship with the number of nodes and the number of pistillate, but a significant and positive relationship with days to the first flower (Table 2). The coefficients varied among weak, moderate, and strong correlations. These indicated that higher gac fruit numbers per plant and total weights per plant result in a higher number of nodes, a higher number of pistillates, and a shorter period of first flower emergence. A similar result was recorded by Kumari et al. (2018) and Manisha and Pal (2014) in cucumbers.

In addition, total weight per plant demonstrated a significant, positive and strong correlation with the number of fruits (R=0.910) and a moderate correlation with the average fruit weight (R=0.612) of gac fruit harvested per plant. These results indicated that the greater the number of fruits harvested, the higher the total weight of fruits per plant. The higher total weight of fruits would also affect the higher average gac fruit weight harvested at maturity index 4. The variables of number of nodes, internodes, and number of pistillate closely relate to photosynthesis synthesis, assimilations, and partitioning (Abd El-Hafeez & Ali, 2013; Silva et al., 2019). This process would affect fruit quality and production due to biomass allocation to source and sink organs. Several studies stated that yield per plant had a significant and positive correlation with the number of fruits per plant (Kumari, Kumar et al., 2018; Manisha & Pal, 2014; Tran, 2017). Moreover, several studies stated that photosynthesis synthesis and assimilations are affected by the application of plant hormones or plant growth regulators, thus improving plant growth and development (Müller & Munné-Bosch, 2021; Shah, 2011). The utilization of plant hormones such as auxin, gibberellin, and cytokinin may be involved in photosynthesis activities and contribute to the improvement of plant growth and development, with higher fruit quality and production.

In the current study context, each plant growth regulator (PGR) applied had specific effects on the internal hormonal balance and, consequently, on the physiological functions of the gac plant. Auxins, like IAA, promote cell elongation and division, which result in increased node formation and earlier flowering. Gibberellins, such as GA, are known to stimulate stem elongation and can increase node numbers and fruit sets under certain conditions. Cytokinins, such as BA, promote cell division and differentiation, enhancing the number of nodes and pistillate flowers. MH, or maleic hydrazide, acts as a growth retardant that enhances branching and node formation by interfering with auxin transport. The internal hormone levels were modulated by applying these extraneous growth regulators, influencing photosynthesis, assimilate partitioning, and overall plant growth and development. This hormonal manipulation ultimately improved the yield and quality of gac fruits in this study.

CONCLUSION

Most growth and development variables such as internode length, ovary diameter, number of fruits, and total fruit weight demonstrated better performances in the gac plants treated with plant growth regulators than in the control. Gac plants treated with plant growth regulators also exhibited faster first flower anthesis, first harvest, and shorter duration from pollination to harvest. Furthermore, the plant growth regulators displayed the most frequent optimal performance in the number of nodes and pistillate, ovary diameter, early first flower anthesis, and number of fruits, which were those with MH at 40 and 80 ppm. It proves that the application of MH at 40 and 80 ppm on the gac plant could significantly

enhance growth and development as well as hasten gac fruit production. The study on the effects of exogenous plant growth regulators on endogenous plant hormones of gac fruit production and quality should thus be explored further in future research.

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Germination Behavior of Deteriorated Shallot Seeds Applied with Zinc as Priming Agent

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ABSTRACT

This study aims to see the effect of applying the seed priming method with the provision of micronutrient zinc (Zn) on the germination performance of two types of shallot varieties from deteriorated botanical seeds. The study was arranged on a completely randomized design with two factors. The first factor was the type of variety consisting of two combinations: Lokananta and Maserati. The second factor is priming, composed of unprimed, hydropriming indole 3 acetic acid (IAA), zinc oxide (ZnO), zinc sulfate heptahydrate ($ZnSO_4.7H_2O$), zinc ethylenediaminetetraacetic acid (Zn-EDTA). The combination of the Maserati variety and Zn-EDTA priming recorded the fastest mean germination time (2.82 days), the highest germination rate index (16.15%/day), and the coefficient velocity of germination (35.51) compared to other treatment combinations. The combination of the Maserati variety and $ZnSO_4.7H_2O$ priming recorded the highest germination percentage (86%) compared to different treatment combinations. Then, the combination of Lokananta and ZnO priming recorded the most increased vigor index (453.20), produced the most extended plumule length (55.30 mm), longest radicle length (11.00 mm), fresh weight (19.00 mg), and dry weight (2.34 mg) compared to other treatment combinations. Combining two varieties with seed priming with Zn improves deteriorated shallot seed germination potential.

Keywords: Path analysis, principal component analysis, seed priming, shallots, variety

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INTRODUCTION

Shallots are one of the primary commodities in Southeast Asia, including Indonesia. Shallots have a high economic value and play a role in shaping the country's inflation. Shallots are rich in polyphenols, organic

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sulfur compounds, and vitamins. These bioactive compounds have antioxidant properties that can reduce the occurrence of various diseases, such as ischemic heart disease, several types of cancer, and atherosclerosis (Salata et al., 2022) so that shallot consumption continues to increase along with the increasing population and the diversity of culinary preparations. The average shallot consumption of the Indonesian population reaches 3.01 kg/capita/year. However, until now, productivity has still been far below the potential yield of shallots. In 2022, the national shallot production reached 1.98 million t/ha, which decreased by 22.23 thousand tons from the previous year. Until now, national shallot productivity has reached 10.71 t/ha, still far from the potential yield of shallots (Central Bureau Statistics of Indonesia, 2023). Therefore, there is a need for intensification efforts in shallot cultivation.

Shallots are generally cultivated using bulbs as planting material. Seeds from bulbs have a short shelf life and limited availability. Currently, the available quality seeds are only 20% of the needs, so the consumption of bulbs and imports fulfills the need for bulb seeds. One of the efforts that can be made to increase shallot productivity is by using true shallot seed (TSS), which has high potential as an alternative to bulb seeds. The advantages of using TSS include: (1) the average seed requirement is 5 kg/ha, (2) relatively low cost, (3) easy transportation, (4) 1–2 years of shelf life, (5) produce healthy bulbs, avoid pathogens, (6) high productivity (Adin et al., 2023).

The problem is more than seed availability. The use of planting material from seeds also faces various obstacles in terms of the growth of seeds and the low quality of seedlings produced in the seeding process. Seeding is a crucial stage because maximum yield production is reflected in the quality of the seedlings. Botanical shallot seeds are very susceptible to quality decline if stored long. Seed germination is a parameter that shows that TSS has decreased; if seed viability is low, seed vigor is also low. Indications of quality decline can be seen from seed growth's low viability and speed (Tanjung et al., 2022). The use of planting materials from seeds needs intensive control to affect the potential quality of seed germination. Seed germination is an essential stage because maximum yield production is reflected in the quality of the seeds. Deteriorating seeds can be improved by applying invigoration technology. Invigoration is a physical, physiological and biochemical treatment that increases seed viability so that it can grow faster and synchronously in diverse environments (Triyadi et al., 2023). The seed invigoration technique that can be used is the seed priming method, a hydration technique that controls water absorption to stimulate seed germination. In the priming process, the physiological conditions of the seeds are controlled, resulting in increased and improved metabolic processes before germination. This method has various benefits, including reducing fertilizer use, increasing production by improving the quality of seed germination, inducing plant resistance, and being cheap and environmentally friendly (Tanjung et al., 2022).

Zinc (Zn), an essential micro-nutrient for shallots, is one of the seed priming agents that can be used. Zn acts as a catalyst and structural constituent in proteins and can affect some biochemical pathways and cell functions such as enzyme activity, deoxyribonucleic acid (DNA) biosynthesis, gene expression, cell division, and defense against oxidative cell damage conditions (Cakmak et al., 2023). It is an essential micronutrient for humans, animals, and plants and a component of enzymes that catalyze plant metabolic reactions. Zn increases plant resistance to disease, plays a role in photosynthesis, maintains cell membrane integrity, is needed in protein synthesis and pollen formation, and increases antioxidant enzymes and chlorophyll levels in plant tissues. Zn deficiency negatively affects plant growth, causing plants to be stunted, have short internodes, small leaves, chlorosis, and delayed maturity. Thus, Zn sufficiency is critical for crop yield and quality (Hacisalihoglu, 2020; Vadlamudi et al., 2020). Saranya et al. (2017) concluded that seeds given Zn are well used to refresh shallot seedlings and get quality seedlings. Priming induces germination metabolic activity and produces glucose, which is used in protein synthesis during germination to increase germination rate and plant growth uniformity. This study aims to see the effect of applying the seed priming method with the provision of micronutrient Zn on the germination performance of two types of shallot varieties from deteriorated botanical seeds.

MATERIALS AND METHODS

Experiment Location

The research was conducted from June to July 2023 at the Laboratory of Mushroom and Biofertilizers, Department of Agronomy, Agriculture Faculty, Hasanuddin University, Makassar, Indonesia. The average laboratory temperature was 26.1°C.

Experimental Design

The research was arranged on a completely randomized design with two factors. The first factor is the type of variety (v) consisting of two varieties: (1) Lokananta (v_0) and (2) Maserati (v_1). The second factor is the type of Zn priming agent (z) consisting of (1) unprimed (z_0), (2) hydropriming (z_1), (3) IAA (z_2), (4) ZnO (z_3), (5) $ZnSO_4.7H_2O$ (z_4), and (6) Zn-EDTA (z_5). Twelve treatment combinations were repeated three times, resulting in 36 observation units.

Preparation for Priming Agents

This study used Zn and IAA priming agents with a solution concentration of 100 ppm. The solution was obtained by dissolving 125.72 mg/L ZnO, 444.04 mg/L $ZnSO_4.7H_2O$, 700.77 mg/L zinc EDTA and 100 mg/L IAA in distilled water solution.

Seed Priming Implementation

The shallot seeds used are Lokananta and Maserati varieties with an expiration date of 15 months. The shallot seeds were then primed according to the treatment. A 4.5 g of seeds were added to each treatment solution in a 1:20 (W/V) ratio in a glass jar connected to an aerator. Then, the seeds were soaked for 20 h. Afterward, the seeds were removed and dried until they reached their initial moisture content—the character of the seed priming solution in Table 1.

Table 1
Character of seed priming solution

Treatments	Electrolyte Conductivity ($\mu S\ cm^{-1}$)		pH		Temperature (℃)		Solute (ppm)	
	X	Y	X	Y	X	Y	X	Y
Unprimed	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Hydropriming	0.00	316.00	7.08	7.12	30.00	28.80	0.00	158.00
Priming IAA	123.00	418.00	6.75	7.24	29.80	28.70	61.00	209.00
Priming ZnO	87.00	536.00	6.93	7.07	29.60	29.70	43.00	268.00
Priming $ZnSO.7H_2O$	521.00	854.00	5.41	6.92	29.70	29.10	260.00	427.00
Priming Zn-EDTA	366.00	719.00	5.74	7.03	29.70	29.00	181.00	359.00

Note. Description: X (before priming), Y (after priming)

Germination Assay

The germination assay was done using a paper method test. Seeds primed are sterilized using 70% alcohol and washed with distilled water. Then, 50 seeds are placed in a petri dish for each treatment. The seeds are then placed in a plant growth chamber (©Labtech made in Indonesia) in the laboratory.

Parameters and Data Analysis

The parameters observed included mean germination time (days), germination percentage (%), coefficient velocity of germination, germination rate index (%/day), vigor index, radicle length (mm), plumule length (mm), fresh weight (mg), and dry weight (mg). These parameters were calculated using the Weerasekara et al. (2021) formula (Table 2). The data collected were then analyzed for correlation, principal component analysis (PCA), path analysis, and analysis of variance. If the data were significant, further tests with Duncan's new multiple range test (DMRT) at a significant level of 5% would be needed to detect the differences among treatments.

Table 2
The observation parameter formula mean germination time, germination percentage, coefficient velocity of germination, germination rate index, and vigor index

Parameters	Formula
Mean Germination Time (days)	MGT = $\frac{\Sigma fx}{\Sigma f}$ Remarks: F = number of seeds germination on day x
Germination Percentage (%)	GP = $\frac{A}{B} \times 100$ Remarks: A = number of seeds germination B = total test seeds
Coefficient Velocity of Germination	CVG = $\left(\frac{G1+G2+G3+\ldots+Gn}{G1T1+G2T2+G3T3+\ldots+GnTn}\right) \times 100$ Remarks: G1/2/3/n= number of seeds germination on day 1/2/3/n T1/2/3/n= observation day
Germination Rate Index (%/day)	GRI = $\frac{G1}{1} + \frac{G2}{2} + \frac{G3}{3} + \ldots + \frac{Gn}{n}$ Remarks: G1/2/3/n= number of seeds germination on day 1/2/3/n
Vigor Index	VI = GP × PL Remarks: GP = germination percentage PL = plumule length

RESULTS

Germination Parameter

Analysis of variance showed an interaction between the use of shallot seed varieties and seed priming agents that significantly affected the mean germination time, germination rate index, and coefficient velocity of shallot seeds (Table 3). The combination of the Maserati variety and Zn-EDTA priming recorded the fastest mean germination time (2.82 days), which was not significantly different from the combination of the Lokananta variety and IAA priming, the highest germination rate index (16.15%/day), and the coefficient velocity of germination (35.51) compared to other treatment combinations.

Analysis of variance showed an interaction between the use of shallot seed varieties and seed priming types that significantly affected the germination percentage, vigor index, and plumule length of shallot seeds (Table 4). The combination of Maserati varieties and $ZnSO_4.7H_2O$ priming recorded the highest germination percentage (86%), which was not significantly different from the combination of Maserati varieties and ZnO priming, Maserati and Zn-EDTA priming, Lokananta and Zn-EDTA priming, Lokananta and ZnO,

Lokananta and hydropriming, but significantly different compared to other treatment combinations. Then, the combination of Lokananta variety and ZnO priming recorded the highest vigor index (453.20) and produced the most extended plumule length (55.30 mm), which was not significantly different from the combination of Lokananta and IAA priming, Lokananta and $ZnSO_4.7H_2O$ priming, Lokananta and Zn-EDTA priming, Maserati and IAA priming, Maserati and ZnO priming, Maserati and $ZnSO_4.7H_2O$ priming, Maserati and Zn-EDTA priming, but significantly different compared to other treatment combinations.

Analysis of variance showed an interaction between the use of shallot seed varieties and seed priming types that significantly affected radicle length, fresh weight, and dry weight of shallot seedlings (Table 5). The combination of Lokananta variety and ZnO priming recorded the most extended radicle length (11.00 mm), which was not significantly different from the combination of Lokananta and IAA priming, Lokananta and $ZnSO_4.7H_2O$ priming, Lokananta and Zn-EDTA priming, Maserati and IAA priming, Maserati and ZnO priming, Maserati and $ZnSO_4.7H_2O$ priming, Maserati and Zn-EDTA priming, but significantly different compared to other treatment combinations, the heaviest fresh weight (19.00 mg), which was not significantly different from the combination of Lokananta and Zn-EDTA priming, Maserati and IAA priming, Maserati and $ZnSO_4.7H_2O$ priming, Maserati and Zn-EDTA priming, but significantly different compared to other treatment combinations, and the heaviest dry weight (2.34 mg) compared to other treatment combinations.

Table 3
The effect of combining two varieties with priming agents on the mean germination time, germination rate index, and coefficient velocity of germination

Combination Treatment		Mean Germination Time (days)	Germination Rate Index (%/day)	Coefficient Velocity of Germination
Lokananta	Unprimed	4.14 ± 0.46^{b}	7.95 ± 0.50^{cd}	24.42 ± 2.68^{bc}
	Hydropriming	5.29 ± 0.32^{b}	8.36 ± 0.89^{bcd}	18.96 ± 1.14^{cd}
	IAA	3.98 ± 0.40^{ab}	10.00 ± 1.40^{bcd}	25.40 ± 2.54^{b}
	ZnO	4.45 ± 0.48^{b}	10.57 ± 0.51^{bc}	22.72 ± 2.43^{bcd}
	$ZnSO_4.7H_2O$	4.90 ± 0.04^{b}	8.61 ± 0.19^{bcd}	20.40 ± 0.18^{bcd}
	Zn-EDTA	5.04 ± 0.43^{b}	9.42 ± 0.68^{bcd}	19.98 ± 1.71^{bcd}
Maserati	Unprimed	7.49 ± 0.94^{c}	1.42 ± 0.53^{e}	13.57 ± 1.71^{e}
	Hydropriming	5.33 ± 0.06^{b}	7.50 ± 0.22^{d}	18.75 ± 0.20^{d}
	IAA	4.93 ± 0.30^{b}	8.09 ± 0.54^{bcd}	20.35 ± 1.25^{bcd}
	ZnO	5.01 ± 0.11^{b}	9.32 ± 0.52^{bcd}	19.95 ± 0.43^{bcd}
	$ZnSO_4.7H_2O$	4.39 ± 0.07^{b}	10.79 ± 0.37^{b}	22.81 ± 0.35^{bcd}
	Zn-EDTA	2.82 ± 0.15^{a}	16.15 ± 1.71^{a}	35.51 ± 1.88^{a}

Note. Mean ± SE values with different letters in the column indicate significant ($p < 0.05$) differences by Duncan's new multiple-range test

Table 4
Effect of combination of two varieties with priming agents on germination percentage, vigor index, and plumule length

Combination Treatment		Germination Percentage (%)	Vigor Index	Plumule Length (mm)
Lokananta	Unprimed	59.00 ± 1.00^{d}	166.30 ± 1.90^{c}	28.20 ± 0.80^{b}
	Hydropriming	76.00 ± 4.00^{abc}	248.32 ± 38.08^{c}	32.50 ± 3.30^{b}
	IAA	70.00 ± 6.00^{c}	359.00 ± 86.36^{ab}	50.60 ± 8.00^{a}
	ZnO	82.00 ± 2.00^{ab}	453.20 ± 0.40^{a}	55.30 ± 1.30^{a}
	$ZnSO_4.7H_2O$	72.00 ± 2.00^{bc}	354.32 ± 12.72^{b}	49.20 ± 0.40^{a}
	Zn-EDTA	81.00 ± 1.00^{ab}	413.84 ± 0.56^{ab}	51.10 ± 0.70^{a}
Maserati	Unprimed	18.00 ± 4.00^{e}	31.62 ± 19.86^{d}	15.90 ± 7.50^{c}
	Hydropriming	72.00 ± 0.00^{bc}	232.56 ± 5.04^{c}	32.30 ± 0.70^{b}
	IAA	69.00 ± 1.00^{c}	356.50 ± 10.70^{ab}	51.70 ± 2.30^{a}
	ZnO	84.00 ± 2.00^{a}	427.80 ± 15.00^{ab}	51.00 ± 3.00^{a}
	$ZnSO_4.7H_2O$	86.00 ± 2.00^{a}	448.08 ± 48.24^{ab}	52.00 ± 4.40^{a}
	Zn-EDTA	81.00 ± 5.00^{ab}	414.90 ± 10.70^{ab}	51.50 ± 4.50^{a}

Note. Mean ± SE values with different letters in the column indicate significant ($p < 0.05$) differences by Duncan's new multiple-range test

Table 5
Effect of combination of two varieties with priming type on radicle length, fresh weight, and dry weight

Combination treatment		Radicle length (mm)	Fresh weight (mg)	Dry weight (mg)
Lokananta	Unprimed	3.70 ± 1.90^{cd}	7.50 ± 0.50^{d}	1.53 ± 0.005^{g}
	Hydropriming	3.90 ± 1.50^{bcd}	8.50 ± 2.50^{d}	1.66 ± 0.010^{fg}
	IAA	7.40 ± 4.60^{abcd}	12.50 ± 1.50^{bcd}	1.82 ± 0.000^{de}
	ZnO	11.00 ± 0.20^{a}	19.00 ± 0.00^{a}	2.34 ± 0.075^{a}
	$ZnSO_4.7H_2O$	10.20 ± 1.00^{ab}	10.50 ± 3.50^{cd}	1.84 ± 0.095^{de}
	Zn-EDTA	9.30 ± 2.30^{abc}	15.50 ± 2.50^{abc}	2.06 ± 0.015^{bc}
Maserati	Unprimed	1.60 ± 0.60^{d}	0.00 ± 0.00^{e}	0.00 ± 0.000^{h}
	Hydropriming	3.30 ± 1.10^{cd}	10.50 ± 2.50^{cd}	1.78 ± 0.060^{ef}
	IAA	6.60 ± 1.00^{abcd}	15.00 ± 0.00^{abc}	1.93 ± 0.000^{cde}
	ZnO	8.60 ± 0.40^{abcd}	12.00 ± 1.00^{bcd}	1.88 ± 0.070^{de}
	$ZnSO_4.7H_2O$	6.60 ± 1.20^{abcd}	17.00 ± 1.00^{ab}	2.12 ± 0.050^{b}
	Zn-EDTA	7.60 ± 1.60^{abcd}	15.50 ± 0.50^{abc}	1.99 ± 0.055^{bcd}

Note. Mean ± SE values with different letters in the column indicate significant ($p < 0.05$) differences by Duncan's new multiple-range test

Correlation Analysis

A correlation map based on color between observation parameters is shown in Figure 1. Correlation value is a parameter used to evaluate the relationship between characters. The correlation value is between -1 and 1, where if it is positive, then if the value of a character increases, it will increase the value of other characters. If it is negative, then an increase in the value of a character will reduce the value of other characters. The correlation coefficient value is in the range of weak (<0.40), moderate (>0.4), and strong (>0.70) (Schober et al., 2018).

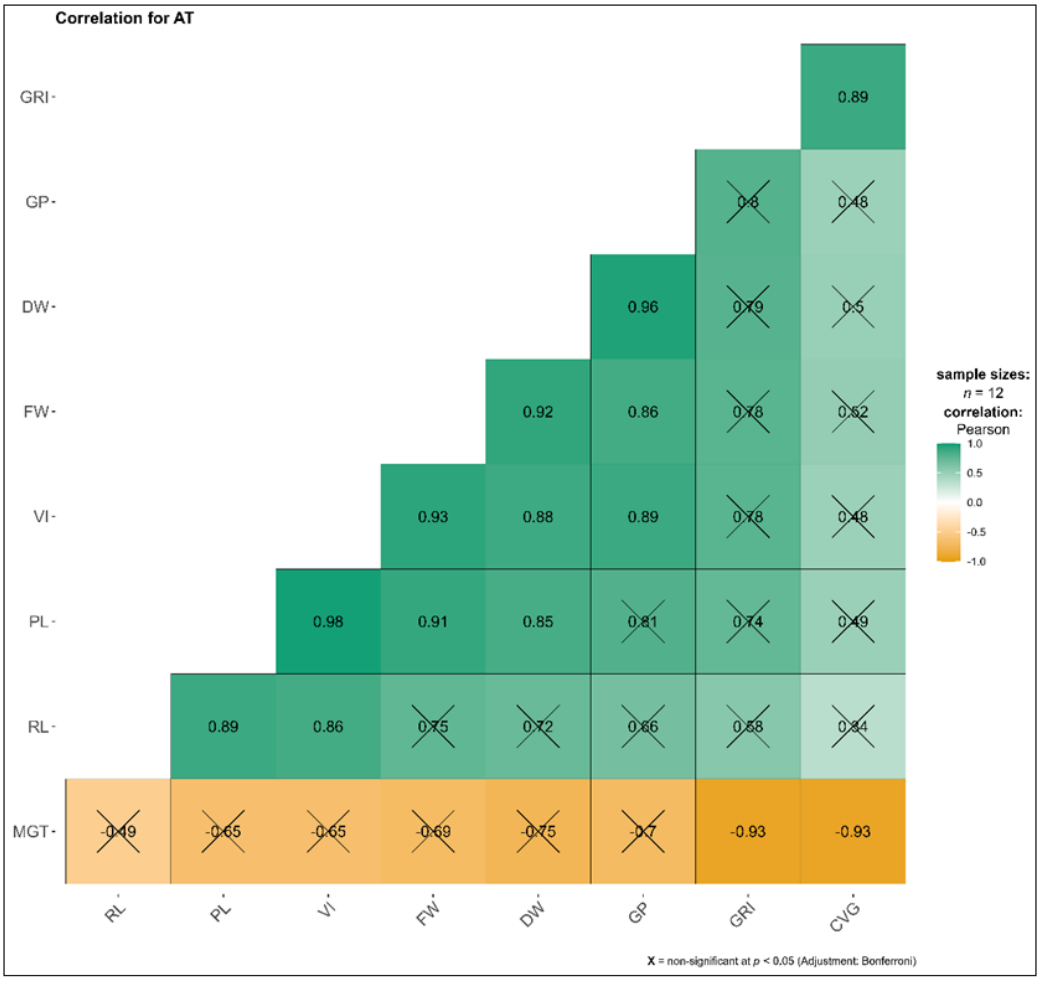

Figure 1. Correlation maps among parameters. (MGT) mean germination time; (RL) radicle length; (PL) plumule length; (VI) vigor index; (FW) fresh weight; (DW) dry weight; (GP) germination percentage; (GRI) germination rate index; (CVG) coefficient velocity of germination *Note*. The x symbol indicates a non-significant relationship between parameters according to the Bonferroni test, as stated in the figure caption

A strong correlation was shown in the relationship parameters between vigor index with plumule length (0.97), fresh weight with dry weight (0.92), radicle length with fresh weight (0.75), radicle length with dry weight (0.71), plumule length with radicle length (0.89), radicle length with fresh weight (0.91), radicle length with dry weight (0. 85), vigor index with radicle length (0.86), vigor index with fresh weight (0.93), vigor index with dry weight (0.88), germination percentage with vigor index (0.88), germination percentage with plumule length (0.80), germination percentage with fresh weight (0.85), germination percentage with dry weight (0.95), germination rate index with coefficient velocity of germination (0.89), germination rate index with germination percentage (0.80), germination rate index with vigor index (0.78), germination rate index with plumule length (0.74), germination rate index with fresh weight (0.77), and germination rate index with dry weight (0.78).

A moderate correlation was shown in the relationship parameters between germination percentage with radicle length (0.66), the coefficient velocity of germination with germination percentage (0.47), the coefficient velocity of germination with vigor index (0.48), the coefficient velocity of germination with radicle length (0.48), the coefficient velocity of germination with fresh weight (0.51), and the coefficient velocity of germination with dry weight (0.50). A weak correlation is shown in the relationship parameter between the coefficient velocity of germination with radicle length (0.33). The relationship between the mean germination time and all parameters showed a negative correlation.

Principal Component Analysis

Based on the principal component analysis in Figure 2, the first principal component (PC1) significantly positively affected germination percentage, dry weight, fresh weight, vigor index, plumule length, and radicle length. PC1 also has a significant negative impact on mean germination time. The second principal component (PC2) significantly positively affects the germination rate index and coefficient velocity of germination. PC2 also has a significant negative impact on the mean germination time. Based on the principal component analysis in Figure 2, the biplot results show that combining two varieties of treatment with seed priming can increase the value of the first principal component. Therefore, seed priming treatment with IAA, ZnO, $ZnSO_4.7H_2O$, and Zn-EDTA on Lokananta and Maserati varieties increases the value of germination percentage, dry weight, fresh weight, vigor index, plumule length, and radicle length compared to water priming and no priming treatments. Biplot results also show that combining two varieties of treatment with seed priming can increase the value of the second principal component. Therefore, seed priming treatment with IAA and Zn-EDTA on Lokananta and Maserati varieties increased the germination rate index and coefficient velocity of germination.

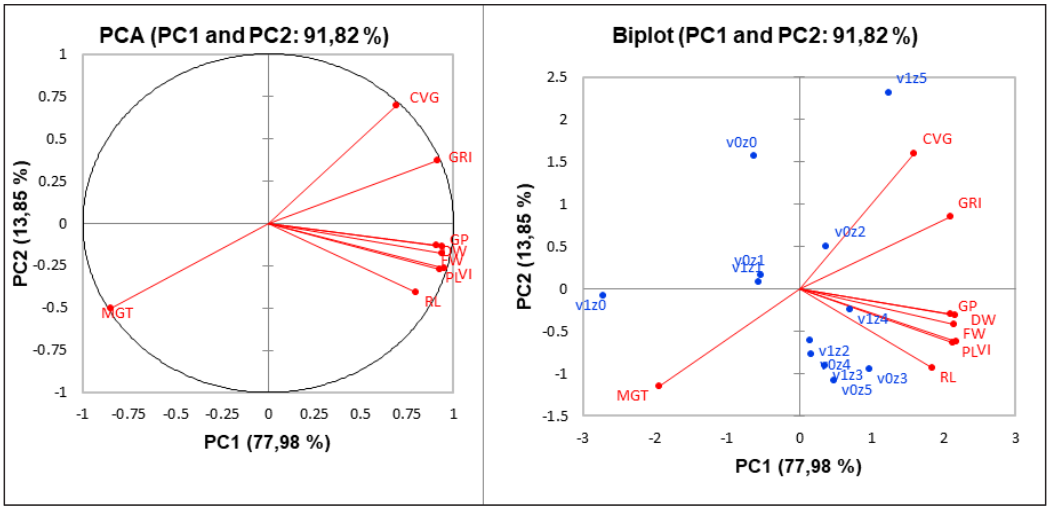

Figure 2. Biplot of shallot seed germination based on PC1 and PC2. MGT=mean germination time; RL=radicle length; PL=plumule length; VI=vigor index; FW=fresh weight; DW=dry weight; GP=germination percentage; GRI=germination rate index; CVG=coefficient velocity of germination. Lokananta + unprimed (v_0z_0), Lokananta + Hydropriming (v_0z_1), Lokananta + IAA priming (v_0z_2), Lokananta + ZnO priming (v_0z_3), Lokananta + $ZnSO_4.7H_2O$ priming (v_0z_4), Lokananta + Zn-EDTA priming (v_0z_5), Maserati + unprimed (v_1z_0), Maserati + Hydropriming (v_1z_1), Maserati + IAA priming (v_1z_2), Maserati + ZnO priming (v_1z_3), Maserati + $ZnSO_4.7H_2O$ priming (v_1z_4), and Maserati + Zn-EDTA priming (v_1z_5)

Path Analysis

Correlation analysis can identify the relationship between two characters but does not explain the relationship. Thus, insignificant correlation coefficient values cannot be taken to show a functional relationship between each variable. Path coefficient analysis explains it by dividing the total correlation coefficient into components that have direct and indirect effects (Waluyo et al., 2022). The path coefficient value, according to Solanki et al. (2015), is divided into very high (>1), high (0.30–0.99), medium (0.2–0.29) and low (0.1–0.19).

The results of path analysis between observation parameters are shown in Figure 3. The value of the observation parameter that has a very high direct effect on the dry weight of seedlings is the germination percentage (1.01). The value of the observation parameter that has a high direct effect on the dry weight of the seedlings is plumule length (0.43) and fresh weight of the seedlings (0.54). Observation parameter values that have a low direct effect on the dry weight of seedlings are the coefficient velocity of germination (0.13) and radicle length (0.19). Parameters of germination percentage, plumule length, and fresh weight of seedlings have a very high direct effect and have a positive value: germination percentage (1.01), plumule length (0.43), and fresh weight of seedlings (0.54). It is in line with the results of the correlation between the dry weight of seedlings and the germination percentage (0.95), plumule length (0.85), and fresh weight of seedlings (0.92). If the

correlation between parameters is almost the same as the direct effect, the correlation can explain the actual relationship and direct selection through these variables will be effective (Waluyo et al., 2022). So, the dry weight of seedlings can be determined by direct selection on germination percentage, plumule length, and fresh weight. In contrast, the dry weight of shallot seedlings can be increased by increasing these growth parameters.

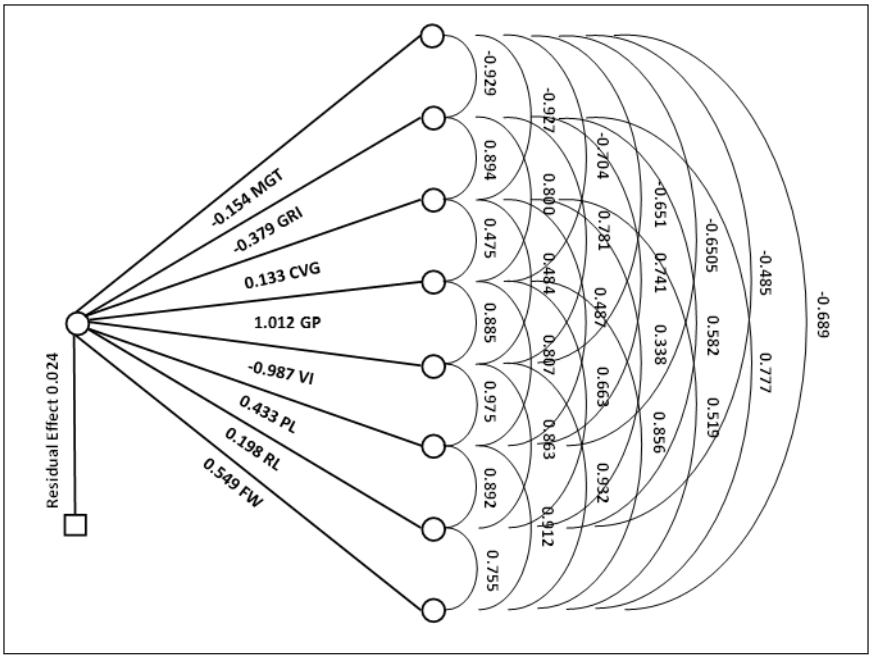

Figure 3. Path coefficient analysis diagram for dry weight of shallot seedlings. MGT=mean germination time; RL=radicle length; PL=plumule length; VI= vigor index; FW=fresh weight; DW=dry weight; GP=germination percentage; GRI=germination rate index; CVG=coefficient velocity of germination

DISCUSSION

The priming method affects the physiological process of seeds, which refers to the amount of metabolic activity in the early stages of seed germination that can be seen from the germination parameters (Choukri et al., 2022). In this case, the germination test showed that combining the Maserati variety treatment and Zn-EDTA priming increased the mean germination time, germination rate index, and coefficient velocity of germination. The differences that occur are due to genetic factors from two different varieties. Yeshiwas et al. (2022) explained that the differences between each variety for plant height and leaf length of shallots are due to differences in genotypes and responses to different environmental conditions. On the other hand, priming with Zn-EDTA is more efficient than other types of priming. Zn-EDTA compounds are more difficult to mobile than ZnO and $ZnSO_4.7H_2O$ due to the high stability constant of the Zn complex (Doolette et al., 2018). The chelated

form of Zn-EDTA has the effect of reducing the mobility of Zn transport in plant tissues and limiting Zn translocation, so it is more beneficial because it can avoid the effect of toxicity on plant tissues, which can further reduce the decrease in Zn availability in plants.

True shallot seeds are highly susceptible to deterioration due to prolonged storage. The decline in seed quality can be seen from the seeds' low germination and growth strength. The germination test showed that the treatment combination of the Maserati variety and Zn-EDTA priming increased the germination percentage, and the combination of the Lokananta variety and ZnO priming increased the vigor index and plumule length of the seedlings. Differences can be seen in unprimed seeds. Two seed varieties have deteriorated due to long seed storage. Then, Hiremat et al. (2018) reported that onion seeds progressively lost their vigor and germination as they age. Germination percentages decreased to 69% and 55%, and the percentage of seedlings emerging in the field to 66% during the storage period of nine months. The results showed that free radicals in aged seeds can cause membrane damage when mitochondrial respiration is activated. Increased reactive oxygen species result from less efficiency in mitochondrial activity. DNA, RNA, proteins, and lipids are essential molecules that can undergo oxidation due to increased reactive oxygen species. Furthermore, the mitochondrial membrane oxidizes, reducing aerobic respiration potential (Ranganathan & Groot, 2023). Priming Zn can increase the germination percentage where Zn has structural and catalytic functions in several proteins and affects several biochemical pathways and cell functions such as enzyme activity, DNA, and protein biosynthesis and maintains defense against oxidative cell damage (Cakmak et al., 2023).

The germination test showed that the treatment combination of Lokananta variety and ZnO priming increased radicle length, fresh weight, and dry weight of seedlings. Zn has a vital role in protein synthesis, where Zn deficiency reduces the rate of protein synthesis and protein concentration in plant tissues. Zn is a structural component in ribosomes. In the absence of Zn, ribosomes will be destroyed but can be reversed when given a supply of Zn. The results showed that the need for Zn in the apical part of the growing plant is about 150 µg compared to the need for the basal part of only about 50 µg (Ender et al., 1983). At the root tip of newly grown plants, there is 220 µg of Zn concentration (Ozturk et al., 2006). Zn concentration is at least 100 µg in shoot meristem tissue, and other meristems are required in the process of protein synthesis, which is translocated by the roots to the shoot meristem through the xylem-phloem network (Cakmak et al., 2023). Protein concentration is reduced in plants caused by Zn deficiency, where there is a decrease caused by the higher rate of RNA degradation due to increased RNAse activity (Sharma et al., 1982). To support the process of DNA translation and transcription, adding an addition is necessary to increase RNA activity significantly.

CONCLUSION

Based on the research that has been done, micronutrient zinc seed priming treatment affects the germination of shallot seeds. Both varieties have different responses to seed priming treatment. The combination of Maserati varieties and Zn-EDTA priming recorded the fastest mean germination time, the highest germination rate index, and the coefficient velocity of germination. The combination of the Maserati variety and $ZnSO_4.7H_2O$ priming recorded the highest germination percentage. Combining the Lokananta variety and ZnO priming recorded the highest vigor index and produced the most extended plumule length. Then, the combination of Lokananta and ZnO priming recorded the most extended radicle length, heaviest fresh weight, and heaviest dry weight.

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Impact of *Paenibacillus polymyxa* Amendment on Soil Bacterial Communities and Physicochemical Properties in Sandy Soil Restoration

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ABSTRACT

Soil infertility is a significant challenge in achieving sustainable agricultural practices. In this regard, the chemical fertilizer usage is not an environmentally friendly solution. Organic amendment and bacterial inoculation can positively restore soil quality, enhancing biogeochemical nutrient cycles. In this study, we assessed the effect of adding plant growth-promoting bacteria (PGPB) alongside organic amendments on the physicochemical parameters of sandy-loam soils. Over a 90-day pot experiment, we measured organic matter accumulation, physicochemical, chemical variation trends and changes in microbial community assemblages. Working on the joint application could have a synergistic effect; different agro wastes spent such as mushroom substrate (SMS), empty fruit bunch (EFB) of palm oil and pineapple leaf (PL) residue was amended with *Paenibacillus polymyxa* ATCC 825 and effective microorganism. Significant changes in soil properties (physicochemical and microbial community) due to the application of *P. polymyxa* and SMS-amended material were observed after incubation. On average, an increase in water holding capacity, soil pH and mineral content availability was significantly higher than other amended materials. Compared to others, the organic amendment significantly increased sandy-soil aggregation content by 44%. In addition, increased taxonomic diversity in phyla composition was observed with an abundance of *Proteobacteria* (33%), *Firmicutes* (18%), *Actinobacteria* (14%), *Bacteriodata* (12%), and *Verrucomicrobia* (6%). The findings indicate that the addition of SMS amendment

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with bacterial inoculation is beneficial for problematic soil recovery. The incorporation of bacterial inoculation, specifically *P. polymyxa* ATCC 825, following organic amendment, seems to have a greater positive effect on the soil characteristics.

Keywords: Agro-waste, microbial community structure, organic amendment, *Paenibacillus polymyxa*, sandy soil

INTRODUCTION

Land desertification is a worldwide issue receiving much attention nowadays, affecting the socio-economic and ecological environment. Unsuitable agricultural practices and anthropogenic activities have resulted in permanent soil fertility degradation. This frequently degraded soil is characterized by decreasing levels of soil organic matter and low fertility as consequences of intensive land use. Generally, sandy soils contain much lower carbon content, high evapotranspiration and poor aggregate stability with coarse-loose particle structure, influencing their water retention and adhesion to any mineral elements (Vityakon, 2007; Herawati et al., 2020). Sandy soil covers 86,000 km^2 in Asia, and that region is not suitable for cropping purposes. In comparison with other soils, tropical sandy soil tends to possess low storage of C content and is presumed to be very fragile to mitigate greenhouse gas (GHG) emissions (Arunrat et al., 2020). As a result, the ecosystem functioning of desertified land is likely unstable, restricting its reclamation to normal conditions. Therefore, the realization of resource utilization for sandy soil restoration offers great significance to reducing soil depletion while achieving environmental protection.

An important step for soil restoration is the identification of feasible amendment strategies. In this regard, the organic amendment seems feasible and practical as the efficacies of physical and chemical methods are doubtly questioned in terms of safety and cost implication (Sahin et al., 2011; Singh et al., 2016). In light of the role played by organic amendment, it is vital to consider this approach as it has been proven to improve aggregate stability and soil organic content (SOC). SOC plays an important indicator in evaluating soil aggregate stability. It is primarily due to increased organic matter content that promotes the proliferation of soil microbial community diversity through decomposition and mineralization processes (Kamolmanit et al., 2013). Having a significant impact on the microbial structure, Tang et al. (2015) demonstrated that organic carbon content has a high correlation with soil respiration rate, with an increase in microbial diversity, thus promoting aggregate formation. The application of organic inputs with good biochemical quality is considered feasible as a means of guaranteeing SOC accumulation in the sandy soil. According to Putasso et al. (2011), organic input with high N content and low cellulose and polyphenol content (tamarind residue) was a suitable option for amendment purposes under long-term field experiments in Thailand as soil stabilization was greatly regulated.

Much work has been conducted on the application of spent mushroom substrate (SMS) or any agro-waste to improve soil aggregate stability, as SMS can act as a cementing agent and composted material (Atallah et al., 2021; Loganathan et al., 2023). Specifically, spent mushroom compost (SMC) applies to establishing weak-structured and physically degraded soil. This positive result was attributed to the high level of organic carbon content and low-strength material properties (Gűműş & Şeker, 2017). The substantial addition of organic amendment input material increases SOC content per se and provides a readily available substrate for soil microbiome.

Native bacteria play an important role in maintaining soil fertility by mineralizing essential nutrients through solubilization and improving nutrient uptake. The application of microbial inoculants consisting of plant growth-promoting bacteria (PGPB) may enhance the mobility of low nutrient supply such as potassium and phosphorus. Following that, a relatively less explored bacterial strain, *Paenibacillus polymyxa*, was reported to have been intrinsically stable and capable of solubilizing phosphorus under extreme conditions (Mohd Din et al., 2020). Besides, a wealth of hydrolytic enzymes avenue together with exopolysaccharides (EPSs) capability from *P. polymyxa* has been reviewed by Daud et al. (2019) to mark the beneficial properties owned by that bacterial strain. In recent years, some scholars have researched the combined effect of microbial inoculation and organic amendment under semiarid conditions. For example, Mengual et al. (2014) have reported that using native rhizobacteria (*Bacillus megaterium* and *Bacillus* sp.) and composted sugar beet residue for soil rehabilitation showed a significant increase in phosphorus availability in the soil as compared to the control. Trivedi et al. (2017) have reported that microbial application has a positive effect on saline-sodic soil fertility with increased harvestable yield after amended soil incubation.

In Malaysia, sandy loam soils with a high volume of sand compared to silt are commonly often improved by organic amendment (Garbowski et al., 2023; Manickam et al., 2015). Additionally, 168 million tons of abundant agro-waste biomass generated from Malaysian agriculture systems have provided a good advantage in the exploitation route into a source of wealth. To elucidate the effect of different organic inputs (spent mushroom substrate [SMS], empty fruit bunch [EFB], pineapple leaf [PL] residues) amended with PGP bacteria on the physicochemical properties of sandy loam soil, we conducted a short-term experiment to quickly observe how these biochemically contrasting organic inputs lead to distinct shifts in microbial community structure and abundance. This study aims to test our hypothesis that increases in soil physicochemical properties are correlated with the selection of various organic materials used in conjunction with the microorganisms studied. Results from this study could provide an empirical solution in using the combination of PGPB and organic residues while minimizing environmental waste for application in a degraded sandy soil area.

MATERIALS AND METHODS

Experimental Site

Soil for our study was collected from the University of Technology Malaysia (UTM) research farm located in Pagoh province, southern state Johor, Malaysia (2°09'19.9"N 102°44'00.2"E) at an average altitude of 22 meters or 72.18 feet above sea level. The soil was taken from a depth of 20 cm. The area has a typically rain-fed attribute throughout the year. The average temperature is 32°C (<28°C minimum and about 40°C maximum). A hydrometer test was used to calculate the fraction of sand, silt, and clay in the soil and determine the texture of the soil using the US Department of Agriculture (USDA) triangle (Barman & Choudhury, 2020). The initial physicochemical properties of the collected soil sample are shown in Table 1. The texture of the soil was classified as sandy-loam soil with proportions: sand: 83%, silt: 9%, and clay: 8%, respectively. The soil used in this study has low aggregate stability and low organic matter content. Different organic inputs were used in this study, namely spent mushroom substrate (SMS), empty fruit bunch (EFB) of palm oil and pineapple leaf (PL) residues.

Table 1
Physiochemical properties of sandy-loam soil used in this study

Parameters	Sandy-loam soil (before treatment)
Moisture content (%)	35.0
pH	3.8
EC (dSm^{-1})	64.3
C/N ratio	0.4
Available P (mg kg^{-1})	29.2
Available K (mg kg^{-1})	399.8

Note. EC=Electrical conductivity; C/N ratio=Carbon to Nitrogen ratio; Available P= Available Phosphorus; Available K=Available Potassium

Experimental Design

The experiment involved different types of soil amendments and was conducted under greenhouse conditions (30°C ± 3). The experiment was arranged in a completely randomized design (CRD) with three replicates for each treatment. The air-dried and ground sandy soil was sieved to pass through a 2-mm sieve, and a total of 2 kg sieved soil was used for each treatment and placed in the pots (size 16' x 16') before being mixed with organic input materials. The effect of inoculant *P. polymyxa* ATCC 825 and Effective Microorganism (EM) applied on each of the organic inputs was assessed on organic matter accumulation, nutrient availability, acidity, water retention and soil aggregation of sandy-loam soil. All the treatments were mixed up with sandy soil in three replicates. Six treatments and their abbreviations are as follows: control (T_0), *P. polymyxa* + SMS (T_1), *P. polymyxa* + EFB (T_2), *P. polymyxa* + PL (T_3), EM + SMS (T_4), EM + EFB (T_5), EM + PL (T_6). All treatments were watered twice a day with 50 ml distilled water to maintain 60% of the maximum water holding capacity. Each inoculant of approximately 60 ml volume was poured once a month, and the control treatments were treated with 50 ml deionized water instead. Soil mixture was incubated for 90 days. After incubation,

soil samples were collected from each pot and mixed according to the treatment applied to ensure homogeneity before analysis.

Inoculants Preparation and Organic Input Material

The plant growth-promoting bacteria (PGPB), *Paenibacillus polymyxa* ATCC 825, was obtained from the American Type Culture Collection (ATCC). The PGPB strain was preserved in sterile cryovial tubes containing nutrient broth (NB) with 20% glycerol (Chemiz, Malaysia) and stored in the deep freezer at -80°C. *P. polymyxa* ATCC 825 was activated on nutrient agar (NA) to prepare the inoculant. NA was composed of (g L^{-1}) yeast extract 2.0 (HiMedia, India), peptone 5.0 (Merck, Germany), sodium chloride 5.0 (Merck, Germany), and agar 15.0 (Sigma-Aldrich, USA). The pH was adjusted to 7 before autoclave and incubated at 30°C. After 24 h of incubation, the cells on the solid medium were collected and further grown in an optimized liquid medium composed of sucrose, 30 g L^{-1} yeast extract (HiMedia, India), 30 g L^{-1} dipotassium phosphate (Merck, Germany), 5.72 g L^{-1} ammonium nitrate (Daejung, Korea), 5 g L^{-1} potassium dihydrogen phosphate (Merck, Germany), 1.9 g L^{-1} and magnesium sulfate (Merck, Germany), 0.5 g L^{-1} for 24 h at 30ºC and under agitation speed 150 rpm. When the growth of *P. polymyxa* ATCC 825 reached the stationary phase after 16 h cultivation with 10^8 Colony Forming Unit (CFU) mL^{-1}, the broth was repeatedly centrifuged and suspended in 100 mM phosphate buffer.

Commercial EM-1 was activated according to the manufacturer's instructions. Activation was carried out by incubating the mixture of EM-1 and sterilized molasses with 20 parts water for 7 days, which comprised approximately 10^7 CFU ml^{-1} of fungi and 10^8 CFU mL^{-1} of bacteria. According to the standard inoculation procedure, 10 ml of activated EM-1 was diluted with 1000 ml distilled water before addition to soil amendments. This activated EM-1 solution was referred to as EM hereafter.

Different agro-waste materials, referred to as organic amendment, were obtained from the nearby fresh market and mass mushroom cultivation farm. Spent mushroom substrate (SMS) from *Pleurotus ostreatus* species was kindly obtained from C&C Mushroom Cultivation Farm, Grisek province, state of Johor, Malaysia. All organic amendments, including EFB of palm oil and pineapple leaf PL residue, were properly ground to 1–2 cm in diameter using the multipurpose grinder. These organic amendments were dried in a 60°C oven. Then, dried organic amendment materials were thoroughly mixed with sandy soil with a ratio of 2:1. The total weight for each pot treatment was approximately 3 kg.

Soil Sampling

Samples were collected 90 days after incubation. Soil samples were collected from all the pots at the soil depth of 0–15 cm depth from the surface and kept in polyethylene bags. All visible vegetation residues were removed from the soil samples. The soil samples

were divided into subsamples for physicochemical and microbial community analysis. One subsample was evenly mixed, air-dried, sieved at 2 mm-sieve, packed in zip lock plastic bags and kept at 4°C prior to commencing soil physicochemical analysis. The other subsample was stored in a -80°C freezer prior to Deoxyribonucleic Acid (DNA) extraction.

Analysis of Soil Physicochemical Properties

The soils were analyzed for pH, Electrical Conductivity (EC), soil moisture, carbon to nitrogen (C/N ratio), and nutrient contents (P: phosphorus and K: potassium). Soil pH and EC values of the soil were measured in a ratio of 1:2 (w/v) soil to water suspension using a pH meter (Mettler Toledo, Germany). 20 g of soil was weighed in a plastic bottle, and 40 ml of distilled water was added. The soil was shaken and left standing overnight for more than 16 h. The sample was shaken overnight before pH and EC values were read. The moisture content was determined by drying the soil sample with an oven (Memmert, Germany) at 105°C until it reached constant weight. About 10g of soil was weighed in a porcelain dish. The oven was heated at 105°C until the weight was constant, and the soil sample was left in the oven for two and a half hours. Then, the soil was cooled in a desiccator. Finally, the soil was weighed and recorded. The C/N ratio was mathematically calculated from organic carbon (OC) and total nitrogen (TN). Organic carbon (OC) and total nitrogen (TN) were analyzed via the Dumas method based on results obtained from the CHN analyzer (PerkinElmer, Model 2400 Series, USA). Other P and exchangeable K contents were measured using inductively coupled plasma-optical emission spectrometry (PerkinElmer, Model Optima 8300, USA).

Dry aggregate stability was determined by placing air-dried samples on a stack of sieves without breaking the soil structure. Then, 75g of soil samples were analyzed through sieving to separate the samples into different aggregate size fractions, including 4000-, 2000-, 1000-, and 250-mm mesh openings. The stack was shaken horizontally, by hand, at a rate of 30 times per minute for 2 min. The resulting aggregate fractions were gently removed from the sieves. The dry-sieving method was repeated with a second sub-sample of dried soil to determine the distribution of aggregate fractions (Castellanos-Navarrete et al., 2013). The calculation of soil aggregation is as follows:

$$W_t\ (g) = (W_{t1} - W_{t2})$$

$$\text{Distribution of aggregates } (\%) = W_t/W_{t3} \times 100$$

Where, Wt = Weight of aggregates in each sieve group (g), Wt_1 = Weight of aggregates in each sieve group plus sieve can (g), Wt_2 = Weight of empty sieve can (g), Wt_3 = Total weight of soil (g).

For water holding capacity analysis, soil samples were thoroughly air-dried before analysis. After plugging one end with cotton, two long cylindrical tubes were filled with the weighed amount (100 g) of soil samples. Both the tubes were clamped in a vertical position. Then, water was slowly poured and allowed to percolate through the soil by gravitational pull. When the soil was saturated, the pouring of water stopped, and the tubes were allowed to stand until all the gravitational water had drained. The wet soil was then taken out from the tubes, and the amount of retained water was determined by weighing and drying. The amount of water retained by 100 g of original dry soil gave the field capacity of the soil, which can be calculated from the following formula according to Brischke and Wegener (2019):

Water holding capacity (%) = (Weight gain at saturation point/Soil dry weight) × 100%

DNA Extraction, 16S rRNA Sequencing and Data Processing

Total DNA from 0.5 g soil samples was extracted using the soil DNA Isolation Kit method (Omega, Norcross, USA). DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) and DNA integrity was detected using 1% agarose gel electrophoresis. Polymerase Chain Reaction (PCR) amplification and high-throughput sequencing were undertaken on 16S rRNA V3-V4 region using primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-ACTCCTACGGGAGGCAGCA-3') (Wang et al., 2015). PCR reactions were performed in triplicate at a final volume of 20 µl containing 4 µl of 5 x FastPfu Buffer, 2 µl of 2.5 mM dNTPs, 0.8 µl of each primer (5 µM), 0.4 µl of FastPfu Polymerase, and 10 ng of template DNA. The PCR reaction procedure was performed as follows: 95°C for 3 min, 30 cycles, 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, 72°C for 10 min. The amplification product was detected using 2% agarose gel electrophoresis. After measuring the concentration of the purified product, the equimolar number was mixed. Sequencing was performed using an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocol of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Operational taxonomy units (OTUs) were clustered at a 97% similarity cut-off using USEARCH (version 7.1). Chimeric sequences were removed. OTUs were classified with the Ribosomal Database Project (RDP) classifier (http://rdp.cme.msu.edu/) against the Silva 16SrRNA database using a confidence threshold of 70% (Quast et al., 2012).

Statistical Analysis

The results were initially collated with Excel 2010 (Microsoft, USA) before analyzing using Statistical Packages for Social Sciences (SPSS, USA). One-way variance (ANOVA) and Duncan's multiple comparisons were performed. Mean comparisons between treatments

were performed at a significant level of $p<0.05$. The Pearson correlation test was performed to determine the relationship. The tables and figures were produced using ORIGIN Pro 8.0 (Origin Lab). The OTU dataset calculates Chao 1 and Shannon-Simpson's diversity indices. Results were visualized by using R 3.2.3.

RESULTS

Changes in Physicochemical Soil Properties After Microbial Inoculation and Amendment with Organic Residues

Changes in soil pH, EC, and moisture content after 90 days of incubation are illustrated in Table 2. Statistical analysis revealed that all treatments with respect to different organic amendments lead to a pH value increase. *P. polymyxa* inoculation with SMS organic amendment significantly increased to neutral pH at 7.0, higher than the previous untreated soil sample. The highest pH was found in EM+SMS (8.4), while the pH in Control+EFB was the lowest at 5.2. While pH value showed an overall increase in all soil samples, soil EC resulted in a stronger reduction, lower than the untreated sample before the experiment started. No obvious differences were observed in EC values between treatments, except 3.8 dSm^{-1} from *P. polymyxa* inoculation and PL amendment. After soil amendment, the changes in moisture content were clearly distinctive among all treatments. As shown in Table 2, higher moisture content (83.9%) was observed in soil amended with PL as organic input, which *P. polymyxa* addition to PL appeared to rank the highest. Similarly, compared to PL amendment, *P. polymyxa* inoculation with EFB amendment significantly increased moisture content very reasonably, higher than those in EFB untreated control and EM+EFB samples, respectively. Here, it is interesting to mention that the increase in moisture content due to SMS amendment, regardless of microbial inoculations, was within suitable range for soil amelioration.

Overall, the dominant size fraction was >2 mm aggregates, accounting for more than 60% of the total dry-stable aggregate (Table 2). The effect of *P. polymyxa* and EM inoculation with SMS amendment on soil aggregation was significantly higher compared to the control. The combination of *P. polymyxa* and EFB resulted in the lowest soil aggregation at 10.2%, whereas *P. polymyxa* with SMS showed much higher aggregation at 44.4%. Looking at EFB and PL as organic amendment materials, EM inoculation increased soil aggregation by 38.9% and 22.9% as compared to the untreated control, respectively. The effect of organic amendment following microbial inoculation on water-holding capacity is illustrated in Table 2. Water holding capacity significantly increased in soil inoculated with *P. polymyxa*, SMS, and EFB as an organic amendment, except for PL residues. Water holding capacity increased significantly with microbial inoculation in *P. polymyxa* and EM, regardless of organic residue variations. Our results revealed that water-holding capacity increments in response to the *P. polymyxa* inoculation; the highest was recorded at 80.2%

Table 2
Effect of organic amendments and microbial inoculation on soil physicochemical properties

Parameters	Organic residues	Microbial inoculation		
		Control	*P. polymyxa*	EM
pH	SMS	8.1 ± 0.1[a]	7.0 ± 0.1[b]	8.4 ± 0.1a
	EFB	5.2 ± 0.1[a]	6.3 ± 0.1[b]	6.1 ± 0.1b
	PL	6.4 ± 0.1[a]	8.1 ± 0.1[b]	7.9 ± 0.1[b]
EC (dSm^{-1})	SMS	2.4 ± 0.2[a]	2.6 ± 0.2[a]	2.4 ± 0.2[a]
	EFB	1.6 ± 0.2[b]	1.4 ± 0.1[b]	1.9 ± 0.1[b]
	PL	3.5 ± 0.3[a]	3.8 ± 0.6[a]	3.3 ± 0.3[a]
Moisture content (%)	SMS	40.3 ± 0.8[a]	48.3 ± 0.1[a]	45.3 ± 0.8[a]
	EFB	16.1 ± 0.2[b]	41.1 ± 0.1[a]	12.5 ± 0.1[b]
	PL	65.9 ± 0.8[a]	83.9 ± 0.6[b]	70.7 ± 0.3[b]
P concentration (mg kg^{-1})	SMS	22.3 ± 0.1[a]	60.3 ± 0.1[b]	27.9 ± 0.1[b]
	EFB	13.2 ± 0.1[a]	35.1 ± 0.1[b]	12.7 ± 0.1[a]
	PL	30.3 ± 0.1[a]	35.5 ± 0.1[a]	25.2 ± 0.1[a]
K concentration (mg kg^{-1})	SMS	173.3 ± 0.1[c]	475.4 ± 0.1[a]	233.7 ± 0.1[b]
	EFB	165.6 ± 0.1[c]	241.3 ± 0.01[b]	210.3 ± 0.1[b]
	PL	242.0 ± 0.1[b]	279.4 ± 0.1[b]	231.1 ± 0.1[b]
Soil aggregation (%)	SMS	15.0 ± 0.1[a]	44.4 ± 0.1[b]	40.7 ± 0.1[b]
	EFB	32.2 ± 0.1[a]	10.2 ± 0.1[b]	38.9 ± 0.2[a]
	PL	15.9 ± 0.1[a]	19.9 ± 0.1[a]	22.9 ± 0.5[a]
Water holding capacity (%)	SMS	37.7 ± 0.9[b]	80.2 ± 0.1[a]	70.2 ± 0.1[a]
	EFB	37.6 ± 0.1[b]	60.1 ± 0.1[a]	30.2 ± 0.1[b]
	PL	30.1 ± 0.1[b]	40.2 ± 0.1[a]	60.1 ± 0.1[c]

Note. SMS=Spent mushroom substrate; EFB=Empty fruit bunch of palm oil; PL=Pineapple leaf residues; EM=Activated EM-1; Significant at $p<0.05$. Within each row, means with different letters are significantly different at $p<0.05$, while means with similar letters are insignificant at $p<0.05$

and 60.1%, respectively. Phosphorus (P) and potassium (K) content increased after SMS amendment and *P. polymyxa* inoculation, much higher than the initial untreated soil sample before amendment took place (Table 2). Except for K concentration, a significant difference with lower reading was detected in the soil sample amended with EFB+*P. polymyxa* and PL+*P. polymyxa* compared to previously untreated soil samples. Meanwhile, the availability of P soil treated by EM regardless of organic inputs compared with the initial available P showed a significant decrease ($p<0.05$). The P contents of soil samples fluctuated but reached the highest value at *P. polymyxa* inoculation of SMS amendment with 60.25 mg kg^{-1}. The lowest P content was recorded at EM inoculation of EFB amendment with 12.65 mg kg^{-1}. The addition of plant-growth-promoting bacteria increased the K concentration in the soil by 475.40 mg kg^{-1}, 241.28 mg kg^{-1} and 279.40 mg kg^{-1} in SMS, EFB and PL amendments, respectively. Compared with EFB, SMS significantly increased the availability of P in soil, while PL was slightly better than control pots in terms of P concentration.

C/N ratio values showed a significant increase in all organic amendments and microbial inoculation over 90 days' incubation compared to the initial untreated control (Figure 1). The maximum values of the C/N ratio were observed in amended soil treated with SMS along with *P. polymyxa* in comparison to other treatments. An improvement up to the recommended value in the C/N ratio was achieved by SMS amendment with both inoculations of *P. polymyxa* (15.6) and EM (18.7), respectively. However, the C/N ratio value in the EFB amendment showed a slight increase to 8.67 from the initial 6.3 in the EM inoculation, and EM+PL increased to 10.4 from the initial 8.03.

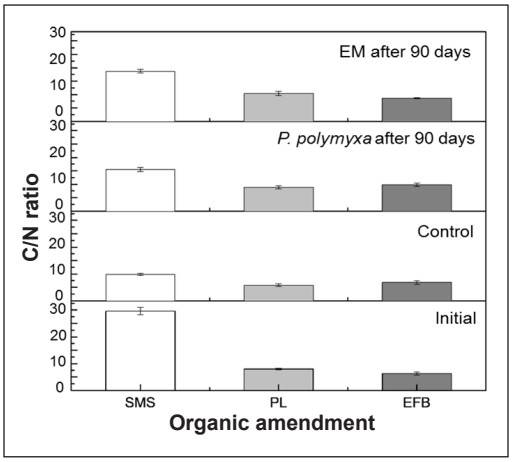


Figure 1. Changes in soil C/N ratio value under different organic amendments. SMS= spent mushroom substrate; PL=pineapple leaf residues; EFB= empty fruit bunch of palm oil before and after 90 days incubation. Error bars represent the standard deviations (n=3), and the bar graphs hide some error bars

Diversity and Taxonomic Composition of the Soil Bacterial Community

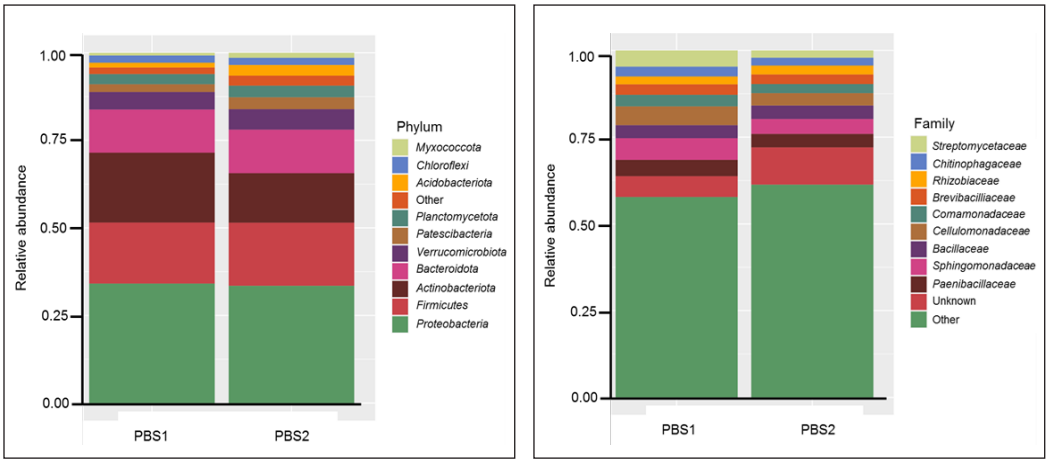
A total of 216,178 bacterial 16S rRNA sequences were obtained from two samples (PBS1: Initial SMS amendment and PBS2: SMS-amended soil with *P. polymyxa* after 90 days) (Table 3). Classified operational taxonomic units (OTUs) belonged to 29 phyla among all treatments based on a cut-off value of 97%. *P. polymyxa* inoculation led to significantly higher bacterial species observed and richness (Chao1) as compared to initial samples. The amendment following inoculation impacted the ACE (abundance-based coverage estimators) index significantly. However, no insignificant differences were recorded in bacterial diversity (Shannon and Simpson). These results show that organic amendment following microbial inoculation changes the diversity of the soil bacterial community. Bacterial communities in soils were dominated by phyla abundance of *Proteobacteria* (33%), *Firmicutes* (18%), *Actinobacteria* (14%), *Bacteriodata* (12%) and *Verrucomicrobia* (6%) (Figure 2a). These taxa accounted for more than 83% of the bacterial sequences in all the treatments.

The relative abundance of *Firmicutes*, *Bacteriodata* and *Verrucomicrobiota* was noticeably a bit increased by 17.4%–18.1%, 12.3%–12.4%, and 5.1%–5.8%, respectively. Other phyla (< 3% abundance, *Myxococcota* and *Chloroflexi*) accounted for 6.1% of total bacteria abundance. At the family level, the ten most abundant were *Paenibacillaceae*, *Shingomonadaceae*, *Bacillaceae*, *Cellulomonadaceae*, *Comamonadaceae*, *Rhizobiaceae*, *Brevibacillaeae Chitinophagaceae* and *Streptomycetateae* (Figure 2b). The relative

Table 3
Summary of high throughput sequencing data and diversity indices

Sample name	No. of sequences	Observed species	Chao1 index	Shannon	Simpson
PBS1	104099	699[a]	701[a]	5.68[a]	0.992[a]
PBS2	112444	863[b]	867[b]	5.79[a]	0.993[a]

Note. Different letters shown after values with the same column indicate significant differences (p<0.05)



(a) (b)

Figure 2. Relative abundance (%) of dominant bacteria taxa. Dominant bacterial phyla (a) and family (b) in both soil samples, PBS1 and PBS2, refer to different soil samples subjected to initial and final samples, respectively

abundance of unknown bacterial groups showed an increment shift by 57.7%–61.3% at the family level after 90 days' amendment. After the amendment and inoculation period, the relative abundances of *Paenibacillaceae*, *Sphingomonadaceae* and *Cellulomonadaceae* significantly decreased by 4.6%–3.9%, 6.2%–4.3%, and 5.4%–3.5%, respectively in soils compared with initial soils.

DISCUSSION

In the present study, changes in the physicochemical properties of soil were likely confirmed, and the usage of SMS, EFB, and PL could improve the pH of acidic sandy loam soil to a neutral condition suitable for any agricultural reclamation. This result was in harmony with the findings by Laurent et al. (2020), as they explained that an increased pH was attributed to a long-term application of the organic amendment. Amendment with organic-rich agro-waste was found to be successful in lowering EC values towards an acceptable limit for agriculture application. Compost with high or low EC was not recommended for crop cultivation as it could potentially cause root injury and burning, whereas low EC affects the

absorption ability of mineral elements from the soil (Manirakiza et al., 2021). Huerta-Pujol et al. (2010) reported that an optimum range of EC for field application should be between 2–4 dS m^{-1}, which was in line with our findings. EC values are expected to decrease with SMS-compost amendment, according to the recent result reported by Carpio et al. (2023). It is commonly known that the fertility of sandy loam soil could be improved by applying organic residue amendment, which was related to stable EC values.

Paula et al. (2017) showed that SMS amendment either by *P. polymyxa* or EM had increased soil moisture content within the allowable range (40%–60%). Instead of improving water-holding capacity, organic residue amendment could affect C allocation and increase soil water dynamics by 27%–37% within a short-term period (Villa et al., 2021). Managing moisture levels leads to a successful decomposition rate following microbial inoculation as it influences the process of organic matter formation. SMS amendment, together with bacterial inoculation in the present study, had a positive effect on the enhancement of the soil's ability to retain water. It indicates that moisture content may indirectly contribute to microbial proliferation, thus accelerating chemical solubility for crop uptake. Additionally, being too humid is unsuitable for decomposition, which may produce odors and potentially harmful gasses.

Our result showed that *P. polymyxa* inoculation enhanced soil aggregation by 44.4%, the highest percentage among the others. Wu et al. (2014) reported that extracellular polymeric substances (EPS) produced by *Paenibacillus* sp. have a role in speeding up sand stabilization and aggregation improvement. Potentially, EPS-producing bacteria improve soil fertility physically and biochemically and can maintain moisture availability during harsh conditions for their survival. These EPSs have been reported in previous reports (Othman et al., 2018) concerning other bacterial species. Even in our current result highlighting the optimal EPS derived from *P. polymyxa*, ATCC 824 appears as a homogenous spherical shape for aggregation improvement in root-adhering soil (Daud et al., 2023). Yet, we believe that EPSs of *P. polymyxa* are involved in ensuring the processes of biofilm flocculation prior to the appearance of other rhizosphere microbial species and adhesiveness of soil particles, as noted by Yegorenkova et al. (2013) and Grinev et al. (2020). Because sandy soil has low clay content, these soil particles could be bound by EPS-derived polysaccharides associated with glycosidic linkages.

Plant residues or any organic biomass input used as an amendment material has been shown to significantly promote the stability of soil aggregates, which bind soil particulates to form stable macro aggregates (Huang et al., 2017; Xue et al., 2022). In support of this, dried-brownish and fine-structured materials such as SMS are applied as composted amendments with the help of microbial inoculation to enhance soil carbon C sequestration. Composting carbonaceous materials could help build productive soil and lead to greater capacity for nutrient storage with subsequent accumulation of organic C (Verchot et al.,

2011). In this way, nutrient loss via leaching could be avoided, and the availability of P concentration could be recovered, as reported by our study. In comparison with EFB, SMS significantly increased soil P availability as follows: *P. polymyxa*+SMS; 60.62 mg kg^{-1}, much better than others. We hypothesized that cellulose-producing *P. polymyxa* would be a critical factor in fastening the decomposition rate for nutrient readiness. Interestingly, even though other organic residue inputs were applied simultaneously to the treated sandy soil, the K concentration in SMS amended with *P. polymyxa* was significantly higher (475.406 mg kg^{-1}) than others, suggesting that K availability was attributed to the large proportion of woody material from composted SMS (Lou et al., 2015). The resultant changes from organic residue amendment typically led to greater plant nutrient availability in the soil.

We observed that bacterial diversity indices evidently increased; the compositions of soil bacterial communities changed in PBS2 compared with those in PBS1 soil. Surprisingly, the organic amendment generated more observable species, increasing the number of species detected. In this present study, the relative abundance of potential beneficial taxa like *Proteobacteria*, *Firmicutes*, *Actinobacteriota* and *Bacteriodata* were the main dominant bacterial group after the treatment with *P. polymyxa*, suggesting that the introduction of *P. polymyxa* facilitated native soil bacteria, which is in accordance with the previous studies (Dai et al., 2017; Suleiman et al., 2019). Reduced abundance of *Actinobacteria* taxa in relation to the final amendment has been associated with the finished degradation of lignocellulose polymer materials (Lacombe-Harvey et al., 2018). In contrast, *Chloroflexi* decreased as a result of competition from similar preferred niches in the soil. Also, the lower presence of *Acidobacteriot*a was likely due to stable soil pH after soil amendment, which was unfavorable for this bacterial phylum (Kielak et al., 2016). It should be noted that their abundance and activities exhibited positive correlations with some soil microorganisms following organic material addition. In response to microbial inoculation and SMS amendment, small-scale disturbances and changes in the structural soil resident bacterial community were observed. However, this phenomenon has been described as a resilient response by the soil microbial community by which they return to their original composition after being 'disturbed' (Lourenço et al., 2018). The relative abundance of *Paenibacillaceae*, *Shingomonadaceae*, *Bacillaceae*, *Cellulomonadaceae*, *Comamonadaceae* and *Rhizobiaceae* at the family taxonomic level is expected to result in enhanced cycling of essential nutrients, which might be crucial in improving soil fertility (Monreal et al., 2017; Schmid et al., 2017; Wang et al., 2021). Although this work provided information about how microbial inoculation and organic amendment affect the resident bacterial communities, future studies should consider comparing the seasonal microbial succession and inter-kingdom interactions in the long-term experiment. Taken together, we believe that organic amendment and PGPB promote a synergistic effect by shifting the soil microecology and ameliorating soil fertility.

CONCLUSION

The application of organic amendment, particularly SMS combined with PGPB and *P. polymyxa* inoculation, seems to be helpful in the reclamation of sandy loam soil by improving physicochemical properties and fertility (status of nutrient concentration and microbial diversity). However, adequate selection of bacterial strains must be taken into consideration when reconstructing this soil restoration technology. It can be concluded that during the decomposition of organic residues in soils, more aggregate formation led to greater persistence of nutrients in the soil and water holding capacity. The data obtained gives complementary insights into the substantial change of the soil-dominant bacterial community structure to applied amendment approaches, which can be correlated with a selection of organic material inputs and studied microorganisms. Prospective analysis should be considered, apart from studying bacterial communities, as well as other inter-kingdom partners in the soil, which are fungi, archaea and protozoa. Although a simple model of potting soil was used, it allows less complex interpretation than in open-field application. The use of carrier-based inoculant technological development should be considered for future investigations.

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Review Article

Tea Polyphenols and Iron Oxide Nanoparticles: Therapeutic Benefits, Microbiota Interactions, and Proteomic Perspectives

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ABSTRACT

Combining green tea polyphenols (GTPs) in iron oxide nanoparticles (IONPs) has attracted significant interest due to its potential therapeutic implications. This review investigates the beneficial effects of conjugating IONPs with polyphenols, highlighting their enhanced bioavailability and efficacy. The relationship between tea polyphenols and intestinal microbiota has been clarified by metagenomics research, highlighting how these relationships improve bioavailability. Moreover, studies elucidating the impact of metallic and magnetic nanoparticles on the composition of the gut microbiota provide insight into their function in regulating microbial diversity. Proteomic analyses have provided valuable insights into the molecular mechanisms underlying polyphenol-metallic nanoparticle interactions, offering a comprehensive understanding of their biological processes at the protein level. The study of polyphenol-nanoparticle interactions using metagenomics and proteomic approaches provides a promising direction for further research into possible medicinal uses and therapeutic applications.

Keywords: Iron oxide, metagenomics, nanoparticles, polyphenols, proteomics

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INTRODUCTION

Numerous studies have shown that the bioactive polyphenols present in tea plants confer significant nutritional and health benefits (Li et al., 2022). Tea polyphenols are a complex set of compounds that consist of approximately 30 types of phenols, with catechins consisting around 30 to 42% of the total polyphenol content (Zhang, Zhang

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et al., 2021). Among these, green tea polyphenols (GTPs) are of great significance due to their strong antioxidant potential and a number of noteworthy chemical and biological properties (Li et al., 2019). One of the main polyphenolic catechins found in green tea, epigallocatechin gallate (EGCG), has drawn interest for its wide range of bioactive characteristics, which include antiviral, antitumorigenic, anti-inflammatory, antibacterial, antioxidative, and antiproliferative effects (Chacko et al., 2010). Furthermore, theaflavins (TFs) and their derivatives have many biological activities, including antibacterial, antiviral, antitumor, and anti-inflammatory qualities (Mhatre et al., 2021).

The presence of galloyl and gallic moieties in the catechin structure has also been associated with specific cytotoxicity towards cancer cells (Karas et al., 2017). Despite promising therapeutic potential, the efficiency of these compounds is not fully evaluated, with occasional pro-oxidant effects and potential medication interactions via enzymatic and microbiota-mediated processes (Galati & O'Brien, 2004). In recent years, nano-delivery systems have gained traction for their capacity to enhance the stability and targeted delivery of bioactive compounds (Zhang, Qiu et al., 2021). Particularly, metal nanoparticles have garnered interest because of their special qualities, which include a high specific surface area and adjustable size and shape, which may improve the bioavailability of bioactive compounds (Meena et al., 2020). However, significant gaps persist in understanding the biological implications of nanomaterials despite their widespread application in various products (Barreto et al., 2020).

Over time, proteomics has seen a steady increase in the application of nanotechnology (Agrawal et al., 2013). The impact of nanoparticles (NPs) on various cells can be seen clearly, simply, accurately, and valuably through proteomics analysis (Abdelhamid & Wu, 2015). As a result, proteomics and nanotechnology have come together to form nanoproteomics, which offers a reliable, real-time analytical platform for sensitively identifying low-abundance proteins (Matarraz et al., 2011). Additionally, alterations in microbial communities are tracked through metagenomics analysis, which examines modifications in various metrics such as microbial biomass, microbial activity rates, or microbial community composition (Lynch et al., 2012). Quantifying community composition is essential for determining how NPs affect the environment, creating toxicity detection tools, and enhancing bioremediation techniques (Afzal & Singh, 2022). The main objective of this review is to thoroughly investigate the current state of science regarding green tea polyphenols iron oxide nanoparticles' advantages in terms of bioavailability. The study focuses on metagenomics insights into the interactions between intestinal microbiota and tea polyphenols, highlighting how these relationships improve bioavailability. In addition, the review explores proteomic issues, thoroughly examining the effects of associations between tea polyphenols and iron oxide nanoparticles on biological systems. Figure 1 highlights the key ideas presented in this review, from the potential medicinal uses

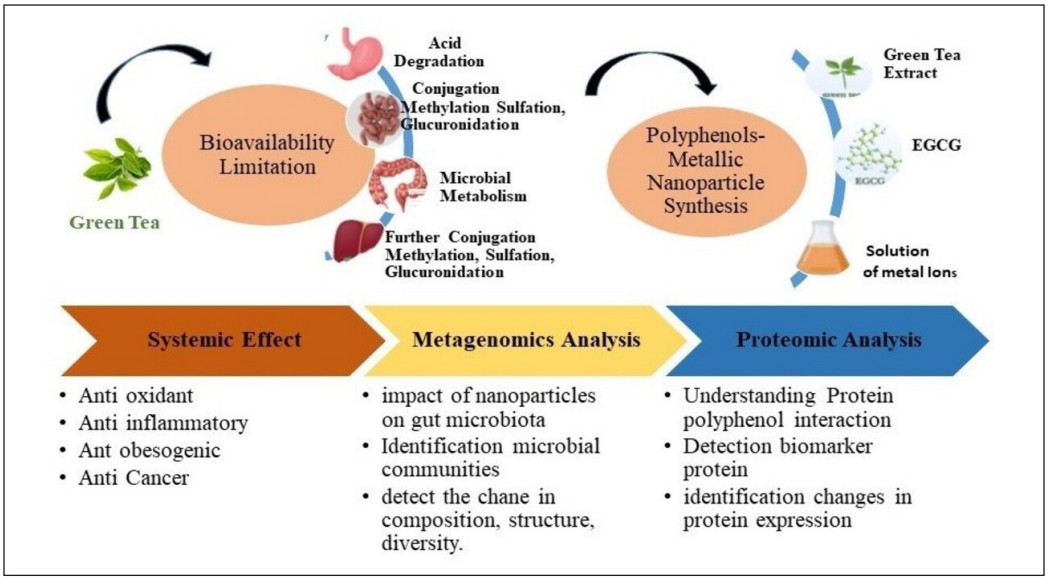

Figure 1. Summary of the key points presented in this review from the potential medicinal uses of tea polyphenols, its bioavailability limitation to nanocarriers using metagenomics and proteomics approaches

of tea polyphenols and their bioavailability limitation to nanocarriers using metagenomics and proteomic approaches.

Green Tea Polyphenols and Iron Oxide Nanoparticles

Tea is one of the most popular beverages made from the leaves of the *Camellia sinensis* plant. Tea has become one of the most widely consumed drinkables in the world for enjoyment and health (Meegahakumbura et al., 2018). Green tea, which is high in polyphenols, has attracted the attention of researchers and scientists for its consumption. Green tea is generally favored over black tea in terms of health benefits (Pasrija & Chinnaswamy, 2015). Green tea also contains a high concentration of flavonoids, amino acids, caffeine, phenolic acids, carbohydrates, and volatile constituents, which exist in their biomolecular form (Mujtaba et al., 2023). A high proportion of the constituents of green tea are catechins, consisting of 80–90% of the total polyphenols. Among the various catechins reported in green tea, epigallocatechin gallate (EGCG) constitutes more than 50% of total catechins. Other green tea polyphenol constituents are flavonols, phenolic acids, and alkaloids such as caffeine (Sinija & Mishra, 2008; Venkata et al., 2018). Due to the beneficial effects of caffeine, flavonoids, and catechins on health, this beverage has been used in natural medicine for thousands of years (Almeida & Figueira, 2013). However, some issues, including low solubility, poor permeability, instability, rapid release, susceptibility to environmental influences and low bioavailability, restrict the use of phenolic compounds in humans (Li et al., 2015).

Encapsulating chemically labile bioactive agents in nanoparticles enhances *in vivo* performance, providing enhanced absorption, decreased toxicity, extended circulation times, and controlled release of bioactive compounds (Li, Jin et al., 2018). Metal nanoparticles have drawn attention due to their large specific surface area, shape, controllable size and enhanced bioavailability of polyphenolic compounds (Sahraeian et al., 2024). Metal oxide and magnetic nanoparticles are utilized in various biomedical applications, including gene therapy, photodynamic therapy, drug delivery, medical imaging, dentistry and wound healing (Zhao et al., 2021). Therapeutic and diagnostic agents can be encapsulated, covalently attached, or adsorbed using iron oxide nanoparticles (IONPs) (Low et al., 2022). IONPs exhibit promising potential as effective drug delivery systems owing to their expansive surface area and diminutive size (Sankaranarayanan et al., 2022). IONPs provide several advantages over free drugs when iron oxide is integrated into drug delivery systems. The conjugated drug can effectively accumulate in tumor sites due to the notable ability of IONPs to be localized (Alphandéry, 2019). For instance, the immobilization nanocarrier of epigallocatechin-3-gallate (EGCG) and iron oxide nanoparticles (Maghemite) is far from auto-oxidation and degradation. With remarkable protein kinase CK2 inhibition, this composite can deliver EGCG to cancer cells (Saha et al., 2023).

IONPs are usually considered biocompatible and widely used in biomedical applications (Mollarasouli et al., 2021). Furthermore, magnetic nanoparticles possess unique characteristics, such as the ease of modification of their surface chemistry to achieve better compatibility and selectivity. Vast amounts of biomolecules aid in capping the metal salts' surfaces and turning them into metallic nanoparticles (NPs) (Sidhu et al., 2022). It has been possible to successfully create different metal oxide nanoparticles through green synthesis using a variety of bio-sources, such as plant extracts and microorganisms like bacteria, fungi, and algae (Chugh et al., 2021). As shown in Table 1, numerous studies are concentrated on synthesizing IONPs using particular bioactive molecules from polyphenols (Jamwal et al., 2018).

Table 1
Synthesis of magnetic nanoparticles using specific bioactive molecules from polyphenols

Polyphenolic compound	Metal nanoparticle	Improved target properties	References
Green tea extracts	Iron oxide (FeONPs)	Synergetic removal of melanoidin from ethanol distillery simulated model wastewater	Akhtar et al. (2023)
Polyphenols in the leaf extract (*Canthium coromandelicum*)	IONPs	Antibacterial activity against *Staphylococcus aureus* and *Salmonella typhi*.	Sudhakar et al. (2021)
Green tea	FeNPs	Removal of metal (loids) in acid mine drainage	Pan et al. (2023)
Tea polyphenols	Zero-valent iron NPs	Electrochemical determination of Hg2+	Bao et al. (2022)

Table 1 *(continue)*

Polyphenolic compound	Metal nanoparticle	Improved target properties	References
Green tea extract	FeNPs	Low ecotoxicological risks and the suitability of these green-synthesized FeNPs for environmental remediation purposes	Plachtová et al. (2018)
Green tea polyphenols	Fe3O4 NPs	Removal of dye pollutants from aqueous solution	Singh et al. (2017)
EGCG	FeNPs	Enhanced photothermic/chemodynamic cancer combination therapy	Su et al. (2023)
Polyphenols and caffeine	FeNPs	Selective removal of cationic dyes	Xiao et al. (2020)

The Beneficial Effects of Iron Oxide Nanoparticle Conjugation with Polyphenol

By using IONPs, a number of polyphenols have been used to boost their efficacy. A study found that the bioavailability of quercetin-conjugated dextran-coated Fe3O4 nanoparticles in the brain is about ten times that of free quercetin (Enteshari Najafabadi et al., 2018). Another study shows that encapsulation of catechin as a potent polyphenol drug in IONPs enhances its bioavailability, suggesting that NPs are an effective vehicle for targeted drug delivery in cancer therapy (Nobahari et al., 2023). EGCG was utilized to synthesize IONPs. These nanoparticles demonstrated minimal organ toxicity and were efficient for combined hyperthermia, drug delivery, and real-time magnetic resonance imaging (MRI) in mice tumor models (Yin et al., 2017). Additionally, dendrimerized magnetic nanoparticles have been created as EGCG delivery vehicles. EGCG-loaded nanoparticles induced human cervical cancer cells to undergo controlled cell death (Aggarwal et al., 2022). Research revealed a robust interaction between the iron oxide nanoparticles and curcumin. It was discovered that curcumin-capped magnetic nanoparticles and blue light irradiation could inhibit *Staphylococcus aureus* up to 60% of inhibition (Cañon-Ibarra et al., 2023).

Metagenomics Reveals Tea Polyphenols' Association with Intestinal Microbiota and Enhanced Bioavailability

Tea polyphenols (TPs) have limited intestinal absorption, contributing to low systemic bioavailability (Liu et al., 2018). In contrast, TPs are highly concentrated in the gastrointestinal tract (GIT), where the intestinal microbiota catabolizes them to phenolic acid (Stalmach et al., 2010). The numerous health advantages and varied bioactivities linked to green tea's polyphenols have led to the application of a number of techniques to study their biochemical mechanisms. Metagenomics has become a prominent method (Garza & Dutilh, 2015). The metagenomics approach includes a sequence of stages, starting with environmental sample collection, then metagenomics deoxyribonucleic acid (DNA) isolation, metagenomics library

creation, screening, and library modification. This technique uses 16S rRNA gene sequencing and whole-genome shotgun sequencing (Ilett et al., 2019).

Green tea supplements high in polyphenols can alter the gut microbiota's composition, increasing beneficial bacteria abundance, particularly *Bifidobacterium* and *Lactobacillus*, highlighting the positive impact of polyphenols on gut microbiota (Yuan et al., 2018). Polyphenols' effects on the gut microbiota of healthy individuals have been clarified by recent clinical interventions using metagenomics technology Table 2.

Gut microbiota can break down the heterocyclic structures of catechins into smaller compounds like phenylvalerolactones (PVLs) and phenylvalericacids (PVLa) through C-ring fission, glyosidic connections and A-ring fission. It is possible for these recently formed microbial metabolites to eventually cross the colon's epithelium and enter the systemic bloodstream (Chen, Zhu, et al., 2020). As main reaction products from catechins, valerolactone and phenolic acids increase, so does their absorption through the intestinal wall. Consequently, these compounds have improved bioavailability and are easier for the large intestine to absorb (Liu et al., 2018). Therefore, it is critical to consider how colonic metabolites and gut microbiota metabolism influence the bioavailability of TPs. According to earlier research on humans, 39% of TPs flavan-3-ol were bioavailable. Nevertheless, the bioavailability rose to 62% when colonic metabolites were taken into account (Calani et al., 2012).

As illustrated in Figure 2, TPs have the potential to improve metabolic health by promoting commensal abundance, decreasing pathobionts, and enhancing the diversity, richness, and metabolic processes of gut microbiota. Gut microbes are essential to maintaining optimal health by promoting healthy digestion, controlling the immune system and averting opportunistic infections (Dey et al., 2021). The catechins in tea polyphenols have been demonstrated to have antimicrobial properties against gut pathobionts and to promote the growth of beneficial bacteria, such as *Lactobacillus/Enterococcus* groups and *Bifidobacterium* spp., while inhibiting the growth of pathogenic bacteria such as *Eubacterium-Clostridium* groups, *Bacteroides-Prevotella*, and *Clostridium histolyticum*. The health advantages could be attributed to the interaction between EGCG and gut flora (Bond & Derbyshire, 2019).

Table 2
Summary of polyphenols' effects on the gut microbiota using metagenomics approach

Polyphenol	Technology used	Objective	Target (Result)	Reference
Oolong tea (OT)	16S rRNA gene sequencing	To examine the interventive effect of daily OT intake on body composition, gut microbiota, metabolic profile, and gastrointestinal functions of healthy subjects.	Changed microbial diversity in the gut. Enhanced the growth of *Bacteroides* and *Prevotella*, reduced the number of Megamonas, and enhanced digestive health.	Li et al. (2023)

Table 2 *(continue)*

Polyphenol	Technology used	Objective	Target (Result)	Reference
Tea polyphenol (TP) and epigallocatechin gallate (EGCG)	16S rRNA	To study how TP and EGCG affect liver fat accumulation and hyperlipidemia using physiology, genomics, and metabolomics.	TP enhanced the number of good bacteria (*Faecalibacterium*, *Parabacteroides*, *Akkermansia*, and *Bacteroides*), and EGCG encouraged the growth of bacteria that produce acid (*Desulfovibrio*, *Butyricimonas*).	Wen et al. (2023)
Dried black raspberry polyphenols	16S rRNA gene	To analyze how polyphenols and gut microbiotas from different sources interact bilaterally.	Identifying the essential bacterial species involved in synthesizing bioactive phenolic metabolites for colonic absorption, such as *B. vulgatus, E. rectale, and F. prausnitzi*i. Furthermore, certain phenolics, such as gallic acid, 3-(4-hydroxyphenyl) propionic acid, and 5-(3,4-dihydroxyphenyl) valeric acid, were able to suppress pathogenic genera while enhancing beneficial ones.	Chan et al. (2023)
Polyphenols from *Gnetum gnemon* Linn. leaves	Next generation sequencing (NGS)	The effects of vacuum-dried *Gnetum gnemon* var. tenerum leaf powder on the characteristics of gut health.	Elevated Bacteroides levels and significantly higher *Bifidobacterium* numbers	Anisong et al. (2023)
Mulberry leaf extracts	16 S rRNA	A multi omics approach that included gut microbiota, transcriptional analysis, and SCFA composition analysis was applied to clarify the precise mechanisms through which particular MLEs affect female obesity models.	Modulatory effects on obesity-related gut microbiota (*Firmicutes*-to-*Bacteroidetes ratio*)	Zhao et al. (2024)
Green tea extract	16S ribosomal RNA	To understand the impact of green tea extract on the composition and metabolism of gut microbiota from people with metabolic syndrome.	According to bioinformatics analysis, *Escherichia* and *Klebsiella*'s relative abundance was generally higher, while *Bacteroides*, *Citrobacter*, and *Clostridium*'s relative abundance was significantly lower.	Zhang, Xu, et al. (2022)

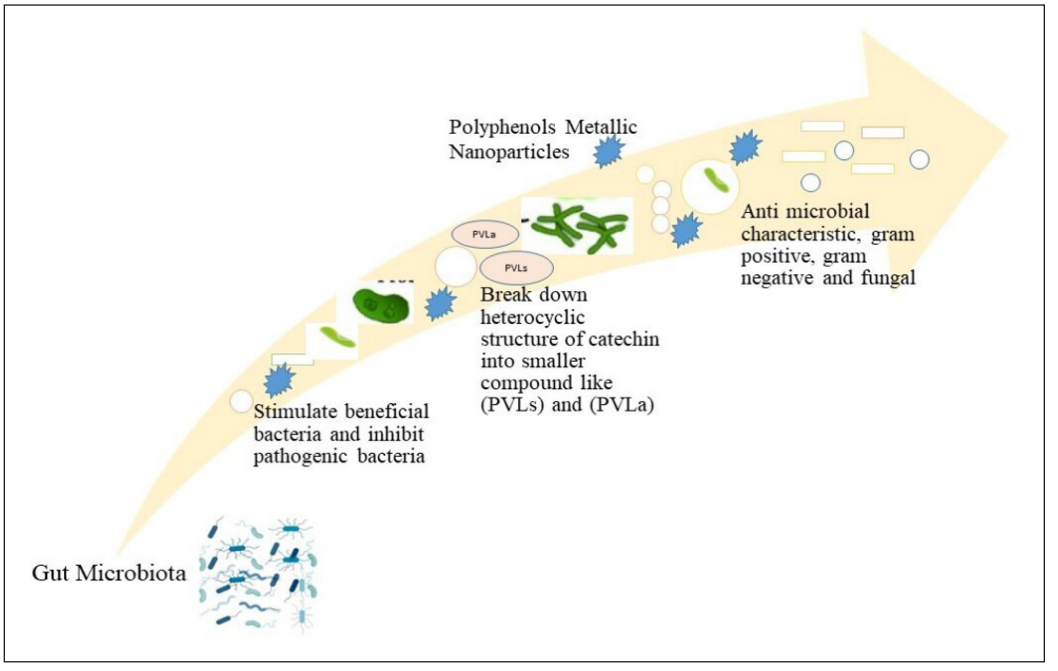

Figure 2. Interaction between polyphenol metallic nanoparticles and gut microbiota

Exploring the Influence of Metallic Nanoparticles on Gut Microbiota

The metagenomics approach has been used to investigate the impact of nanoparticles on gut microbiota, providing enhanced awareness of these particles' effects on microbial communities (Ma et al., 2023). Most nanomaterials possess antibacterial characteristics that effectively protect against common bacteria. As demonstrated in Table 3, metal oxide

Table 3
The effect of metallic nanoparticles on gut microbiota

Metallic nanoparticles	Gut microbiota	Effect	Reference
Titanium Dioxide (TiO_2 NPs)	*Lactobacillus*, *Firmicutes*, and *Proteobacteria*	Microbiota diversity and composition	Sohm et al. (2015)
TiO_2 NPs	*Proteobacteria*	Microbiota composition and structure	Li, Yang, et al. (2018)
Silver Nanoparticles (Ag NPs)	*Lactobacillus and E. coli*	Microbiota diversity and composition	Williams et al. (2015)
Zinc Oxide Nanoparticles (ZnO NPs)	*Lactobacillus*	Microbiota diversity and composition	Zhu et al. (2023)
Copper-loaded chitosan nanoparticles (CNP-Cu)	*Bifidobacterium and Lactobacillus*	Microbiota abundancy	Han et al. (2010)
Nano-Al(2)O(3)	*Firmicutes, Proteobacteria* and *Bacteroidetes*	Microbiota structure	Zhang, Li, et al. (2022)

nanomaterials such as nano-TiO_2, ZnO, and Ag_2O have the capability to inhibit common bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, and *E. coli* (Hajipour et al., 2012). Gram-positive, Gram-negative, and fungal microorganisms are poisoned by nano-TiO_2 and ZnO (Daou et al., 2018).

Investigating the Impact of Polyphenol-Metallic Nanoparticle Interaction from a Proteomic Perspective

Recent developments in proteomics have resulted in the identification of numerous biomolecules, primarily proteins, as disease markers for diagnosing and detecting infectious diseases, autoimmune disorders, and cancer (Mazzara et al., 2015). Research on tea has extensively used metabolomics and proteomics techniques, which can offer a thorough understanding of biological processes at the "protein-metabolite" level (Chen, Shi, et al., 2020) for instance, applying a proteomic approach allowed for identifying changes in the expression of multiple tumor-associated proteins in A549 cells following treatment with green tea (Lu et al., 2009; Singh et al., 2010). Additionally, a study was carried out employing mass spectrometry and a 2D gel-based proteomic analysis to examine the effects of green tea polyphenols (GTPs) on ovariectomized rats. The results demonstrated the potential estrogenic effects of GTPs and their antioxidant qualities, as demonstrated by the downregulation of catechol-O-methyl transferase and the upregulation of adenosine triphosphate synthase and superoxide dismutase-1 (Shao et al., 2011). Furthermore, mass spectrometry and two-dimensional gel electrophoresis were used to measure colon protein expression. Protein identification in response to green tea polyphenols revealed a decreased abundance of transcripts and proteins linked to fibrinogenesis and immune and inflammatory response pathways (Barnett et al., 2013).

Over the years, there has been a steady increase in nanotechnology applications in proteomics (Li et al., 2013). By using nanoparticles in proteomics (nanoproteomics), the proteome could be explored, which could provide the basis for the identification of biomarkers and result in the identification of numerous proteins in intricate biological materials (Abdelhamid & Wu, 2015). The research employed proteome modulation caused by curcumin nanoformulation was investigated using quantitative proteomic methods based on Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS). The result confirmed that curcumin nanoformulation positively influenced the expression of several proteins involved in TGF-β-mediated fibrosis (Ceccherini et al., 2023).

In addition, several studies have used proteomic analysis to examine cellular response to metal nanoparticles. For instance, there was significant deregulation of different pathways related to protein homeostasis, namely eIF2, eIf4/p70S6K, and unfolded protein response signaling after exposure to ZnONPs (Doumandji et al., 2020). Moreover, proteomic information on silver nanoparticles exposed HepG2 cells verifies that these metallic

nanoparticles caused changes in inflammatory responses, mitochondrial dysfunction, posttranslational protein modification, redox stress, and other cellular parameters (Braeuning et al., 2018; Gao et al., 2022). Similarly, using *Withania coagulans* plant extract for the biological synthesis of the Fe2O3NP, proteomic analysis revealed that chemical Fe2O3NPs produced 41 differentially expressed proteins, compared to 103 produced by biological Fe2O3NPs. These proteins could be used in therapeutic and diagnostic approaches (Hasan et al., 2023).

Limited proteomic studies have focused on green tea and metallic nanoparticles. A study confirmed that green tea-synthesized magnetic nanoparticles accelerate the microwave digestion of proteins, as analyzed by MALDI-TOF-MS (Sharma & Tapadia, 2016). One intriguing aspect of green synthesis and outer layer covering in nanoparticles (NPs) is that the metallic core is hidden by the organic corona in the NPs, making the metallic core biocompatible (Spagnoletti et al., 2021).

CONCLUSION

This review emphasizes various advantages and possible synergies of iron oxide nanoparticles and green tea polyphenols combination. This conjugation offers enhanced therapeutic potential owing to its targeted delivery and increased bioavailability. Furthermore, metagenomics research highlights the connection between intestinal microbiota and tea polyphenols, indicating possible consequences for improved bioavailability and gut health. Studies on how the makeup of the gut microbiota is affected by metallic nanoparticles provide more illumination on how these particles affect microbial diversity. The biological processes of polyphenol-metallic nanoparticle interactions have been comprehensively understood at the protein level through proteomic analyses, yielding valuable insights into the molecular mechanisms underlying these interactions. Developing new therapeutic approaches may be aided by additional research using proteomics and metagenomics techniques, which may offer deeper insights into the molecular pathways underlying interactions between polyphenols and metallic nanoparticles.

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Secretory Cells in *Etlingera elatior* (Jack) R. M. Smith (Zingiberaceae): Morphology, Histochemistry, and Essential Oil Composition

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ABSTRACT

Etlingera elatior is a perennial, aromatic herb with attractive flavour and fragrance afforded by organic compounds that are stored and released from specialised structures; however, the secretory structures remain undefined. Thus, this study was carried out to determine the secretory cells in the leaves, inflorescences, and peduncles of *E. elatior* by using scanning electron and light microscopies. Histochemical tests were performed to localise and ascertain the nature of secretion materials, while gas chromatography-mass spectrometry was used to characterise the composition of essential oils (EO). Findings indicate the presence of a heterogeneous mixture comprising EO, mucilaginous and/or lipophilic substances in the secretory cells. A total of 50 compounds were identified in the EO, with the predominance of alcohol. The presence of several terpenic compounds (*α*-pinene, (*E, E*)-*α*-farnesene, *(E)*-caryophyllene, *E*-*β*-farnesene, *(E)*-nerolidol) suggests a potential involvement of the secretory structures in plant signalling. The widespread distribution of secretory cells throughout the plant tissues indicates adaptive features of the plant's secretory system. These cells emerged as the main secretory system of *E. elatior* that renders the EO.

Keywords: Gas chromatography-mass spectrometry, light microscopy, scanning electron microscopy, secretory cells, secondary metabolites

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INTRODUCTION

The aromatic properties of a plant are afforded by the presence of secretory structures as the site capable of synthesising, accumulating, and/or storing volatile organic compounds. The plant volatiles comprised of secondary metabolites that constitute the "essence" of the plant (Elshafie et al., 2023),

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imparting not only a strategic adaptation for the plant to compensate its immobility for mediating with external cues such as in plant-herbivore and plant-pollinator interactions (Lucas-Barbosa et al., 2016) but also provide human with valuable natural plant products for multitudes of purposes, from medicine to flavours and fragrances (Lee & Ding, 2016).

Ecologically, specialised structures such as glandular trichomes, secretory cavities, and oil cells produce specific secretion products that are often tailored to fulfil the functional aspects of defence against herbivory attacks or even attracting pollinators. Their localisation in the plant body also varies with their function, consistent with their diverse structures (Watts & Kariyat, 2021). For instance, an adaptive feature exists in the structural and localisation of floral nectaries in the legume flower with their specific pollinators (Sinjushin et al., 2022). The glandular trichomes of *Etlingera elatior* true flowers release mucilage, terpenes, and phenolic compounds that are able to secure its anthesis. In contrast, the non-glandular trichomes were involved in the floral development by providing physical and mechanical protection to the flowers (Lee & Ding, 2024).

Alternatively, the mixtures of secondary metabolites offer humans as sources of medicinally important substances or compounds with high biotechnological interests. The specialised cells possess the remarkable capability to manufacture metabolites in large quantities relative to their microscopic size, thereby presenting it as a potential utility as "green factories" for molecular farming and functional targets for plant metabolic engineering (Huchelmann et al., 2017; Muthulakshmi et al., 2023). Therefore, a detailed description of the localisation, morphology and histochemistry of the secretory structures would provide not only useful information for ecological, taxonomic, and chemosystematic purposes (Cassola et al., 2019) but also a new opportunity, particularly for plant breeders to exploit the plant by modifying gland metabolism to enhance yield and improve the composition of essential oil (EO) for commercial production (D'Amelia et al., 2021).

Etlingera elatior, commonly known as torch ginger, is an aromatic herb native to Southeast Asia. The plant comprises a leafy and flowering shoot system that grows in a clump of pseudostems emerging from the rhizome. The leafy shoot can grow up to 3-4 m in height, whereas the flowering shoot comprises an inflorescence borne on a peduncle that can reach up to 1.5 m (Choon & Ding, 2017) (Figures 1A-B). In Malaysia, the inflorescence bud is a staple ingredient for food flavourings in popular local cuisines such as 'asam laksa', 'nasi kerabu' and 'nasi ulam' (Choon & Ding, 2016). Despite the extensive characterisation of the chemical profile of its EO (Bezerra-Silva et al., 2016; Juwita et al., 2018; Sungthong & Srichaikul, 2018), identification of the type and morphological characteristics of the secretory structures that afforded their aromatic properties remains poorly understood. Besides, the biological activities of EO derived from torch ginger have indicated their potential for various pharmacological purposes (Juwita et al., 2018). Bezerra-Silva et al. (2016) demonstrated the promising potential of the EO to deter ovipository activities against

Aedes aegypti, the vector of the dengue virus. Most recently, the EO of torch ginger was incorporated into a starch-based edible film as an active packaging to improve the quality of chicken meat during the chilled storage period (Marzlan et al., 2022).

This study aims to identify the micromorphological characteristics of the secretory structures in leaves, inflorescences, and peduncles of *E. elatior*. We also investigated the secretory structures' histochemical contents and the chemical composition of the EO.

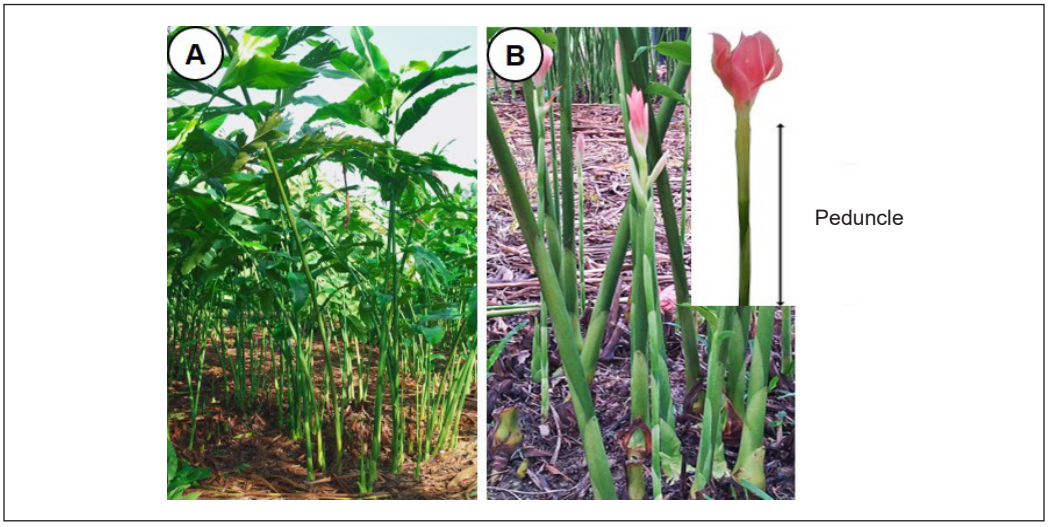

Figure 1. General view of *Etlingera elatior* plant. (A) Leafy shoot and (B) inflorescence shoot with a long peduncle

MATERIALS AND METHODS

Plant Materials

The *E. elatior* young, fully expanded leaves (Figure 2A) and inflorescence shoot at full bloom stage (Figure 2B), along with its peduncle, were collected from five-year-old plants grown in Field 2, Faculty of Agriculture, Universiti Putra Malaysia (3°00'28' N, 101°42'10' E). The sample collection was carried out between eight and nine in the morning during sampling days. The true flowers of the fully bloomed *E. elatior* inflorescence were removed to obtain its inflorescence axis (Figure 2C).

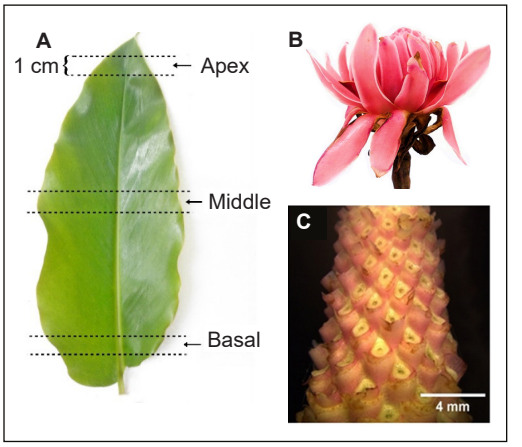

Figure 2. A young and fully expanded *Etlingera elatior* leaf lamina; (B) A full bloom of *E. elatior* inflorescence indicated by the opening of the true flowers in dark red colour (arrow); and (C) The inflorescence axis of *E. elatior* after the removal of true flowers

Scanning Electron Microscopy (SEM)

The middle part of the leaf, inflorescence axis, and peduncle were fixed in FAA (10% formaldehyde, 5% acetic acid, 50% ethanol, which were purchased from Sigma Aldrich®, Germany) for 24 hr and placed in a vacuum to remove air from the tissue as described by Lee and Ding (2024). The samples were rinsed thoroughly with distilled water and then post-fixed in 1% osmium tetraoxide (Sigma Aldrich®, Germany) for 24 hr. After a series of dehydration in graded ethanol (50%, 60%, 70%, 80%, 90% and 100%), the samples were subjected to critical point drying (Leica EM CPD030, Vienna) and then sputter-coated (Bal-Tec SCD 050, Netherlands) with gold. Observations were carried out using JEOL JSM-5610V SEM (JEOL Ltd., Japan) at an accelerating voltage of 15 kV.

Histochemical Analysis

Fresh plant materials were hand-sectioned using ethanol-cleaned razor blades and then subjected to the following histochemical tests: Nadi reagent for EO and terpenes (Caissard et al., 2004), ruthenium red for mucilage and pectin (Johansen, 1940) and Sudan IV for lipids (Jensen, 1962). Distilled water and lipid removal solution of methanol, chloroform, water and chloride acid mixture (66:33:4:1) (Machado et al., 2006) were performed as positive and negative control procedures, respectively. All sections were mounted on a glass slide with a cover slip and then examined with a light microscope (Meiji Techno, Japan) equipped with a digital single-lens reflex (DSLR) camera (Olympus E-420, Japan).

EO Extraction and Chemical Analysis

The EO from fresh leaves, inflorescence, and peduncles were extracted in triplicate by hydro distillation using Dean-Stark apparatus for 4–5 hr. The yields were determined as % w/w on a fresh weight basis. Collected EO were first diluted in 1:100 HPLC grade methanol (Sigma Aldrich®, Germany) prior to analysis by gas chromatography coupled with mass spectrometry (GC-MS). The EO of 1 µl was injected into a GCMS-QP2010 Ultra (Shimadzu Co., Japan). The chemical composition was separated on a BPX5 silica column (30 m length × 0.25 mm internal diameter × 0.25 µm film thickness). The temperature conditions were programmed as follows: initial temperature of the column oven at 50°C, heated to 300°C at the rate of 3°C per minute, and then held constant at 300°C for a further 10 min. Helium was used as a carrier gas. Mass spectra were recorded with an ion source temperature of 200°C and interface temperature of 250°C. The mass scan parameters included a start time of 2.50 min and an end time of 93.0 min, and the data were collected at a to-charge ratio (*m/z*) between 40 and 700. Identification of the individual compounds was based on a comparison of their retention times and mass spectra with those from the National Institute of Standards and Technology (NIST) 08, Flavour and Fragrance Natural Synthetics and Compounds (FFNSC) version 1.3 and Wiley 229 registry of the mass spectral library. The

relative abundance of each compound in EO was quantified by dividing the area response of a particular peak by the absolute responses from peak areas of the total ion chromatogram.

Data Analysis

The diameter of secretory structures was measured on the digitally recorded micrographs using Image J software (National Institute of Health, Bethesda, MD, USA). The means were calculated from n = 10 measurements ± SD.

RESULTS

Morphology and Distribution of the Secretory Structures

The secretory cells of *E. elatior* were scattered on the spongy mesophyll of leaves and the ground tissues of the inflorescence axis and peduncle (Figures 3A-C), each measuring an average diameter size of 27.0 ± 3.2, 35.1 ± 1.9 and 29.4 ± 2.7 µm, respectively (Table 1). The translucence of the oil accumulating in a sac-like structure further augmented the appearance of isodiametric-shaped secretory cells that can be readily distinguished from the neighbouring cells (Figures 3B-C). Superficial observation of the secretory cells revealed that a membrane binds an extra-plasmatic space containing the secretory materials similar to a large central vacuole. Upon detailed examination, the cells consisted of tripartite cell walls: (1) outer wall, an intermediary suberised wall that enclosed the oil, and (2) inner wall with tiny protuberance identified as cupule (Figure 3D).

Table 1
Diameter of Etlingera elatior *secretory cells at different plant parts*

	Plant parts		
	Leaves	**Inflorescence axis**	**Peduncle**
Secretory cell diameter (µm) ± SD	27.0 ± 3.2	35.1 ± 1.9	29.4 ± 2.7

Note. SD = Standard deviation; n = 10

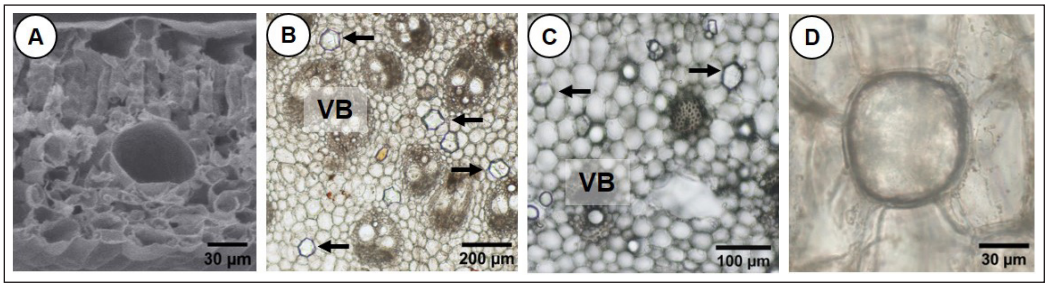

Figure 3. Secretory cells of *E. elatior*. (A) Scanning electron micrograph of leaf transverse section showing the secretory cell in spongy mesophyll. (B–C) Distribution of secretory cells (arrows) occurring alongside the vascular bundles (VB) in the (B) inflorescence axis and (C) peduncle. (D) Isodiametric shape and translucence of secretory cell accumulating secretory content in a sac-like structure

Histochemical Analysis

The secretory materials contained in the secretory cells reacted strongly with the Nadi reagent and Sudan IV tests (Figures 4A–B). The reaction produced intense colourations that indicated the presence of EO and lipophilic substances. The secretory contents, however, yielded negative reactions with Ruthenium red for mucilage. During the Sudan IV histochemical test, observation revealed that the oil droplets were liberated to the adjacent cell via an aperture identified as a cupule (Figure 4C). The paradermal section of the peduncle revealed six to eight elongated cells radially surrounding the secretory, forming a rosette (Figure 4D). Surface analysis of the peduncle showed that the secretory cells were lodged as slight protrusions on the epidermal surface (Figure 4E).

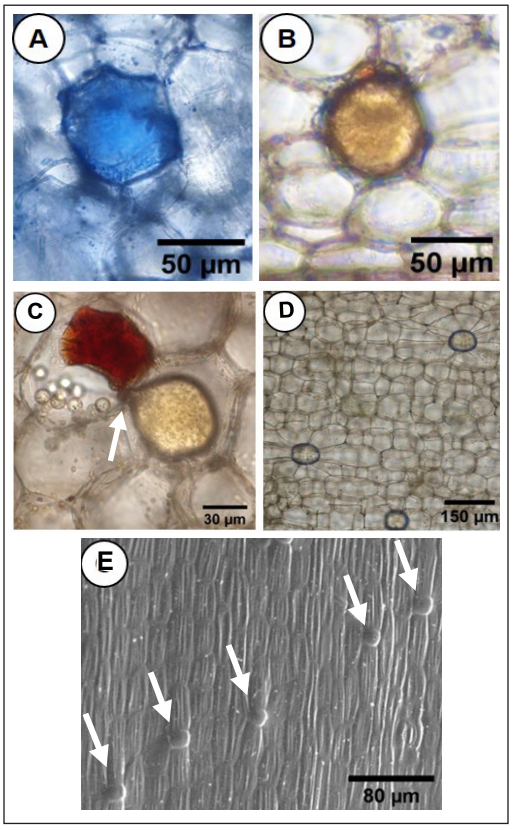

Figure 4. Histochemistry of the secretory structures. (A-B) Secretory cells tested positive for (A) essential oils and (B) lipophilic substances after strong reactions from the Nadi reagent and Sudan IV, respectively. (C) Oil droplets stained with Sudan IV exit the cell into the adjacent cell via a tiny aperture identified as a cupule (arrow). (D) The paradermal section of the peduncle shows the secretory cells surrounded by 6–8 cells arranged radially, forming a rosette. (E) Scanning electron micrograph of peduncle surface showing the secretory cells slightly lodged as protrusions (arrows)

EO Yield and Chemical Composition

The EO obtained from fresh leaves yielded 0.11%, whereas the inflorescence and peduncle yielded 0.09% and 0.05%, respectively (Table 2). A total of 50 constituents were identified in the EO, accounting for 96.6%–99.3% of the total compositions. Overall, the EO from aerial parts of *E. elatior* were rich in alcohols and aldehydes. The major compounds detected in the EO were 1-dodecanol (32.1%–36.9%), n-dodecanal (10.2%–33.9%), dodecyl acetate (2.9%–9.2%) and n-tetradecanol (3.4%–5.5%).

The EO from different plant parts can be distinguished by the higher occurrences of dodecyl acetate (9.2%), *(E)*-caryophyllene (6.3%), n-tetradecanol (5.5%), 1-decanol (4.9%) and *(E)*-*β*-farnesene (3.6%) in the leaves compared to inflorescences and peduncle (Table 2). In the flowering shoot, the occurrences of n-dodecanal (27.4%–33.9%) and dodecanoic acid (4.8%–4.9%) were higher in the inflorescences and peduncles compared to the leaves (2.1%).

Table 2
GC-MS profiles of essential oils from leaves, inflorescences, and peduncles of Etlingera elatior

	Name	RT	RI	Relative abundance (%)		
				Leaves	Inflorescence	Peduncle
1	*α*-Pinene	8.0	936	-	0.4	4.9
2	D-Limonene	9.9	1034	-	-	0.5
3	*cis*-Pinocamphone	19.3	1191	0.2	-	-
4	*α*-Terpineol	20.2	1210	0.5	-	-
5	*n*-Decanal	20.5	1217	1.0	2.6	1.6
6	1-Decanol	23.6	1286	4.9	4.2	1.9
7	2-Undecanone	24.6	1304	0.7	1.2	1.4
8	Methyl myrtenate	24.9	1309	0.3	-	-
9	2-Undecanol	25.0	1312	-	0.2	-
10	Undecanal	25.4	1316	-	0.1	0.9
11	Geranic acid methyl ester	26.0	1332	0.3	-	-
12	*β*-Elemene	28.9	1396	0.2	-	-
13	*cis*-Dodec-5-enal	29.2	1398	-	0.2	0.4
14	*(Z)*-9-Tetradecenal	29.5	1410	0.3	-	-
15	*n*-Dodecanal	30.0	1427	10.2	27.4	33.9
16	*(E)*-Caryophyllene	30.3	1433	6.3	1.3	0.4
17	*(E)-β*-Farnesene	31.6	1459	3.6	-	-
18	*(E, E)-α*-farnesene	31.7	1496	-	0.4	0.4
19	α-Humulene	32.0	1468	0.7	0.2	-
20	(Z)-8-Dodecen-1-ol	32.3	1476	2.2	1.4	0.4
21	1-Dodecanol	32.9	1496	32.2	36.9	32.1
22	2-Tridecanone	33.7	1512	1.4	1.3	1.3
23	*E*-Nerolidol	36.4	1573	0.4	-	-
24	Dodecanal dimethyl acetal	36.8	1581	0.4	-	-
25	Dodecanoic acid	37.0	1585	2.1	4.9	4.8
26	Dodecyl acetate	38.2	1620	9.2	5.7	2.9
27	Tetradecanal	38.7	1625	-	0.4	1.1
28	Tetradec-(9Z)-en-1-ol	40.4	1682	1.9	0.5	0.3
29	Dec-*(5Z)*-en-1-yl acetate	40.5	1792	2.5	-	-
30	*cis-9*-Tetradecen-1-ol	40.6	1677	2.8	1.7	1.0
31	*n*-Tetradecanol	41.2	1689	5.5	4.4	3.4
32	*trans*-Farnesol	42.6	1729	0.1	-	-
33	Tetradecanoic acid	44.4	1779	-	0.3	1.1
34	*(Z)*-5-Tetradecen-1-yl acetate	45.0	1794	0.3	-	-
35	*(E)*-9-Tetradecen-1-ol acetate	45.4	1804	-	0.2	-
36	Tetradec-*(9E)*-en-1-yl acetate	45.4	1805	0.6	-	-
37	Eicosyl acetate	45.9	1818	1.2	-	-
38	Trifluoroacetoxy hexadecene	46.0	1813	-	0.7	-

Table 2 *(continue)*

	Name	RT	RI	Relative abundance (%)		
				Leaves	Inflorescence	Peduncle
39	Phytol	55.9	2117	0.5	-	-
40	Tricosyl heptafluorobutyrate	65.3	2422	-	0.4	0.3
41	Tricosyl trifluoroacetate	67.8	2512	-	0.3	
42	Dodecanoic acid, dodecyl ester	69.2	2576	1.9	1.0	0.7
43	Tetracosyl trifluoroacetate	70.0	2619	-	-	0.5
44	1,54-Dibromo-tetrapentacontane	70.2	2425	0.4	-	-
45	1-Hentetracontanol	73.6	2622	0.3	-	-
46	Dodecanoic acid, tetradecyl ester	74.4	2771	-	0.6	-
47	13-Bromotetradecanoic acid	75.7	2855	1.1	-	-
48	Myristyl myristate	74.3	2776	0.5	-	-
49	Hexadecanoic acid, dodecyl ester	79.4	2973	-	0.2	-
50	Triacontyl heptafluorobutyrate	82.8	3558	-	-	0.5
	Total identified (%)			96.9	99.3	96.6
	Oil yield (%)			0.11	0.09	0.05
	Acids			2.1	5.3	5.9
	Alcohols			49.5	49.1	39.2
	Aldehydes			11.5	30.6	37.5
	Diterpene			0.5	0.0	0.0
	Esters			15.6	6.9	3.6
	Ketones			2.2	2.4	2.7
	Monoterpenes			0.8	0.4	5.4
	Sesquiterpenes			11.3	1.9	0.7
	Others			3.4	2.7	1.7

Note. RT=Retention time; RI=Retention indices relative to BPX5 column; Dash indicates not detected

Further, the EO profiles from the flowering shoot can be distinguished by the higher monoterpene constituents of α-pinene in the peduncles (4.9%) compared to the inflorescences (0.4%).

DISCUSSION

Morphological Characteristics of Secretory Structures

The secretory cells found in *E. elatior* corresponded to the morphological characteristics described in the leaves of *Laurus nobilis* (Maron & Fahn, 1979) and *Piper umbellatum* (Marinho et al., 2011) and flower of *Magnolia sirindhorniae* (Ghosh et al., 2021) in which these plants are notable species with aromatic properties. The secretory cells are characterised by extra-plasmatic space enclosed by a membrane similar to a large central vacuole where the secretion is accumulated. The tri-lamellar structure is a typical

feature that differentiates oil-secreting from mucilage-secreting cells, with the latter only characterised by an outer cellulosic wall (Marinho et al., 2011). The presence of a suberised wall presumably acts as a seal to the oil cell to prevent leakage of potentially toxic substances to the surrounding cells (Evert, 2006).

Interestingly, the occurrence of cupule has been a subject of debate, with some researchers indicating their presence as a fixation artefact where convincing evidence must be supplemented with ultrastructural studies as demonstrated by Maron and Fahn (1979) and Marinho et al. (2011) on oil cells of *L. nobilis* and *P. umbellatum*, respectively. Our finding, on the contrary, accords with Geng et al. (2012) on observing cupule under the light microscope as opposed to requiring ultrastructural studies. Our investigation using fresh, free-hand sections of the plant materials may have resulted in minimal changes in the structure and dimension of the tissue and, hence, affording a close *in situ* observation of the morpho-characteristic of the tissues compared to the conventional FAA or other fixative solutions that may lead to misinterpretation of tissue shrinkage as artefacts. Furthermore, documentation of tissue morphology depends on the methods of tissue processing with solvent fixation using methanol, which has been reported to preserve tissue better than conventional FAA and/or glutaraldehyde-based fixation that usually causes tissue shrinkage (Talbot & White, 2013).

The discovery of the release method of secretion materials from secretory cells via an aperture is unexpected. One possible reason to attribute the observation is the affinity of Sudan IV to react with the lipophilic nature of the secretory contents (Jensen, 1962). While it may be beneficial to perform an ultrastructural examination of the secretory structures to elucidate the secretion mechanism and transport of the oil in and out of the cells, it goes beyond the scope of this study. Notwithstanding, further comparison between secretory cells with their content liberated versus intact revealed that oil droplets exited out to the adjacent cells via the cupule. Therefore, we hypothesise the newly discovered function of the cupule as an aperture that allows the release of secretion content. Previously, the cupule has been functionally described as a peg that attaches the oil sac to the wall (Geng et al., 2012; Postek & Tucker, 1983).

Functional Significance, Taxonomic Relevance, and Potential Exploits

The present study is the first report to comprehensively characterise the secretory structures, especially in Zingiberaceae. The occurrence of secretory cells in all of the plant parts examined in *E. elatior* indicates their prevalence and, therefore, emerges as the main secretory structures of the plant. Their presence as an internal secretory system suggests reservoirs of chemical defence that can be dispensed immediately upon herbivory attack or injury to the organ (Costa et al., 2021; Kromer et al., 2016).

Furthermore, chemical analysis of the EO, particularly from the inflorescence, revealed the presence of several compounds that are commonly indicative of plant signalling,

such as *α*-pinene, *(E,E)*-*α*-farnesene and *(E)*-caryophyllene. These compounds have been shown to either attract pollinating bees (Giuliani et al., 2020; Perkins et al., 2023), enhance the repellence of aphids (Ahmed et al., 2019; Dieudonné et al., 2022), or attract natural enemies (Ninkuu et al., 2021).

The secretory cells in the leaves, inflorescence axis, and peduncle of *E. elatior* in this study further underline its importance as a structure taxonomically acquainted with Zingiberaceae, thereby corroborating the previously reported in ginger (*Zingiber officinale*) and shell ginger (*Alpinia zerumbet*) (Remashree et al., 1999; Jezler et al., 2013; Victorio et al., 2011)

CONCLUSION

The present study is the first report to comprehensively characterise secretory structures in Zingiberaceae. Secretory cells have emerged as the main secretory system of *E. elatior* that produces EO, and their presence is widely distributed throughout the leaves, inflorescences, and peduncles. Although an elaborate field experiment is necessary to demonstrate the mechanism of plant signalling in mediation with their environment, the present study has established the foundation for further research avenues, particularly those that utilise secretory structures for agricultural advancement.

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Growth Performance and Survival of Red Tilapia (*Oreochromis* spp.) Larva Rearing in Floating Hapa-canvas in Kenyir Lake, Terengganu

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ABSTRACT

Water quality in the larvae-rearing environment plays a significant role in its growth and survival, especially in an open water body system. The effect of hapa placed in a tank-like structure made of canvas (hapa-canvas; HC) on the growth performance and survival of red tilapia larvae reared in Kenyir Lake was evaluated in wet and dry seasons compared to hapa without canvas as a control. The experiment was conducted for five weeks, and the stocking density of larvae in wet and dry seasons was 1500 larvae/m^3 and 400 larvae/m^3, respectively. Larvae were fed with commercial powdered feed until satiation twice daily and sampled weekly. The final weight of larvae reared in HC in the wet (603.3 ± 25.9 mg) and dry (1,308.7 ± 60.7 mg) seasons were higher (p<0.05) than control (398.7 ± 68.0 mg and 807.3 ± 47.9 mg; respectively). The survival rate was also higher (p<0.05) in HC (68.6 ± 7.3% and 87.7 ± 1.7%) compared to control (7.3 ± 1.0% and 74.8 ± 0.3%) in wet and dry seasons, respectively. Thus, hapa-canvas may be a good alternative to rear tilapia larvae in an open water body based on its growth performance and survival rate.

Keywords: Canvas, dry season, stocking density, temperature, wet season

INTRODUCTION

Kenyir Lake, located in Terengganu, Malaysia, is the largest man-made lake in Southeast Asia and was built for electricity generation. Under the government initiative, up to 2000 floating cages were assembled in the aquaculture industrial zone to boost freshwater fish production with red tilapia, *Oreochromis* spp. becomes the dominant fish cultured in Kenyir Lake. According to annual statistical data, red tilapia is among the three major freshwater species being cultured in Malaysia (Department of

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Fisheries, 2022). Several issues arise regarding the inconsistency of tilapia seed supply, either from the quantity or quality aspect. High mortality of fish is usually caused by stress due to the long distance of travel and fish handling during packaging for transportation because fish seed hatcheries are typically situated far away from the culture site (Honryo et al., 2018; Husen et al., 2021). Thus, cage operators have been encouraged to produce fish seeds in the cage system.

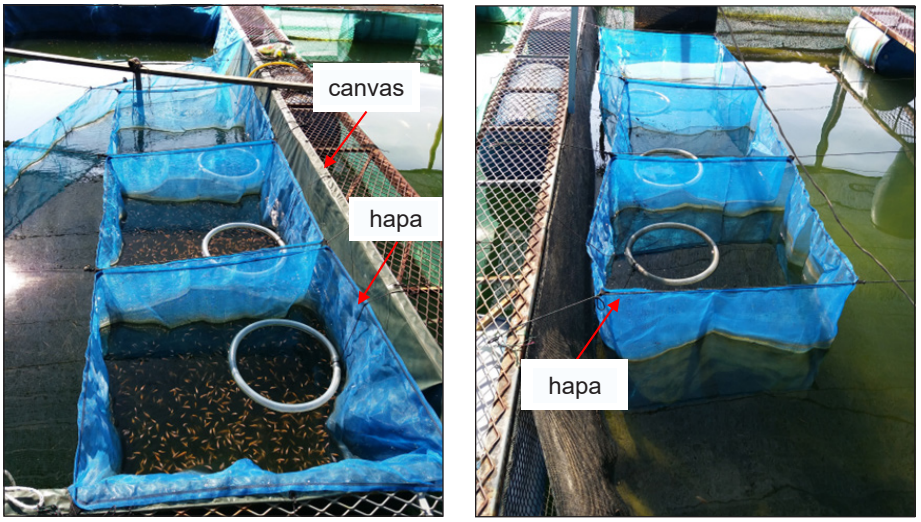
Water quality in Kenyir Lake falls into Class II based on the National Water Quality Index by the Department of Environment, Malaysia (Subramaniam et al., 2023). Kenyir Lake is an oligotrophic lake because of its low primary productivity, nutrient content, and algal production (Suratman et al., 2019) and is illustrated as 'clear water.' According to Ismail et al. (2016), the Secchi disc reading of Kenyir Lake was 425 cm deep, indicating the water's clarity. In the natural habitat, phytoplankton serves as a major food source for a wide range of aquatic organisms, including fish fry, especially after the egg-yolk development of the fish larvae has been completed (Raja et al., 2018). Phytoplankton is consumed by the zooplankton, which then becomes a feed source for the fish fry in the food web (Soukaina et al., 2022). The growth and diversity of the phytoplankton depend on the water condition (Jewson et al., 2015) and nutrient availability.

Water consists of microalgae or phytoplankton, which is beneficial for fish rearing, especially during the nursery stage (Chen & Zeng, 2021), often referred to as 'green water.' In this study, the canvas was used as an impoundment in cages to entrap nutrients and induce the development of 'green water' for rearing red tilapia larvae in cages. A fine mesh of nylon net called hapa was inserted into the canvas, called hapa-canvas. Early-stage larval rearing becomes a challenge in floating cages due to 'clear water,' which indicates low natural food availability and direct sunlight penetration into the waterbed, affecting larvae viability (Pan et al., 2020). Therefore, the objective of this study was to evaluate the effect of using hapa-canvas on the growth performance and survival rate of red tilapia larvae reared at the cage culture of Kenyir Lake.

MATERIALS AND METHODS

Study Site and Experimental Set-up

The location of the study was at Como River, Kenyir Lake, Terengganu (5°00'N, 102°48'E). Kenyir Lake has an average depth of 37 m and 260 km^2 of surface area in a 38,000-ha area (Freshwater Fisheries Research Centre [FFRC], 1995). Cages with a dimension of 6 m × 6 m × 3 m were used. Nylon canvas (6 m × 3 m × 1.5 m) was hanging in the cage frame before hapa (1 m × 1 m × 1 m) was put inside the canvas, known as hapa-canvas (HC). HC provided an enclosed water system to entrap nutrients and induce the development of green water, while hapa without canvas was used as a control (Ctl) (Photo 1). In this study, hapa was made of nylon with 20 strands per inch mesh size.



(a) (b)

Photo 1. (a) Hapa-canvas and (b) hapa without canvas acted as a control at the end of the experiment

Fish Stocking and Sampling

An average of 14 ± 1 mg and 17 ± 1 mg of tilapia larvae were stocked at 1500 larvae/m^3 and 400 larvae/m^3 in the wet and dry seasons, respectively. Larvae were weighed using an analytical balance (ELB2000, Shimadzu) before being stocked into the hapa. A total of 100 larvae were assigned into a group of ten at each time of weighing and sampled weekly. Larvae were supplied by Telaga Juta Solution (M) Sdn. Bhd. that produces red tilapia fry at their cage facility. The difference in stocking density between the two seasons was based on the larvae's availability when the experiment started. The first experiment was conducted in an inter-monsoon to wet season (September till October 2019) with a stocking density of 1500 larvae/m^3 in hapa-canvas (HC-W) compared to control (Ctl-W) without canvas. The second experiment was conducted in the dry season (July until August 2020) with a stocking density of 400 larvae/m^3 in hapa-canvas (HC-D) compared to the control (Ctl-D) without canvas. Larvae were fed with marine fish feed (Cargill; 38%–40% crude protein) twice daily until satiation. The rearing was conducted for five weeks. At the end of the experiment, all surviving fish in the treatment and control group were harvested, counted, and weighed in a group of twenty to determine their survival and final body weight. Treatment and control were performed in triplicate.

Water Analysis

Water quality was monitored biweekly using Horiba LAQUAact DO-110 portable water quality meters for measuring dissolved oxygen (DO) and temperature. In contrast, a Horiba LAQUAtwin pH sensor was used to determine the pH. Water quality parameters during

the study period were reported in the range of 26.8°C –30.0°C, pH 7.1–7.6, and 5.4–6.5 mg/ml of dissolved oxygen. Plankton samples were taken at 8 a.m. at the end of the experiment (fifth week) using a plankton net with a mesh size of 35 µm. The plankton net was placed for five minutes in the HC and Ctl, respectively, horizontally 0.5 m below the water surface. Then, 100 ml of the water sample in the sampling bottle was transferred to a plastic sampling bottle. A few drops of formalin were put in the samples for preservation. A 1 ml water sample was drawn into the Sedgwick-Rafter chamber for cell counting under a compound microscope (Motic BA200) using 10× and 20× magnification lenses.

Parameter Observed

All parameters related to fish growth were calculated, including the body weight (BW), specific growth rate (SGR) and survival rate (SR). These parameters were calculated based on Equations 1 and 2:

$$SR(\%) = 100 \times \left(\frac{Final\ \ count}{Initial\ \ count}\right) \quad [1]$$

$$SGR\ (\%)\ per\ day = \frac{log_{final\ weight} - log_{initial\ weight}}{time_{days\ of\ rearing}} \times 100 \quad [2]$$

Statistical Analysis

Data on growth and survival were analyzed using an independent *t*-test between treatments in the same season. All analysis was performed using Statistical Product and Service Solutions (SPSS) version 20 software for Windows (SPSS Chicago, IL, USA), and values were presented as means ± SEM (standard error of means). All statistical analyses were tested with a significance level of $\alpha = 0.05$ ($p<0.05$).

RESULTS

The mean body weight and survival of red tilapia larvae at harvest reared in hapa-canvas for both stocking densities (HC-W and HC-D) were higher than in control (Ctl-W) and Ctl-D) (Table 1). Red tilapia larvae achieved the highest mean body weight of 1.310 ± 0.250 g reared at 400 fish/m^3 in HC-D, while the lowest was 0.313 ± 0.115 g of red tilapia fry raised in Ctl-W at 1500 larvae/m^3. In the fifth week of rearing, the mean body weight of red tilapia larvae was significantly higher ($p< 0.05$) in hapa-canvas for both seasons compared to the control. At the end of rearing, the survival rate of larvae in hapa-canvas was highly significant ($p<0.05$, 0.001) during the wet season and significant in the dry season ($p<0.05$, 0.010) when compared to the control. The SGR was significantly higher ($p<0.05$) in HC-D when compared to control (Ctl-D); however, it was not significantly different ($p>0.05$) in the wet season.

The growth of red tilapia larvae in both hapa-canvas (HC-W and HC-D) significantly increased (p<0.05) after the second (HC-W) and third week (HC-D) of rearing at 1500 larvae/m^3 and 400 larvae/m^3, respectively (Figure 1). The final body weight of red tilapia larvae at a lower stocking rate (dry season) showed a higher result than at a higher stocking rate (wet season) after five weeks of rearing.

Water samples from hapa-canvas and control were checked for collective phytoplankton; however, no plankton was identified. The phytoplankton was not reported in the first experiment (wet season) due to sample degradation during preservation. The phytoplankton and zooplankton density in HC-D was 356 ± 19 individuals/ml and 433 ± 22 individuals/ml, respectively, and significantly (p<0.05) higher when compared to Ctl-D, which was 130 ± 13 individuals/ml and 59 ± 22 individuals/ml; respectively.

Table 1
Comparison of body weight, survival rate and specific growth rate of tilapia larvae reared in different seasons in hapa-canvas and control

	Wet Season			Dry Season		
	Ctl-W	**HC-W**	**p-value**	**Ctl-D**	**HC-D**	**p-value**
Stocking density, larvae/m^3	1500			400		
Initial BW, mg	13.9 ± 0.3	13.6 ± 0.3	0.664	17.0 ± 0.6	17.3 ± 1.2	0.815
Final BW, mg	398.7 ± 68.0^a	603.3 ± 25.9^b	0.048	807.3 ± 47.9^a	1308.7 ± 60.7^b	0.003
SR, %	7.3 ± 1.0^a	68.6 ± 7.3^b	0.001	74.8 ± 0.3^a	87.7 ± 1.7^b	0.010
SGR	9.5 ± 0.5	10.9 ± 0.2	0.070	11.4 ± 0.2^a	12.7 ± 0.1^b	0.006

Note. Values are the mean of three replicates ± SEM. The means on the same row within the same season, the different superscripts are significantly different (p<0.05). BW=Body weight; SR=Survival rate; SGR=Specific growth rate

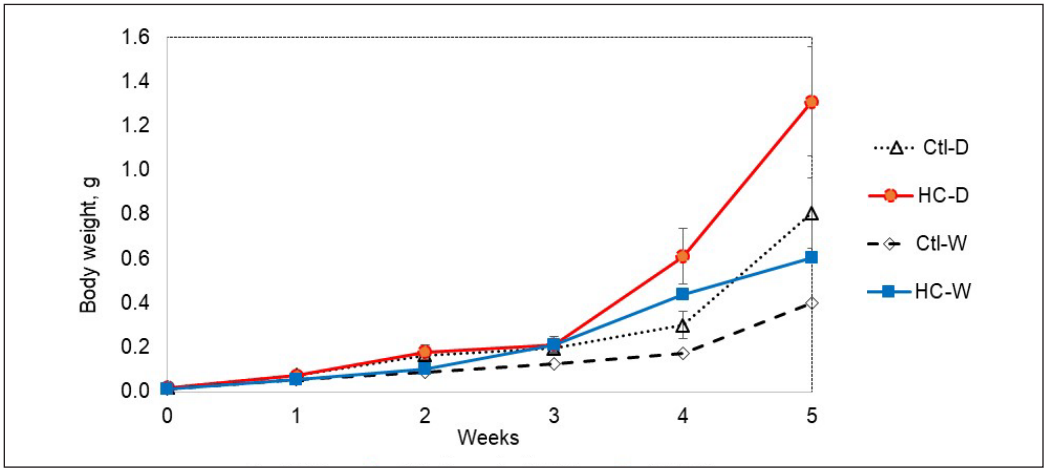


Figure 1. Growth performance of tilapia larvae rearing in hapa-canvas compared to control during wet inter-monsoon (HC-W and Ctl-W) and dry (HC-D and Ctl-D) seasons

The trend of environmental temperature was higher for eight days or points in the dry season compared to the wet season (Figure 2). The mean temperature was 28°C in both seasons. The same trend was also recorded for wind speed, where the wind speed was higher during the dry season (5.9 m/h) compared to the wet season (4.2 m/h). The highest wind speed was 10.1 m/h. The average precipitation was 11.3 mm for the wet and 8.1 mm for the dry season. The highest precipitation was 23.7 mm during the experimental period.

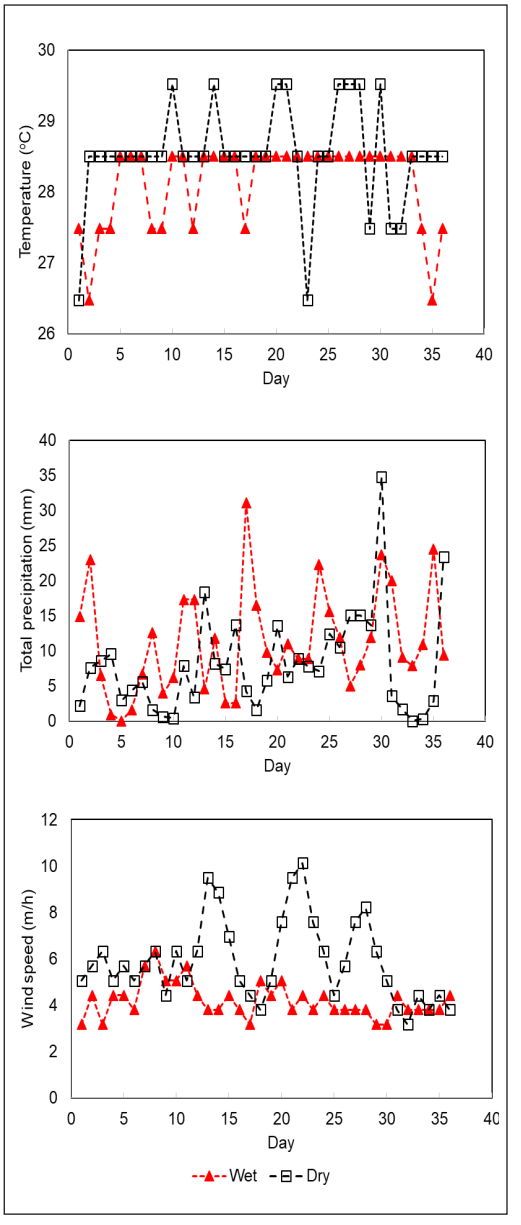


Figure 2. Trends of temperature, total precipitation, and wind during the inter-monsoon (wet) and dry seasons (Kuala Berang, Terengganu, 2023)

DISCUSSION

Kenyir Lake's water bodies are associated with 'clear water,' which scarcely holds onto the nutrients from the aquaculture activities that provide a platform for phytoplankton to bloom. The development of green water in hapa-canvas might contribute to better growth (final mean weight and SGR) and survival of red tilapia larvae during the rearing period compared to the control hapa. Basford et al. (2021) found that the growth and survival of *Portunus armatus* larvae in green water were superior to those of the crabs that were reared without green water. The higher density of phytoplankton and zooplankton in the hapa-canvas compared to the control might offer natural feed availability for larvae to graze on. Post-larvae of *Penaeus monodon* showed better growth performance and survival after having a diet supplemented with algae and co-feeding with *Artemia* (Jaseera et al., 2021). Meanwhile, the mean body weight of larvae reared in HC-D was higher than HC-W due to a negative correlation between stocking density and weight increment of the fish (Ani et al., 2022).

The formation of green water, which contains phytoplankton, was faster at higher stocking density, which could possibly cause the difference in mean body weight after two weeks of rearing compared to low stocking density, which occurred after the third week. More solid waste and excess feed were expected to be produced from 1500 larvae/m^3 than 400 larvae/$m^{3,}$ which would then serve as nutrients for phytoplankton to bloom. Excess feed and fish waste can fertilize the pond and increase fish production (Boyd et al., 2020). The most important elements the phytoplankton requires for growth and reproduction are nitrogen and phosphorous (Cremen et al., 2007). According to Zhang et al. (2022), tilapia affected the water quality by increasing the amounts of total nitrogen, total dissolved nitrogen (TDN), NH^{4+}, and total suspended solids in the mesocosms study.

Larvae are fragile, and exposure to extreme environments can have a fatal impact. The canvas surrounding the hapa provided a conducive, secure, and stable environment for the larvae rearing, thus not affected by the water current or turbulence during the wet season. It was observed that larvae reared in hapa-canvas showed higher survival in the wet season than in the control. This study was conducted in the inter-monsoon seasons of September to October, entering the wet season. The drastic changes in water quality or turbulent water can be avoided in the hapa-canvas compared to the control hapa. Rain coming with the heavy wind could cause water turbulence in Kenyir Lake and affect larvae in the control hapa more than in the hapa-canvas. Hence, the turbulence during heavy rain could possibly contribute to higher survival in HC-D than in HC-W. Wind speed seems to have a minimal impact on water bodies compared to precipitation or rainfall. Abdulqadir et al. (2016) have reported that weather conditions seem to be the major factor contributing to the fluctuation of water quality parameters and primary production in the man-made lakes in Selangor. They found that water parameters such as pH, water temperature and alkalinity were changed due to the impact of rainfall dilution. In addition, green water also acts as a shade to protect fish from direct light (Sanaye et al., 2014), reducing the stress on the fish larvae.

CONCLUSION

Hapa-canvas is a suitable tool for rearing tilapia larvae in an open or large water body because it can help develop ‘green water’ and reduce the impact of drastic changes in the surrounding environment due to weather changes. Further research can be conducted on the effects of water quality parameters, plankton distribution, and other factors affecting fish growth, such as stocking density and depth of hapa-canvas.

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Pigeon Pea (*Cajanus cajan*) Leaf Flavonoid Production at Different Cow Manure Rate Application and Pruning Height

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ABSTRACT

Pigeon pea (*Cajanus cajan*) seeds are widely consumed as a staple food in numerous countries, with leaves recognized for various medicinal values, particularly in flavonoid production. Therefore, this study aimed to investigate leaf flavonoid production in pigeon peas through recurrent harvesting at different heights and the application of cow manure rate. The experiment was carried out in a Split Plot Design, with the main plot consisting of three levels of cow manure at 0, 15, and 30 tons/ha, while the subplots comprised no pruning, 100 and 125 cm above the ground. The findings indicated no observed interaction between cow manure and pruning concerning the variables studied. Recurrent harvesting with 30 tons of cow manure/ha produced a total leaf flavonoid of 2228.3 mg quercetin equivalent (QUE)/100 g leaf dry weight. It showed that 30 tons of cow manure/ha needed to be reapplied for three consecutive harvests since the value declined from the second to the third harvesting. Pruning at different heights above ground produced no significant differences in total leaf flavonoid per plant, with a range of 841.10–1,539.00 mg QUE/100 g leaf dry weight.

Keywords: Manure reapplication, medicinal plant, recurrent harvesting, quercetin

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INTRODUCTION

The pigeon pea (*Cajanus cajan*) is a flexible legume that has served as a dietary staple

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in diverse cultures for centuries. This legume is a significant protein source in numerous tropical and subtropical regions, including India, Africa (Fuller et al., 2019), and Indonesia. Primarily grown as seeds, the other parts of pigeon peas, such as leaf, can be used as a medicinal plant (Aja, Igwenyi et al., 2015; Fuller et al., 2019). Based on comparative analysis, the leaf of pigeon peas contains higher concentrations of bioactive compounds than the seeds. Specifically, the levels of flavonoids were found to be 423.75 ± 57.81 and 31.08 ± 8.20 mg/100g in leaf and seeds, respectively. Tannins were 31.55 ± 2.67 and 17.30 ± 0.47 mg/100g, alkaloids had values of 3118.86 ± 79.35 and 385.54 ± 75.15 mg/100g, saponins were 51.21 ± 4.66 and 1.82 ± 0.29 mg/100g, cyanogenic glycosides were 43.91 ± 5.99 and 12.42 ± 1.84 mg/100g, glycosides were 3.55 ± 1.98 and 3.80 ± 1.01 mg/100g, and anthocyanins were 8.35 ± 0.172 and 4.75 ± 0.174 mg/100g in leaf and seeds, respectively (Aja, Alum et al., 2015). A previous study reported that repeated doses of a combination of pigeon pea leaf extract and ginger rhizome showed no toxic effect on rats (Wresdiyati et al., 2023). Various solvent extracts obtained from different parts of pigeon peas, including leaves, roots, stems, and seeds, have also been assessed for phytochemical composition and biological activities. Flavonoid evaluations as a therapeutic agent included antioxidant, antimicrobial, antidiabetic, neuroprotective, and anti-inflammatory effects, showing the medicinal properties and therapeutic potential of plants (Fuller et al., 2019; Gargi et al., 2022; Oke, 2014; Ullah et al., 2020).

Pigeon peas thrive in warm and semi-arid conditions, showing their suitability for tropical and subtropical climates. It grows optimally in areas with temperatures ranging from 20°C to 30°C (68°F to 86°F) and requires well-drained soil (Abebe, 2022). Although pigeon peas can endure different soil types, optimal performance is mostly observed in sandy loam or loamy soils within a pH range of 6.0 to 7.0. The potential to withstand dry periods makes pigeon peas suitable for regions with irregular rainfall (Musokwa & Mafongoya, 2021). Additionally, pigeon peas can fix atmospheric nitrogen through symbiotic relationships with certain bacteria (Fossou et al., 2016).

Fertilization practices for pigeon peas should be focused on soil characteristics and specific regional requirements, particularly the Nitrogen Phosphorus Potassium (NPK) fertilizer (Ahmed et al., 2021). Typically, phosphorus shows a positive response from pigeon peas, which require enough calcium, potash, and magnesium for proper growth and development (Pal et al., 2011). Moreover, the balanced use of fertilizers, adding organic matter and nitrogen-fixing ability contribute to this versatile legume's productive and environmentally friendly cultivation. Mago and Bunga (2020) stated that there is no significant increase in pigeon pea seed productivity with the application of 2.5 kg cow manure m^{-2}, equivalent to 25 tons per ha.

Nutrient levels play a crucial role in determining the yield of pigeon pea cultivars. Based on previous studies, applying 45 kg N/ha of urea significantly enhanced the dry matter of pods and grain yield. The combination of urea at 45 kg N/ha with 10 tons of manure/ha showed positive effects on the growth parameters, including stems and branches, pods dry

matter, as well as the number of primary branches. Additionally, applying 120 kg P205/ha in the form of TSP contributed to increased grain yield. Plants grown during short rains also showed greater height compared to those cultivated in long rains (Mukindia, 1992). In sandy loam soil during summer plantations, the fertilization of pigeon pea plants with 25 m^3/fed of cow manure resulted in a significant increase in fresh green forage for animals, reaching 21.55 tons/fed. Subsequent fertilization with mineral nitrogen at a rate of 80 kg/fed also contributed to enhanced forage production (El-Seifi et al., 2013).

Pigeon pea leaf has been produced on nodes, which remains unaffected by season and plant density of 4±33 plant m^{-2}. The rate of senescence of the main stem node, regarding thermal time, was also unaffected by plant density and growth duration (Ranganathan et al., 2001). In one of the references, an inorganic fertilizer consisting of 100 kg/ha with 19% nitrogen (N), 38% phosphorus pentoxide (P_2O_5), and 7% sulfur (S) was applied. Subsequently, harvesting was performed with three repetitions by cutting the plant 50 cm above the ground during the flowering stage. The results showed that plots with wider interrow spacing had higher levels of leaf crude protein and in vitro digestible organic matter compared to those with narrower row spacing (Mekonen et al., 2022). Recurrent harvesting with different cutting intervals affected vegetative growth variables and forage yield (Abidinsyah et al., 2020), and the cutting height of 50 cm above the ground with 4 weeks harvesting interval produced the highest fodder yield (Bode et al., 2018). Cow manure, recurrent harvesting, and cutting height are supposed to influence secondary metabolites, including flavonoids, as was found in waterleaf (*Talinum triangulare*) (Saleh et al., 2014). Flavonoids, naturally occurring compounds synthesized in various plant tissues, demonstrate significant antioxidant capabilities. They effectively regulate reactive oxygen species (ROS) buildup by scavenging them upon their formation. As a result, these antioxidant compounds play a crucial role in enhancing plant stress resilience (Dias et al., 2021). The flavonoids can be used as a bioactive marker to standardize the bioactive compounds related to medical benefits, including antioxidant and antidiabetic activities (Al-Masri et al., 2023; Ullah et al., 2020). Consequently, through organic cultivation, this study aimed to determine the best fertilizer rate and pruning height for leaf and flavonoid production in pigeon peas.

MATERIALS AND METHODS

This study was carried out at the IPB Biopharmaceutical Cultivation Conservation Unit Research Station, Bogor, Indonesia, from March to December 2023. The materials used included semi-determinate pigeon peas from Lombok, West Nusa Tenggara, Indonesia, and cow manure. The experiment was conducted using a Split Plot Design, with the main plot having three levels: cow manure 0, 15, and 30 tons/ha, while the subplots included no pruning, 100 and 125 cm above the soil. Each experimental unit comprised 8 plants, and the entire setup included three replications, resulting in 216 plants.

Cow manure analysis showed a pH of 7.41, containing 37.41% C-organic, N-total of 1.90%, P_2O_5 1.50%, K_2O 2.13 mg/100g, Ca^{2+} 1.52%, and Mg^{2+} 0.54%. The soil analysis showed that the pH was 4.44 (low), containing 2.22% C-organic (medium), N-total 0.27% (medium), P 3.54 ppm (very low), Ca 3.87 cmol $^{(+)}$/kg (low), Mg 1.18 cmol $^{(+)}$/kg (medium), K 0.15 cmol $^{(+)}$/kg (low), and cation exchange capacity of 24.43 cmol $^{(+)}$/kg (medium).

In this study, one and half months old seedlings were transplanted to the field. Initially, land clearing was carried out by cleaning weeds, followed by planting holes, and the application of cow manure according to the treatment rate, the whole rate at one time, with a spacing of 1 m x 1.5 m. Harvesting was performed by cutting the shoot or above-ground part of the plant-based on specified treatment. Observations were made monthly on production components in leaf number, plant height, branch number, relative growth rate (RGR), and net assimilation rate (NAR). According to the experimental procedure, RGR and NAR for the treatment without pruning would be carried out every month for 6 months, while for pruning height treatment of 100 and 125 cm, sampling for RGR and NAR was performed until flowering time (the RGR and NAR computed in days). Leaf harvesting was carried out when 50% of the population of plants had started to flower at a height of 30 cm from the initial pruning height (3 months after planting; MAP). Leaf harvesting is executed by pruning the plant according to the pruning height. The leaf was weighted and expressed as plant weight. At the second harvest (5 MAP), leaf NPK was analyzed with N using the Kjehldahl method, while P and K applied the fresh ashing method with a mixture of Nitric Acid and Perchloric Acid. Furthermore, with modification, flavonoid analysis was conducted using a spectrophotometer UV-VIS Shimadzu UV-1280 (Japan), according to Vongsak et al. (2013). The modification executed is the reduction in absorbance resulting from the reaction reduced by the absorbance of the sample that was not reacted with Aluminum Chloride. The addition of potassium acetate was adjusted according to the method of Chang et al. (2002) to reduce turbidity due to reaction with $AlCl_3$. The flavonoid concentration is multiplied by the leaf dry weight to find the flavonoid produced per plant. The third harvest was carried out at 7 MAP, and the data obtained were subjected to analysis of variance. The Duncan Multiple Range Test followed it with a significant level of α 5% for further analysis and comparisons.

$$\text{Relative growth rate /RGR} = (\ln W2 - \ln W1)/ (t2 - t1) \quad [1]$$

Remarks: $W1$ and $W2$ = plant dry weights at times $t1$ and $t2$; t=months

$$\text{Net assimilation rate /NAR} = (W2 - W1)/ (t2 - t1) \times (\ln LA2 - \ln LA1)/ (LA2 - LA1) \quad [2]$$

Remarks: $W1$ and $W2$ = plant dry weights at times $t1$ and $t2$
$LA1$ and $LA2$ = leaf area at times $t1$ and $t2$

RESULTS AND DISCUSSION

No interaction between cow manure and pruning affected all the variables observed. Table 1 shows the climatic conditions during the experiment, indicating that the drought season in Bogor usually lasts from May/June to September. Except for September 2023 (87.3 mm/month), rainfall intensity in all months is considered wet or rainy season, although the temperature is relatively high. With enough rainfall, these conditions significantly enhanced pigeon peas' vegetative growth.

Table 1
Climatic conditions during the growing season of March–October 2023

	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
T (°C)	25.7	26.7	27.0	26.5	26.3	26.3	26.8	27.6
Tmax (°C)	31.3	32.6	32.9	32.5	32.3	33.1	33.7	34.6
Tmin (°C)	22.2	22.9	23.5	23.1	22.4	21.9	21.5	23.1
RF (mm)	325.8	312.8	294.2	310.7	134.4	134.4	87.3	180.7
RH (%)	86.1	83.7	82.4	83.8	79.5	76.0	71.8	74.4
SD (h)	4.9	6.1	6.2	7.1	6.9	7.8	8.1	7.7

Note. T=Average temperature; Tmax=Temperature maximal; Tmin=Temperature minimum; RF=Rainfall; RH=Relative humidity; SD=Sunshine duration (Indonesia Meteorology, Climatology, and Geophysical Agency, 2023)

A previous study in Gunung Kidul, Yogyakarta, Indonesia, with less optimal soil conditions and low rainfall intensity, showed the survival of 30 black pigeon pea types (Yuniastuti et al., 2020). However, this study was conducted in Bogor, which is characterized by a better soil analysis that contributes to plant growth. The soil analysis showed a low pH of 4.44, containing 2.22% C-organic (medium), N-total of 0.27% (medium), P 3.54 ppm (very low), Ca 3.87 cmol $^{(+)}$/kg (low), Mg 1.18 cmol $^{(+)}$/kg (medium), K 0.15 cmol $^{(+)}$/kg (low), and cation exchange capacity of 24.43 cmol $^{(+)}$/kg (medium).

In plants without pruning, the application of manure to pigeon peas did not affect the RGR and NAR from 1 to 6 MAP. The significant fast growth rate, as shown by the RGR in Figure 1 and NAR in Figure 2, was observed during the initial 1 to 2 MAP, followed by a decline to 6 MAP. These semi-determinate plants elongated with higher height produced branches with leaves on the nodes, which started flowering at 3 MAP and produced pods with more branches. The flowering reduced the vegetative growth rate due to the movement of the sink from leaf buds to flowers or pods. The results showed that different growth stages have various photosynthate translocations, with the blooming stage being lower (Isobe et al., 2020). In contrast, pruning or harvesting in this experiment removed the flowers, causing increased biomass production, as observed in *Hesperaloe funifera* (Agavaceae) (McLaughlin, 2003). The integrative developmental stages identified in mango included

growth asynchronisms between two topologically connected organs: the vegetative axis and leaf. This phenomenon was explained by examining the coordinated development between the vegetative axis and leaf during various stages of growth (Dambreville et al., 2015).

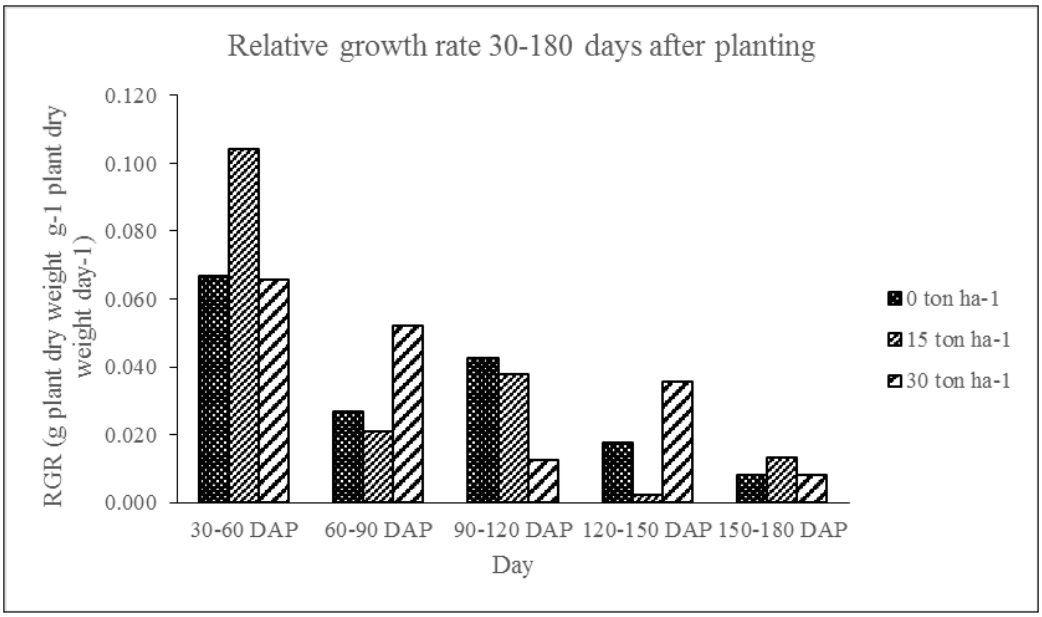

Figure 1. Relative growth rate 30–180 days after planting (DAP) with different cow manure rate

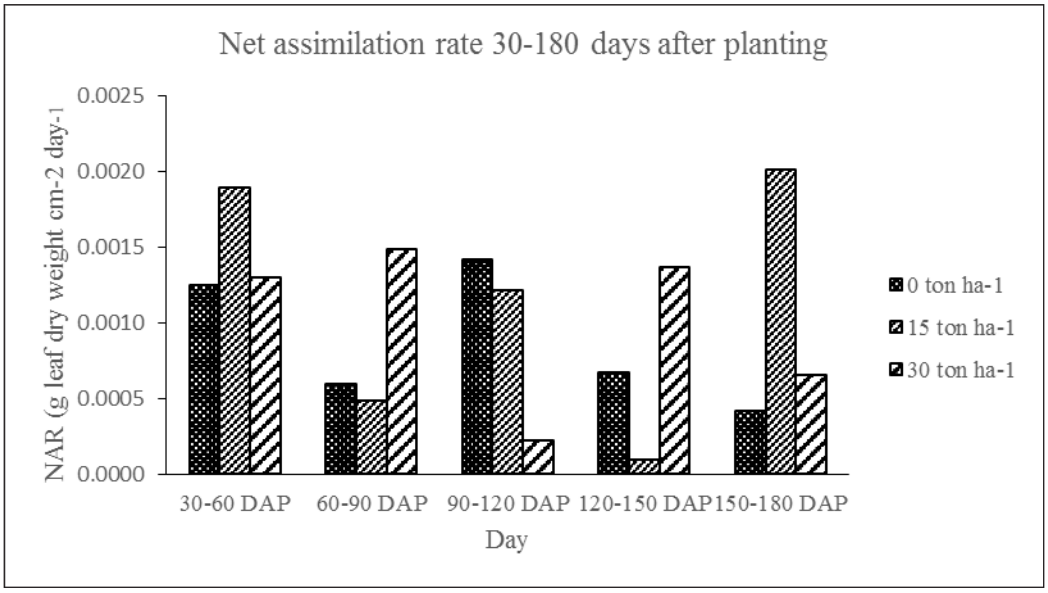

Figure 2. Net assimilation rate 30–180 DAP with different cow manure rate

Plant heights ranging from 1 to 6 MAP remained unaffected by the application of cow manure. Nonetheless, notable disparities in plant height were observed at 5 MAP following two rounds of harvesting at 3 and 5 MAP, particularly evident in Table 2. Based on observation, the data showed that pruning at 100 and 125 above ground produced significantly lower heights of 53.22 and 44.15%, respectively, compared to 6 MAP. Before the first harvesting, approximately 3 MAP 30 tons of cow manure/ha had 145.81 and 39.39% significantly higher leaf numbers than without or 15 tons of cow manure/ha, respectively. At 4 MAP, 30 tons of manure/ha produced 83.02% significantly higher leaf numbers than without manure, as shown in Table 3.

Table 2
Plant height 1–6 Months After Planting (MAP) with different cow manure rates or pruning heights

Treatment	Plant Height					
	1 MAP	2 MAP	3 MAP	4 MAP	5 MAP	6 MAP
	……cm……					
Cow manure rate						
0 ton ha^{-1}	86.20	150.01	202.30	189.04	235.36	188.45
15 ton ha^{-1}	74.83	147.36	208.86	186.2	219.11	180.08
30 ton ha^{-1}	85.11	158.12	212.82	207.61	225.74	182.48
Pruning height (cm)						
0	82.71	153.64	215.96	237.8	262.37a	271.94a
100	78.47	148.11	198.59	174.71	199.54b	127.20c
125	84.96	153.74	209.42	170.33	218.51b	151.87b

Note. Numbers followed by different letters in the same column indicate significantly different results in the 5% DMRT test

Table 3
Leaf number 1–6 Months After Planting (MAP) with different cow manure rates or pruning height

Treatment	Average leaf number					
	1 MAP	2 MAP	3 MAP	4 MAP	5 MAP[1)]	6 MAP[1)]
Cow manure rate						
0 ton ha^{-1}	29.2	154.5c	352.4b	264.4b	444.2	235.7
15 ton ha^{-1}	24.0	190.2b	466.2b	375.6ab	462.5	239.8
30 ton ha^{-1}	32.3	259.6a	649.8a	483.8a	549.5	222.6
Pruning height (cm)						
0	30.0	202.0	516.6	667.6a	602.2a	368.0a
100	27.1	192.4	464.2	156.3c	316.7b	110.3c
125	29.1	209.8	487.6	299.9b	537.3a	219.8b

Note. Numbers followed by different letters in the same column indicate significantly different results in the 5% DMRT test; [1)]=Transformation $\sqrt{x}+0.5$

The growth of pigeon peas for leaf harvesting follows a sequential condition influenced by plant height due to node growth from the stems and branches. According to Ranganathan et al. (2001), longer internodes would reduce node number, while higher pruning height produced lower leaf number. In this study, pruning caused leaf number differences from 4 to 6 MAP. A significantly higher leaf number was found at 6 MAP on plants without pruning, 100, and 125 cm pruning above the ground, as shown in Table 3. Plants without pruning produced significantly higher leaf numbers of 70.02 and 40.27% compared to 100 and 125 cm above ground, respectively.

In this study, 30 tons of manure per hectare influenced branch number compared to the control at 2, 3, and 5 MAP, with a significant increase of 40.74, 43.35, and 115.09%, respectively. Although harvesting at 3 MAP produced a similar branch number at 4 MAP, higher manure rates at 5 MAP were more significant compared to the control, as shown in Table 4. The results also showed that branch numbers were affected by cow manure after 2 months of harvesting. This phenomenon suggested a time lag in nutrient usage due to the slow release (Prado et al., 2022; Saputra et al., 2019) of cow manure (Prado et al., 2022).

Table 4
Branch numbers 1–6 Months After Planting (MAP) with different cow manure rates or pruning height

	Average branch number					
Treatment	**1 MAP**	**2 MAP**	**3 MAP**	**4 MAP[1)]**	**5 MAP[1)]**	**6 MAP[1)]**
Cow manure rate						
0 ton ha^{-1}	4.3	10.8b	14.3b	24.3	26.5b	29.0
15 ton ha^{-1}	4.6	14.4a	18.2ab	32.0	42.7ab	37.3
30 ton ha^{-1}	5.9	15.2a	20.5a	42.5	57.0a	35.6
Pruning height (cm)						
0	5.0	14.0	18.4	17.5b	17.5c	17.5c
100	4.8	12.4	16.4	29.3b	41.8b	30.6b
125	5.1	14.0	18.2	52.0a	66.9a	53.8a

Note. Numbers followed by different letters in the same column indicate significantly different results in the 5% DMRT test; [1)]=Transformation $\sqrt{x}+0.5$

After the first harvest at 3 MAP, pruning height significantly produced higher branch numbers at 4, 5, and 6 MAP. Branch numbers for pruning height of 100 cm and 125 cm at 6 MAP, which recorded an increase of 74.86% and 207.43%, were significantly higher compared to the control. Furthermore, recurrent harvesting of pigeon peas showed affected plant height, leaf, and branch number (Tenakwa et al., 2022).

Leaf increased with manure application, including fresh stem and dry weight, as shown in Figures 3A and 3B. In the first harvest, the application of 30 tons of cow manure/ha produced leaf fresh and dry weight, which was significantly higher at 125.74 and 174.94%

compared to those without manure. Furthermore, the total leaf freshness of 554.59 g/plant and the dry weight of 204.24 g/plant were significantly higher than the control, 108.93 and 115.68%, respectively. Plants with and without cow manure had the highest leaf dry weight in the second harvest, which significantly declined in the third harvest. It showed that after the second harvest, the nutrients from cow manure decreased, indicating the need for reapplication.

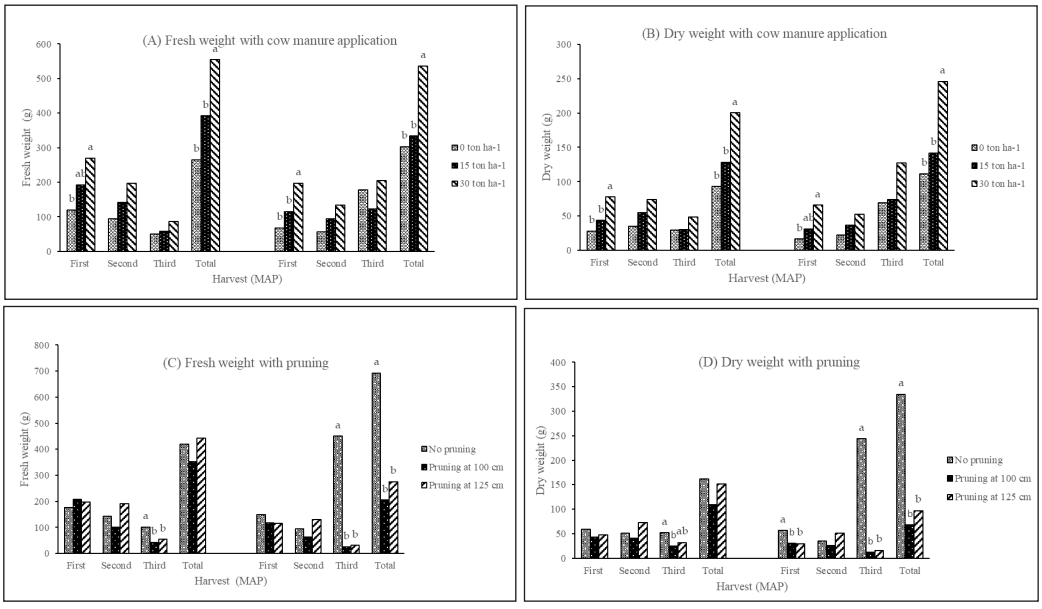

Figure 3. Fresh (A) and dry weight (B) with manure application; fresh (C) and dry weight (D) with pruning. Bars with different letters in the same harvesting time above them are significantly different in the 5% DMRT test

Total stem fresh (536.55 g/plant) and dry weight (245.82 g/plant) with 30 tons of cow manure/ha was significantly higher than control at 77.10 and 119.93%, respectively. During the second harvest, the application of cow manure and pruning height did not affect the leaf weight, including stem fresh and dry weight. At the third harvest, no significant increase was observed in the fresh and dry weight of the leaf and stem. However, it was observed that recurrent harvesting led to an increase in the fresh and dry weight of the stem.

Plants without pruning produced significantly higher leaves and stem fresh weight at third and total harvest, as shown in Figure 3C. The absence of pruning resulted in significantly higher leaf dry weight during the third harvest. Additionally, stem dry weight was significantly higher on the first, third, and total harvest when no pruning was implemented, as shown in Figure 3D. This phenomenon could be accepted, as harvesting at a certain height reduced harvest weight. Tenakwa et al. (2022) stated that the cutting

regime for pigeon peas significantly affected the biomass yield, while further cutting at 20 weeks after planting (WAP) produced more biomass compared to 12 WAP.

The results also showed that leaf and stem weight decreased from the first to the second harvest, while a significant increase was observed at the third harvest, contributing to total stem weight. Without pruning, plants in the generative phase produced more stems than leaves. Furthermore, harvesting at 100 cm above ground produced insignificant lower fresh and dry leaves compared to 125 cm above ground. The presence of more branches due to pruning contributed to harvesting younger leaves with higher water content.

The application of cow manure significantly increased leaf N and P, as added fertilizers met the crop's nutrient requirement. However, no substantial effect was observed on leaf K, as shown in Table 5. The results showed that cow manure supplied 37.41% C-organic, N-total 1.90%, P_2O_5 1.50%, K_2O 2.13%, Ca^{2+} 1.52%, and Mg^{2+} 0.54%. Leaf N with the application of 15 and 30 tons of manure/ha was 7.12 and 9.87%, significantly higher than control. Furthermore, leaf P with 30 tons of cow manure/ha was 14.29% significantly higher compared to without manure. In soybean leaf, nutrient sufficiency for N, P, K, Ca, and Mg was 3.86–4.57%, 0.31–0.37%, 1.83–2.07%, 0.94–1.13%, and Mg 0.44–0.53% (Souza et al., 2020). Based on soybean reference, pigeon pea leaf N was sufficient with 30 tons of cow manure/ha application, while P and K were insufficient. It showed that the application of cow manure at 30 t/ha could not supply the P needed by pigeon peas, playing a fundamental role in regulating abiotic stress tolerance (Khan et al., 2023). Despite the low nutrients found in cow manure (Prado et al., 2022), N, P, and K content contributed to the growth and leaf harvest in pigeon peas. Adding organic matter also contributes to long-term nutrient availability and soil health, which may require fertilization (Goldan et al., 2023). In mung beans, N, P, and K presence facilitated root development, flowering, and pod formation (Yin et al., 2018). On other species, organic matter increased the growth variables, namely plant height, branch number, leaf freshness, and dry weight, such as in *Vernonia amygdalina* (Tjhia et al., 2018).

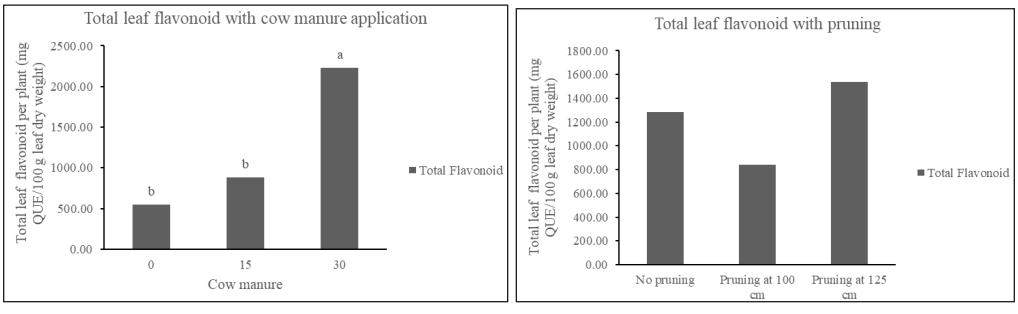

Figure 4. Total leaf flavonoid per plant from 3 times harvesting with cow manure application (left) and pruning height (right). Bars with different letters above them are significantly different in the 5% DMRT test

Table 5
Leaf NPK content at 5 Months After Planting (MAP) and leaf total flavonoid per plant for three harvesting with different manure rates or pruning height

Treatment	N	P	K	Leaf total flavonoid
	……% dry weight……			mg QUE/100 g leaf dry weight
Manure rate				
0 ton ha^{-1}	3.65b	0.21 b	0.80	523.3b
15 ton ha^{-1}	3.91a	0.22ab	0.78	618.9b
30 ton ha^{-1}	4.05a	0.24 a	0.86	993.5a
Pruning height (cm)				
0	3.73	0.22	0.73	725.3
100	3.96	0.23	0.90	630.4
125	3.92	0.21	0.81	780.0

Note. Numbers followed by different letters in the same column indicate significantly different results in the 5% DMRT test; QUE = quercetin equivalent

Total leaf flavonoid production per plant from three times harvesting with application of cow manure and pruning height was shown in Figure 4. The application of 30 tons of cow manure per hectare showed a significantly higher value compared to both without manure and 15 tons, accounting for an increase of 115.68 and 55.88%, respectively. Higher cow manure dosage contributed to greater nutrients available for plants. Adding 25 tons of cow manure per ha does not increase pigeon pea seed production in India (Mago & Bunga, 2020), but no data was found for fodder production. The Latosol soil in Indonesia has low organic matter (Adhi et al., 2017; Suminar et al., 2017), which shows the need for manure application. A minimum of 5 t cow manure/ha should be applied (Hatibie & Garantjang, 2022). Leaf total flavonoids for 30 tons of cow manure/ha application also showed a significantly higher value, as presented in Table 5. In *Centella asiatica*, repeated harvesting with organic manure showed higher phytoconstituents, such as flavonoids (Bhattacharya et al., 2017).

This value could be explained by the significantly higher leaf N and P. Specifically, N as part of the chlorophyll molecular formula contributed to the photosynthetic processes that produced plant growth and biomass (Ebrahimi et al., 2023). P plays a crucial role in energy transfers, photosynthesis, as shown by Kayoumu et al. (2023), nutrient flow, and plant growth (Khan et al., 2023). In this study, the analysis of the dry leaf harvesting from Figures 1A and B showed that for the third harvesting, the application of 30 tons of cow manure/ha was insufficient, indicating the need for manure reapplication. Additionally, pruning with different heights above ground produced no significant differences in total flavonoid production in the leaves per plant.

CONCLUSION

This study showed that leaf flavonoid production in pigeon peas using 30 tons of cow manure/ha resulted in 2228.3 mg QUE/plant. The results showed the need for reapplication of 30 tons of cow manure/ha three times harvesting, as the value declined from second to third harvesting. Additionally, pruning at various heights above the ground did not result in significant differences in leaf total flavonoid production per plant, with values ranging from 841.10 to 1,539.00 mg QUE/plant.

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Evaluating AedesTech Mosquito Home System (AMHS) Effectiveness on *Aedes* Mosquitoes

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ABSTRACT

Ovitrap deployment stands as a viable strategy for *Aedes* mosquito control. This study evaluated the efficacy of an autodissemination ovitrap called AedesTech Mosquito Home System (AMHS), which incorporates pyriproxyfen. The study encompassed laboratory trials. Within the laboratory trials, our investigations unfolded across two species of mosquitoes: *Aedes albopictus* and *Aedes aegypti*. Three distinct facets were explored in the laboratory trials: the influence of an attractant on the oviposition, the effect of trap positioning on oviposition, and the selection of oviposition sites. Our laboratory results indicated that the Mosquito Home Aqua (MHAQ) solution with attractant consistently attracted *Ae. aegypti* effectively (Welch's Analysis of Variance) F (2,68.66) =5.22, p=0.01). However, its efficacy with *Ae. albopictus* was suboptimal compared to other treatments (Two-way ANOVA, F=0.16, df=2, p>0.05), highlighting the need for considering additional attractants. Notably, the placement of AMHS exhibited no discernible impact on its attractiveness for both mosquito species (T-test, p>0.05), underscoring the flexibility in trap deployment. The occurrence of simultaneous oviposition choices within the same replicates hinted at the possibility that the existing attractant in MHAQ did not significantly influence oviposition (p> 0.05). Therefore, eliminating the attractant is suggested to reduce the cost of AMHS production. Overall, our investigation underscores the promising potential of AMHS for *Aedes* control, especially *Ae—aegypti* and substantiated by robust statistical evidence gleaned from this controlled laboratory study.

Keywords: *Aedes*, dengue, mosquito, ovitrap, pyriproxyfen

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INTRODUCTION

Entomological studies encompass laboratory and field testing and are indispensable for comprehensively assessing the effects of control methods on *Aedes* mosquitoes (Ferguson et al., 2008). Field testing, for example, can be employed to compare the efficacy of an ovitrap with other methods in identifying *Aedes* abundance (Gao et al., 2019). Meanwhile, a laboratory study, such as determining the most attractive colour of traps, can potentially influence the future design of the ovitrap (Khan et al., 2023).

Laboratory testing has many advantages for checking the efficacy of any methods in controlling *Aedes* mosquitoes. One of the advantages of laboratory testing is that it allows for the controlled selection of mosquito strains with a wide range of insecticide resistance phenotypes and genotypes (Thornton et al., 2020). Furthermore, laboratory testing can establish the concentration range that effectively kills *Aedes* mosquitoes at any stage of their development, unaffected by any uncontrolled factor such as rain (Reza & Ilmiawati, 2020). Laboratory testing is usually less laborious and time-consuming than field testing, as evidenced by a study in the Philippines where ten locations were used for field testing and conducted over a year (Gualberto & Demayo, 2022).

Numerous laboratory studies demonstrate the testing of various instruments and chemicals to control *Aedes* mosquitoes by exploiting their oviposition behaviour (Musunzaji et al., 2023; Snetselaar et al., 2014; Tawatsin et al., 2019). For example, an ovitrap using carpet shell extract as an attractant was proven to effectively draw in dengue vectors *Aedes albopictus* and *Aedes aegypti* for oviposition in a laboratory setting (Tawatsin et al., 2019). Another study showed that *Piper betle* L. essential oil concentration can act as a repellent against the oviposition of *Ae. aegypti* in a laboratory setting (Martianasari & Hamid, 2019). A separate study proved the attractiveness of banana infusion as a potential attractant for *Ae. aegypti* oviposition activity by assessing the number of eggs deposited after four days (Musunzaji et al., 2023).

This study utilised the AedesTech Mosquito Homes System (AMHS) trap, an ovitrap capitalising on *Aedes* mosquito oviposition behaviour. It utilises a 'lure and kill' strategy with an undisclosed mosquito lure agent (Lim, C. H, personal communication, September 22, 2020). The device includes an auto-dissemination feature that enables female mosquitoes to unintentionally spread the pyriproxyfen insecticide to other breeding sites (Man et al., 2020). Pyriproxyfen, which is an insect growth regulator that disrupts juvenile mosquito development by mimicking juvenile hormones, has been proven to change both the ethology and physical characteristics of *Ae. aegypti* (Campos et al., 2023; Fansiri et al., 2022; Fiaz et al., 2019).

Previous studies on AMHS in laboratory settings were limited. Only one study by Mohd Ngesom et al. (2021) explored the effects of different Mosquito Home Aqua Solution (MHAQ) dosage levels on *Ae. aegypti*, including their emergence, autodissemination

events, preference for MHAQ over water, direct impact on larvae, and effects on fecundity and fertility. MHAQ are the solution containing pyriproxyfen that was used with AMHS, and it was observed to cause a shrinkage in the wing length of *Ae. aegypti* mosquitoes (Mohd Ngesom et al., 2021). It is known that the wing measurement can be used as an indicator of body size (Yan et al., 2021). The body size is crucial because smaller mosquitoes lead to repeated hematophagy, elevating the risk of virus transmission through heightened interactions with humans (Tchouassi et al., 2022).

This study was performed to investigate the efficacy of AMHS traps under controlled laboratory conditions, bolstering the findings of the preceding research. This research primarily aimed to establish the effects of attractant on oviposition, optimal trap placement position, and oviposition selection in the AMHS in response to the attractant and variant of trap placements.

MATERIALS AND METHODS

This experimentation seeks to replicate outdoor settings with alterations made in accordance with the indoor setting under laboratory conditions with the methodology proposed by Roque and Eiras (2008) and the World Health Organization (WHO, 2018) with few alterations on the oviposition substrate and time of exposure. These studies encompassed two *Aedes* species, *Ae. aegypti* and *Aedes albopictus*. This inclusion was motivated by the prevalence of *Ae. aegypti* and *Ae. albopictus* in regions on Penang Island afflicted by dengue, as highlighted in the work of Hashim et al. (2019).

Ethical Approval

The procedures involving the use of rats for blood feeding in this study were approved by the Universiti Sains Malaysia Institutional Animal Care and Use Committee (USM IACUC) under animal ethics permission number USM/IACUC/2019/(117)(990).

Study Conditions

The testing was conducted in a spacious 30m^3 room size chamber in Laboratory 304A, Vector Control Research Unit (VCRU), Universiti Sains Malaysia. The laboratory contained two air-conditioned to control and maintain under the environment ambient between 25°C–29°C and 60%–100% humidity, providing an ideal setting condition for the experiment. The chamber featured 17 small opening windows around the walls as well as a door for entering the chamber. A small opening on the front door was used to release gravid females during the study, following Roque and Eiras (2008) and World Health Organization (WHO; 2018) methods. The researcher also used the front door to enter and exit the chamber before and after each replicate for setup and cleanup. During Study 1 and

Study 3, the researcher used the front door for OviTo linen collection and replacement at each time interval. To minimise disturbance, entry and exit were swift, with care taken to avoid direct contact with mosquitoes. Egg counting was performed outside the chamber. The chamber floor is white to enhance visibility and facilitate accurate counting of the mosquitoes, whether in the dead, knockdown, or alive state (Stupp et al., 2020).

AedesTech Mosquito Home System (AMHS) Trap

AedesTech Mosquito Home System (AMHS) traps were supplied by One Team Networks Sdn. Bhd. as an autodissemination trap (Figure 1). It comprises a black polyethene opaque bucket with dimensions of 19.70 cm (height) × 11.00 cm (bottom width) × 14.61 cm (top width) and features a plum-coloured lid. The Mosquito Home Aqua (MHAQ) solution also sponsored the trap, containing 400 ppm pyriproxyfen. Each trap was equipped with OviTo linen, a towel that allows mosquitoes to lay eggs and was used for data collection (Figure 2[a]). The dimensions of the OviTo linen are 7.5 cm in length and 17.5 cm in width (Figure 2[b]). The MHAQ solution bottle is centrally positioned and can be readily secured and detached from the bucket base. The flow of the MHAQ solution is facilitated by gravity.

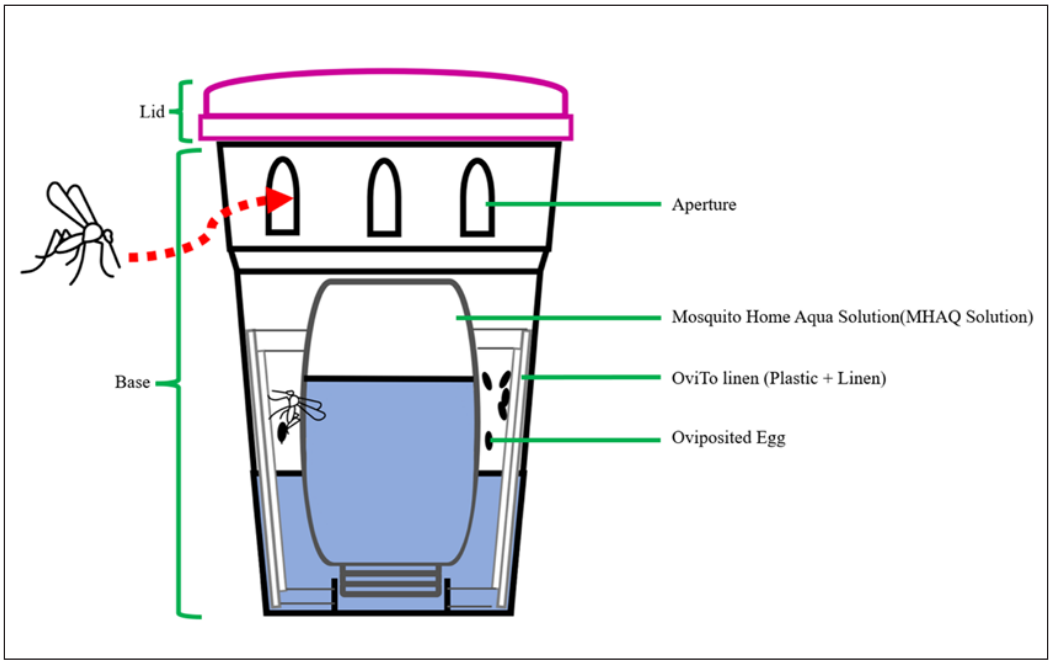

Figure 1. A concise visual representation showcasing the AedesTech Mosquito Home Trap equipped with OviTo Linen and MHAQ solution

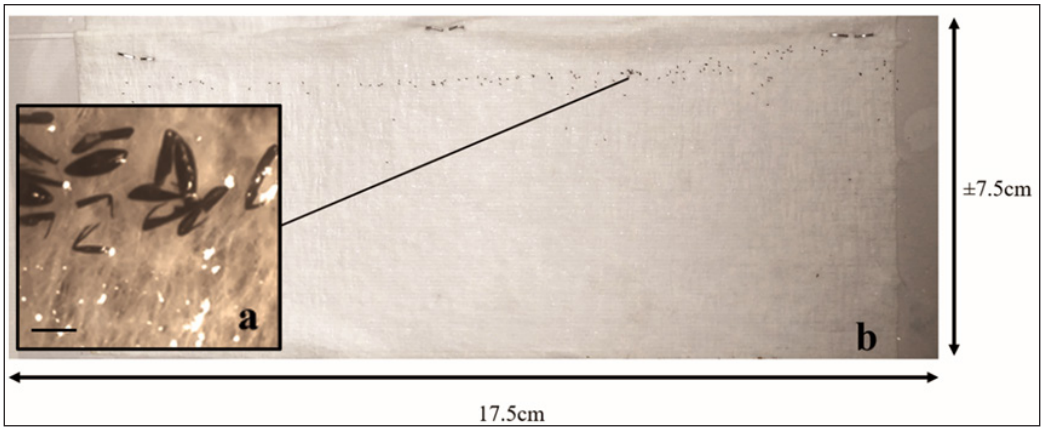

Figure 2. (a) Mosquito eggs attached to the OviTo linen (oviposition strip used in AedesTech Mosquito Home Trap) under a dissecting microscope. Scale bar = 500 µm. (b) Mosquito eggs attached to the OviTo linen and the linen's size

Gravid Female Mosquitoes

Six to eight-day-old gravid female *Ae. aegypti* and *Ae. albopictus* mosquitoes were prepared for the study by culturing eggs from a susceptible lab strain sourced from the Vector Control Research Unit (VCRU). The *Aedes* species was identified using the key by Rueda (2004). *Aedes* mosquito eggs were submerged in seasoned water trays and sorted according to species. They were kept in a controlled lab at 27 ± 2ºC with a 12:12 (L: D) light-dark cycle and 80%–90% humidity to ensure the successful hatching of larvae (Hogg & Hurd, 1997; WHO, 2018; Zuharah & Lester, 2010). A measured quantity of approximately one gram of larval nutrition, comprising a finely powdered amalgamation of dog biscuits, beef liver, yeast, and milk powder in a 2:1:1:1 ratio, was administered bi-daily (Ahbirami et al., 2014). The aqueous medium in the tray was renewed preceding each feeding session (Dieng et al., 2018, 2019).

Pupae were collected in 250 ml plastic containers filled with aged tap water and then transferred to collapsible breeding cages, each with a dimension of 30 cm^3 per layer and equipped with a screen mesh. The adults had continuous access to a 10% sugar solution (Dieng et al., 2017). Upon reaching six to eight days of adulthood, a rat was secured in a wire mesh and introduced into a breeding cage for an hour, allowing 100–200 female mosquitoes to feed on the rat (Buckner et al., 2017; WHO, 2018). The 50 selected females for each replicate were those who had taken their first blood meal after 48 hours to 96 hours before the experiment (WHO, 2018). Fully gravid females were identified by observing whitish eggs within their abdomens (Rebollar-Téllez et al., 1995; Santos et al., 2019).

Half of the gravid females were utilised to obtain eggs for the culture of the next generation, which was intended for use in upcoming replicates. These gravid females were

left in collapsible breeding cages and provided with an oviposition substrate comprising a piece of Smith Filter Papers 102 Qualitative and a black-coloured tin filled with 200 ml seasoned water (Maïga et al., 2017; Thavara et al., 2004; Yap et al., 1995).

Study Design

Three types of study were performed: (1) Effect of an attractant on female mosquitoes' oviposition, (2) Effect of trap position on female mosquitoes' oviposition, and (3) Oviposition selection by gravid females. There were three different treatments using the AedesTech Mosquito Home System (AMHS): (1) AMHS with Mosquito Home Aqua solution (MHAQ) containing an attractant, (2) AMHS with MHAQ without an attractant, and (3) a control group containing seasoned water only. All the solutions were used at a volume of 500 ml. The attractant consisted of the MHAQ provided by the One Team Network Sdn. Bhd. and the ingredients are unknown. However, the only information provided is that the attractant was derived from natural resources. The traps for this study were strategically positioned, ensuring a minimum distance of 1m between each other (Roque & Eiras, 2008; WHO, 2018).

The free-flying technique was utilised in all replicates with the gravid mosquitoes as a subject for testing following the study by Roque and Eiras (2008) and WHO (2018). A total of 50 gravid female mosquitoes were introduced into a room-sized chamber for each replication (WHO, 2018). The mosquitoes were released at the centre of the chamber from a 350 ml plastic container with a lid that was opened using a thread tied to the lid through the small opening attached to the front door. All the studies were run separately for triplicates. The data collection for all studies consisted of three distinct assessments: mean number of mosquito eggs, Hatching Index, and Emergence Rate. Manual counting for the eggs attached to the OviTo linen to determine the mean number of mosquitoes' eggs oviposited was performed thrice by two people using a magnifying glass (Gopalsamy et al., 2021). After all studies, all released mosquitoes were recaptured, and their status (alive, dead, gravid) was recorded. To ensure the validity of the assay, at least 50% of the released female mosquitoes were recaptured.

Study 1: Effect of An Attractant on Female Mosquitoes' Oviposition

In this study, we conducted an assessment within a room chamber, following the arrangement depicted in Figure 3(a). Two AMHS traps with MHAQ were placed at the horizontal position of east and west, whereas the resting place without MHAQ was placed vertically based on the study by Roque and Eiras (2008). Three treatments were run separately by replacing the treatment set with (1) AMHS with MHAQ (with an attractant), (2) AMHS with MHAQ (without an attractant), and (3) control (contained seasoned water). The AMHS traps were lined with the OviTo linen as a substrate for *Aedes* mosquito oviposition.

Subsequently, 50 gravid female mosquitoes were released freely through the small opening attached to the door. Following Roque & Eiras (2008), the OviTo linen in the AMHS trap was evaluated at 30, 60, 90, 120, 150, and 180 minutes to count deposited eggs. A researcher entered the chamber briefly at each interval to collect and replace the OviTo linen, except at the final 180-minute mark. Throughout the assessment, six replicates were carried out for each treatment. Before the next round of bioassays, the remaining mosquitos were removed. Notably, the assessment was carried out on *Ae. aegypti* and *Ae. albopictus* separately for all three treatments.

All the *Aedes* eggs laid on the Ovito linen throughout this study were checked for the mean number of eggs attached to OviTo linen, hatching and emergence index. The culture was separated between replicates, treatments, times, and *Aedes* species. After tallying the egg counts on the OviTo linen for all replicates at each time and counting for mean eggs oviposited, each OviTo linen was submerged in a tray containing seasoned water separately and was cultured following the methods in

Gravid Female Mosquitoes

The hatched larvae were counted, and the Hatching Index (HI) was determined using Equation 1. Subsequently, these larvae were nurtured until adulthood to calculate the Emergence Rate (ER) using Equation 2. The HI were counted based on Yazan et al. (2020):

$$\text{Hatching Index} = \frac{\text{No of egg that hatched}}{\text{Total no. of egg counted}} \times 100\% \text{Hatching Index} = \frac{\text{No of egg that hatched}}{\text{Total no. of egg counted}} \times 100\% \quad [1]$$

The ER were calculated and modified using percentage of emergence based on Gualberto and Demayo (2022):

$$\%\ \text{Emergence} = \frac{\text{No. of adults emerge}}{\text{Total no. eggs counted}} \times 100\% \%\ \text{Emergence} = \frac{\text{No. of adults emerge}}{\text{Total no. eggs counted}} \times 100\% \quad [2]$$

Study 2: Effect of Trap Position on Female Mosquitoes' Oviposition

A stratified random design was employed for trap placement in the study to minimise factors like position biases. Within the room-sized chamber (Figure 3 [b]), traps were positioned in two separate positions: horizontal (West to East) and vertical (North to South), according to the study by Roque and Eiras (2008).

The assessment utilised two AMHS traps with a MHAQ solution containing an attractant and two additional resting place AMHS traps for each position set (Figure 3 [b]). The study was run separately for two treatments with the same setting: AMHS with MHAQ (without an attractant) and control (with seasoned water only).

Each trial involved releasing 50 gravid female mosquitoes into the chamber cage. Each replicate was conducted for 17 hours, starting at 16:00 hours, and the traps were monitored the following morning at 9:00 am, which was aligned with the WHO protocol (WHO, 2018). This timing aimed to maximise heat stress and align with the biting cycle of *Aedes* mosquitoes. The OviTo linen was evaluated for the number of eggs present. Three replications were done for each position. The assessment was carried out separately for *Ae. aegypti* and *Ae. albopictus*.

In Study 2, procedures akin to Study 1 were followed, involving inspecting *Aedes* eggs on the OviTo linen for hatching and emergence. Cultures were segregated by replicates, treatments, position, and *Aedes* species, omitting a time-based culture. Following egg counts for each replicate to calculate the mean number of eggs oviposited, linens were individually immersed in a tray with seasoned water, per the methods outlined in *Gravid Female Mosquitoes*. Hatched larvae were calculated to determine the HI using Equation 1, followed by their maturation to adulthood for calculating the ER using Equation 2.

Study 3: Oviposition Selection by Gravid Females

This investigation was carried out to assess oviposition selection influenced by the presence of an attractant and the variation in positions of traps for *Aedes* mosquito oviposition. This part of the assessment has two types of variants for trap placement to minimise factors like position biases using a stratified random design. Two types of AMHS traps with and without attractants were placed in the chamber across each other, as shown in Figure 3(c). The assessment utilised two AMHS traps with MHAQ solution containing an attractant, along with two additional AMHS containing no attractant were used for each variant setting.

Fifty fully gravid female mosquitoes were released into the room chamber through the opening at the chamber door. Based on Roque and Eiras (2008), the OviTo linen in the AMHS trap was inspected for egg deposition at 30, 60, 90, 120, 150, and 180-minute intervals. To switch the linen, a researcher briefly accessed the chamber at each time point, excluding the 180-minute mark. Entry and exit were rapid to reduce disturbance, and egg counting was performed outside the chamber. Each variant was replicated three times. The assessment was carried out separately for *Ae. aegypti* and *Ae. albopictus*. Upon completing the Variant 1 setting assessment, the methods were repeated by replacing all the trap placements according to the Variant 2 setting.

Aedes eggs attached on the OviTo linen were counted to the mean number of eggs oviposited according to each replicate. Then, we assessed the HI and ER of *Aedes* eggs on

Ovito linen as in Studies 1 and 2. *Aedes* eggs were cultured on OviTo linens considering replicates, variants, treatments, times, and *Aedes* species. This culturing followed the methods outlined in *Gravid Female Mosquit.*

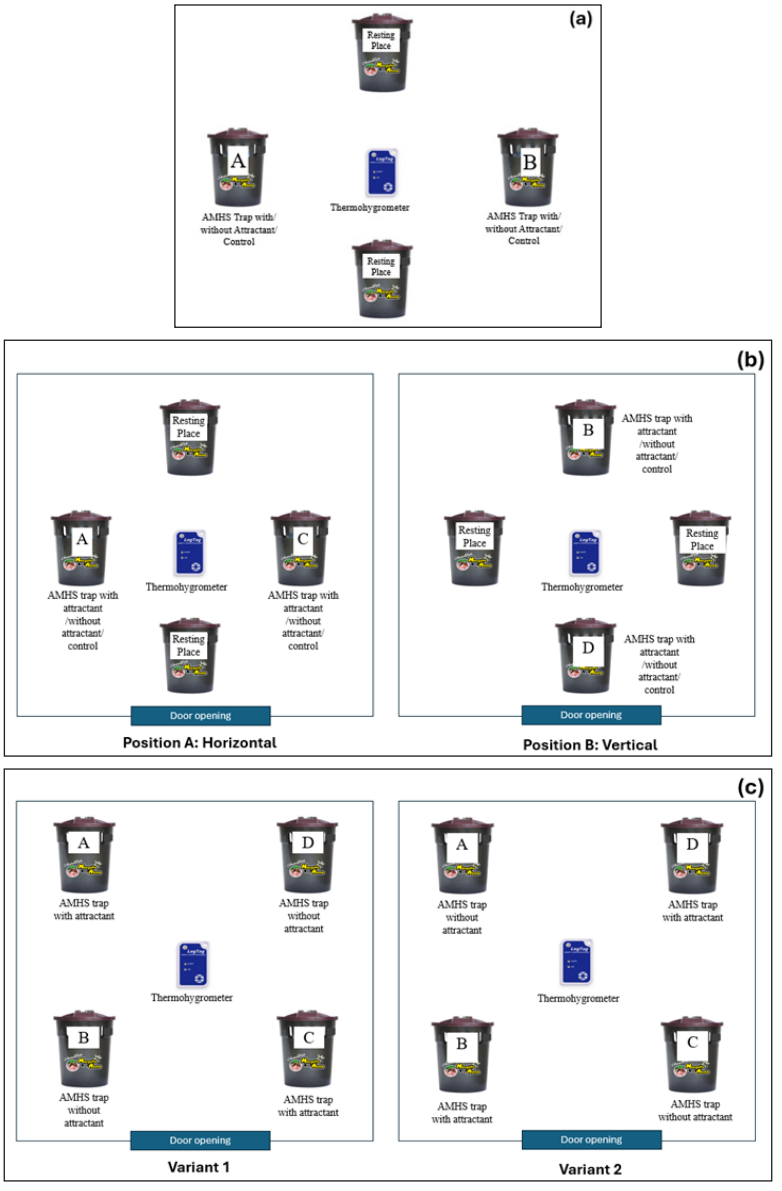

Figure 3. (a) The position of traps in the room chamber during Study 1. The study was run separately using three treatments: Aedes Mosquito Home System (AMHS) without an attractant, AMHS with an attractant, and control. (b) Treatment traps and resting traps with the thermohygrometer were placed in the middle of the room chamber for Study 2. The study was conducted separately using three treatments: AMHS without an attractant, AMHS with an attractant, and a control group in two positions. (c) The placement of treatment traps containing an attractant and without an attractant in two different variant settings in Study 3

Laboratory Condition

Temperature and humidity were carefully monitored using the Log Tag Analyzer®. Optimal conditions were maintained throughout the trials, with the temperature at around 27°C ± 2°C and the relative humidity between 60% and 80%. Additionally, a balanced 12-hour light-dark cycle (12L:12D) was implemented. These controlled conditions allowed for consistent and reliable observations and measurements during the study, minimising the influence of external factors.

Statistical Analysis

The data analysis for this study was performed utilising Statistical Package for the Social Sciences (SPSS) Version 25. All the data collected, including the mean number of eggs, HI, and ER, were subjected to the Shapiro-Wilk test to assess their distribution characteristics. All data were log-transformed using (ln(x+1)) to achieve normal distribution. Subsequently, a two-way analysis of variance (ANOVA) was independently conducted for the following data, as shown in Table 1. Then, the data were further analysed using Tukey's HSD multiple comparison test.

Table 1
List of data that were analysed using Two-way Analysis of Variance (ANOVA) in this chapter

Study	Species	Factor (s)		
		Dependent	**Fixed**	
1	*Aedes albopictus*	The mean number of eggs oviposited	Time	Treatment
		Hatching Index		
		Emergence Rate		
	Aedes aegypti	Emergence Rate		
2	*Aedes aegypti*	Hatching Index	Position	Treatment
		Emergence Rate		
3	*Aedes albopictus*	Hatching Index	Time	Variant_ Treatment
		Emergence Rate		
	Aedes aegypti	The mean number of eggs oviposited		
		Hatching Index		
		Emergence Rate		

Meanwhile, Welch's ANOVA was employed for analysis since some data did not meet homogeneity assumptions. The data underwent log transformation using (ln(x+1)) to achieve a normal distribution. Subsequently, ANOVA analyses that included Welch as an option in the statistics selection were conducted for this study. Following this, post-

hoc analysis using Games-Howell multiple comparisons was performed for datasets with more than two groups.

This study also employed independent *t*-tests for Study 2 to compare the mean numbers of eggs oviposited and the position of the trap. Initially, the data normality was confirmed using the Shapiro-Wilk test. Independent *t*-tests were then used to evaluate the influence of trap position on oviposition by gravid mosquitoes. These tests compared mean egg numbers in horizontal and vertical positions for *Ae. albopictus*.

RESULTS

Study 1: Effect of an Attractant on Female Mosquitoes' Oviposition

Effect of an Attractant on Female Mosquitoes' Oviposition, Hatching Index, and Emergence Rate in Aedes albopictus

Initially, the MHAQ with attractant had a higher mean number of eggs (2.17) compared to the MHAQ without attractant (0.42) and the control (0.75), as shown in Figure 4(a). However, this trend was inconsistent over subsequent checks, as depicted in Figure 4(a). This part uncovered the impact of the attractant in the MHAQ on *Ae. albopictus* oviposition and revealed no significant effect (Two-way ANOVA, F=0.16, df=2, p>0.05). This finding implies that the presence of an attractant has no discernible impact on oviposition in preference of *Ae. albopictus*.

Further analysis of the three-hour trials across six time points using two-way ANOVA also indicated no distinct trend in the mean number of eggs oviposited by *Ae. albopictus*. Showing that *Ae. albopictus* did not exhibit any time preferences for oviposition. The analysis highlighted that time did not significantly impact the results as the p-value was at the threshold and not less than 0.05, confirming these observations (Two-way ANOVA, F=2.29, df=5, p=0.05). Similarly, the treatments within each time point showed no difference (Two-way ANOVA, F=0.84, df=10, p>0.05). These findings collectively suggest that neither time nor treatment, individually or in combination, significantly influences the oviposition preference of *Ae. albopictus*.

The HI and ER data did not exceed 0.2% for all times and treatments. The highest recorded values were 0.19% for HI (observed during 60 minutes in MHAQ without attractant) and 0.08% for ER (noted during 120 minutes in the control). Most remaining data consistently recorded 0.00% for both HI and ER across all times and treatments, showing no variation.

Following these, we concluded that the presence of attractant in the MHAQ solution did not result in any significant differences in the HI and ER across different times (Two-way ANOVA, HI: F=0.53, df=5, p>0.05; ER: F=0.41, df=5, p>0.05), treatments (Two-way ANOVA, HI: F=0.47, df=2, p>0.05; ER: F=0.89, df=5, p>0.05), or when comparing

treatments within each time (Two-way ANOVA, HI: F=1.68, df=10, p>0.05; ER: F=1.07, df=10, p>0.05).

Effect of an Attractant on Female Mosquitoes' Oviposition, Hatching Index, and Emergence Rate in Aedes aegypti

Regarding treatment preferences alone, without considering time, *Ae. aegypti* demonstrated a significant inclination toward MHAQ with an attractant (28.65) compared to MHAQ without an attractant (5.29) (Welch's ANOVA F (2, 68.66) = 5.22, p = 0.01). However, this preference did not extend to the control (12.80). This shows that the presence of an attractant in MHAQ does affect the oviposition of *Ae. aegypti*.

When considering both timing and treatment, *Ae. aegypti* exhibited its highest mean oviposition rate at the 30-min mark, depositing an impressive 111.00 eggs in MHAQ with attractant, surpassing counts in both MHAQ without attractant (0.92) and the control (0.42) (Figure 4[b]). These results were significant according to Welch's ANOVA analysis (F [2,5.46] =7.48, p=0.03), but further checking due to homogenous violation with Games-Howell's Post-hoc Test showed no significance between the treatments (p>0.05). However, the preference of *Ae. aegypti* for MHAQ with an attractant (7.42) shifted to the control (37.92) at the 60-minute mark (Welch's ANOVA, F (2,9.01) =7.48, p=0.33) (Figure 4[b]). Subsequent observations revealed fluctuating preferences between MHAQ with the attractant and the control with no significant preference shown (Welch's ANOVA, p>0.05). Suggesting no preference time for the *Ae. aegypti* for ovipositing.

In this part, we researched the impact of treatment on the HI of *Ae. aegypti*. The HI of *Ae. aegypti* in MHAQ without attractant (0.10%) was significantly higher than in MHAQ with attractant (0.01%) and the control (0.04%) (Welch's ANOVA analysis; F (2, 51.23) = 3.69, p=0.03).

We also investigated the HI of *Ae. aegypti* over time in comparison to each treatment—the data for these metrics consistently registered values below 0.20%. Notably, the highest HI values were observed at 120 min, with a reading of only 0.19% in MHAQ without attractant, while the remaining data ranged from 0.00% to 0.17%. This pattern suggests that time does not exert a significant effect on the HI of *Ae. aegypti* in any treatments. This observation was further supported by Welch's ANOVA analysis for each treatment within each time, which indicated that time has no statistically significant impact on the HI of *Ae. aegypti* across all treatments (30 min: F (2, 6.67) = 1.54, p=0.28; 60 mins: F (2,7.78) = 0.64, p=0.56; 90 mins: F (2, 9.51) = 0.16, p=0.85; 120 mins: F (2, 7.48) = 0.77, p=0.50; 150 mins: F (2,8.89) = 0.29, p=0.76; 180 mins: F (2, 9.40) = 0.18, p=0.84).

The highest recorded ER in *Ae. aegypti* was 0.12% in MHAQ without attractant during the 120 min. It implies that approximately 99.88% of the larvae were inhibited from progressing into adult mosquitoes, indicating a notably low ER across all periods. Conversely, the lowest recorded ER was 0.00%, signifying that none of the hatched

larvae emerged as adults. Notably, there were no obvious fluctuations in the recorded ER values. This observation is reinforced by the result of Two-way ANOVA, which indicates that neither time nor treatment significantly influences the ER (Two-way ANOVA, ER: F=0.95, df=5, p>0.05), treatments (Two-way ANOVA, ER: F=2.72, df=2, p>0.05), nor when comparing treatments within each time (Two-way ANOVA, ER: F=0.68, df=10, p>0.05). It underscores the conclusion that neither time nor treatment or a combination of both exerts a substantial impact on the ER of *Ae. aegypti*.

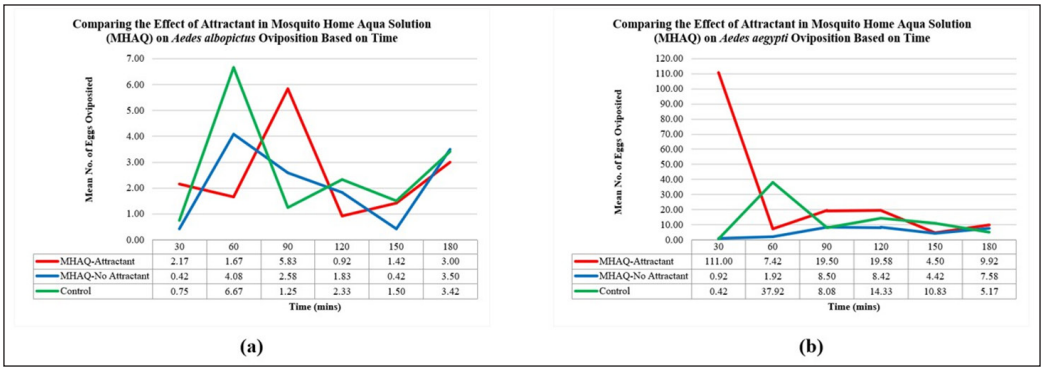

Figure 4. (a) The comparison of the oviposition of *Aedes albopictus* in all treatments at each time. (b) The comparison of the oviposition of *Aedes aegypti* in all treatments at each time

Study 2: Effect of Trap Position on Female Mosquitoes' Oviposition

Effect of the Position of Trap, Hatching Index, and Emergence Rate in Aedes albopictus

Within the control group, the *Ae. albopictus* egg oviposition data in the horizontal position were significantly higher than those in the vertical position (T-test, F=1.47, df=4, p=0.01). Specifically, the mean number of eggs oviposited in the horizontal position stood at 408, a significant contrast to the 105 recorded in the vertical position, as shown in Figure 5(a). This disparity indicates that the horizontal position yielded approximately four times as many eggs as the vertical position in control.

In the context of MHAQ with attractant, the mean number of eggs deposited in the horizontal position (96) was observed to be higher than that in the vertical position (55) (Figure 5[a]). However, despite this difference, no statistically significant variations were found in egg deposition between the horizontal and vertical positions within MHAQ with an attractant (t-test, F=3.43, df=4, p=0.22). Furthermore, there were no statistically significant differences in egg deposition between the vertical and horizontal positions in MHAQ with non-attractant (T-test, F=0.35, df=4, p=0.51). Thus, the oviposition of *Ae. albopictus* in MHAQ remains unaffected by suggesting a particular position, regardless of the presence or absence of an attractant.

In this part, we explored the impact of various treatments on the HI and ER of *Ae. albopictus*. Notably, *Ae. albopictus*'s HI in MHAQ without attractant (0.35%) significantly exceeded that in MHAQ with attractant (0.01%) and the control group (0.01%). The same situation occurred with the ER of *Ae. albopictus.* Welch ANOVA analyses confirmed the statistical significance of these differences for both HI (F [2, 8.76] = 11.63, p = 0.003) and ER (F (2, 8.96) = 17.23, p = 0.001). These findings suggest that attractant presence does not impact HI and ER in *Ae. Albopictus,* reducing these entomological parameters.

Subsequently, an investigation into the positional impact on the HI and ER of *Ae. albopictus* was undertaken. The recorded data spanned from 0.00% to 0.50% for HI and 0.00% to 0.24% for ER. In the MHAQ with attractant, both horizontal and vertical positions yielded identical HI results (0.01%) (Welch's Analysis, F (1, 4.00) = 0.54, p = 1.00). No significant difference was observed in terms of position for ER in the MHAQ with attractant, with the horizontal position marginally higher at approximately 0.01% compared to the vertical position (0.00%) (Welch's ANOVA, F (1, 2.63) = 0.310, p = 0.62). These findings indicate no observable contrast in the Hatching Index and Emergence Rate readings of *Ae. albopictus* when positioned vertically or horizontally in any treatment.

Effect of the Position of Trap, Hatching Index, and Emergence Rate in Aedes aegyptii

The data on the mean number of *Ae*. *aegypti* eggs oviposited in all treatments did not show a significant difference between both horizontal and vertical positions (t-test, p>0.05). As illustrated in Figure 5(b), although the non-attractant treatment exhibited a higher mean number of eggs collected at the vertical position (367) compared to the horizontal (94), this difference was not statistically significant (t-test, F=3.24, df=4, p= 0.28). It implies no inclination towards any specific position within the various treatments.

The positional orientation, treatment variations, and their combined influence on the HI and ER in *Ae. aegypti* were thoroughly examined. Results from the Two-way ANOVA revealed no significant effects on HI (Position: F=0.89, df=1, p>0.05; Treatment: F=1.07, df=2, p>0.05; Combination: F=0.15, df=2, p>0.05) or ER (Position: F=0.51, df=1, p>0.05; Treatment: F=2.04, df=2, p>0.05; Combination: F=0.16, df=2, p>0.05) based on position, treatment, or their combination.

The HI demonstrated a range from 0.01% to 0.15%, with the highest value recorded at 0.15% in the horizontal position within MHAQ without attractant. Similarly, the peak value for ER was observed in the horizontal position in the control group (0.13%), while the lowest was noted in the vertical position in MHAQ with an attractant (0.00%). In conclusion, neither the position, treatment type nor their interaction significantly influences the HI and ER in *Ae. aegypti*.

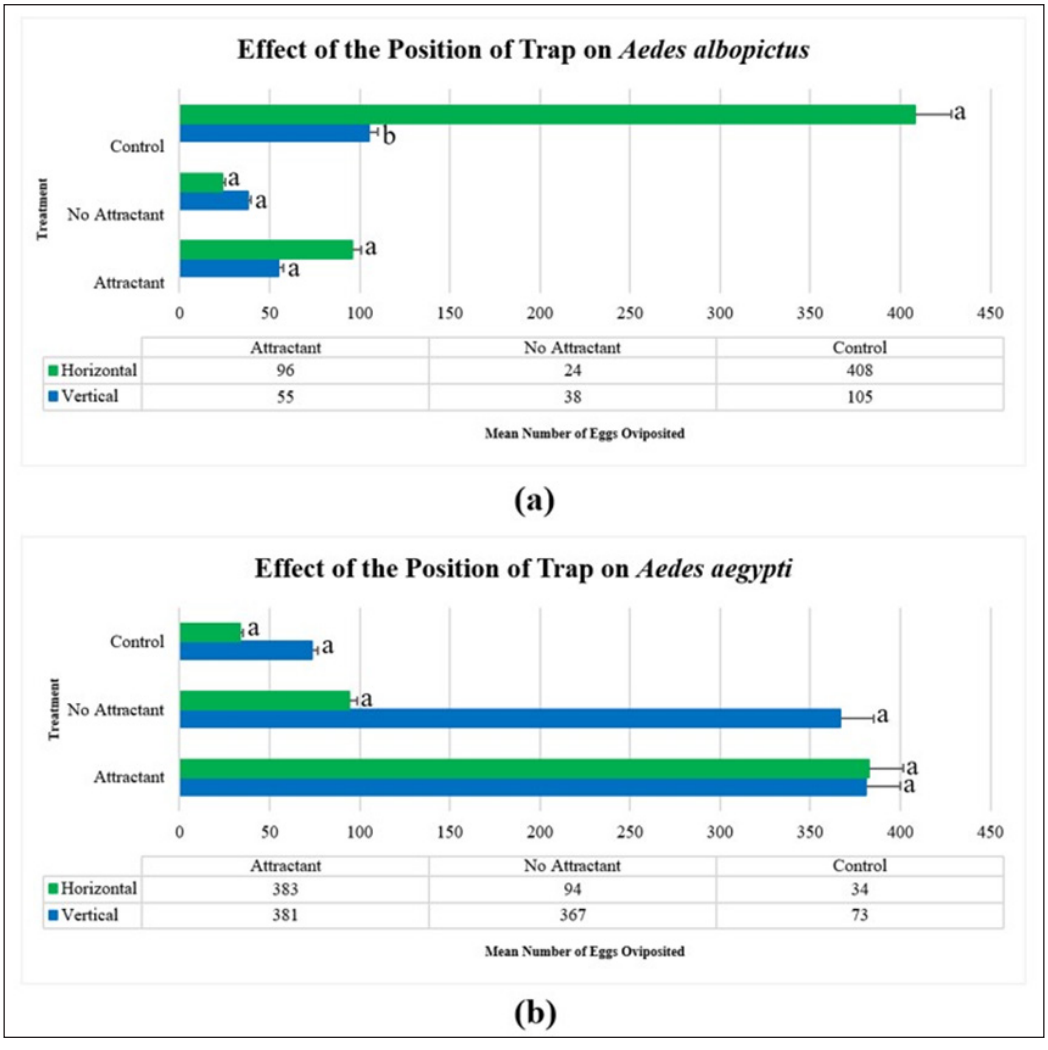

Figure 5. (a) The effects of trap position in each treatment on *Aedes albopictus* oviposition. (b) The effects of the position of the trap in each treatment on *Aedes aegypti*
Note. The same small letter shows no significant differences within the position of trap placement

Study 3: Oviposition Selection by Gravid Females

Oviposition Selection by Gravid Females, Hatching Index, and Emergence Rate in Aedes albopictuS

According to the graph in Figure 6(a), the mean number of eggs oviposited by *Ae. albopictus* in MHAQ with attractant was higher at all time points, except at 180 min, compared to MHAQ without attractant. This trend was observed for both Variant 1 (red line) and Variant 2 (red dashed line) in the attractant treatments versus Variant 1 (blue line) and Variant 2 (blue dashed line) in the non-attractant treatments. However, when we

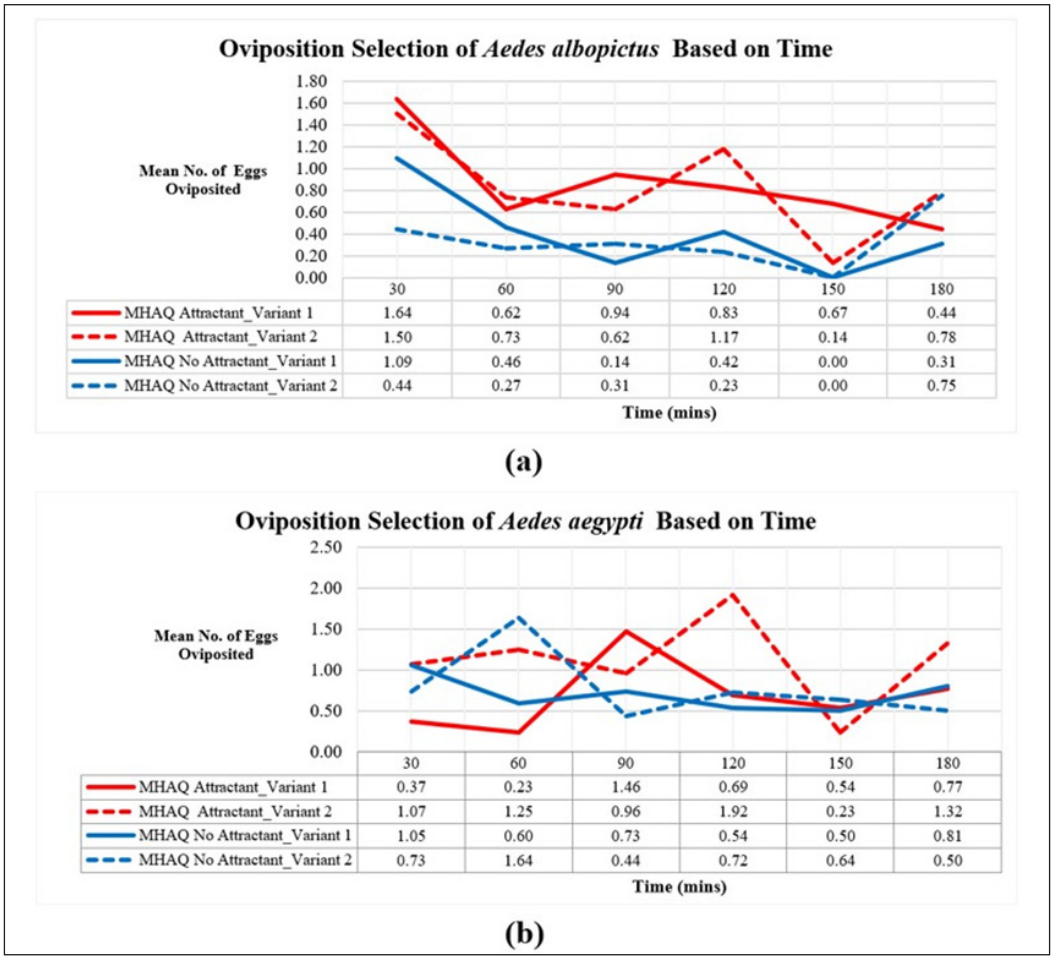

Figure 6. (a) Oviposition selection of *Aedes albopictus* based on time for MHAQ for different variants. (b) Oviposition selection of *Aedes aegypti* based on time on two variant types in MHAQ

differentiated according to the treatment and variant, there was no significant difference in the oviposition selection in *Ae. albopictus* as detailed in Table 2 (Welch ANOVA, F (3, 37.24) = 3.07, p>0.05). Suggesting there is no preference site for oviposition over the treatment and variant in *Ae. albopictus*.

However, when comparing the oviposition selection based on time, the mean number of eggs oviposited within the first 30 min was higher in comparison to the rest of the observation points (p<0.05). Specifically, the mean number of eggs laid within the first 30 min (1.17) was significantly greater than the count at 150 min (0.20) shown in Table 2. The Welch ANOVA supported these results (F (5, 30.42) = 3.36, p = 0.02). Thus, *Ae. albopictus* prefers ovipositing in the initial half-hour of trap introduction compared to the 150-min mark.

Table 2
Comparison of mean number of eggs oviposited by Aedes albopictus *using Welch's ANOVA analysis according to treatment and time in Study 3*

Treatment	**Mean no. of eggs oviposited**
MHAQ with attractant_Variant 1	0.86 [a]
MHAQ with attractant_Variant 2	0.83 [a]
MHAQ without attractant_Variant 1	0.40 [a]
MHAQ without attractant_Variant 2	0.33 [a]
Mean ± SE	0.60 ± 0.08
Time (min)	Mean no. of eggs oviposited
30	1.17 [ad]
60	0.52 [ac]
90	0.50 [ac]
120	0.66 [ac]
150	0.20 [bc]
180	0.57 [ac]
Mean ± SE	0.60 ± 0.08

Note.
*Welch's ANOVA was run for a mean number of eggs oviposited by *Ae. albopictus* with time as a factor. Then, the analysis was repeated with treatment as a factor
**The same small letter shows no significant differences within the treatment/time of the trap within a column. ***Significant result with $p<0.05$

The HI recorded ranged from 0.00% to the highest recorded at 0.14%, as indicated in Table 3. The peak Hatching Index (HI) occurred at 30 min in variant 1 with an attractant, 90 min in variant 2, and 120 min in variant 2 without an attractant. However, the HI in *Ae. albopictus* were not affected by the time (Two-way ANOVA, HI: $F=0.58$, $df=5$, $p>0.05$) treatment (Two-way ANOVA, HI: $F=0.70$, $df=3$, $p>0.05$) and the combination of both (Two-way ANOVA, HI: $F=1.31$, $df=15$, $p>0.05$).

Correspondingly, the ER of *Ae. albopictus* exhibited a similar range in line with the HI. These findings suggest that inhibition of *Ae. albopictus* adults emerged with the highest inhibition values, reaching 100.00% observed in almost all treatments and times, except for a few instances (six data points), as listed in Table 3. However, ER in *Ae. albopictus* were not affected by the time (ER: $F=0.56$, $df=5$, $p>0.05$), treatment (ER= $F=0.58$, $df=3$, $p>0.05$) and the combination of both (ER: $F=1.22$, $df=15$, $p>0.05$). In conclusion, the HI and ER of *Ae. albopictus* were unhindered by the time, and the treatment was introduced despite the presence of a high percentage of inhibitions.

Table 3
Comparison of the hatching index and emergence rate according to time and treatment for Aedes albopictus *and* Aedes aegypti *in Study 3*

Time (mins)	Treatment	Species			
		Aedes albopictus		Aedes. aegypti	
		Hatching index (%)	Emergence rate (%)	Hatching index (%)	Emergence rate (%)
30	Variant 1 with Attractant	0.14 [a]	0.09 [a]	0.00 [a]	0.00 [a]
	Variant 1 without Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
	Variant 2 with Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
	Variant 2 without Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
60	Variant 1 with Attractant	0.02 [a]	0.02 [a]	0.14 [a]	0.00 [a]
	Variant 1 without Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
	Variant 2 with Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
	Variant 2 without Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
90	Variant 1 with Attractant	0.00 [a]	0.00 [a]	0.14 [a]	0.00 [a]
	Variant 1 without Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
	Variant 2 with Attractant	0.14 [a]	0.13 [a]	0.00 [a]	0.00 [a]
	Variant 2 without Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
120	Variant 1 with Attractant	0.02 [a]	0.02 [a]	0.00 [a]	0.00 [a]
	Variant 1 without Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
	Variant 2 with Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
	Variant 2 without Attractant	0.14 [a]	0.14 [a]	0.00 [a]	0.00 [a]
150	Variant 1 with Attractant	0.00 [a]	0.00 [a]	0.07 [a]	**0.07** [a]
	Variant 1 without Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
	Variant 2 with Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
	Variant 2 without Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
180	Variant 1 with Attractant	0.05 [a]	0.05 [a]	0.00 [a]	0.00 [a]
	Variant 1 without Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
	Variant 2 with Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
	Variant 2 without Attractant	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]
Mean ± SE		0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.00 ± 0.00

Note:
*Two-way ANOVA was run separately for the hatching index and emergence rate with time and treatment as factors
**Analysis was run separately according to species of mosquitoes
***The same small letter shows no significant differences within the position of trap placement
***Significant result with $p<0.05$

Oviposition Selection by Gravid Females, Hatching Index, and Emergence Rate in Aedes aegypti

Figure 6(b) illustrates the oviposition selection behaviour of *Ae. aegypti*, revealing no specific trend throughout the conducted experiment. Upon the initial introduction of the traps, MHAQ with attractant-Variant 2 (1.07) exhibited the highest mean number of eggs oviposited, followed by MHAQ without attractant-Variant 1 (1.05), MHAQ with attractant-Variant 1 (0.37), and MHAQ without attractant-Variant 2 (0.73) (Table 4). Subsequently,

Table 4
Comparison of the mean number of eggs oviposited according to time and treatment for Aedes aegypti *in Study 3*

Time (min)	Treatment	Mean no. of eggs oviposited
30	Variant 1 with Attractant	0.37 [a]
	Variant 1 without Attractant	1.05 [a]
	Variant 2 with Attractant	1.07 [a]
	Variant 2 without Attractant	0.73 [a]
60	Variant 1 with Attractant	0.23 [a]
	Variant 1 without Attractant	0.60 [a]
	Variant 2 with Attractant	1.25 [a]
	Variant 2 without Attractant	1.64 [a]
90	Variant 1 with Attractant	1.46 [a]
	Variant 1 without Attractant	0.73 [a]
	Variant 2 with Attractant	0.96 [a]
	Variant 2 without Attractant	0.44 [a]
120	Variant 1 with Attractant	0.69 [a]
	Variant 1 without Attractant	0.54 [a]
	Variant 2 with Attractant	1.92 [a]
	Variant 2 without Attractant	0.72 [a]
150	Variant 1 with Attractant	0.54 [a]
	Variant 1 without Attractant	0.50 [a]
	Variant 2 with Attractant	0.23 [a]
	Variant 2 without Attractant	0.64 [a]
180	Variant 1 with Attractant	0.77 [a]
	Variant 1 without Attractant	0.81 [a]
	Variant 2 with Attractant	1.32 [a]
	Variant 2 without Attractant	0.50 [a]
Mean ± SE		0.82 ± 0.10

Note:
* Two-way ANOVA was run separately for the Hatching Index and Emergence Rate with time and treatment as factors
**The same small letter shows no significant differences within the time/treatment of the trap within a column
***Significant result with $p<0.05$

the highest mean number of oviposited eggs alternated between MHAQ without attractant-Variant 2, MHAQ with attractant-Variant 1, and MHAQ with attractant-Variant 1 in the following time intervals. Analysis of these data suggests that there is no specific preference for oviposition in *Ae. aegypti* concerning time (Two-way ANOVA, F=0.57, df=5, p>0.05), treatment (Two-way ANOVA, F=1.16, df=3, p>0.05), or treatment within each time (Two-way ANOVA, F=0.90, df=15, p>0.05. Consequently, we conclude that neither time, treatment, nor their combinations significantly affect the oviposition selection in *Ae. aegypti*.

The recorded range of HI spans from 0.00% to 0.14% in Variant 1, with an attractant at 60 min and 90 min (Table 3). These results indicate a range of inhibitions for the conversion of *Ae. aegypti* eggs to larvae varying from 100% to 99.86%. As evaluated through Two-way ANOVA, the time factor did not significantly influence the HI in *Ae. aegypti* (HI: F=0.64, df=5, p>0.05). Similarly, the treatment introduced also failed to yield a significant effect (HI: F=2.82, df=3, p=0.05), and the combination of both factors exhibited no substantial impact (HI: F=0.64, df=15, p>0.05).

Simultaneously, the ER of *Ae. aegypti* displayed a range from 0.00% to 0.07%, resulting in the inhibition of *Ae. aegypti* emergence as adults, ranging from 100% to 99.93%. Surprisingly, in *Ae. aegypti,* all larvae failed to reach adulthood except for variant 1, with attractant in 150 min at 0.07%, as listed in Table 3. Emergence Rate (ER) in *Ae. aegypti* showed no significant differences in times (ER: F=1.00, df=5, p>0.05), treatments (ER: F=1.00, df=3, p>0.05) and time x treatments (ER: F=1.00, df=15, p>0.05). In summary, the time and the administered treatment showed no significant impact on the HI and ER of *Ae. aegypti*.

DISCUSSION

Examining ovitrap efficacy is a well-established practice in numerous studies (Ritchie et al., 2014; Tawatsin et al., 2019; Withanage et al., 2020). This study specifically investigates the efficacy of the AMHS, employing the MHAQ-containing PPF. The findings indicate a significant preference for *Ae. aegypti* oviposition in the MHAQ with attractant compared to the MHAQ without attractant. However, this preference lacks statistical significance compared to the control, despite a higher oviposition rate in the MHAQ with attractant compared to other treatments. It unveils the specific preferences of *Ae. aegypti* towards the attractant, evident in the mean egg count of 28.65 eggs, almost six times more than that of MHAQ without attractant, which recorded 5.29 eggs. In contrast, *Ae. albopictus* did not exhibit any significant preference for oviposition in either treatment. It appears that MHAQ with attractant is less attractive to *Ae. albopictus*. These outcomes contribute valuable insights into species-specific attractiveness, which addresses a notable gap in prior laboratory evaluations (Mohd Ngesom et al., 2021; Yazan et al., 2020).

An attractant can be described as a substance or factor attracting mosquitoes toward a specific location, such as an AMHS trap (Mwingira et al., 2020). In this study, the manufacturer undisclosed the attractant used. Attractants strategically utilised the communication methods of either the same or different species, which involved using semiochemicals (El-Ghany, 2020). Pheromones are a distinctive category of semiochemicals that play a crucial role in linking communication with individuals of the same species (Rizvi et al., 2021). In the context of *Aedes* mosquitoes, an attractant which is a sex pheromone named heptacosane, has been demonstrated to enhance the sterile insect technique (Wang et al., 2023). Remarkably, several attractants have proven effective in ovitraps, including infusions of *Leucaena leucocephala* (Barreto et al., 2020; Ridha et al., 2020).

Due to the lower attractiveness of attractant in the product towards *Ae. albopictus* than *Ae. aegypti*, our results spark an interest in the manufacturer's focus on attracting *Ae. albopictus* over *Ae. aegypti* for MHAQ's future development (Lim Chee Hwa, personal communication 2023). Towards the MHAQ, several lures could be used to tackle the low level of attractiveness of *Ae. albopictus*. Studies have identified effective attractants for *Ae. albopictus*, including sodium chloride solution (0 to 2.0% dilution), lactic acid bacteria infusion, and a 2-Hydroxyethylcellulose-based hydrogel formulation (Friuli et al., 2022; Guo et al., 2022; Suria et al., 2022).

Interestingly, the rationale behind *Ae. aegypti's* preference for AMHS traps over *Ae. albopictus* may stem from a species-specific response to MHAQ concentration, as hypothesised in a study on volatile organic compounds (VOC) influence on both species' sensory perception (Hutcheson et al., 2022). Unlike *Ae. albopictus*, which can sense and perceive any concentration range of the VOCs, *Ae. aegypti* appears more restricted, favouring only a specific concentration range. Outside this concentration level, both heightened and reduced concentrations are not effectively sensed by *Ae. aegypti* (Hutcheson et al., 2022). Alternatively, *Ae. albopictus* required more days for egg maturity than *Ae. aegypti* (Tsunoda et al., 2020). In this study, both species were used for experimentation mostly after a 48-hour blood feeding session, shorter than the durations used in the experiment, which is 96 hours for *Ae. albopictus* and 72 hours for *Ae. aegypti*. Thus, the number of eggs laid by these species is affected due to the suspected incomplete egg maturation.

By comparing each treatment's position for both species, MHAQ with attractant indicated flexibility in room-size cage positioning for evaluating gravid females' responses. No significant differences were observed in the present study, which aligned with cautionary notes on position biases by Roque and Eiras (2008). Daily repositioning of ovitraps in laboratory studies is a common practice to minimise bias, as observed in studies involving *Culex* spp. and *Anopheles* spp. (Borel et al., 2021). Similarly, a prior AMHS laboratory study also took meticulous precautions by regularly altering the ovitrap's position (Mohd Ngesom et al., 2021).

Through repositioning, we confirmed that the position does not contribute to the attractiveness or deterrence of the AMHS trap as the bias has been minimised, as mentioned in Borel et al. (2021) and Eiras et al. (2021). It facilitates future users of AMHS traps to position the traps flexibly when creating floor plans for deployment. For example, users can place them alongside one side of the corridor or arrange them in pairs on both sides. Consequently, expenses can be minimised by reducing the number of traps used by planning the least number of traps that should be used as the trap can be placed adaptably without any concern on positioning. Economic efficiency is crucial when executing methods for mosquito control (Hustedt, 2020; WHO, 2012).

Building on this understanding, a previous laboratory study conducted in Brazil explored spatial orientation and found no significant difference in oviposition rates when gravid mosquitoes were introduced into four types of placements. However, a notable preference emerged when considering the vertical position, with a higher propensity for oviposition observed at point C compared to point D (Roque & Eiras, 2008). In a separate study, *Ae. aegypti* was revealed to distinctly favoured ovipositing in ovitrap placed in the corner as opposed to the central position, leading to the collection of more than 85.00% of eggs in the corner position (de Jesus et al., 2020).

In our study, the position of the traps at the horizontal or vertical position has less impact on the oviposition of both mosquito species, *Ae. albopictus* and *Ae. aegypti*. In the case of a favourable position in any treatment, more precautions will be needed for the subsequent study. One possible explanation for any favourable outcome could be the slightly higher temperature and humidity experienced during testing in the horizontal position, which has been proven to impact the oviposition of both *Ae. albopictus* and *Ae. aegypti* (Thongsripong et al., 2023). It only requires a one-degree Celsius rise, which can induce a more than fourfold increase in the oviposition of *Ae. aegypti*, while another study predicts a 50% or more rise with the same temperature increase (do Nascimento et al., 2022; Gimenez et al., 2020). These results were also supported by a field study conducted in Parana State, Brazil, which demonstrated that the rise in temperature has a significant impact on the oviposition rate of *Aedes* spp. (Souza et al., 2022).

Notably, when given a choice between two types of treatment selections, both *Aedes* species showed no preference over MHAQ with attractant or without attractant in terms of oviposition sites. Despite the presence of an attractant, the lack of significant differences in the mean number of eggs oviposited between the two treatments in both variants suggests that the attractant used in MHAQ may not be influential for oviposition. Thus, it opens the possibility for its elimination to reduce costs, aligning it with consumer preferences for lower-priced alternatives. Notably, farmers in Besur Village, Lamongan, Indonesia, have already shifted away from costlier chemical pesticides, opting for more economical biological pesticides (Afandhi, 2020). Another reason that could explain the lack of a subtle

effect of the attractant may be attributed to the colour of the AMHS trap itself, which is black. Research has shown that black-coloured ovitraps were preferred over red or other colours tested (Marin et al., 2020; Tsunoda et al., 2020). Besides, the cylindrical design of the AMHS, paired with the incorporation of OviTo linen, could potentially enhance its overall attractiveness. It was aligned with a study that showed the attractiveness of a tube-shaped ovitrap lined with a propagation towel has a higher oviposited egg in comparison to paddles or styrofoam pieces (Velo et al., 2016). However, more studies must be conducted to find a more attractive substance in MHAQ.

Another potential factor contributing to the absence of specific selection by *Aedes* mosquitoes when presented with two treatment selections is the competition among breeding sites. In Study 3, four oviposition traps were compared to two in Study 1 and Study 2 in each replicate. This increase resulted in a higher number of available oviposition sites, leading to heightened competition among the traps. The competition of breeding sites emerges as a potential concern, highlighted in several field studies (Brisco et al., 2023; de Resende et al., 2013; Moura et al., 2020).

In Study 3, *Ae. albopictus* exhibited a higher number of eggs laid in the traps at the early test time of 30 min. This situation might be attributed to a fading attraction to the breeding site over time. This observation aligns with research on a species of *Aedes* mosquito, indicating a heightened oviposition within the initial introduction to the breeding site followed by a subsequent decline. While there is no recent data depicting the interplay between the introduction time to the ovitrap and the mean number of eggs laid, previous studies only highlighted the correlation between the mean egg count and the ovitrap design, attractiveness, or efficacy of insecticides compared to water (McGaughey & Knight, 1967; Mohd Ngesom et al., 2021; Parker et al., 2017; Tawatsin et al., 2019).

A human entering the chamber during OviTo linen collection in Study 1 and Study 3 could impact mosquito oviposition behaviour, as it may disturb the mosquitoes. However, this method remains the most direct way to measure treatment efficiency over time. Potential disturbances include mosquitoes flying away or altering their flight patterns. For example, non-blood-fed female *Ae. aegypti* has been shown to fly more vertically in response to human presence (Poh et al., 2017). However, this concern is mitigated in our study, as the *Ae. aegypti* used were blood-fed. Meanwhile, a study has shown that *Ae. albopictus* mosquitoes aged 10–15 days are sensitive to human scent (Drago et al., 2021). Given that our study utilised mosquitoes aged six to eight days, this issue is irrelevant.

Lima-Camara et al. (2014) found *Ae. aegypti* and *Ae. albopictus* females exhibit reduced locomotion after insemination and blood-feeding compared to unmated, unfed females. Since our study used blood-fed and after-inseminated mosquitoes, their reduced locomotion further lessens the impact of human presence. Additionally, a study noted that blood-fed female *Ae. aegypti* mosquitoes have reduced sensitivity to human odours important for

host-seeking but increased sensitivity to odours that help locate egg oviposition sites (Chen et al., 2019). Thus, human presence is less concerning in our study, as blood-feeding may have already diminished their sensitivity to human odours.

Even though the impact of human presence seems minimal, several precautions were still taken to further minimise potential disturbances during chamber entry. A rapid entry and exit practice was employed, with the researcher quickly replacing the OviTo linen and conducting counts outside the chamber. Only one researcher, who handled all chamber entries for every replicate, wore full protective gear, including a long-sleeved lab coat, long pants, gloves, closed shoes, long socks, a face mask, and a fully covered head, with only the eyes and forehead exposed. The lab coat blends with the floor and is light in colour, making it less attractive to mosquitoes (Benz et al., 2024). This helped reduce both visual and olfactory attraction. Precautions were also taken to gently brush off any mosquitoes before exiting. Multiple replications were conducted to average out variability, as replication is crucial for ensuring scientific reliability (National Academies of Sciences Engineering and Medicine, 2019).

In the context of hatching and adult inhibition observed across all studies, the Mosquito Home Aqua Solution (MHAQ) with attractant notably impacted both *Ae. albopictus* and *Ae. aegypti*, with the highest recorded inhibition, reaching 100.00% for both species. This observation aligns with prior investigations on pyriproxyfen intervention, underscoring the significance of species-specific dosage requirements and asserting the cross-species efficacy of an insecticide effective against both *Ae. albopictus* and *Ae. aegypti* as well (Gómez et al., 2011). Moreover, these findings regarding the effectiveness of MHAQ-containing pyriproxyfen (PPF) in impeding adult emergence are supported by earlier research associated with the AMHS (Harburguer et al., 2016; Iyaloo et al., 2021; Mohd Ngesom et al., 2021; Yazan et al., 2020).

Another factor that reduces adults' hatching index and emergence rate is using OviTo linen in the traps. *Aedes* spp.'s inherent ability to hatch in water is a key element in the control traps (Prameswarie et al., 2023). The rapid 24-hour hatching capability and the possibility of pre-submersion hatching on OviTo linen, influenced by moisture, could explain the situation (Ninditya et al., 2020). *Aedes* spp. exhibit breeding adaptability in minimal water, but OviTo linen, despite retaining moisture, lacks sufficient volume of water for larvae survival, prompting premature hatching and subsequent mortality due to inadequate water volume (Dharmamuthuraja et al., 2023; Owolabi & Bagbe, 2019; Ratnasari et al., 2020).

Overall, this investigation accentuates the necessity for ongoing exploration and optimisation of attractants within the AMHS, especially for *Ae. albopictus*. It also emphasises the efficacy of the MHAQ with attractants in inhibiting the hatching and emergence of both species, providing pivotal insights essential for refining effective mosquito control strategies.

CONCLUSION

This study examines the effectiveness of the AMHS with MHAQ in attracting and controlling *Ae. albopictus* and *Ae. aegypti*. While MHAQ consistently entices *Ae. aegypti*, its performance with *Ae. albopictus* is suboptimal, prompting consideration of additional attractants. Simultaneous oviposition choices in the same replicates suggest that the current attractant in MHAQ may not influence oviposition, raising the possibility of cost-effective elimination. The positioning of the AMHS does not affect its attractiveness, indicating flexibility in deploying the trap. This research offers nuanced insights for optimising ovitrap efficacy in comprehensive mosquito control strategies.

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Review Article

Reproductive Biology of Several *Garcinia* Species of Agricultural Importance in Malaysia

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ABSTRACT

The *Garcinia* genus belongs to the Clusiaceae or Guttiferae family, comprising 350 species. *Garcinia* is a large genus, and it is widely distributed in tropical environments, especially in the Southeast Asia region. Despite their wide distribution in Malaysia, information on their reproductive biology is still lacking. This overview highlights the distribution and the reproductive system of several *Garcinia* species in Malaysia, which are *Garcinia mangostana* var. *mangostana*, *Garcinia celebica*, *Garcinia mangostana* var. *malaccensis*, *Garcinia prainiana*, *Garcinia cowa*, *Garcinia atroviridis* and *Garcinia parvifolia.* Apomixis, specifically agamospermy in *Garcinia* species, is widely acknowledged by previous research. Apomixis develops a distinctive mechanism in gametophytic and sporophytic types, and it is molecularly triggered either by hybridisation or polyploidisation. *G. mangostana* var. *mangostana* is classified under obligate apomict due to male sterility caused by the alteration of tapetum during pollen development. On the other hand, the occurrence of male trees, male fertility and pollen viability are the important features that consider *G. celebica, G. mangostana* var. *malaccensis*, *G. prainiana, G. cowa, G. atroviridis* and *G. parvifolia* to be facultative apomict. Besides, the seeds of *Garcinia* species are recalcitrant and possess low resistance to desiccation. The size and moisture content of the seeds can influence germination ability. The overall information of this review can help explore the life cycle of *Garcinia* trees to facilitate conservation programs along with the agricultural benefits.

Keywords: Botany, conservation biology, floral biology, *Garcinia* species, plant reproduction, reproductive biology

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INTRODUCTION

Garcinia species are mostly agamospermy, either facultative or obligate (Richards, 1990a). Scientifically, agamospermy is a form of apomixis in which seed is produced asexually rather than sexually (Ha et al., 1988). In the common condition of most angiosperms, the facultative apomixis plant undergoes both sexual and asexual fertilisation to produce embryos. According to Richard (1990b), most *Garcinia* species are facultative apomixis, including *Garcinia celebica*, *Garcinia mangostana* var. *malaccensis, Garcinia cowa* and *Garcinia atroviridis* with the occurrence of male trees. Another species, *Garcinia prainiana*, is a potential facultative apomict, proved through genetic variation analysis (Rohani et al., 2021). On the other hand, obligate apomixis undergoes adventive embryo development (Kant & Verma, 2012) that occurs asexually within the tissue of ovules. *G. mangostana* var. *mangostana* is an obligate apomict that potentially bears no morphological and genetic variation (Mansyah et al., 2010). It can be inferred that all of the offspring of *G. mangostana* var. *mangostana* are identical to their mother plant.

The reproductive biology of *Garcinia* species is another study area with significant research gaps. *Garcinia* species has ecological and economic importance, yet there is limited understanding of many aspects of its reproductive biology. Plant reproductive biology serves as primary knowledge to identify reproduction types and capabilities through a sexual and asexual mechanism. This understanding can improve plant conservation and agricultural technology in conventional and non-conventional breeding, such as genetic selection, mutation, somaclonal variations, genomic sequence-based application and physical maps (Ahmar et al., 2020), especially on the common cultivated and rare *Garcinia* species. Some *Garcinia* species are economically important in the agricultural sector, such as *G. mangostana* var. *mangostana*. In contrast, the others might be critically endangered due to the loss of biodiversity, especially *G. cowa*. The failure of conservation of the threatened plants in nature due to lack of regeneration success can be avoided by employing the knowledge of reproductive biology (Marbaniang et al*.,* 2018). The underutilised species, such as *G. celebica, G. atroviridis* and *G. parvifolia*, perhaps are important for commercial development and local uses (Khoo et al., 2010).

This review aims to provide the available research documentation on the reproductive biology of several *Garcinia* species that encompass morphologies of flowers and fruit, the occurrence of apomixis, pollen viability and seed germination ability. The overall information regarding the distribution and the reproductive system of *G. mangostana* var. *mangostana, G. celebica, G. mangostana* var. *malaccensis, G. prainiana*, *G. cowa, G. atroviridis* and *G. parvifolia* will be brought to light for identification of the reproductive capability for agricultural interest and plant conservation.

CULTIVATION OF *GARCINIA* SPECIES

As proposed by Yaacob and Tindal (1995), *G. mangostana* var. *mangostana* is native to Southeast Asia. Due to its pleasant taste, *G. mangostana* var. *mangostana* is a well-known tropical fruit that locals highly prefer. Malaysia, Indonesia, Thailand and the Philippines are the major cultivating countries of *G. mangostana* var. *mangostana. G. mangostana* var. *mangostana* is scattered in other tropical regions, namely Northern Australia, South America and Tropical Africa (Cruz, 2001). Furthermore, the growth and cultivation of *G. celebica* occur in Malaysia, Thailand, Vietnam, Cambodia, Borneo, Andaman, Nicobar Island, Kerala and Tamil Nadu, India (Lim, 2012b; Nazre, 2010; Nazre et al., 2018). *G. prainiana* is usually found in Malaysia, specifically in Pahang, Kelantan, Terengganu and Perak (Azuan et al*.,* 2015). The information on the cultivation areas of *G. mangostana* var. *malanccensis, G. cowa, G. atroviridis* and *G. parvifolia* is scarce.

ECOLOGY OF *GARCINIA* SPECIES

Plant reproductive traits are important in the correlation studies between ecological interactions and evolutionary change. The environmental diverseness in abiotic climate conditions highly regulates the extent of sexual dimorphism in the dioecious system (Puixeu et al., 2019). In addition, the ecological well-being of plants is affected by climate change (Pareek et al., 2020) and soil (Bitew & Alemayehu, 2017). The breeders need to consider these factors for cultivation purposes in the agriculture sector and plant conservation. *Garcinia* species are commonly distributed in lowland tropical areas, and most of the species occur in a particular region, especially in Southeast Asia (Sweeney, 2008). Table 1 shows the overall ecological and cultivation description of *G. mangostana* var. *mangostana, G. celebica, G. mangostana* var. *malaccensis, G. prainiana, G. cowa, G. atrovirdis* and *G. parvifolia*.

A prolonged drought followed by rain in July-August led to increased leaf flushing rather than flowering, limiting fruit production of *G. mangostana* var. *mangostana* in Thailand (Apiratikorn et al., 2012). Unfavourable conditions also will cause slow growth and the contamination of yellow latex on fruit. According to Mansyah et al. (2003), heavy rainfall and higher relative humidity will lead to more yellow latex within the fruit's endocarp. Moreover, the level of yellow latex is also affected by the calcium concentration in the soil (Martias et al., 2021). According to Lim (2012a), *G. mangostana* var. *mangostana* grows better on organic-rich soils such as sandy loams and moderately lateritic and volcanic soils with proper drain conditions.

Garcinia celebica is known as seashore mangosteen or “beruas” in Malaysia. The name of seashore mangosteen is based on its tropical distribution from the seaside region and morphological resemblances with *G. mangostana* var. *mangostana* (Nazre, 2010). *Garcinia celebica* is native to Malaysia, Cambodia, Thailand, and Vietnam (Lim, 2012b)

and has largely spread from coastal to highland regions in tropical countries (Nazre, 2018). Due to its nature, *G. celebica* can adapt to sandy, rocky and acid clay soils, heavy rainfall, and drought environments and is salt-tolerant (Lim, 2012b). *Garcinia mangostana* var. *malaccensis* is regarded as one of the possible ancestors of *G. mangostana* var. *mangostana* (Richards, 1990b). *Garcinia mangostana* var. *malaccensis* is native to Peninsular Malaysia, Sumatra and Brunei and is found in lowland and highland tropical forests (Lim, 2012c; Nazre et al., 2018). *Garcinia mangostana* var. *malaccensis* is well-adapted to organic-rich soils and has a tropical climate with hot and wet conditions (Lim, 2012c). The study of the reproductive biology of *G. celebica* and *G. mangostana var. malaccensis* is essential due to their significant role in determining the ancestor of *G. mangostana* var. *mangostana*.

Garcinia prainiana (cerapu), or button mangosteen, is believed to be indigenous to Peninsular Malaysia and Thailand (Mohd Khairuddin et al., 2018). Besides, *G. prainiana* and *G. mangostana* var. *mangostana* share a similar condition for growth, which is in lowland and hill areas where it needs well-distributed rainfall, warm temperature, well-drained and porous soils and fairly acid clay loams that are rich in organic matter (Lim, 2012d). The current status of *G. prainiana* under the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (April 2024) is registered as lower risk. *G. prainiana* is an underutilised species (Azuan et al., 2015), resulting in limited agricultural yield and research studies. The potential of underutilised species remains largely untapped due to limited scientific understanding, especially concerning their reproductive biology. The reproductive biological study of underutilised species encompasses their potential and incorporates them into mainstream agriculture and conservation.

Garcinia cowa Roxb. known as kandis in Malaysia, is natively from Southwest India, East India, Nepal, Andaman and Nicobar Island, Yunnan in China, as well as Southeast Asia including Myanmar, Thailand, Laos, Vietnam, and Northern Peninsular Malaysia (Lim, 2012e). Like other *Garcinia* species, *G. cowa* commonly occurs in tropical evergreen, sand, and dry deciduous forests, especially in Thailand. The current status of *G. cowa* corresponds to *G. prainiana* under the IUCN Red List of Threatened Species (April 2024). Based on the assessment by Deepu and Geethakumary (2020), *G. cowa* is listed as one of the endangered species mainly caused by deforestation and habitat destruction; for endangered species like *G. cowa*, effective conservation strategies might require a deep understanding of its reproductive biology.

Garcinia atroviridis Griff. ex T. Anderson is locally known as "asam gelugor" in Malaysia. *G. atroviridis* is an indigenous species from Peninsular Malaysia, Thailand, Myanmar, and India, and it is found in humid weather in lowland and highland rainfall regions (Lim, 2012f). *G. atroviridis* is widely distributed and essential to Indonesia's forest (Bayu, Lestami et al., 2018). *G. parvifolia* (Miq.) is native to tropical regions: Thailand, Malaysia, Sumatra, Java, Brunei, Kalimantan, Sulawesi, Maluku Islands and New Guinea

(Lim, 2012g). *G. parvifolia* or cherry mangosteen is also locally known as "asam kandis" or "takob akob", especially in Sabah and "asam kundong" in Sarawak (Hassan et al., 2013). In the agroecological part, the distribution of wild *G. parvifolia* is favourable toward humid tropical areas such as peat swamp and lowland forests through submontane with hillsides and ridges as well as well-drained, alluvial sites and along rivers (Lim, 2012g; Hassan et al., 2013). Both *G. atroviridis* and *G. parvifolia* have agricultural benefits. It is necessary to acknowledge the reproductive biology of these *Garcinia* species to enhance productivity, sustainability, and resilience in the agricultural system.

REPRODUCTIVE BIOLOGY OF *GARCINIA* SPECIES

The reproduction process is an important phase in the life cycle of an organism, and it also can be a fundamental aspect of the evolutionary system (Li et al., 2018). The sexual reproduction cycle in most angiosperm involves the development of an embryo (embryogenesis) and endosperm as an outcome of the fusion of a male gamete (sperm) and a female gamete (egg cell) together with the combination of two polar nuclei and a male gamete in the embryo sac respectively (Ao, 2020).

Apomixis is closely related to sexual reproduction; however, the embryo undergoes (1) the exclusion of meiosis throughout embryo sac development (apomeiosis), (2) the development of the embryo out of an unfertilised egg cell (parthenogenesis) and (3) the development of endosperm either through fertilisation or non-fertilisation (Hand & Koltunow, 2014). The change of epigenetic regulatory pathway determines Apomixis as an outcome of polyploidisation and hybridisation that can generate unreduced female gametophytes (Hojsgaard & Hörandl, 2018) as most of the natural apomictic individuals are polyploids and hybrids (Barke et al., 2018). Polyploidy disintegrates genetic self-incompatibility (SI) by initiating a reproductive block on the diploid progenitor, and hence, pseudogamous apomictic plants can be self-fertile (Hojsgaard & Hörandl, 2018).

Apomixis is classified into two types: (1) sporophytic and (2) gametophytic (apospory and diplospory). The reproduction in sporophytic apomixis occurs concurrently with normal sexual reproduction (Figure 1). However, the adventive embryo emerges somatically from the integumental or nucellus tissue through mitotic division. As a result, multiple embryos or polyembryony in sporophytic apomixis are developed. The formation of polyembryony in sporophytic apomixis is prevalent in Rutaceae, such as *Citrus* species (Yuantao et al., 2021). Brukhin (2017) stated that the maturation of diploid adventive embryos is competitive, and it relies on sexually-originated endosperm to gain nutrients by repressing sexual embryos. Occasionally, the endosperm can autonomously evolve independently of fertilisation. This condition is termed autonomous apomixis (Cardoso et al., 2018).

Meanwhile, in gametophytic apomixis, both apospory and diplospory share a similar pathway where the unreduced embryo sac is developed via mitosis with suppression

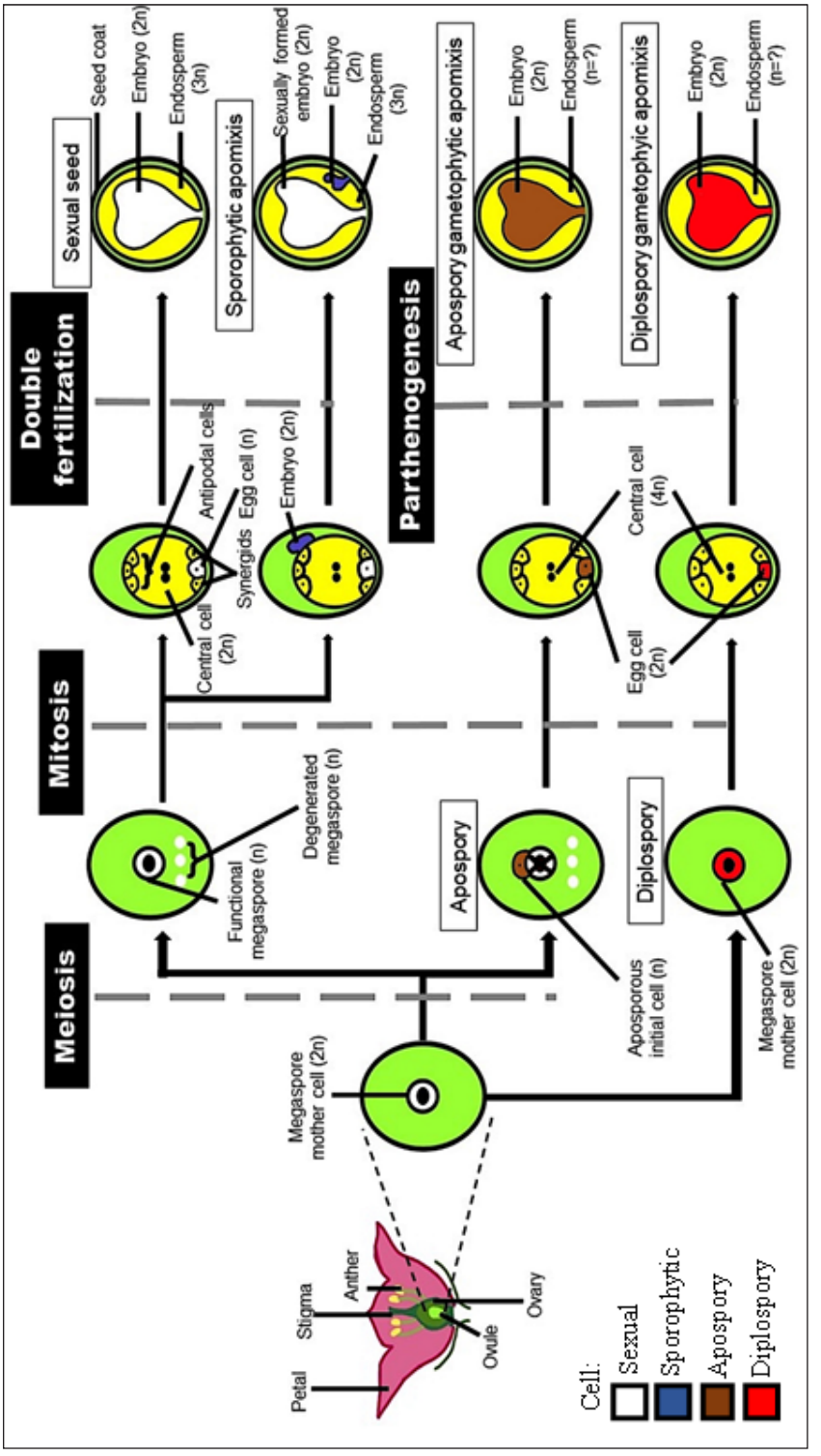

Figure 1. Process of sexual and apomixis seed development. The illustration is based on the description from Hand and Koltunow (2014) with slight modifications. A dashed line represents the processes and pathways. Colours represent cells that develop the embryo: sexual (white), sporophytic (blue), apospory (brown), and diplospory (red). The ploidy level of cells (n) is displayed as a question mark

of meiosis (apomeiosis) in the absence of double fertilisation and later undergoes parthenogenesis to form a diploid embryo (Hand & Koltunow, 2014). Parthenogenesis occurs synergically with apomeiosis and autonomous endosperm formation (absent or present of central cell fertilisation) (Vijverberg et al*.,* 2019). Functional megaspore mother cells from meiosis division in apospory degenerate, leaving only diploid somatic apospory initial cells to further develop mitotically into the embryo (Figure 1). Meanwhile, the megaspore mother cell in the diplospory develops into the embryo sac through mitotic division during the whole process. The development of endosperm in gametophyte apomixis differs in the ploidy level of cells (n) with or without the occurrence of fertilisation.

Obligate Apomixis

Generally, obligate apomixis occurs without the fusion of female and male gametes, which might be caused by altering certain components during the reproductive phase. The reproduction system of *G. mangostana* var. *mangostana* is categorised under obligate apomixis, as confirmed by Lim (1984) and Richard (1990a), due to the absence of male trees (Whitmore, 1973). Male *G. mangostana* var. *mangostana* was last spotted by Idris and Rukayah (1987), and it is uncommon due to the cultural actions of the locals that chop down the trees because they believe male trees are non-beneficial (Nazre, 2014). Thus, the progenies of apomictic *G. mangostana* var. *mangostana* should be genetically identical to the mother plant (Koltunow et al., 1995) because no depletion of chromosome number and egg fertilisation occurred to form an embryo (den Nijs & van Dijk, 1993). It also results in a lack of genetic variability. However, in some cases, a morphological variation of *G. mangostana* var. *mangostana* happens depending on its locality. In addition, the mechanism of male flowers in terms of morphology and pollen development of *G. mangostana* var. *mangostana* has been described (Awachare & Upreti, 2019; Nazre, 2014; Sutthinon et al., 2019).

Reproductive System of Garcinia mangostana var. mangostana: *Flowers, Fruits, and Seeds*

A large flower of *G. mangostana* var. *mangostana* consists of four fleshy petals, yellowish green in colour and pinkish or reddish toward the margin, and it also bears infertile staminodes (Awachare & Upreti, 2019; Lim, 2012a; Sulassih et al., 2013; Yuniastuti, 2010). In the report of Nazre et al. (2018), the calyx of *G. mangostana* var. *mangostana* is thicker than other *Garcinia* species with ovate to obovate or concave in shape (Table 2). The female flowers consist of a large sessile or subsessile discoid stigma (Richards, 1990b; Te-chato, 2007) and blunt star-shaped or broadly ovoid ovary with 4–8 chambers, and the inflorescence is usually in triads (Nazre et al., 2018; Yuniastuti, 2010). A male flower has a fungiform pistillode with basal encircled by 4-angled stamen bundles (Nazre, 2014).

Most wild species have a long flowering cycle, and the flowering period takes place more than once a year. In the case of *G. mangostana* var. *mangostana*, the flowering period occurs in March–April and July–September (Te-chato, 2007). The flowering begins after the vegetative growth flushes depending on the environmental states, the total flushes and post-dry weather (Awachare & Upreti, 2019). As Yuniastuti (2010) reported, the average time taken for a flower of *G. mangostana* var. *mangostana* (Jogorogo, East Java province, Indonesia) to bloom perfectly was 24–25 days. The flower has a perfectly functional female part but has male sterility due to male reproductive degradation (Awachare & Upreti, 2019; Sobir & Poerwanto, 2007; Suthhinon et al., 2019).

During the initial stages of flowering in *G. mangostana* var. *mangostana*, the development of stamens is stunted and aborted (Awachare & Upreti, 2019). It has been discussed that the male sterility of *G. mangostana* var. *mangostana* experienced pollen abortion during micro gametogenesis due to alteration of tapetum (Shi et al., 2010), which resulted in microspore degradation and failed formation of male gamete (Suthinon et al*.,* 2019). Specifically, cellular deprivation due to a slight accumulation of starch on disintegrated microspore and degenerated microspore mother cell has caused early cell death of tapetum with acutely shape alteration of mitochondria (Suthinon et al., 2018a; Suthinon et al*.,* 2019). It was stated that the microspore mother cell in *G. mangostana* var. *mangostana* contained insufficient Golgi apparatus and vesicles together with unusual callose wall build-up that resulted in unsystematic cytoplasm (Sutthinon et al., 2019). Furthermore, microspore degeneration occurs at all stages of development (Suthhinon et al., 2019) but precisely during meiosis at stage VI (Yapwattanaphun et al*.,* 2008). The microspore tetrads alongside total callose depositions were infrequently detectable (Sutthinon et al*.,* 2018b). Few persistent free microspores initiated from meiosis were noticeable, with an unusual shape during the late microspore development stage but completely degenerated later during vacuolate and bicellular stages (Nuanjunkong & Meesawat, 2010). Besides, the pollen viability (0.1-1%) was extremely low (Te-chato, 2007), which strongly justifies the male sterility theory on *G. mangostana* var. *mangostana*.

The fruits are globose in shape, smooth surface and large, thick with dark purple or red pericarp, which contains a sweet and sour taste, and fleshy and snowy white aril (Nazre, 2014; Sulassih et al., 2013; Techato, 2007; Yuniastuti, 2010). Yuniastuti (2010) mentioned that the fruits also contain more than one light brown seed and are ellipsoid in shape. *G. mangostana* var. *mangostana* also displays certain variations in fruits. Based on the report from Mulyono et al. (2021), six varieties of *G. mangostana* var. *mangostana* in Indonesia named “Kaligesing”, “Puspahiyang”, “Wanayasa”, “Ratu Kamang”, “Ratu Tembilahan”, and “Lingsar” exhibited different fruit traits in terms of size, shape, weight and flavour. It was concluded that all varieties may adapt differently in the same environmental state. The differences in the morphological features of fruit in *G. mangostana* var. *mangostana*

(taper and round flat downside in shape), aril (cream-coloured and pure-white), seed (ellipse, oval, long and irregular in shape) were reported in Langkat region, North Sumatra, Indonesia (Syahputra et al., 2021). Meanwhile, in the Philippines, the fruit characteristics of *G. mangostana* var. *mangostana*, including shape, size, pericarp thickness and ripened taste between two territories, namely Quezon in Luzon Island and Davao-Zamboanga in Mindanao Island, were highly comparable to each other. These characteristics also nearly resembled *G. mangostana* var. *malaccensis* from different geological areas (Berame et al*.,* 2020).

The fruits of *G. mangostana* var. *mangostana* are likely to mature in the absence of crossbreeding (Richards, 1990a; Te-chato, 2007). The time for *G. mangostana* var. *mangostana* to begin fruiting is relatively slow. Fruiting was rare before the age of 12 (Lim, 1984), but it started to fruit regularly at 18 to 20 (Richards, 1990b). Te-chato (2007) also reported that developing unfertilised ovaries into mature fruits took 4 to 6 months. The fruit of *G. mangostana* var. *mangostana* also has a few locules that formed completely developed ovules with liquid endosperm residuals consisting of plant growth hormones (auxin, cytokinin, gibberellin, abscisic acid and jasmonic acid), while the remaining locules contained a deflated ovule that will cease to growth (Yapwattanaphun et al., 2014). Besides, the fertility of apomictic seeds of *G. mangostana* var. *mangostana* is sustainable, as Yuniastuti claims (2010). The apomictic seeds appear to be polyembryony (Suhendra & Mustamu, 2018), which means multiple seedlings can emerge from one seed.

A long interval of seed dormancy before germination is one of the common traits in the agamospermy plant (Ha et al., 1988). However, in the report of Normah et al. (2016), the mean germination time (MGT) in *G. mangostana* var. *mangostana* was reported to be 24 days along with *G. celebica* (22.2 days), *G. atroviridis* (25 days) and *G. prainiana* (47 days). A short germination time in *G. mangostana* var. *mangostana* is likely caused by the high level of moisture content that could accelerate the germination ability even though the seeds have short-term viability (Oliveira & Valio, 1992). The moisture content (61.12% of fresh weight) in the larger seed (length:width=1.9 ± 0.03 cm: 1.3 ± 0.03 cm) of *G. mangostana* var. *mangostana* was the highest; however, due to desiccation, the moisture content constantly decreased beyond 30% after 72 hours which dropped its germinability drastically (Normah et al., 2016). Besides, the seeds from *Garcinia* species, especially *G. mangostana* var. *mangostana*, are recalcitrant (Yuniastuti, 2010), which means the seeds are susceptible to losing viability during cold storage together with low survivability on desiccation (Normah et al., 2016).

Seed sizes and nutrient reservation also play a role in seed germination. The variation in seed size possesses distinct levels of starch and energy reservation that could enhance germination expression and primary development of seedlings (Steiner et al., 2019)—in the case of *Garcinia* species, *G. mangostana* var. *mangostana, G. celebica, G. atroviridis* and

G. prainiana similarly contained a large amount of lipid and calcium oxalate despite being different in sizes and moisture content, except for the high level of starch which was found in *G. mangostana* var. *mangostana* only (Normah et al., 2016). The high accumulation of energy metabolites could increase *G. mangostana* var. *mangostana* germinability. As stated by Goh et al. (2019), the seeds can germinate easily upon imbibition along with the optimum level of seed metabolism, unlike other *Garcinia* species that need a period of dormancy breaking, such as *G. cowa* (Liu et al., 2005).

Facultative Apomixis

Facultative apomixis is very common in an angiosperm plant where both apomixis and sexual reproduction are concurrent in the presence of male plants. The level of residual sexuality in facultative apomicts is highly varied, which enhances the heterogeneity of genotypic and phenotypic (Majeský et al., 2017). As mentioned before, *G. celebica, G. mangostana* var. *malaccensis, G. prainiana*, *G. cowa, G. atroviridis* and *G. parvifolia* are considered facultative apomixis based on the reproductive system reported by previous studies.

Reproductive System of Garcinia celebica*: Flowers, Fruits and Seeds*

The female flowers of *G. celebica* are solitary, axillary, borne singly, lack staminode and yellowish-green petals (Sulassih et al., 2013), with a large fungiform stigma and globose or ovoid ovary (Table 2). The male flowers are axillary in fascicle, slightly smaller, sessile and borne vertically in clusters, thinly coriaceous sepals, usually aromatic, cream in colour with numerous 4-lobed stamens connected at the base of pistillode on top of the petals (Nazre et al., 2018; Richards, 1990c).

Unlike *G. mangostana* var. *mangostana*, *G. celebica* is considered a facultative apomixis when it bears fertile female and male flowers. As reported by Richards (1990c), female inflorescence buds produce one or two flowers and reach anthesis six days following bud initiation, whereas male inflorescence developed earlier by producing three to seven flowers that open consecutively over four days, followed by pollen release on two straight mornings. In addition, the flowering period of *G. celebica* is longer, occurring from January to June (Richard, 1990c). Besides, the increase in chances for sexual reproduction in *G. celebica* is likely due to a high number of male flowers compared to female flowers (Nazre et al., 2018).

In a previous study reported by Suthhinon et al. (2019), during an anthesis phase, the complete flower of *G. celebica* gives rise to a standard male gametophyte with normal development of microspore mother cell containing packed cytoplasm and plentiful Golgi apparatus as well as vesicles that lead to the effective pollen yield. Unlike the premature cell death of tapetum that occurred in *G. mangostana* var. *mangostana*, the degradation of

tapetum for *G. celebica* occurred after meiosis II, which is parallel with the total enclose of callose wall surrounding microspore tetrads (Sutthinon et al., 2018b). A normal starch build-up occurs primarily at the microspore cell stage and reaches its highest peak during the unicellular microspore phase (Sutthinon et al., 2018a). It is consistent with the outcomes where *G. celebica* provided 68% viability and 68% germination through tetrazolium test (TTC) assay and in vitro germination (Sutthinon et al., 2018b). Pollen viability and development can justify the functionality of *G. celebica* as a perfect and fertile male plant.

The fruits of *G. celebica* have thin pericarp, deep red when matured, sub-globose to ellipsoid, and smooth surface with creamy white and sour aril (Sulassih et al., 2013) (Table 3). The recalcitrant seeds of *G. celebica* were found to be the largest in terms of seed length (2.5 ± 0.05 cm) and seed width (1.4 ± 0.04 cm) and obtained the second-highest moisture content (51.52% of fresh weight) from the study conducted by Normah et al. (2016). The seed size and moisture content could affect the percentage of seed germination. The higher rate of germination is comparable to the larger seed size (Yousif, 2010); a similar case happened in *G. mangostana* var. *mangostana.* The bigger seeds of *G. celebica* reserved relatively 20%–60% moisture content at 96 hours desiccation with 95.6% germination, which was the highest, followed by *G. mangostana* var. *mangostana* (75.6%)*, G. atroviridis* (57.3%) and *G. prainiana* (48.9%) (Normah et al*.,* 2016).

Reproductive System of Garcinia mangostana var. malaccensis*: Flowers and Fruits*

The male flowers of *G. mangostana* var. *malaccensis* have pinkish-red petals, pistillode fungiform but sometimes can be absent, and long and slender stamen is slightly 4-angled or comical-cylindrical, whereas the female flower has a corrugated surface stigma (Nazre, 2014; Nazre et al., 2018) (Table 2). The fruits are reddish pink to dark purple, ovoid to globose, corrugated surface with snowy white and sour taste of aril (Sulassih et al., 2013; Nazre, 2014) (Table 3). In addition, the size of its mature fruit is smaller than *G. mangostana* var. *mangostana* but similar to *G. celebica* (Taher et al*.,* 2012). There is no data concerning the specific details of pollen viability and *G. mangostana* var. *malaccensis* seed germination. According to Nazre (2014), the previous studies of *G. mangostana* var. *malaccensis* done by Ha et al. (1988) and Richards (1990a) were not properly documented due to species misidentification.

Reproductive System of Garcinia prainiana*: Flowers, Fruits, and Seeds*

Garcinia prainiana is regarded as a dioecious plant whereby the male and female flowers are situated on different plants, as stated by Rohani et al. (2021). The flowers of *G. prainiana* are axillary in dense clusters, consisting of five rounded sepals with green to pink in colour and five rounded petals with rose pink to yellow; male flowers have compacted stamens with yellow anthers, whereas female flowers are large and no stamens were found (Azuan

et al., 2015; Rohani et al., 2021) (Table 2). Like *G. celebica*, *G. prainiana* exhibits viable pollen from the male plants. To justify this, Rohani et al. (2021) have revealed the high pollen viability (77.6 ± 9.68%) and germination (60.95 ± 15.87%) of *G. prainiana.* Hence, it shows that *G. prainiana* is a facultative apomictic with both functional male and female plants with well-evolved ovules (Rohani et al., 2021). In addition, most of the sexual pollination in plants will rely on biotic and abiotic approaches to ensure the proper dispersal of pollen. A group of insects were found on the male flower of *G. prainiana*, assuming that the pollination probably occurred between male and female plants (Rohani et al., 2021).

The fruits of *G. prainiana* are yellowish-orange in colour, rounded but rather flattened soft and thin rind; the pulp is orange in colour, 4–7 segmented, and acidic sweet taste (Azuan et al., 2015; Mohd Khairuddin et al., 2018) (Table 3). For seed development, based on the study by Normah et al. (2016), *G. prainiana* contained a smaller size (length:width= 1.1 ± 0.02 cm:0.9 ± 0.01 cm) and thickest testa (147.35 ± 1.24 μm) with 47 days mean germination time which was the slowest compared to *G. mangostana* var. *mangostana, G. celebica* and *G. atroviridis* (22 to 25 days). The slow germination of *G. prainiana* is probably due to the test itself. Testa in higher plants protects the embryo against biotic and abiotic conditions during seed storage by limiting water permeability or mechanically restricting the radicle protrusion (Debeaujon et al., 2000).

Reproductive System of Garcinia cowa: *Flowers, Fruits, and Seeds*

The flowering period of *G. cowa* usually occurs from July until September, similar to *G. mangostana* var. *mangostana* (Te-chato, 2007). *G. cowa* also develops distinctive features for flower morphology. It bears male flowers consisting of four yellow petals with clustered stamens, with four unified fascicles developing an innermost capitate quadratic clump of 40 to 50 anthers. In contrast, female flowers are solitary, axillary with fused staminodes in the lower half, and the surrounding ovary base consists of an ovoid ovary and four to eight radiate ridged and papillate stigma (Te-chato, 2007; Lim, 2012e) (Table 2). As reported by Richards (1990a), *G. cowa* is likely to be facultative apomixis due to the occurrence of the male plant. Besides, pollen of *G. cowa* exhibited 96%–100% of viability (Te-chato, 2007), which strongly supported the claim by Richards (1990a).

In addition, according to Lim (2012e), the fruits of *G. cowa* are subglobose to globose and oblique in shape, green when unripe, and the matured fruits turn to yellow or pale orange with five to eight furrows close to the top, and remaining stigma lowered on the small black persistent calyx. The young fruit has an elongated oval shape and changes into a round shape during maturation (Roy et al., 2010) (Table 3). Moreover, the seeds of *G. cowa,* immersed in dull orange and sour pulp, are great in size and trigonous. Due to their large size, the matured seeds of *G. cowa* provided moisture content roughly at 50% fresh weight (Liu et al., 2002), and the seeds were desiccation-tolerant. However, they

immediately decreased seed viability under 4°C and died almost at 17% of moisture content (Liu et al., 2005). Hence, the seeds of *G. cowa* might be regarded as tropical recalcitrant due to low endurance to desiccation and chilling imbibition. Seed dormancy is a common phenomenon in all plants that affect germination ability. To break dormancy and promote germination, seed pre-treatment, such as scarification, is required by providing water permeability through the seed coat and initiating imbibition (Ardiarini et al., 2021). In the case of *G. cowa,* the seeds have long dormancy up to 8–11 months with 252 days of MGT, and unsuccessful germination was reported after the dispersion at 30°C for 120 days (Liu et al., 2005). The removal of the seed coat of *G. cowa* resulted in the decline of MGT to 13 days at 30°C (Liu et al., 2005).

Reproductive System of Garcinia atroviridis: *Flowers, Fruits, and Seeds*

The flowering period of *G. atroviridis* occurs from July to September, usually five to six years after planting (Pangsuban et al., 2009; Te-chato, 2007). The flowers of *G. atroviridis* are terminals and pedicellate, consisting of four yellow, spreading spherical and concave sepals as well as dark red or crimson, obovate, and fleshy petals with yellowish colour at the edge (Bayu, Febrianti, & Damanik, 2018b; Lim, 2012f) (Table 2). Male flowers are in small, flowered racemes with various stamens in whorls around the pistillode. In contrast, the female flowers are solitary, large, and have 8–16 celled ovoid ovaries with deep red pileate, sub-tetragonal, convex, or sessile stigma with corrugated surface, and staminode connected to an annulus (Te-chato, 2007; Lim, 2012f).

Garcinia atroviridis is gynodioecious that carries both female and hermaphrodite flowers, and it depicts a female-biased ratio when the hermaphrodite flower produces less to no fruit while the female flower contains a bigger ovule that develops a bigger fruit with more seeds (Pangsuban et al*.,* 2009). On top of that, the clustered hermaphrodite flowers have a long filament and plenty of fertile pollens, but they encounter early drops compared to female flowers (Bayu, Febrianti, & Damanik, 2018b). *G. atroviridis* is presumably classified as facultative apomixis, although male trees are rare (Richards, 1990a). The pollen viability of *G. atroviridis* was reported to be 3%–5% (Te-chato, 2007) owing to its influence on the female-biased ratio. Besides, based on the *in vitro* germination evaluation, *G. atroviridis* showed high pollen viability (79.5%) a day after anther dehiscence but gradually lost about 50.0% (17 days) due to pollen half-life and then followed by non-germinated pollen grains prior to 25 days after dehiscence (Pangsuban et al., 2009).

The immature green fruits of *G. atroviridis* turn to vivid yellow when matured with sunken globose in shape, widely lowered hollow apex that consists of 12–16 ribs, slightly grooved, segregated segments, sustained calyx and corolla, thick husk as well as contain flattened seeds encircled by sour and bright orange arillode (Lim, 2012f; Te-chato, 2007) (Table 3). Based on the study by Pangsuban et al*.* (2009), the apogamy treatment (bags

without pollination) on *G. atroviridis* resulted in a high fruit drop ratio, less and smaller fruit production, and low quantity and quality of seeds compared to open and hand pollination treatments. Regardless, this apogamy treatment on *G. atroviridis* carried out the asexual development of fruit (17.5%) and seed with a 2.82 ± 0.99 (7) average number per ripe fruit based on the data obtained from the same study. In addition, the smaller recalcitrant seeds (Length:width= 1.3 ± 0.03 cm:0.6 ± 0.01 cm) of *G. atroviridis* showed 57.3% of germination and 25 mean germination time (Normah et al., 2016). A small seed will likely have a rapid moisture content loss that causes low desiccation resistance (Wen & Cai, 2014). In the case of *G. atroviridis*, the moisture content was reported to be 34.92% in fresh weight and rapidly dropped at 96 hours of desiccation (Normah et al., 2016).

Reproductive System of Garcinia parvifolia*: Flowers, Fruits, and Seeds*

The flowers of *G. parvifolia* are unisexual, bisexual, polygamous, pedicellate, solitary, or axillary in fascicles (2–12) with four yellow to dull orange sepals, large petals, the male flower has no pistillodes, whereas the female flower consists of 7–12 staminodes (Lim, 2012g) (Table 2). *G. parvifolia* is shown to be facultative apomixis, and the reproduction behaviour depicts gametophytic apomixis, while *G. mangostana* var. *mangostana* and *G. celebica* reproduce through sporophytic apomixis (Dike et al., 2020).

The young dull green fruit turns to yellow or orange when matured, depressed globose in shape that comprises a sunken top with a persistent small stigma, thin peel, and small seed surrounded by white and moderately acidic arils (Lim, 2012g) (Table 3). *G. parvifolia* exhibits apomixis behaviour when seed germination occurs without males, parthenogenesis happens, undeveloped proembryos are actuated as well as single embryos can generate many seedlings (Ha et al., 1988; Richards, 1990a). The seed germination was revealed to be a *Garcinia* type when the emergence of plumule and radicle occurs in two opposite ways, similar to *G. mangostana* var. *mangostana, G. celebica G. atroviridis* and *G. prainiana* (semi-*Garcinia*), as documented by Normah et al. (2016).

Table 1
The overall description of the distribution of selected Garcinia *species*

***Garcinia* species**	**Ecological aspects**			
	Origin	**Cultivation**	**Climate**	**Soil**
Garcinia mangostana var. *mangostana*	Southeast Asia (Yaacob & Tindal, 1995)	Malaysia, Indonesia, Thailand, Philippines, Northern Australia, South America and tropical Africa (Cruz, 2001)	Non-seasonal wet tropical and hot and humid weather (Richard, 1990b; Júnior et al., 2019)	Rich in organic matter and well-drained. Sandy loams, lateritic and volcanic soils. (Lim, 2012a)
Garcinia celebica	Malaysia, Cambodia, Thailand and Vietnam. (Lim, 2012b)	Malaysia, Thailand, Vietnam, Cambodia, Borneo, Andaman, Nicobar Island, Kerala and Tamil Nadu, India (Lim 2012b; Nazre 2010; Nazre et al., 2018)	Tropical (Lim, 2012b)	Sandy, rocky and acid clay soils (Lim, 2012b)
Garcinia mangostana var. *malaccensis*	Peninsular Malaysia, Sumatra and Brunei (Lim, 2012c)	NI	Tropical. Hot and wet condition (Lim, 2012c)	Organic rich soils (Lim, 2012c)
Garcinia prainiana	Peninsular Malaysia and Thailand (Mohd Khairuddin et al., 2018)	Pahang, Kelantan, Terengganu and Perak (Azuan et al., 2015)	Tropical (Lim, 2012d)	Well-drained and porous soils, fairly acid clay loams (Lim, 2012d)
Garcinia cowa	Southwest India, East India, Nepal, Andaman and Nicobar Island, Yunnan (China), Myanmar, Thailand, Laos, Vietnam, and Northern Peninsular Malaysia (Lim, 2012e; Richard, 1990)	NI	Tropical (Lim, 2012e)	NI
Garcinia atroviridis	Peninsular Malaysia, Thailand, Myanmar and India (Lim, 2012f)	NI	Humid weather in lowland and highland rainfall region (Lim, 2012f)	NI
Garcinia parvifolia	Thailand, Malaysia, Sumatra, Java, Brunei, Kalimantan, Sulawesi, Maluku Islands and New Guinea (Lim, 2012g)	NI	Tropical. Humid conditions such, as in peat swamps and lowland forests (Hassan et al., 2013; Lim, 2012g)	Well-drained, alluvial sites and along rivers (Hassan et al., 2013; Lim, 2012g)

Note. NI indicates that no information

Table 2
The summary of the flower parts of selected Garcinia *species*

***Garcinia* species**	**Flower parts**						
	Inflorescence	**Petal**	**Sepal**	**Stamen**	**Pistillode**	**Stigma**	**Ovary**
Garcinia mangostana var. *mangostana*	Inflorescence triads (Nazre et al., 2018)	Fleshy Yellowish green and pinkish toward the edge (Awachare & Upreti, 2019; Sulassih et al., 2013; Yuniastuti, 2010)	Ovate to obovate or concave Thick (Nazre et al., 2018)	4-angled (Nazre, 2014) Pollen viability = 0.1-1% (Te-chato, 2007)	Fungiform (Nazre, 2014)	Large sessile or subsessile Discoid (Osman & Milan, 2006; Richards, 1990b; Te-chato (2007)	Blunt star-shaped or broadly ovoid 4–8 chambers (Yuniastuti, 2010)
Garcinia celebica	Solitary, axillary (female) Axillary, borne singly (male) (John et al., 2008; Nazre et al., 2018; Richards, 1990c)	Yellowish green (Sulassih et al., 2013)	Thinly coriaceous (John et al. 2008; Nazre et al., 2018; Richards, 1990c;)	4-lobed (Nazre et al., 2018) Pollen viability and germination = 68% (Sutthinon, Samuels, & Meesawat, 2018a)	NI	Large Fungiform (John et al., 2008; Nazre et al., 2018; Richards, 1990c;)	Globose to ovoid (John et al., 2008; Nazre et al., 2018; Richards, 1990c;)
Garcinia mangostana var. *malaccensis*	NI	Pinkish red (Nazre, 2014)	NI	Long and slander 4-angled or comical-cylindrical (Nazre, 2014; Nazre et al., 2018)	Fungiform (Nazre, 2014)	Corrugated surface (Nazre, 2014)	NI

Table 2 *(Continue)*

***Garcinia* species**	**Flower parts**						
	Inflorescence	**Petal**	**Sepal**	**Stamen**	**Pistillode**	**Stigma**	**Ovary**
Garcinia prainiana	Axillary in clusters (Azuan et al., 2015; Rohani et al., 2021)	Five rounded Rose pink to yellow (Azuan et al., 2015; Rohani et al., 2021)	Five rounded Green to pink (Azuan et al., 2015; Rohani et al., 2021)	Compacted (Azuan et al., 2015; Rohani et al., 2021) Pollen viability and development = 77.6±9.68 and 60.95±15.87%, respectively (Rohani et al., 2021)	NI	NI	NI
Garcinia cowa	Solitary or axillary (female) (Lim, 2012e)	Yellowish (Te-chato, 2007, Lim, 2012e)	NI	Clustered Pollen viability = 96%–100% (Te-chato, 2007)	No pistillodes (Lim, 2012e)	Radiate ridged and papillate stigma (Lim, 2012e)	Ovoid (Lim, 2012e)
Garcinia atroviridis	Terminal and pedicellate Racemes (male) Solitary (female) Clustered (hermaphrodite flower) (Bayu, Febrianti, & Damanik, 2018b; Lim, 2012f)	Dark red or crimson, obovate and fleshy petals with yellowish colour on the edge (Bayu, Febrianti, & Damanik, 2018b; Lim, 2012f)	Four yellow, spreading spherical and concave sepals (Bayu, Febrianti, & Damanik,(2018b; Lim, 2012f)	Stamens in whorls round (Te-chato, 2007; Lim, 2012f) Pollen viability = 3-5% (Te-chato, 2007), 79.5% (a day after anther dehiscence), 50.0% (17 days), no pollen germinated (25 days) (Pangsuban et al., 2009)	Pistillodes present (Lim, 2012f)	Deep red pileate, sub-tetragonal, convex or sessile stigma with corrugated surface (Lim, 2012f; Te-chato, 2007)	Ovoid (Lim, 2012f)

Table 2 *(Continue)*

***Garcinia* species**	**Flower parts**						
	Inflorescence	**Petal**	**Sepal**	**Stamen**	**Pistillode**	**Stigma**	**Ovary**
Garcinia parvifolia	Pedicellate, axillary or solitary in fascicles (Lim, 2012 g)	Large (Lim, 2012 g)	Four in total and yellow to dull orange (Lim, 2012 g)	Female flower consists of 7–12 staminodes (Lim, 2012 g)	No pistillodes (Lim, 2012 g)	NI	NI

Note. NI indicates that no information

Table 3
The summary of the fruit parts of selected Garcinia *species*

***Garcinia* species**	**Fruit parts**				
	Shape	**Colour**	**Taste**	**Aril**	**Seed**
Garcinia mangostana var. *mangostana*	Globose (Nazre, 2014; Richards, 1990b; Sulassih et al., 2013; Te-chato, 2007; Yuniastuti, 2010) Obovoid with the pointy end (Mesta var.) (Osman & Milan, 2006)	Dark purple or red (Nazre, 2014; Richards, 1990b; Sulassih et al., 2013; Te-chato, 2007; Yuniastuti, 2010)	Sweet and sour (Nazre, 2014; Richards, 1990b; Sulassih et al., 2013; Te-chato, 2007; Yuniastuti, 2010)	Fleshy Snowy white (Nazre, 2014; Richards, 1990b; Sulassih et al., 2013; Te-chato, 2007; Yuniastuti, 2010)	Light brown Ellipsoid (Yuniastuti, 2010) Recalcitrant Large *Garcinia*-type of germination Germination rate=75.6% (24 days; Normah et al., 2016)
Garcinia celebica	Sub-globose to ellipsoid (John et al., 2008; Sulassih et al., 2013)	Deep red (John et al., 2008; Sulassih et al., 2013)	Sour (John et al., 2008; Sulassih et al., 2013)	Creamy white (John et al., 2008; Sulassih et al., 2013)	Recalcitrant Large *Garcinia*-type of germination Germination rate = 95.6% (22 days) (Normah et al., 2016)
Garcinia mangostana var. *malaccensis*	Ovoid to globose (Nazre, 2014; Sulassih et al., 2013)	Reddish pink to dark purple (Nazre, 2014; Sulassih et al., 2013)	Sweet and sour (Nazre, 2014; Sulassih et al., 2013)	Snowy white (Nazre, 2014; Sulassih et al., 2013)	NI

Table 3 *(Continue)*

***Garcinia* species**	**Fruit parts**				
	Shape	**Colour**	**Taste**	**Aril**	**Seed**
Garcinia prainiana	Rounded but rather flattened (Azuan et al., 2015; Mohd Khairuddin et al., 2018)	Yellowish orange (Azuan et al., 2015; Mohd Khairuddin et al., 2018)	Acidic sweet (Azuan et al., 2015; Mohd Khairuddin et al., 2018)	Orange (Azuan et al., 2015; Mohd Khairuddin et al., 2018)	Recalcitrant Small Thick testa Semi *Garcinia*-type of germination Germination rate = 48.9% (47 days; Normah et al., 2016)
Garcinia cowa	Subglobose to globose and oblique (Lim, 2012e; Roy et al., 2010)	Yellow or pale orange (Lim, 2012e)	Sour (Lim, 2012e)	Dull orange (Lim, 2012e)	Trigonous' Recalcitrant Large Mean germination time = 252 days (without scarification) and 13 days (with scarification) (Liu et al., 2002; Liu et al., 2005)

Table 3 *(Continue)*

***Garcinia* species**	**Fruit parts**				
	Shape	**Colour**	**Taste**	**Aril**	**Seed**
Garcinia atroviridis	Sunken globose, widely lowered hollow apex that consists of 12–16 ribs, slightly grooved and segregated segments (Lim, 2012f)	Vivid yellow (Lim, 2012f)	Sour (Lim, 2012f; Techato, 2007)	Bright orange (Lim, 2012f)	Flattened (Lim, 2012f) Recalcitrant Small *Garcinia*-type of germination Germination rate = 57.3% (25 days; Normah et al., 2016)
Garcinia parvifolia	Depressed globose and sunken top (Lim, 2012 g)	Yellow or orange (Lim, 2012 g)	Moderately sour (Lim, 2012 g)	White (Lim, 2012 g)	Small *Garcinia*-type of germination (Vogel, 1980)

Note. NI indicates that no information

CONCLUSION

This present overview has provided an insight into the reproductive system of *G. mangostana* var. *mangostana, G. celebica, G. mangostana* var. *malaccensis*, *G. prainiana, G. cowa, G. atroviridis* and *G. parvifolia*. To summarise, a tropical environment provides well-distributed *Garcinia* species, perhaps due to suitable ecological conditions such as temperature, weather and soil properties. On top of that, apomixis is a valuable discovery as it can alter normal sexual reproduction. Based on previous findings, *G. mangostana* var. *mangostana* is reported to be an obligate apomict due to male sterility resulting from a cellular alteration in tapetum that gives rise to abnormal pollen development. Meanwhile, a male flower of *G. celebica* is functionally fertile due to normal pollen development and viability similar to *G. prainiana* and *G. cowa* which these species display a facultative apomixis together with *G. atroviridis* and *G. parvifolia*. Moreover, the seed size, moisture content, and vulnerability towards desiccation are the key factors that could influence the viability and germinability of *Garcinia* species in this overview study. This available information could perhaps serve to further understand plant conservation and breeding approaches to improve the quality and yield of *Garcinia* plants.

To date, several comprehensive studies of reproduction are yet to be documented. There is limited information on the timing and duration of flowering periods and how environmental factors influence these. Effective conservation strategies for *Garcinia* species are scarce, especially endangered ones with restricted distributions. Pollen development and viability studies can further determine male fertility, particularly in *G. mangostana* var. *malaccensis* and *G. parvifolia*. In addition, more studies regarding seed development and germination capability are required on these two species. The influence of seed scarification, especially on *G. prainiana*, can be evaluated for future research since the seeds require a long period to germinate. These recommendation studies could help ascertain a possible solution to fulfil agricultural and plant conservation demands.

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Review Article

Food Wastes for Enhancing Soil and Crop Productivity in Tropical Acid Soils

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ABSTRACT

Excessive inorganic chemical fertilizers could cause negative environmental effects, such as soil quality degradation. In contrast, organic bio-fertilizers contain beneficial bacteria and fungi that foster soil health and crop yield, presenting a sustainable and eco-friendly alternative for crop production. This review evaluated different types of potential food waste in Brunei Darussalam for producing effective bio-fertilizers. Along with the benefits, limitations, challenges, and suggestions for bio-fertilizers (Produced from food wastes) application in Brunei Darussalam were subsequently outlined. The literature search was limited to 2009 to 2024, using relevant keywords to extract information from online databases such as Scopus, Mendeley, Science Direct, Elsevier, and Google Scholar. Additional searches were performed to retrieve grey literature. This review revealed that food wastes such as eggshell wastes, washed rice water, fruits, vegetables, and animal wastes have positive effects on improving soil and crop productivity. Bio-fertilizers provide many benefits in terms of environment, socio-economic, soil and crop productivity and disease resistance. The few limitations of bio-fertilizers are heavy metal contents, low macro-nutrient content, and large quantities required for field application. These limitations can be further researched to improve the accessibility and quality of bio-fertilizer production. Currently, the implications of organic bio-fertilizers (Produced from food wastes) in Brunei Darussalam are limited because of a lack of information and awareness on bio-fertilizer use. Thus, this comprehensive review of bio-fertilizers (made of food waste) may benefit the agricultural sector of Brunei Darussalam and beyond.

Keywords: Bio-fertilizers, crop productivity, food wastes, soil productivity, sustainable agriculture

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INTRODUCTION

Sustainable agriculture is one of the most effective methods to consistently fulfill the Sustainable Development Goals (SDGs). These include poverty and hunger eradication, good nutrition and health, quality education, economic and social progress, peace and security, and environmental preservation. Amidst the growing concerns about chemical fertilizers, bio-fertilizers can sustainably enhance agricultural soil and crop productivity (Arjjumend et al., 2020). It is essential because bio-fertilizers have bacteria and fungi, which are reputed to promote plant growth and health. Moreover, they have the ability to increase crop yield through environmentally friendly means. A substance composed of living microorganisms that colonize the rhizosphere or plant internal tissues and stimulate plant growth when applied to soils, seeds, or plant surfaces is known as a 'bio-fertilizer'. The bacteria or fungi in bio-fertilizers often fix nitrogen, solubilize phosphate, oxidize sulfur, produce plant hormones, or decompose organic substances. Therefore, bio-fertilizers are able to enhance nutrient cycling, which translates into optimal crop growth and development (Pirttilä et al., 2021). Furthermore, bio-fertilizers have a positive impact on crop productivity and soil health. In addition to enhancing nutrient availability and increasing soil organic matter abundance, they neutralize the harmful effects of chemical fertilizers because organic wastes and the microorganisms in bio-fertilizers are compatible (Areeshi, 2022).

Food wastes have the potential to facilitate the production of bio-fertilizers since these wastes could effectively speed up microbial metabolism and are bio-degradable (Areeshi, 2022). Food waste is the portion of food that is not consumed by anyone and is often referred to as food loss or unconsumed food. Any stage of the food chain system, including production, processing, distribution, retail and consumption, may result in food waste. Along the food chain, food waste ranges from 30% to 40% (Kang et al., 2021). Kumar et al. (2017) reported that food waste is generated by households in 42% of cases, food industries in 39%, and distribution in 5% of cases (Kumar et al., 2017). Food waste has the worst impact on the environment and the agricultural business, especially if not properly composted. Over 1.3 billion tons of food are wasted yearly (Paritosh et al., 2017). More than half (2.3 billion tons) of the world's yearly production of grain crops is equivalent to the amount of food wasted every year (Kang et al., 2021). In Asia, developing countries discard approximately 11 kg of food per person, whereas developed nations waste approximately 80 kg per person (Food and Agriculture Organization of the United Nations [FAO], 2013).

The Association of Southeast Asian Nations (ASEAN) population produces approximately 83 kg of food waste per capita per year, the largest type of waste generated (approximately 6/10 of total waste) for the whole country combined (United Nations Environment Program [UNEP], 2017). Approximately 32% of the total food waste is produced in Brunei Darussalam (Kon, 2022). Food waste is one of the major issues in ASEAN, particularly in small countries such as Brunei Darussalam.

This review is significant because it focuses on recent studies on bio-fertilizers, which are likely to benefit Brunei Darussalam's agricultural industry and elsewhere, especially farmers, as readily available low-cost input. It could also serve as an alternative to chemical fertilizers. Currently, composts, which are produced from organic wastes, are the most common type of fertilizers used in Brunei Darussalam and are a more environmentally friendly alternative to chemically produced fertilizers. Although composting wastes could increase agricultural output, it does not have the same impact as applying organic bio-fertilizers, which contain several beneficial microorganisms capable of improving soil and crop health. Bio-fertilizers produced from food waste should be implemented in Brunei Darussalam, especially by the local farmers. The gap this review fills could facilitate introducing and adopting bio-fertilizers used in soil and soilless agriculture. Therefore, this review discusses different types of food waste in Brunei Darussalam (as a case study) and elsewhere, which could or are used to produce effective bio-fertilizers for soil and crop improvement.

METHODOLOGY

Relevant literature was searched using keywords to extract from online databases such as Scopus, Mendeley, Science Direct, Elsevier, and Google Scholar. Additional searches included grey literature sources such as government documents, reports, and conference proceedings. The literature search was limited to 2009 to 2024. The inclusion criteria were based on publication years and topic relevance. Studies were excluded if they were duplicates and non-relevant to the topic. The keyword search combined the terms 'food wastes,' 'organic,' and 'bio-fertilizer.' Data were extracted from the following elements: author, publication year, and key findings. Information extracted from the studies was synthesized using thematic analysis to identify common themes and patterns across the literature, after which key findings were summarized. The synthesis provided a comprehensive overview of the current research on bio-fertilizers produced from food wastes in Brunei Darussalam (as the main case study).

Food Wastes Issues in Brunei Darussalam

Currently, municipal solid waste has become a major concern in Brunei Darussalam because this type of waste generation has increased significantly, partly because of rapid urbanization, industrialization, population growth, and improved lifestyle. Among the ASEAN countries, Brunei Darussalam is the second country to Singapore, which generates more solid waste per capita. Only 6% of the total waste generated is used to produce compost, and 70% of the solid waste goes directly to Brunei Darussalam's six landfills. In contrast, other standard methods dispose of the remaining wastes. Approximately 400 to 500 tonnes of waste are delivered daily to the country's landfills (Wong, 2020). Consequently,

the country's biggest landfill at Sungai Paku, Brunei Darussalam, has greater than 90% of the total waste and is predicted to be full by 2030 if effective recycling strategies are not introduced and adopted (Dariah et al., 2022; Sulaiman et al., 2023). A growing concern for effective waste management and utilization is the Gross Domestic Product (GDP) growth of 2.3%, which occurred between 1999 and 2007, and the continued increase in the number of businesses registered, for example, 2,577 businesses in 1998 to 7,240 a decade later (Shams et al., 2014).

From a 2005 survey in Brunei Darussalam, the most prevalent composition of solid waste was food scraps (36%), followed by plastic waste (18%) (Shams et al., 2014). Furthermore, including imports from other countries, retailers in Brunei Darussalam produced up to BND 1000 worth of spoiled food products per month. One of the country's biggest supermarkets discards 2 kg to 3 kg of spoilt food every two days, for a monthly total of 45 kg of trash (Dariah et al., 2022). The data and statistics suggest that food waste is becoming one of the most significant waste issues in Brunei Darussalam, and this challenge needs to be fixed urgently. Therefore, this review provides a practical and sustainable approach for overcoming the food waste issues in Brunei Darussalam and other countries by transforming the waste into bio-fertilizers to guarantee agricultural sustainability.

Sustainability Development Goals (SDGs) in Brunei Darussalam

In 2015, the United Nations adopted the Sustainable Development Goals (SDGs) as a universal call to end poverty, protect the planet, and ensure the world's peace and prosperity by 2030. The 17 SDGs are linked because they acknowledge that actions in one area impact outcomes in others and that development must balance social, economic, and environmental sustainability (United Nations Development Program [UNDP], 2022). Brunei Darussalam seeks to improve on its Millennium Development Goal accomplishments to enable more progress towards the 2030 Agenda for Sustainable Development and SDGs. The 2030 Agenda supplements Brunei Darussalam's national goal, Wawasan (Vision) Brunei 2035, which aspires to develop a high-quality of life population and a dynamic and sustainable economy with knowledgeable, highly skilled, and competent workforce by 2035 (United Nations [UN], 2020). There are nine focused goals in Brunei Darussalam, out of which the two goals related to food and related matters relevant to this review are discussed.

Zero hunger is the goal two of the SDGs**.** In line with this goal, a more sustainable food system in Brunei Darussalam is being expedited. Achieving sustainable food security in Brunei Darussalam has been a challenge. For example, the agricultural sector contributes only 0.54% of the country's GDP (Department of Agriculture and Agrifood, 2022). To feed approximately 445,440 Bruneians (Ministry of Finance and Economy [MOFE], 2022), the government of Brunei Darussalam has made efforts to become self-sufficient in food production. In recent times, the agricultural industry of Brunei Darussalam has

been improving because of the expansion of farmland and more resource support. Brunei Darussalam's self-sufficiency level in the poultry business was estimated to be 89.5% in 2018, or an output of 2538 thousand tonnes. The local output for 2019 was estimated to be 24.58 thousand tonnes, an increase in poultry output of approximately 1.4%. Brunei Darussalam's self-sufficiency levels in fruits and vegetables are 37% and 47%, respectively. The country imports approximately 30,000 tonnes of rice annually from Cambodia, Thailand, and Vietnam (Pehin & Basir, 2021). To this end, urgent action should be taken to enhance the domestic crop output to decrease food shortage issues in Brunei Darussalam now and in the future.

Responsible consumption and production are goal 12 of the SDGs. To be in tune with this goal, the government of Brunei Darussalam utilizes a national strategy to improve consumption and production behavior to protect the environment. An example is the efforts of Green Brunei. This social enterprise supports environmental sustainability, such as support the Plastic Bottle Free Initiative run by the Department of Environment, Parks, and Recreation (JASTRe) and the Green Protocol initiated by the Brunei Darussalam National Council on Climate Change (BDNCCC) (Prime Minister's Office, 2021). Although Brunei Darussalam's efforts in waste recycling are commendable, food wastes continue to be a significant issue because they are disposed of in conventional landfills, a practice that is not environmentally friendly because it could increase greenhouse emissions. Given that food wastes account for the bulk of the waste produced in Brunei Darussalam, it is more practical to recycle them by producing soil amendments/conditioners or organic fertilizers, which are rich in nutrients and organic matter for organic farming systems. There is a need to address Brunei Darussalam's food waste issues and educate farmers and the general populace on recycling food waste into organic fertilizers and related farm inputs for achieving sustainable agriculture through regenerative or climate-smart agriculture. With these efforts, there could be significant curtailment in the food waste issues in Brunei Darussalam besides contributing to achieving goal 12 (Responsible consumption and production) of the SDGs before 2035.

Food Wastes Issues and Management on Tropical Acid Soils

This review uses Malaysia as an example of a tropical country with acid soils comparable to those of other countries. Malaysia produces approximately 16,688 tonnes of food waste daily, which is related to the growing urbanization of the country (Shukla et al., 2024). Food waste in Malaysia is generated by households, agricultural, manufacturing, and domestic operations (Hashim et al., 2021; Ong et al., 2018). With the agricultural sector in Malaysia producing over 1.2 million tonnes of agricultural waste annually, waste management is topical (Omar et al., 2023). The palm oil industry produces the highest and greatest agricultural product, totaling 19.96 million metric tons. Approximately 90% of palm oil is

lost as waste. Thus, only 10% of palm oil is produced (Awalludin et al., 2015). In 2012, the oil palm biomass (Fronds, trunk, empty fruit bunches, oil palm fibers, and shells) was 83 million tons (Ong et al., 2018). Furthermore, the canning industry for pineapples typically generates a significant amount of waste. Although pineapple production is less than palm oil (0.45 million metric tonnes), 30% to 50% of it is waste (Ong et al., 2014). Food waste in Malaysia is disposed of as municipal solid waste (Thi et al., 2015).

Despite being an unfavorable alternative for waste management, Malaysia and many other countries continue to choose the landfilling approach since it is inexpensive and requires little technical expertise to dispose of food waste (Kamaruddin et al., 2017). Nowadays, landfills in Malaysia account for 80% of the country's food waste production (Abd Ghafar, 2017). However, second-generation waste management techniques have been developed that turn food waste into products with additional benefits, like flavors, chemicals, and biofuels (Ong et al., 2018).

In Malaysia, examples of successful food waste valorization from oil palm wastes include bio-fertilizer production, while pineapple wastes include flavoring compounds (such as vanillin) and biogas generation. Oil palm biomass, particularly Empty Fruit Bunches (EFB), contains significant nutrients like Nitrogen, Phosphorus, and Potassium (NPK) and lignocellulosic components that make it suitable for use in the manufacturing of biofertilizers (Mahmud & Chong, 2021). Composting EFB using biotechnological techniques can produce nutrient-rich biofertilizers by contributing to nitrogen fixation and enhancing nutrient content. It could be a viable and sustainable alternative to chemical fertilizers for increasing soil fertility and crop yield. As for pineapple, waste, especially the peels, is being used for vanillin production (Zubaidah & Karim, 2024). These peels are rich in phenolic compounds like ferulic acid. Ferulic acid from pineapple peels was fermented using *Aspergillus niger* to produce vanillin for the flavoring compound. Additionally, Aili Hamzah et al. (2024) found that pineapple waste could be a useful substrate for biogas production with improved pretreatment methods. Nevertheless, innovative food waste collection management measures must be further developed to accomplish such valorization. Malaysia has yet to establish a defined food waste management system (Ramli et al., 2020) as Brunei Darussalam.

Food Wastes Effects on Soil Health and Quality

Nutrient-deficient soils can benefit from food waste, which is typically high in nitrogen (N). Dehydrated vegetable food waste has been reported by O'Connor et al. (2022) to have a high amount of plant-available N (1.71 g kg−1) and total N (3.25%), making it suitable as a fertilizer to enhance crop growth and development (Palansooriya et al., 2023). A study by Lee et al. (2019) revealed that although there was no difference in the Na/K ratio in plant tissue among the treatments, the use of food waste compost enhanced the amounts

of the macronutrients N, Phosphorus (P), and Potassium (K) in grain and plant residues. After crop harvest, compost from food waste increased the soil's pH, electrical conductivity (EC), total carbon (TC), and available P contents. In particular, it was observed that the application of compost increased the soil's cation exchangeable capacity (CEC) and exchangeable sodium percentage (ESP) contents, regardless of the water content. Applying compost made from food waste to the soil positively impacts the availability of nutrients and organic matter (Lee et al., 2016), both of which significantly enhance soil health. Both saturated and unsaturated soils improved from the addition of food waste compost, which enhanced the carbon and nitrogen contents and improved soil quality (Lee et al., 2019).

Food Wastes Effects on Crop Yields

Adequate amounts of organic food waste affect soil properties and significantly contribute to plant growth and development. Adding organic food waste to other materials improves soil fertility, plant development, yield, and NPK uptake of stevia crops. A 50% organic-N from isolated soy protein, eggshell, potato peels, banana peels, and green pea peels) resulted in the highest fresh plant, dry plant, and dry leaf weights, whereas 50% organic-N from isolated soy protein, eggshell, and banana peels and 50% organic-N from isolated soy protein, eggshell, and green pea peels also showed positive effects on plant growth compared to without fertilizer and 100% NPK chemical. The increase in crop yield caused by the inclusion of organic amendments is related to the contribution of organic food waste in enhancing soil physical and chemical properties, boosting biological activity, organic matter, and nutrient availability (NPK) (Youseff et al., 2020). Improved soil fertility as a result of these effects led to a boost in overall crop yield and growth. Furthermore, these results supported the findings of Hossain et al. (2017), who found that organic wastes promote plant growth and have a favorable effect on soil's biological, chemical, and physical properties.

Soil Fertility and Bio-fertilizers in Brunei Darussalam Agriculture

From the research of Azffri et al. (2022) on paddy soils, approximately 64% of the soils studied were very acidic and unsuitable for rice growing. Apart from low pH, the soils are high in iron, aluminum, and sulfur but are low in nutrients and nutrient-holding capacity. Fertilizers are a common management practice to improve soil fertility and crop productivity. However, excessive use of chemical fertilizers degrades not only soil health but also negates the quality of the environment. Excessive use of chemical fertilizers has been implicated in the ongoing global greenhouse effects and water contamination/ pollution through excessive nitrogen and phosphorus fertilizers in particular. Hence, there is a call to introduce bio-fertilizers in Brunei Darussalam agriculture. Organic farming is the best approach among farming systems because it uses natural resources, including

useful bacteria, plant and animal wastes, and organic materials (Wazir et al., 2018). Organic farming encourages using bio-fertilizers to reduce the negative impacts of the widespread use of chemical fertilizers agrochemicals, and other associated environmental problems. Bio-fertilizers have been developed as potential eco-friendly solutions for crop development and plant protection for organic farming (Parewa et al., 2021), as bio-fertilizers are biological products that can improve soil fertility. They enrich soils with microorganisms to produce organic nutrients besides being reputed for reducing plant diseases (Mulyani et al., 2017). Selected food wastes for the production of bio-fertilizers are subsequently discussed.

Food Wastes for Bio-fertilizers Production

A summarized finding on food waste for bio-fertilizer production is shown in Table 1. Hen egg is a significant food source whose annual output grew from 1.3 million tons in 2010 to 1.6 million tonnes in 2020 (FAO, 2022). Brunei Darussalam's total egg consumption was 173.91 million (Department of Agriculture and Agrifood, 2022). Approximately 250,000 tonnes of eggshell waste were generated and typically discarded on land (Andrade et al., 2022). Eggshell waste is a rich lime source, stabilizing the pH of acid soils. It contains 95% calcium carbonate, making it suitable for plant fertilizer use. Moreover, they are rich in uronic acid, sialic acid, and amino acids. Furthermore, they are rich in macronutrients and micronutrients, both essential for plant growth and development. The macronutrients include potassium, nitrogen, calcium, magnesium, and phosphorus, whereas the micronutrients include zinc and chloride (Wijaya & Teo, 2019). According to Wazir et al. (2018), red clover plants cultivated on soil mixed with eggshells grew approximately 10 mm bigger than those grown on soil without eggshells because of the high calcium in the eggshells. Calcium serves multiple purposes for plants, such as aiding enzyme formation, promoting root growth, and facilitating nitrate uptake. Additionally, it can neutralize soil acidity. It is also a vital component of plant cell walls. The mixture of algae C. vulgaris in soil and eggshell wastes was advantageous for tomato mineral enrichment. It also increases calcium content in tomatoes (Ertürk, 2020). According to Ahmed et al. (2021), powdered eggshells can be transformed into liquid calcium acetate fertilizer by reacting with acetic acid. As an amendment, calcium acetate does not acidify soils because of its relatively neutral pH (7.6 in a 0.2 M solution) besides enhancing calcium solubility (Ahmed et al., 2021).

Table 1
Summarized main findings for effects of different types of food wastes on soil and crop productivity

Classification of food wastes	Food wastes	Main findings	References
Eggshell wastes		Plants cultivated on soil mixed with eggshells grew approximately 10mm bigger than those grown on soil without eggshells.	Wazir et al. (2018)
		The mixture of algae C. vulgaris in soil and eggshell wastes was found to be advantageous for tomato mineral enrichment as it increases calcium content in tomatoes	Ertürk (2020)
Washed rice water (WRW)		Fermented WRW, as compared with unfermented WRW, had higher elemental concentrations, especially N (59.7%), P (60.2%), and K (25%).	Nabayi et al. (2021a)
		WRW improves crop height, stem diameter, and yield. Bacteria can suppress plant diseases, create phytohormones and siderophores, solubilize potassium and phosphate, and fix nitrogen.	Sairi et al. (2018)
Fruit wastes	Banana peels	A banana bio-fertilizer of 5 mL to 20 mL increased the germination of black gram because of the growth promoters and nutritive components such as Potassium (K) in the banana bio-fertilizer.	Sogani (2023)
		Tryptophan in banana peels improves numerous physiological processes, including the growth of wheat and periwinkle plants.	Hussein et al. (2019)
	Pineapple peels	Ultisol soils, characterized as marginal areas with low organic matter content, may benefit from this liquid organic fertilizer. The best outcomes for the growth and production of long bean plants were obtained using a liquid organic fertilizer at a concentration of 450 ml/L	Nurcholis et al. (2020)
Vegetable wastes	Potato peels	Proteins and soil microorganisms break down abundant proteins, and starches break down to produce high nitrogen content.	Priyanga et al. (2016)
		When applied to soils, the slurry from potato peels biogas plant (anaerobic digester) is a beneficial bio-fertilizer and replenishes soil nutrients.	Muhondwa et al. (2015)

Table 1 *(Continue)*

Classification of food wastes	Food wastes	Main findings	References
	Onion peels	1% onion peel extracts significantly stimulated root and shoot length.	Rajput et al. (2022)
		The onion peel water has the potency to reduce the severity of plant infection, increase plant growth, flowering, and commencing plant regeneration, which has several chemical constituents such as flavonoids, phenols, tannins, and others that are good for plants.	Patil et al. (2021)
	Garlic peels	The highest stimulatory effect of 1% garlic peel extract occurred in both fenugreek root and shoot length, with the root length and shoot length increase of 16% and 9%, respectively.	Patil et al. (2021)
		Garlic peels enhance the fermentation quality of high-moisture silages by boosting Lactobacillus abundance and reducing the relative abundance of Clostridium	Chen et al. (2021)
Animal wastes	Bone meals	Spring barley biomass steadily absorbed more P and N in response to increased Meat and Bone Meal (MBM) dosage. In comparison with mineral fertilization (40 kg P/ha), the favorable effects of the highest MBM dose (117 kg P/ha) on P absorption were statistically significant. From a two-year field experiment, MBM provides an important source of N and P for spring barley produced for fodder	Nogalska (2016)
		When regularly treated with fish fertilizers, plants respond rapidly, enabling them to grow vigorously. Fish offal fertilizers can benefit all fruits, plants, flowers, and vegetables because they can be administered *via* their leaves or soil treatment.	Lema & Degebassa (2013)
Tea wastes		Balance soil pH levels. Its high Tannic acid content can counteract soil acidity.	Wazir et al. (2018)

Nabayi et al. (2021a) reported that fermented WRW, as compared with unfermented WRW, had higher elemental concentrations, especially nitrogen (59.7%), phosphorus (60.2%), and potassium (25%). This significant difference is related to the presence of beneficial microorganisms, including Bacillus velezensis, Klebsiella pneumoniae, and a variation of Enterobacter spp., which are N-fixing and P- and K-solubilizing bacteria (Nabayi et al., 2021a). Furthermore, research revealed that watering plants with WRW improves height, stem diameter, and yield of crops such as tomato, water spinach, eggplants, pak choy, lettuce, mushroom, adenium, chili, and mustard greens. Plant growth-promoting bacteria (PGPB), such as Lactobacillus and Bacillus spp., have been discovered in WRW (Sairi et al., 2018). These bacteria can suppress plant diseases, create phytohormones and siderophores, and solubilize potassium and phosphate in addition to fixing nitrogen (Nabayi et al., 2021a).

Fruit and vegetable wastes account for approximately 46% of all production waste (1400 million tons). In the European Union (EU), household waste is more than 17 billion kg of fresh produce yearly, or 35.3 kg per person, 14.2 kg of which can be prevented (De Laurentiis et al., 2018). The fruit and vegetable industries produce more trash than other food-processing industries do, with peelings contributing between 25 to 30% of that waste, followed by seeds, skins, shells, pods, cores, pulp, and pomace (Nirmal et al., 2023). Daily, fruit peel wastes are increasing, both at home and in the workplace. Individuals regularly remove fruit peels and discard them as waste. Because of their high biodegradability and fermentability, fruit wastes cause significant environmental problems, including water and soil pollution, the greenhouse effect, eutrophication, global warming, and other health issues (de Medeiros et al., 2020). It is a crucial issue that needs to be properly controlled to keep the environment free of pollutants, particularly at the industrial level (Jariwala & Syed, 2016). Fruit peels are high in plant essential macro and micronutrients (Ibrahim et al., 2016). Considering the fact that fruit scraps have minerals vital for plant growth, they are used as fertilizers and soil amendments to improve soil fertility and enhance soil microbiota (Dayarathna & Karunarathna, 2021).

Banana fruits are ranked first for the major type of local fruit production in Brunei Darussalam, with a production of 1,673,424 kg in 2022 (Department of Agriculture and Agrifood, 2022). It suggests that banana consumption in Brunei Darussalam is such that it could contribute to high banana peel waste disposal in this country. Banana peels are commonly discarded as waste because they form an integral part of the fruit. However, banana peels have several beneficial properties for transformation into bio-fertilizers as soil amendments. Banana peels are rich in nitrogen, phosphorus, potassium, and micronutrients (iron, manganese, zinc and copper) (Aboul-Enein et al., 2016). The nutrients in banana peels increase plants' defense against infection (Wazir et al., 2018). The high quantities of K, growth stimulants, and amino acids such as L-tryptophan in banana peels have

significant effects on several biological characteristics of plants, including a greater rate of seed germination (Sogani, 2023).

From a recent study, a banana bio-fertilizer 5 to 20 ml increased germination of black gram (Sogani, 2023) because of the growth promoters and nutritive components such as Potassium (K) in the banana bio-fertilizer. Hussein et al. (2019) found that tryptophan in banana peels improves numerous physiological processes, including the growth of wheat and periwinkle plants. Tryptophan also influences plant development and metabolism under water stress, and it also increases the physiological availability of water and nutrients in addition to its effect on boosting endogenous hormone levels. Hence, it might encourage cell division and/or cell expansion, subsequently promoting growth.

Pineapple fruit is one of the major types of local fruit production in 2022 in Brunei Darussalam, with an estimated total quantity of 223,443 kg (Department of Agriculture and Agrifood, 2022). The disposal of pineapple peel waste is on the increase. The use of pineapple waste as a liquid organic fertilizer is essential. The pineapple peel is rich in sugars and carbohydrates. Approximately 81.72% of the pineapple peel is made up of water, followed by 20.87% crude fiber, 17.53% carbs, 4.41% protein, and 13.65% reducing sugar (Sutikarini et al., 2023). Pineapple peels are also rich in nutrients, as presented in Table 2 (Susi et al., 2018). Ultisol soils, characterized as marginal areas with low organic matter content, may benefit from this liquid organic fertilizer. The best outcomes for the growth and production of long bean plants were obtained using a liquid organic fertilizer at a concentration of 450 ml/L (Nurcholis et al., 2020).

The production and processing of horticulture products, especially vegetables, have expanded significantly to meet the increasing demand brought on by the growing population and altering dietary preferences (Sagar et al., 2018). Substantial nutritional, financial, and environmental issues have emerged because of significant losses and waste in the fresh and processing industries. According to the Food and Agriculture Organization of the United Nations (FAO), losses and waste in fruits and vegetables are the largest of all food kinds. They could be up to 60% (Sagar et al., 2018). Industrial vegetable wastes (VWs) can be bio-fertilizers through anaerobic digestion (Pilarska et al., 2017). With the participation of microorganisms, including bacteria and archaea, to decompose organic matter in anoxic circumstances, anaerobic digestion

Table 2
Nutrients present in pineapple peel (Liquid fertilizer) (Susi et al., 2018)

Parameters (units)	Values
Organic Carbon, C (%)	3.10
Nitrogen, N (%)	1.27
Phosphorus, P (ppm)	23.63
Potassium, K (ppm)	8.25
Calcium, Ca (ppm)	27.55
Magnesium, Mg (ppm)	137.25
Sodium, Na (ppm)	79.52
Iron, Fe (ppm)	1.27
Manganese, Mn (ppm)	28.75
Copper, Cu (ppm)	0.17
Zinc, Zn (ppm)	0.53

(AD) breaks down complex organic materials in VWs to produce biogas as a substitute for biofuel. Bio-fertilizers can be developed from the final effluent from anaerobic digesters (Chakravarty & Mandavgane, 2020).

Bio-fertilizers have been successfully produced from potato peels. Proteins and starch, which are abundant in potato peels, are broken down by soil microorganisms to produce fertilizers with high nitrogen content (Priyanga et al., 2016). The bacterial count in vermicompost made from potato peel-fed earthworms (*Pheretima elongata*) was higher than in nearby soil (Pandit et al., 2012). When applied to soils, the slurry from potato peel biogas plant (anaerobic digester) serves as a beneficial bio-fertilizer and replenishes soil nutrients (Muhondwa et al., 2015). Similarly, 45 days of water fermentation produced bio-fertilizers from potato peels, legume peels, cow manure, tulsi leaves, and neem leaves. The physicochemical characteristics of a strawberry fruit and vegetative growth demonstrated an overall improvement after applying this bio-fertilizer (Javed et al., 2019).

Onion peels have significant amounts of carbohydrates (82.15%), biopolymers (93%), protein (3.06%), ashes (5.93%), and fiber (7.78%). Moreover, they are rich dietary fibers (1:13; SDF: IDF), with the major portion being insoluble dietary fibers (IDF) consisting of 41.1% α-cellulose, 16.2% hemicellulose, and 38.9% lignin. Furthermore, onion peels have significant antioxidant capacity (76% suppression of DPPH) (Cavalheiro et al., 2020). From the study of Patil et al. (2021) on the germination of selected seeds using water extracts of onion peels, one percent onion peel extracts significantly stimulated root and shoot length of falooda and garden cress seeds, with a slight increase in shoot length of garden cress seeds from 6.33 cm to 6.54 cm.

According to Rajput et al. (2022), a white-colored fungal infection on the stem area of the Tulsi plant (*Ocimum Tenuiflorum*) prevented plant growth because the plant's growth was halted. The white-colored fungal infection was reduced after applying onion peel water to the diseased areas of plants for 21 days to 25 days. New green leaves were also produced at the plant's tip, indicating that the plant's regrowth process had begun. The onion peel water has the potency to reduce the severity of plant infection, increase plant growth, flowering, and commencing plant regeneration, which has several chemical constituents such as flavonoids, phenols, tannins, and others that are good for plants (Rajput et al., 2022). Thus, onion peels can serve as an organic fertilizer used in farming systems to improve soil and crop productivity.

Approximately 20 million tons of garlic (*Allium sativum L.*) are produced worldwide each year as a flavoring component. Garlic peels and straws are examples of garlic waste, which constitutes approximately 25% to 30% of the weight of the raw material and are usually dumped or incinerated, which may pollute the environment (Chen et al., 2021). Garlic peels provide essential nutrients for plant growth and development because they are rich in vitamins, antioxidants, and nutrients such as fiber, calcium, iron, potassium, and magnesium (Patil et al., 2021). According to Patil et al. (2021), the highest stimulatory effect

of 1% garlic peel extract occurred in both fenugreek root and shoot length, with the root length and shoot length increase of 16% and 9%, respectively. The newest findings indicate that garlic peel may impact bacterial communities. Additionally, garlic peels enhance the fermentation quality of high-moisture silages by boosting Lactobacillus abundance and reducing the relative abundance of Clostridium (Chen et al., 2021). Therefore, garlic peels are another source for producing organic fertilizers to improve soil and crop productivity.

Although the effects of animal waste on humans and the environment are significant, they have several benefits. Animal waste can be utilized as an alternative source of animal feed, a food supply for plants, a growing medium for earthworms, and as a source of energy in the form of methane gas. However, environmental pollution may result from animal manure, which is not properly treated (Said, 2019).

Bones have been mixed with other ingredients to produce organic fertilizers. Nutrient elements such as calcium and phosphorus in bone meal are essential for the growth and development of plants (Said, 2019). One of the by-products of the rendering industry is meat and bone meal (MBM). It is a valuable source of nutrients for the growth of plants because it provides 8% N, 5% P, 1% K, and 10% Ca (Kivelä et al., 2015). In a study by Nogalska (2016), spring barley biomass steadily absorbed more P and N in response to increased MBM dosage. Compared with mineral fertilization (40 kg P/ha), the favorable effects of the highest MBM dose (117 kg P/ha) on P absorption were statistically significant. From a two-year field experiment, MBM provides an important source of N and P for spring barley produced for fodder (Nogalska, 2016).

Fish waste, known as "fish offal," includes the viscera, head, trimmings, and gut of fish. These fish parts are commonly removed for human consumption. The minerals in the planet's oceans are all present in fish, making fish offal fertilizers important sources of nourishment for soil and plants. Like other organic fertilizers, fish offal is environmentally safe; it is not drained easily and stays in the soil for a long time. Furthermore, it does not harm aquatic environments. The productivity of tomato plants increased after applying the organic fertilizer from fish offal. At later stages, tomatoes with fish offal fertilizer grew better compared with those fertilized with a chemical fertilizer. The fertilizers produced from fish offal provide a balance of all the 18 nutrients that are recognized to be important for crop growth besides being rich in protein and nitrogen. The ratio of N, P, and K in fish fertilizers is commonly 10:6:2. When plants are treated with fish fertilizers regularly, they respond rapidly, enabling them to grow vigorously. All fruits, plants, flowers, and vegetables can benefit from fish offal fertilizers because they can be administered *via* their leaves or soil treatment (Lema & Degebassa, 2013).

Boiled Tea waste (the leftovers after making tea) can be utilized to balance soil pH levels because of its high Tannic acid content, which can counteract soil acidity. Tea leaves have essential nutrients such as K, P, and N for plant growth and development. When tea is applied to soils, it increases the soil's capacity to hold nutrients to promote the growth

and development of plants. The decomposition of tea leaves on the ground enhances soil nutrients, thus supporting the growth of useful microorganisms and facilitating soil oxygenation. This response reinforces plants' roots. Wasted tea leaves are a source of nitrogen (N), crucial for several physiological functions (Wazir et al., 2018). Bloom (2015) reported that besides regulating overall plant growth, N stimulates the absorption and use of other nutrients such as K and P.

Anaerobic Digestion during Bio-fertilizer Production

Anaerobic digestion (AD) utilizes organic materials such as food waste and manure as common substrates to generate sustainable energy and nutrient-rich effluents. Anaerobic digestion systems could reduce CH4 and N2O emissions in dairy operations by 60% and 70%, respectively. Additional environmental advantages are obtained if the effluent from AD (digestate) is used as a fertilizer, such as avoiding emissions from synthetic fertilizers. Several studies have indicated that digestate has good fertilizer potential with equivalent or better yields when fertilized with digestate instead of synthetic fertilizer or undigested animal manures or slurries (Barzee et al., 2019). Cheong et al. (2020) state that heat-treated food waste anaerobic digestate (FWAD) increased pH. In contrast, total ammonia nitrogen decreased with increasing heat of temperature treatments because heating removes carbon dioxide in addition to stripping of ammonia. If the pH of the heat-treated digestates is noticeably higher than the ideal pH (5-7 pH) for cultivating xiao bai cai, there is a risk of nutrient toxicity in the soil and poor nutrient uptake during plant growth. Total ammonia-nitrogen (TAN) readings in raw animal manure and anaerobic digestates formed from animal manure freshly produced in a functional anaerobic digestor are high in total ammonia nitrogen in digestates or equivalent. As reported by Cheong et al. (2020), AD of food waste produces fertilizers with higher TAN because processed food waste used in the production of FWAD commonly has larger quantities of undigested nitrogenous material (For example, uneaten meat) than animal manure produced after nutrient assimilation. This necessitates dilution before it is used to grow crops.

Valuable-added Products Produced from Food Wastes in Brunei Darussalam

A Brunei-based company, Biofield Solutions, has successfully turned food waste into organic compost fertilizer as its main product. Their product, which is high in organic matter and full of beneficial microorganisms, assists in improving plant growth and soil health and minimizes soil acidity while enhancing plant nutrient use efficiency (Faisal, 2023). They collaborate closely with commercial farmers, as approved by Brunei Darussalam's Department of Agriculture and Agrifood, to enable them to migrate to sustainable agricultural practices using organic fertilizers.

Food Wastes Management Initiatives in Brunei Darussalam

To support Brunei Darussalam's sustainability goal, Biofield Solutions and Brunei Shell Marketing Company Sendirian Berhad (BSM) signed an agreement for a local composting campaign in 2023. The campaign involves collecting food waste, turning it into organic compost, and then distributing it to local farmers to generate a sustainable food and agricultural cycle ("BSM, Biofield Solutions partner for composting initiative", 2023). Furthermore, the company collaborated with major food and beverage (F&B) businesses in Brunei, such as McDonald's Brunei and Royal Brunei Catering (RBC), to manage waste by introducing a system in their kitchen that turns non-organic and organic waste into high-grade fertilizers (Mahmud, 2023). Recently, Biofield Solutions also offered a service whereby they collect and convert leftover food, including fruits, vegetables, meat, seafood, and other food waste, into commercialized quality fertilizer products. The listed information is provided on their company website (Biofield Solutions, n.d.).

Bio-fertilizers on Soil Physical Properties

Bio-fertilizer has a positive effect on the soil bulk density and porosity. Incorporating beneficial microorganisms and organic amendments resulted in a significant decrease in soil bulk density, according to a study by Abd El-Hamid et al. (2013). This decrease is attributed to the enhanced buildup of organic carbon content in the soil, leading to higher pore space, which consequently increases the porosity and water-holding capacity of the soil (Patel et al., 2024; Abd El-Hamid et al., 2013). Additionally, lower bulk density values are beneficial for soil health, promoting better water infiltration, gas exchange, and root growth, ultimately improving crop production yields and quality (Patel et al., 2024).

Bio-fertilizers on Soil Chemical Properties

Apart from improving soil organic matter, humic substances, and cation exchange capacity, soil total N was significantly changed after applying bio-fertilizer. A dose of NPK fertilizer caused lower total N than when organic fertilizer was used. For example, the bio-fertilizer increased the total N in the soil by 1.51% to 18.01%. Moreover, the organic fertilizer's bio-fertilizer potential K, sorption K, potential P, sorption P, total N, and pH were more effective. It was concluded that the bio-fertilizer could replace 25% to 50% of the NPK fertilizer in soil (Mulyani et al., 2017).

Bio-fertilizers on Soil Biological Properties

Soil microflora such as bacteria and fungi with a body size of <5 μm are related to several key soil functions such as soil fertility, nutrient cycling, and decomposition of inorganic and organic substances (Singh et al., 2020). When organic bio-fertilizer is applied to soils, the

living microorganisms in the fertilizer colonize the region of the soils or the interior of the plant and directly influence root secretions and associated microorganisms (rhizosphere). This further accelerates plant growth by increasing the supply or availability of primary nutrients (Shaji et al., 2021). Soil microorganisms are inadequate when decomposing lignin, and bacteria are typically less capable of lignin degradation than fungi (Du et al., 2018). Therefore, using bio-fertilizers breaks down organic matter and involves plant hormone and enzyme synthesis in plants (Shaji et al., 2021). Bacterial species such as *Cytophaga, Sporocytophaga*, and *Polyangium* (Du et al., 2018), which naturally occur in soils, play a significant part in the breakdown of plant materials and the conversion of other organism's wastes that contain plant nutrients in an unusable form into a form that plants may utilize. Nitrogen is an important nutrient that bacteria cover into ammonium and nitrate ions. Nitrogen-fixing bacteria utilize the atmospheric nitrogen (N_2) and convert it into ammonia (NH_3) through cellular processes. Other organisms can utilize the ammonium ion (NH_4^+) and nitrate (NO_3-) that are produced when nitrifying bacteria combine ammonia with water (Rajan, 2021). According to Wang et al. (2017), organic fertilizers significantly increase the relative abundance of the *Proteobacteria phylum* and *Betaproteobacteria* and *Deltaproteobacteria classes* which are saprotrophic attributed and favored by nutrient-rich conditions with high carbon content. Thus, this accelerates microbial processes in the soil, which in turn enhances the availability of nutrients in a form readily absorbed by plants, suggesting that the microorganisms in bio-fertilizers contribute to restoring soils' natural nutrient cycle and increasing soil organic matter (Shaji et al., 2021).

Fungi are primarily responsible for the breakdown of cellulosic and hemicellulosic materials in aerobic and mesophilic environments. *Trichoderma, Penicillium, Aspergillus and Fusarium* are the most common fungal species in soils responsible for cellulose and hemicellulose degradation (Shaji et al., 2021). Mycorrhiza is the term known for the mutualistic association between fungi and plants that benefits both parties because the hyphae's high surface area to volume ratio enables fungi to easily reach and distribute important nutrients to the plants. In exchange, the fungi receive food from the plants in the form of glucose (Rajan, 2021). According to Wang et al. (2017), the relative abundance of Agaricomycetes fungi increased in paddy soil following the application of an organic fertilizer. Some Agaricomycetes species are referred to as ectomycorrhizal fungi because they mobilize nutrients from organic substrates to aid in the growth of plants. It is because the plants feed the fungi glucose, which enables the fungi to provide nutrients (which they receive) for promoting plant growth (Shaji et al., 2021).

Besides converting nutrients into usable forms, microorganisms also serve as a food supply for a huge percentage of the soil's macro-fauna. The nutrients that the bacteria produce is needed by macro-fauna. Earthworms, ants, and termites are examples of soil macrofauna (Body width more than 2 mm) that can significantly affect soil microbial

communities *via* soil structure, water flow, and nutrient dynamics (Fonte et al., 2012). In most ecosystems, earthworms are a significant part of the soil fauna communities and a dominant part of the macrofauna biomass (Bhadauria & Saxena, 2009). The earthworms dig through dirt, causing tunnel formation, and this enables other organisms to move around, further loosening the soil to improve its structure. Additionally, water enters these spaces, causing the soil to absorb water, which many species, including earthworms, require. There is far less runoff on the surface when water is absorbed by the soil (Rajan, 2021). Earthworm activity is beneficial because it enhances soil nutrient cycling by rapidly incorporating detritus into mineral soils. Mucus production is linked to water excretion in earthworm guts and boosts the activity of other beneficial soil bacteria. The production of organic matter comes afterward; hence, in terms of the short term, there are significant amounts of nutrients (N, P, K, and Ca) that are readily assimilated by plants in, for example, fresh cast depositions (Bhadauria & Saxena, 2009). Earthworm biomass and reproduction increased because organic wastes from organic-based fertilizers provide more available nutrients for enhancing earthworm growth and reproduction (Bhat et al., 2018). Thus, the number of earthworms increases with increasing excretion of castings. They also balance soil pH levels, retaining moisture, improving drainage, and controlling pathogens (Rajan, 2021).

Mendes et al. (2011) demonstrated that certain microbial groups associated with plant disease suppression in the soil microbiome (such as *Pseudomonas*, *Streptomyces*, *and Flavobacterium*, among others) are increased using bio-fertilizers. Measures that promote the activity of these soil-borne microbial groups to control plant diseases are beneficial. Furthermore, little is understood about the organic bio-fertilizer components directly producing disease-suppressive effects after application. The bio-control agent itself, the physical-chemical makeup of the compost substrate, and the microbial population that lives there are among the components (Tao et al., 2020).

The extensive enzymatic activities in soils affect their fertility and the ecological changes in their environment when waste organic materials occur in soils. Following the application of organic materials, sandy soils become richer in nutrients, besides improving soil nutrient sorption. Dehydrogenases are effective markers that impact the contamination of biotic soil components (Kalembasa & Symanowicz, 2012). Most soil enzyme activities, including the activity of α-glucosidase, increase when organic amendments are used. Although the network analysis among the soil ions is less complex with organic amendment, it was more complex between the soil ionome (Mineral nutrient composition of living organisms in soils) (Huang & Salt, 2016). It partly explains why the enzyme activities in soils amended with organic amendments are greater compared with chemical fertilization. Feng et al. (2016) opined that the α-glucosidase this analysis identifies stabilizes soil ion availability. Some soil enzymes activated by organic inputs improve soil productivity (Feng et al., 2016).

Merits of Bio-fertilizers

Bio-fertilizers are noted for enhancing plant and soil immunity, resulting in a boost in yield quality. Arjjumend et al. (2020) revealed that using microbial bio-fertilizers improves tolerance to several diseases and climatic changes apart from improving humus and enzymatic activities. Furthermore, the microorganisms in the bio-fertilizers improve fixing atmospheric N for crop use besides mitigating soil salinity (Arjjumend et al., 2020). Bio-fertilizers are reputed not only for their potential, cost-effective, environmentally friendly merits, and renewable source of plant nutrients for replacing chemical fertilizers, but they are also noted for their effectiveness in remediating soil pollution soil. Soil is a non-renewable resource, so it is essential to remediate polluted areas to avoid soil deterioration. Bio-fertilizers have been utilized to restore the fertility of chromium-polluted soils. The use of harmful metal-polluted water for irrigation has a significant impact on crop productivity. Bio-fertilizer-based remediation of polluted areas is a critical and significant method for environmental sustainability (Pandey & Singh, 2019). In addition, applying excessive amounts of chemical fertilizers causes the release of harmful greenhouse gases into the atmosphere and the eutrophication of our waterways (Sedlacek et al., 2020). Bio-fertilizers act on a synergistic or antagonistic reaction. As a result, the fermentation associated with this process transforms plant leaves and fruits inedible to pathogenic microbes (Arjjumend et al., 2020), thus making plants more resistant to diseases, drought, and frost. Using bio-fertilizers improves soil fertility and minimizes residues by 60%, making it more resistant to fungal diseases, drought, and infections (Arjjumend et al., 2020).

Food Waste Management Issues

Food waste management is becoming a growing concern worldwide (Du et al., 2018), especially in Brunei Darussalam. A potentially successful alternative strategy for valorizing food waste is converting the food waste stream into bio-fertilizers. It lowers the impact of waste disposal on the environment, increases revenue for the food processing sector, benefits agricultural farming systems, and decreases the excessive use of chemical fertilizers (Du et al., 2018). An effective strategy for food waste disposal, such as recycling or valorization, will significantly improve waste management issues in Brunei, especially food waste.

To effectively manage compostable wastes, organic fertilization using food waste should be taken into consideration because it improves crop yield and soil fertility. Utilizing fertilizers made from food waste can be a substitute for chemical fertilizers. These fertilizers can increase vegetable crops' yield and improve soil physical properties (Dlamini et al., 2021). It has been proven that continuous applications of organic fertilizer made from food waste enhance soil quality, increase crop yield, and even promote the growth of soil bacteria. Additionally, it was reported that applying food waste to soil enhances its physical, chemical, and biological characteristics and the growth and development of a

variety of crops, including rice, tomato, pak choi, and common bean (Kang et al., 2021). Direct application of food waste as fertilizer is not possible because it contains salt, which is the major deterrent to fertilization using food waste. Consequently, utilizing food waste to produce organic bio-fertilizers is more sustainable and efficient than conventional chemical fertilizers in agriculture (Kang et al., 2021)—moreover, increased soil and crop productivity guarantees sustainable food security.

Food waste disposal in landfills has several detrimental effects on the environment. Anaerobic degradation of food waste in landfills, for example, releases ammonia, methane, and volatile fatty acid emissions, as well as a high chemical oxygen demand (Fisgativa et al., 2016). Furthermore, there is a significant chance that nearby surface and groundwater will be contaminated by landfill leachates (Bolan et al., 2014). There have been promising research outcomes on converting food waste into value-added products such as bio-fertilizers, biofuels, and new and existing chemicals (Lin et al., 2013). Compared with chemical fertilizers, digested food waste fertilizers should have several environmental merits because high-quality energy is gained in the production process, and the nutrients are preserved within the effluent, the digestate (O'Connor et al., 2020). When food wastes are used to produce organic bio-fertilizers, the approach decreases food waste issues, reducing harmful greenhouse emissions and pollutants.

Given that food waste poses a challenge in Brunei Darussalam, utilizing food waste as the primary component of organic bio-fertilizers will undoubtedly benefit the country. The more food wastes are utilized to produce bio-fertilizers, the costs of importing chemical fertilizers will be reduced. Due to their effectiveness and low cost, organic bio-fertilizers made from food waste can boost agricultural production, increasing the country's income and GDP and encouraging the development of a sustainable economy. Additionally, excess food wastes can be transformed into biofuels and several bio-products aside from transforming them into organic fertilizers. It can further contribute to Brunei Darussalam's improvement in economic sustainability. The value of producing bio-fertilizer from food waste has been underestimated in contrast to biofuel and biochemical production. The total value of the worldwide market for bio-fertilizers was approximately $2.3 billion in 2020, and it is expected to grow to $3.28 billion by 2027 (Mahmud & Chong, 2021). By reducing the demand for synthetic chemical fertilizers and replacing them with bio-fertilizers derived from food waste, the environmental impact of food waste will significantly decrease and directly enhance food production (Du et al., 2018).

Limitations of Bio-fertilizers

According to a study by Du et al. (2018), one of the main concerns about bio-fertilizers safety is their heavy metal contents. Heavy metals, including cadmium, chromium, copper, lead, mercury, nickel, and zinc, are common in food wastes, particularly Municipal Solid

Wastes (Abdullah et al., 2016). With the potential for metals to accumulate in plant roots, reside in the soil, and contaminate groundwater, applying a bio-fertilizer with a high metal content could contaminate arable lands. Heavy metal concentrations in food processing waste and properly separated Organic Fraction of Municipal Solid Waste are typically lower (Govasmark et al., 2011). According to several studies on bio-fertilizers produced from food wastes (Rigby & Smith, 2013), the concentration of heavy metals is lower than the maximum permissible level. Nevertheless, heavy metals in bio-fertilizers must be controlled to comply with the increasingly stringent environmental protection rules.

A study by Patel et al. (2014) demonstrated that soils treated with bio-fertilizer had the lowest total nitrogen content compared to control (untreated soils) and soils treated with chemical fertilizers. The total phosphorus content in soils treated with chemical fertilizer was higher compared with bio-fertilizer. Both N and P are included as the three major macro-nutrients of NPK (Nitrogen, Phosphorus, and Potassium), which are needed for crop production, improving the quality and yield of crops. Thus, they are required by plants in large quantities (Zewide & Reta, 2021). It is why when these essential nutrients are deficient in soils, crop yields and soil fertility decline (Patel et al., 2014; Zewide & Reta, 2021) because of the decrease in the uptake of the essential macro-nutrients by crops (Carvajal-Muñoz & Carmona-García, 2012).

Bio-fertilizers naturally have lower macronutrient concentrations of NPK than commercialized chemical fertilizers (Mulyani et al., 2017). Therefore, agriculture requires larger bio-fertilizer volumes (Carvajal-Muñoz & Carmona-García, 2012). This implies that production costs will increase because of the additional workforce, transportation, and facilities required to maximize the effectiveness of bio-fertilizers for soil and crops in field applications.

Challenges and Suggestions

The practical part of waste management in Brunei Darussalam involves collecting and disposing of generated waste, including door-to-door pick-ups. Under the Department of Environment, Parks and Recreation (DEPR) and the Ministry of Development, a list of registered waste collectors is provided on their website (Sulaiman et al., 2023). However, from the listed registered waste collectors by DEPR, there is still no specific waste collection for the food waste category because they are categorized as general waste. Each type of waste, particularly biodegradable and non-biodegradable, should be separated before being disposed of. Since food waste is still a significant problem, Brunei Darussalam's policymakers should impose a stricter law for food waste disposal. In South Korea, for example, the government banned food disposal in landfills in 2005. The government was establishing the foundation for a national waste disposal system. Each Korean resident was obliged to dispose of their food waste responsibly and pay per weight beginning in 2013.

The government assesses fees for non-compliance. Residents were obligated to dispose of their food waste in a machine bin opened by a radio-frequency identification (RFID) chip. After scanning the chip, the lid opens, and the food waste is weighed by the bin, after which the residents are charged based on the weight (Marshall, 2022).

To obtain sufficient food waste from local households, commercial premises, and public areas in Brunei Darussalam, the relevant authorities, such as the Department of Environment, Parks and Recreation (JASTRe), could work with relevant agencies imposing or introduce food waste recycling or food waste disposal bins for public usage and food wastes pick-up collection services. The food wastes will then be collected and transferred to a composting facility and the Department of Agriculture and Agrifood Brunei for further processing to produce beneficial bio-products such as organic bio-fertilizers. When collected for recycling, organic waste can be put to several uses. In-vessel composting, open-air windrow composting, or anaerobic digestion (where organic matter is decomposed in a container without oxygen), contribute to producing useful products such as bio-fertilizers and biogas during composting. Moreover, the government of Brunei and relevant private sectors could educate the public on the significance of food waste issues, how it harms the environment, and how it contributes to climate change. Also, research grants could be provided to produce bio-fertilizers from food waste on a commercial scale. By raising public awareness, it will be easier to encourage locals to contribute to reducing Brunei Darussalam's issues with food waste in Brunei Darussalam. Food waste can be used to generate environmentally friendly jobs, establish a renewable resource, provide security of product supply, address public concerns about environmental issues such as climate change, encourage research into more innovative technologies, and create fertilizers and products for the agricultural and industrial sectors (Xiong et al., 2019). Valorization is necessary as food waste is indeed unavoidable.

CONCLUSION

Bio-fertilizers have significant positive effects on soil productivity (soil physical, chemical and biological properties), which consequently improves crop yield and productivity. In soil physical properties, the presence of fungi in organic bio-fertilizer, when added to soil, helps boost plant performance and development. As for soil chemical properties, after being applied with bio-fertilizer, there will be increased absorption of N, P, and K nutrients in the soil, allowing crops to yield more and grow quicker. In terms of soil biological properties, the presence of beneficial micro and macro-organisms in soil increases when bio-fertilizer is applied, which maintains the overall fertility and structure of the soil. When applied with bio-fertilizer, there will be an increase in soil microbiome containing plant disease suppression, which may control plant diseases. Additionally, the soil becomes more vigorous and stable due to increased enzymatic activity. However, bio-fertilizers

have a few limitations, including heavy metal content, insufficiency in macro-nutrient requirements, and large quantities required for field application. These limitations can be further researched to improve the accessibility and quality of bio-fertilizer production. Currently, the implications of organic bio-fertilizers are still a new concept in Brunei's agriculture sector, which might be due to insufficient knowledge, awareness, and confidence in bio-fertilizer use. Considering that food wastes possess many benefits (as previously outlined) and commonly have been used directly as compost, there are not many research studies conducted in Brunei Darussalam on the use of food wastes to produce organic bio-fertilizers. It needs to be tested in the laboratory and in the field in Brunei Darussalam on the effects of organic bio-fertilizers on soil and crop productivity to address the research gaps of application of organic bio-fertilizers production (produced from food wastes) to soils.

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Application of *Eucheuma spinosum* for Enhancing the Nutritional Value of Tempeh

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ABSTRACT

The research aims to enhance the nutritional value of tempeh, a traditional fermented food, by incorporating *Eucheuma spinosum*. Different concentrations of *E. spinosum* seaweed were added to tempeh to investigate its effect on nutritional composition and sensory attributes. With a 30% *E. spinosum* sp. addition, tempeh exhibited the highest dietary fiber content (17.99%) and significant changes in protein, carbohydrates, water, fat, and ash. However, no significant differences were observed in the hedonic test, indicating similar sensory preferences among the tempeh variations. Among the different concentrations tested, tempeh with a 20% *E. spinosum* addition was preferred in terms of sensory attributes. The findings suggest that adding *E. spinosum* seaweed can effectively increase the dietary fiber content of tempeh without compromising its overall acceptability. The research highlights the potential of *E. spinosum* seaweed as a supplementary ingredient for enhancing the nutritional value of traditional foods like tempeh.

Keywords: Dietary fiber, *Eucheuma spinosum*, nutritional value, tempeh

INTRODUCTION

Tempeh, a popular food in Indonesia, has consistently increased consumption. In 2022, the frequency consumption of tempeh per individual reached 4–5 times per week (Astuti et al., 2023). Besides its delicious taste and affordability, tempeh is highly regarded for its nutritional value. Made from soybeans fermented with the help of *Rhizopus* sp., it is rich in vegetable protein, vitamins, minerals, isoflavones, and antioxidants. The fermentation process

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enhances its digestibility because the enzyme activity hydrolysis complex compounds into simpler ones, allowing for easier absorption in the body.

Tempeh is a nutrient-rich food containing various beneficial elements for body health. Every 100 g of tempeh provides 17.40 g of protein, 8.23 g of fat, 1.33 ash, 7.30 g of carbohydrate, and 2.5 g of fiber (Rizal et al., 2022; Tan et al., 2024). While tempeh is protein-rich, it has a relatively low fiber content. Fiber, an equally essential component for the body, is a complex carbohydrate usually found in plants. Its functions include optimizing food absorption, promoting intestinal health, aiding digestion, regulating blood sugar, reducing cholesterol and cardiovascular risks, preventing gastrointestinal issues, maintaining body weight, and strengthening the immune system (Mîndrican et al., 2022). Insufficient fiber intake can lead to constipation, unstable blood sugar levels, weight gain, fatigue, weakened immunity, and heightened inflammation risks (Tanes et al., 2021).

Seaweed is a nutrient-rich food source due to its abundant minerals, vitamins, essential minerals, dietary fiber, protein, essential amino acids, and polyphenols, which exhibit antioxidant and anti-inflammatory properties. Its high dietary fiber and low fat make it a healthy, nutritious, and low-calorie food, widely used as a substitute in various food products (Lomartire et al., 2021). Sofiana et al. (2020) found that seaweed has higher levels of soluble fiber compared to land plants like beans, fruits, and cereals, which primarily contain insoluble fiber. Seaweed typically contains 25%–70% total dietary fiber and 50%–80% dietary fiber. However, the fiber content may vary depending on the specific seaweed type, such as *Laminaria* sp. (36% food fiber), *C. racemosa* (33%–40%), *Ulva lactuca* (38%–43%), *E. cottonii* (15.5%) (Praveen et al., 2019), and *E. spinosum* (21.5%) (Diharmi et al., 2019).

Eucheuma spinosum, a type of red algae, is being utilized as an additional source of income by seaweed farmers. Its carrageenan content has applications in various industries, including cosmetics, pharmaceuticals, textiles, and food (Kurniawan & Managi, 2018). Studies regarding the application of *E. spinosum* are still limited. Sagita et al. (2023) applied *E. spinosum* in pudding to improve the texture and be organoleptically acceptable. Iwada et al. (2021) stated that *E. spinosum* could improve the texture and increase the fiber and protein content in local Riau Regency food called "pekdos." There has yet to be any research regarding the application of *E. spinosum* in making tempeh, so this is the first time this research has been performed. Therefore, the research aimed to enhance the nutritional value of tempeh by incorporating *E. spinosum* seaweed.

METHODS

Materials

The study utilized half-dried *E. spinosum* sourced from seaweed farmers in Southern Lampung, Indonesia, as well as soybeans and Raprima brand tempeh yeast obtained from Bandarjo Traditional Market, Semarang, Indonesia.

Seaweed Preparation

The initial step involved preparing the *E. spinosum* seaweed by washing it three times to remove any remaining salt and dirt. Subsequently, the soaking process followed the method used in the research by Siah et al. (2014), employing a ratio of 1:15 (dry seaweed to water) and a soaking time of 117 min. The research required 200 g of dried seaweed using 3,000 ml of water. The next stage involved blanching, which aimed to deactivate the enzymes in the seaweed (Xiao et al., 2017) and was accomplished by placing the seaweed in boiling water for 4 min before draining and allowing it to cool. Finally, the seaweed underwent size reduction by blending until it achieved a slightly smooth consistency.

Tempeh Production with *Eucheuma spinosum* Seaweed Addition

The tempeh-making process follows the guidelines set by the Rizal et al. (2022) with some modifications. The modification was on boiled time, where a traditional processor just boiled once, whereas in the study, boiling was done two times to clean the seeds. The initial step involved sorting and washing the soybean seeds under running water. The soybeans were then boiled for 30 min using a 1:2 ratio of soybeans to water. After boiling, the soybeans were soaked overnight using the remaining cooking water to create an acidic environment. The following day, the soybean skin was peeled off by submerging the soybeans in water and squeezing them until the skin separated, leaving skinless pieces. The soybean pieces were washed again and steamed for 30 min until cooked. Once cooked, the soybeans were lifted and placed in a wide container with a perforated bottom to allow water to drain. The next step involved adding 1 g of tempeh yeast per kilogram of soybeans and thoroughly mixing until evenly distributed.

Subsequently, the seaweed substitution was blended into concentrations of 0% (K), 10% (A), 20% (B), and 30% (C). The seaweed was thoroughly mixed with the other ingredients, wrapped in plastic, and sealed. Toothpick holes were then made approximately 1 cm apart for gas space movement. The raw tempeh was left to ferment on shelves with perforated bottoms. Typically, mold growth occurs within 48 h at room temperature; after this period, the tempeh was ready for analysis.

Crude Protein Content

The protein content was analyzed using the Kjeldahl method (Association of Official Analytical Chemists, 1995), consisting of three main stages: destruction, distillation, and titration. The analysis started by preparing a 0.5 g sample, which was then combined with 0.5 g of selenium catalyst and 10 ml of concentrated sulfuric acid (H_2SO_4) (Sigma Aldrich, USA). The destruction stage was carried out until the solution became clear green. Subsequently, distillation was conducted by adding 100 ml of distilled water, 40 ml of 45% NaOH, 5 ml of 4% H_3BO_3 trap, and two drops of Methyl Red and Methylene

Blue indicators (Sigma Aldrich, USA). The distillation results were titrated using 0.1 N Hydrogen chloride until the solution changed to purple.

$$\text{Crude Protein Content (\%)} = \frac{(mL\ HCl\ sample - mL\ HCl\ blanko) \times N\ HCl \times 6.25 \times 14}{mg\ sample} \times 100\%$$

Crude Fat Content

First, 10 ml of H_2SO_4 (SigmaAldrich, USA) was placed into a Gerber tube. Then, 11 ml of the sample was poured into the same Gerber tube, and 1 ml of isoamyl alcohol was added. The Gerber tube was capped tightly and flipped to thoroughly mix the solution. Next, the tube was centrifuged for 4 min and placed in a water bath at 60°C–63°C for 5 min. The fat content was then determined by reading the results (Association of Official Analytical Chemists, 1995).

Total Moisture Content

An empty cup was dried in an oven at 105°C for 15 min and then weighed to determine the water content. Next, 5 g of tempeh samples were placed in the cup and dried in an oven at 105°C for 6 h or until a constant weight was achieved. The cup was then placed in a desiccator for 30 min before being weighed again. The water content can be calculated by dividing the weight of the sample after drying with the initial weight of the sample multiplied by 100% (Association of Official Analytical Chemists, 1995).

Total Ash Content

A clean cup was heated in a furnace at 400°C for 1 h to determine the ash content (Association of Official Analytical Chemists, 1995). The cup was carefully removed from the furnace using pliers and allowed to cool in a desiccator for 1 h. The weight of the cup after cooling (a) was recorded. A 5 g tempeh sample with a known weight (b) was placed into the porcelain cup. The cup with the sample was then placed back into the furnace for 1 h. Afterward, the cup was taken out of the furnace and allowed to cool in a desiccator. Finally, the cup's weight with the sample residue (c) was recorded. The ash content can be calculated by dividing the difference between c and a with b multiplied by 100%.

Total Carbohydrate Content

The carbohydrate analysis procedure follows the carbohydrate content analysis method (Association of Official Analytical Chemists, 1995). Carbohydrate content differs from 100% minus the total water, ash, protein, and fat content.

Total Dietary Fiber Content

The Association of Official Analytical Chemists (1995) measured dietary fiber. A sample weighing 0.5 g was placed in an Erlenmeyer glass and mixed with 50 ml of phosphate buffer and 0.1 ml of alpha-amylase enzyme and heated on a magnetic stirrer at 100°C for 30 min with occasional stirring. After cooling, 20 ml of distilled water, 5 ml of 1N HCl, and 1% pepsin enzyme (1 ml) were added (Sigma Aldrich, USA). The mixture was heated again at 100°C for 30 min. Then, 5 ml of 1N NaOH and 0.1 ml of beta-amylase enzyme were added. The Erlenmeyer glass was sealed and heated at 100°C for 1 h. The solution was then cooled and filtered using pre-weighed constant filter paper, and the residue was washed with 10 ml of ethanol (twice) and 10 ml of acetone (twice). The filtrate was dried overnight in an oven at 105°C, cooled in a desiccator, and weighed to determine the insoluble food fiber. The filtrate was weighed to 100 ml, and 400 ml of 95% ethanol was added. After settling for 1 h, the solution was filtered, washed with 10 ml of ethanol (twice) and 10 ml of acetone (twice), and dried overnight in an oven at 105°C. The final weight was obtained by weighing the dried sample in a desiccator, representing the dissolved fiber. The total dietary fiber was calculated by adding insoluble fiber and soluble fiber.

Sensory Acceptance

The sensory acceptance was analyzed using the hedonic test. The hedonic aimed to determine consumer preference test was conducted following the methodology outlined by (García-Gomez et al., 2022). A total of 35 semi-trained panelists participated in the evaluation of tempeh substituted with seaweed. The panelists assessed the level of acceptance for appearance, texture, flavor, and aroma using a 9-point scale, where 1 represented "very strongly dislike" and 9 represented "very strongly like." Panelists were instructed to cleanse their mouths with water to neutralize lingering tastes and ensure the taste test's accuracy.

Fatty Acid Analysis

Fatty acid analysis was performed using a Gas Chromatography-Mass Spectrometer (GCMS-QP2010S Shimadzu, Japan) based on Purnamayati and Kurniasih (2020). It used an Rtx column with dimensions of 30 m x 0.25 mm and 0.25 µm film thickness. A total of 1 µl of sample was injected into a column whose temperature had been set to 70°C with a temperature increase of 5°C per minute. The temperature increase is regulated until it reaches 300°C. Fatty acid analysis used a detector set to a temperature of 250°C with a pressure to separate methyl esters of 13.7 kPa—the fatty acid data obtained then identified using the mass spectra database in the Willey 229 dictionary.

Microstructure with Scanning Electron Microscopy

Microstructural analysis was performed using scanning electron microscopy (SEM JEOL JSM 5310 LV, USA), based on Kustyawati et al. (2018). Several samples were placed in the tub and then coated with gold using a generator equipped with a vacuum pump for 20 min. The microstructure was observed at 5000x magnification.

Statistical Analysis

The study used a completely randomized design with triplicates. The data were then analyzed using Analysis of Variance (ANOVA) and processed with the IBM SPSS Statistics application (version 23). Significant differences were further tested using the Tukey test.

RESULTS AND DISCUSSION

Table 1
Chemical characteristics of Eucheuma spinosum *tempeh*

Sample	Crude protein content (%)	Crude fat content (%)	Total moisture content (%)	Total ash content (%)	Total carbohydrate content (%)	Total dietary fiber content (g/100g Carbohydrate)
K	20.19 ± 0.67^{d}	7.38 ± 0.35^{d}	64.93 ± 0.24^{a}	0.94 ± 0.01^{a}	6.55 ± 0.06^{a}	10.87 ± 0.06^{a}
A	16.71 ± 0.24^{c}	6.61 ± 0.13^{c}	67.60 ± 0.08^{b}	1.02 ± 0.01^{b}	8.04 ± 0.17^{b}	14.40 ± 0.34^{b}
B	14.60 ± 0.74^{b}	5.62 ± 0.13^{b}	69.24 ± 0.06^{c}	1.08 ± 0.01^{c}	9.44 ± 0.19^{bc}	17.27 ± 0.07^{c}
C	11.57 ± 0.18^{a}	4.34 ± 0.20^{a}	74.74 ± 0.46^{d}	1.22 ± 0.01^{d}	10.12 ± 0.86^{cd}	17.99 ± 0.17^{c}

Note. Data in triplications ± deviation standard. Data followed by a different number in the same column show a significant difference (p <0.05). K= tempeh with no *E. spinosum*; A= tempeh with *E. spinosum* 10%; B= tempeh with *E. spinosum* 20%; C= tempeh with *E. spinosum* 30%

Crude Protein Content

Protein is a source of amino acids with C, H, and O elements, which are not present in fats or carbohydrates. While fishery products like fish and shrimp are known to have relatively high protein content, seaweed differs in its protein content. Biancarosa et al. (2017) stated that the protein content in seaweed can vary depending on the species. The red algae species *P. dioca* has a relatively high protein content of 20.6%, whereas the brown algae species *A. nodosum* tends to have a lower percent protein content of 3%. *E. spinosum,* which belongs to the red algae category, has a very low protein content compared to *P. Dioca*, which has only 1.67% protein content. Generally, red algae have higher protein content compared to green and brown algae. The protein concentrations in red algae typically range from 5.2% to 40% (dry weight), as Vega et al. (2020) noted. However, the digestibility of protein in algae is lower than animal protein. It may be attributed to the high concentration of dietary fiber present in algae, which limits the accessibility of digestive and proteolytic enzymes

to the protein. The presence of dietary fiber in seaweed can affect protein digestion and absorption in the human body.

Tempeh is a food product known for its high protein content. The primary source of protein in tempeh is soybeans, which have one of the highest protein levels among crops. Soybeans have a protein content of approximately 34.95% (Tahir et al., 2018). However, during the cooking process of tempeh, which involves heat treatment, the protein content decreases to around 20.19%. The decrease in protein content can also be influenced by other additives used in the tempeh-making process.

In the study mentioned, adding seaweed to tempeh significantly affected the final product's protein content (Table 1). Tempeh had the highest protein content of 20.19% without any additional seaweed. On the other hand, tempeh, with the highest addition of *E. spinosum* seaweed, at 30% concentration, had the lowest protein content of 11.57%. Tempeh with 10% and 20% seaweed additions had 16.71% and 14.60% protein levels, respectively. These results were higher than Cornelia and Kartika (2022), who added 50% seaweed flour to tempeh and achieved protein levels of 12.36%. The decrease in protein levels in tempeh with seaweed addition can be attributed to the relatively low protein content. The protein content of seaweed was influenced by factors such as organic compounds in the marine water, current, and salinity of the water where seaweed grew. Fresh seaweed mainly consists of water, accounting for about 80%–90% of its composition, while the protein and fat content is relatively small, as noted by (Sofiana et al., 2020).

Crude Fat Content

Seaweed generally tends to have a low-fat content, and the fatty acid composition of seaweed is significant for health. Seaweed fats are rich in omega-3 and omega-6 fatty acids, which play crucial roles in various bodily functions, such as forming brain tissue membranes, nerves, eye retina, blood plasma, and reproductive organs. Seaweed contains omega-3 fatty acids such as eicosatetraenoic acid (EPA), which amounts to around 24% of the fatty acids in *Eucheuma* sp. (Mohamed et al., 2012). In line with the research of Vega et al. (2020), all types of algae have a low-fat content; however, the fat content is of high quality in terms of nutritional value. Belghit et al. (2017) state that red algae have a higher concentration of amino acids such as glutamate, ornithine, citrulline, serine, and glycine than green and brown algae. Amino acids derived from red algae protein digestion can be affected by various antinutritional agents such as polyphenols, polysaccharides, and glycoproteins.

Soybean tempeh has low fat, dietary fiber, and carbohydrate content (Romulo & Surya, 2021). Compared to beef, tempeh generally contains higher amounts of nutrients, except for fat. During the fermentation process of tempeh, there is an increase in the unsaturation of fats, leading to an increase in polyunsaturated fatty acids (PUFAs), such as oleic and

linolenic acids (Damanik et al., 2018). According to Table 1, it can be observed that tempeh, without the addition of Spinosum seaweed, had the highest fat content of 7.38%, and the addition of Spinosum seaweed significantly reduced it. A concentration of 10% Spinosum seaweed resulted in a fat content of 6.61%, 20% *E. spinosum* had a fat content of 5.62%, and 30% Spinosum had the lowest fat content of 4.34%. The higher concentrations of *E. spinosum* seaweed led to lower fat content in the tempeh. The fat content from adding seaweed in this study was lower than in Cornelia and Kartika (2022), who used *K. alvarezii* seaweed, resulting in a fat content of 14.68% in tempeh. The low-fat content in tempeh, with the addition of *E. spinosum* seaweed, makes it a suitable and healthy alternative for consumption. Furthermore, it can be included in a balanced diet menu, contributing to a well-rounded and nutritious meal plan (Vital et al., 2018).

Total Moisture Content

Seaweed is known to have a high-water content, typically around 90% in its fresh form (Djaeni & Sari, 2015). However, some types of seaweed are distributed in dry form. The use of spinosum seaweed in this study is one such example. According to research by Ahmad et al. (2012), the water content in fresh seaweed generally ranges from 76% to 96%. Different types of seaweed may have varying water contents, with *K. alvarezii* typically having a lower water content compared to Caulerpa and Laurencia. Tempeh itself also has a relatively high-water content. Without the addition of Spinosum seaweed, the water content in tempeh is reported to be 64.93% (Table 1). As the concentration of Spinosum seaweed increases, the water content in tempeh also increases. The addition of 10% Spinosum seaweed resulted in a water content of 67.60%, 20% Spinosum had a water content of 69.24%, and 30% *E. spinosum* had the highest water content of 74.74%. The increase in water content in tempeh is attributed to the inclusion of wet seaweed, which contributes to the overall water content of the tempeh. It is worth noting that this study reports lower water content compared to the research conducted by Yulia et al. (2019), who stated a high-water content of 82.96% in tempeh. The difference in water content could be attributed to variations in processing methods, such as boiling, steaming, soaking, and adding ingredients with high water content. High water content in tempeh can create favorable conditions for the growth of microorganisms, including bacteria, which can potentially lead to food spoilage, as mentioned by Kustyawati et al. (2018).

Total Ash Content

Ash content refers to the total amount of minerals or inorganic components in food. Although present in small quantities, these minerals are very useful (Afify et al., 2017). The ash content in seaweed typically ranges from 17.3% to 44.5%, varying among species and depending on the habitat of the seaweed (Bikker et al., 2020). The high ash content

can be attributed to the passive absorption of ions through charged polysaccharides in the seaweed cell wall, active absorption, and residual salts associated with the biomass (Olsson et al., 2020). In the case of Spinosum seaweed, it has an ash content of 22.82%.

In this study, the ash content in tempeh was relatively low. Without adding *Spinosum* sp, tempeh had the lowest ash content at 0.94% (Table 1). With the addition of Spinosum, the ash content increased. The ash content was 1.02% with 10% addition, 1.08% with 20% *E. spinosum* addition, and the highest ash content of 1.22% was observed with 30% Spinosum addition. The data indicates that higher concentrations of *E. spinosum* added result in higher ash content in tempeh. The ash content in this study is lower than that in the research conducted by Tahir et al. (2018), where tempeh had an ash content of 4.12%. The difference can be attributed to the processing of soybeans, as raw soybeans have a higher ash content of 4.48%, which decreases during processing into tofu (0.8%) and tempeh (0.78%). The decrease in ash content during processing is due to the loss of solids during soaking and cooking (Damanik et al., 2018). Adding *E. spinosum* seaweed to tempeh increases ash content due to the high mineral content present in seaweed.

Total Carbohydrate Content

Seaweed content is high in polysaccharide compounds. Polysaccharides are extensively used in the food industry as thickeners and gelling agents. Incorporating seaweed into food products can enhance their functionality and texture (Rioux & Turgeon, 2015). Polysaccharides derived from seaweed, such as agar, carrageenan, and alginate, have been widely commercialized in food, biotechnology, biomedicine, and textiles. The carbohydrate content of seaweed has gained attention as a functional food ingredient, and these carbohydrates exhibit various biological activities, including anti-implantation, anticoagulant, antioxidant, anti-proliferative, and immunostimulant properties. Moreover, these polysaccharides are also considered dietary fiber, and the consumption of dietary fiber has been associated with positive impacts on human health (Lafarga et al., 2020). The carbohydrate content contained in *E. spinosum* seaweed is 40%.

Total carbohydrates are carbohydrate complexes of starch, sugar and dietary fiber (Compaore-Sereme et al., 2022). Tempeh is known for its high nutritional composition, and its carbohydrate content typically ranges from 6 to 10% (Rizal et al., 2022). The fermentation process involved in tempeh production enhances the digestion of soy carbohydrates (Syida et al., 2018). In the case of tempeh with the addition of *Spinosum* sp. seaweed, differences in carbohydrate levels were observed in the carbohydrate levels. Without adding *E. spinosum*, tempeh had a carbohydrate content of 6.55%, which increased with the addition of *E. spinosum* (Table 1). The concentrations of 10%, 20%, and 30% *E. spinosum* resulted in carbohydrate levels of 8.04%, 9.44%, and 10.12%, respectively. The increase in carbohydrate content can be attributed to the enzyme activity produced by the

microbes involved in the fermentation process. These enzymes have catabolic properties, meaning they can break down complex compounds into simpler ones easily digested. The microbes utilize the chemical components in the substrate as an energy source for their growth and reproduction. The increase in carbohydrates was due to the addition of *E. spinosum*. Sofiana et al. (2020) stated that fresh *E. spinosum* contains 6% carbohydrates on a wet basis or 41.18% on a dry basis. Based on the analysis, it is evident that the highest carbohydrate content was achieved by adding 30% Spinosum, while tempeh had the lowest carbohydrate content without adding *E. spinosum*. The data indicates that the concentration of *E. spinosum* added influences the carbohydrate levels in *E. spinosum* tempeh, with higher concentrations resulting in higher carbohydrate content.

Total Dietary Fiber Content

The addition of *E. spinosum* seaweed concentration significantly affects the fiber content of the produced tempeh, as described in Table 1. Dietary fiber is crucial for the digestive system, influencing water absorption. Dietary fiber can help lower blood sugar, insulin, triglyceride, and cholesterol levels. There are two types of dietary fiber: insoluble and soluble (Raposo et al., 2016). Insoluble fiber, like cellulose, hemicellulose, and lignin, cannot be digested and is insoluble in water. Soluble fiber, such as pectin, Arabic gum, biological polysaccharides, and synthetic polysaccharides, cannot be digested but mostly dissolves in water (Yang et al., 2017). The consumption of high-fiber foods, including seaweed, has positive effects on the body, reducing the risk of colon cancer, constipation, hypercholesterolemia, obesity, and diabetes (Lafarga et al., 2020).

Dietary fiber is part of carbohydrates that cannot be digested in the small intestine (Compaore-Sereme et al., 2022). Therefore, the results show that dietary fiber is part of the total carbohydrates. The dietary fiber content of ordinary tempeh is 10.87% and increases with the addition of *E. spinosum* seaweed. The higher the concentration of seaweed added, the higher the dietary fiber content in the tempeh. For example, adding 30% *E. spinosum* resulted in the highest dietary fiber content of 17.99%. The finding aligns with a study by Fauzi et al. (2023), which demonstrated that adding seaweed to rice improved the dietary fiber content of seaweed rice. Furthermore, the dietary fiber content in the research was higher than in another study by Cornelia and Kartika (2022), who added 50% seaweed to tempeh and achieved a dietary fiber content of 9.88%. Seaweed is rich in soluble fiber, such as agar, alginate, and carrageenan, constituting about 50%–70% of the fiber content in seaweed (Stévant et al., 2020). These soluble fibers can help reduce cholesterol levels in the blood and lower the risk of heart disease. Additionally, seaweed contains pigments, often referred to as "green food," which serve as functional food or supplements rich in natural fiber nutrients. These pigments have various health benefits, including their potential as anti-cancer agents and detoxifiers and aid in wound healing and digestion (Okolie et al.,

2017). Seaweed also contains bioactive compounds that contribute to its health benefits. These compounds are formed through secondary metabolic processes, including alkaloids, flavonoids, tannins, terpenoids, and steroids (Safia et al., 2020). These bioactive components further enhance the nutritional and therapeutic value of seaweed.

Sensory Acceptance of *Eucheuma spinosum* Tempeh

Based on the data in Table 2, the overall evaluation of the tempeh samples' appearance, aroma, texture, and flavor indicated a positive response, falling within the "like" category.

Table 2
Sensory acceptance of Eucheuma spinosum *tempeh*

Sample	Appearance	Aroma	Texture	Flavor
K	7.48 ± 1.28[a]	6.81 ± 1.64[a]	6.81 ± 1.35[a]	7.65 ± 1.01[d]
A	7.06 ± 1.48[a]	6.74 ± 1.69[a]	6.68 ± 1.62[a]	6.69 ± 1.46[b]
B	7.29 ± 1.29[a]	6.84 ± 1.26[a]	6.97 ± 1.49[a]	7.26 ± 0.96[c]
C	6.81 ± 1.44[a]	6.19 ± 1.72[a]	6.13 ± 1.45[a]	6.03 ± 1.40[a]

Note. Data in triplications ± deviation standard. Data followed by a different number in the same column show a significant difference (p <0.05). K= tempeh with no *E. spinosum*; A= tempeh with *E. spinosum* 10%; B= tempeh with *E. spinosum* 20%; C= tempeh with *E. spinosum* 30%

The tempeh without adding Spinosum was preferred in terms of appearance, as it resembled regular tempeh (Table 2). The most favorable aroma was found in tempeh with a 20% *E. spinosum* addition, as it retained the typical tempeh aroma while also having a distinctive aroma. The texture of tempeh with a 20% *E. spinosum* concentration was preferred due to its denser and chewier texture. The most preferred flavor was observed in tempeh without *E. spinosum* addition, followed by tempeh with a 20% *E. spinosum* addition. The 20% seaweed tempeh had a more desirable flavor compared to the 10% *E. spinosum* tempeh, which was perceived as less flavorful. It should be noted that the appearance, aroma, texture, and flavor of the tempeh can vary depending on the amount of *E. spinosum* added. Tempeh with a 30% *E. spinosum* addition tended to be less favorable (although still falling within the "like" category) compared to lower additions. The attribute was a less attractive appearance, less dominant soybean tempeh aroma, a mushier texture, and less desirable flavor. Despite these differences, the overall liking level of tempeh products with different *E. spinosum* additions was relatively similar as they all fell within the "like" category.

Fatty Acid of *Eucheuma spinosum* Tempeh

Table 3
Fatty acid of Eucheuma spinosum *tempeh*

Type	Parameters	K	A	B	C
Saturated fatty acid	Myristic acid	0.33	0.28	0.29	0.33
	Palmitic acid	12.76	12.3	12.55	12.24
	Heptadecanoic acid	0.72	0.67	0.66	0.27
	Arachidic acid	0.61	0.58	0.52	0.45
	heneicosanoate acid	0.25	0.18	0.25	0.25
Unsaturated fatty acid	Palmitoleic acid	0.12	0.11	0.11	0.76
	Linolelaidate acid	25.64	25.03	27.09	26.8
	Linolenic acid	49.1	50.38	48.36	48.74
	Eicosenoic acid	5.00	5.14	4.42	4.49
	Docosahexaenoic acid	0.13	0.16	0.14	0.13

Note. K= tempeh with no *E. spinosum*; A= tempeh with *E. spinosum* 10%; B= tempeh with *E. spinosum* 20%; C= tempeh with *E. spinosum* 30%

The most dominant saturated fatty acid was palmitic acid, followed by heptadecanoic and arachidic acid (Table 3). Tempeh without the addition of *E. spinosum* seaweed had more palmitic acid compared to tempeh with the addition of *E. spinosum*. Palmitate acid is a saturated fatty acid found in food and synthesized endogenously. Although often thought to be involved in adult chronic disease, palmitic acid is an essential component of membrane lipids, secretion, and transport, which plays a role in forming proteins and molecules (Innis, 2016). Heptadecanoic acid and Arachidic acid have the highest values for tempeh without adding seaweed, while the lowest was for tempeh with a 30% *E. spinosum* addition. The heptadecanoic acid in the control tempeh had quite a lot of differences from the tempeh with the addition of 30% spinosum. The control tempeh had a Heptadecanoic acid value of 0.72%, while tempeh, adding 30% spinosum, was 0.27%. Arachidic acid in tempeh decreased with the addition of spinosum seaweed to tempeh.

An analysis of unsaturated fatty acids in tempeh shows that the most dominant is linolenic acid, followed by linolelaidate acid and eicosenoic acid. Damanik et al. (2018) stated that soybeans are a source of linoleic, oleic, and linolenic acids. Soybeans usually contain 25-64% linolenic acid, 1-12% linoleic acid, and 11-16% oleic acid. The linolenic acid in tempeh in this study was lower than in soybeans. It is due to the processing of tempeh, such as soaking, boiling, and fermenting. Heat heating can convert fatty acid components into volatile compounds such as aldehydes, ketones, acids, and hydrocarbons. The compounds evaporate if given heat treatment, reducing fatty acids (Nguju et al., 2018). The highest linolenic acid content in this study was in tempeh, with the addition of 10% spinosum of 50.38%.

In comparison, the highest linolelaidate acid was found in Spinosum tempeh 20%, namely 27.09%. Eicosenoic acid and Docosahexaenoic acid had the highest values in tempeh with the addition of 10% *E. spinosum*. Linolenic acid has many benefits for the body. Linolenic acid is more effective than linoleic acid for lowering triglycerides in the blood. The linolenic acid is usually most commonly found in fish oil; the linolenic acid content in fish oil is very high, so it needs dosage and doctor's supervision to consume it. Linolenic acid is not as high as fish oil in tempeh, so it can be more freely consumed in large quantities without reducing its benefits (Klek, 2016).

Microstructure of *Eucheuma spinosum* Tempeh

SEM (Scanning Electron Microscopy) is an analysis used to determine microscopic magnification by showing detailed visual images of particles with high quality and spatial resolution of the appearance of a material (Akhtar et al., 2018). The results of SEM analysis with the addition *of E. spinosum* to tempeh are shown in Figure 1.

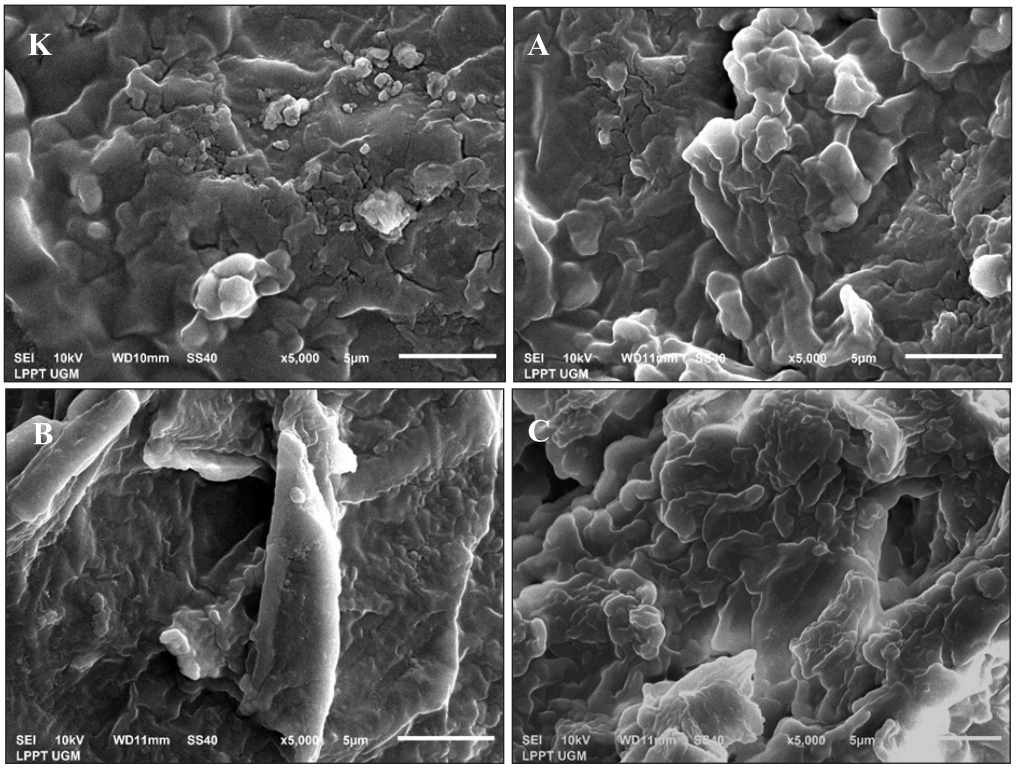

Figure 1. Microstructure of *E. Spinosum* Tempeh. K= tempeh with no *E. spinosum*; A= tempeh with *E. spinosum* 10%; B= tempeh with *E. spinosum* 20%; C= tempeh with *E. spinosum* 30%

The results show that the higher the addition of the *E. spinosum* concentration, the more in amount and more significant the cavities formed between the tissues. The phenomena are caused by adding *E. spinosum* seaweed, which causes the formed tempeh to have more cavities than regular tempeh. Kustyawati et al. (2014) stated that tempeh, without treatment, usually has smaller inter-tissue cavities than treated tempeh. The cavity will affect the texture of a product; the smaller the cavity, the denser the texture will be.

CONCLUSION

The research showed that the addition of *E. spinosum* seaweed to tempeh has a significant impact on its nutritional composition. With a 30% addition, tempeh has the highest dietary fiber content at 17.99%, while the protein content decreases to 11.57%. The fat content is low at 4.34%, and the water content increases to 74.74%. The carbohydrate content increases to 10.12%, and the ash content increases to 1.22%. The addition of *E. spinosum* seaweed significantly affects the levels of food fiber, protein, carbohydrates, water, fat, and ash in tempeh. However, no significant differences were observed in the hedonic test, indicating that the overall liking for tempeh with different *E. spinosum* additions was similar. Furthermore, among the different concentrations of *E. spinosum* seaweed added to tempeh, the 20% addition was preferred in terms of sensory attributes compared to the 10% and 30% additions. It suggests that the 20% Spinosum addition balanced sensory appeal and nutritional benefits. Overall, incorporating Spinosum seaweed into tempeh offers the potential for enhancing its nutritional composition, particularly in terms of fiber content, without compromising sensory acceptance.

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Effect of Seed Priming on Growth of *Andrographis paniculata* and Production of Andrographolide Compound

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ABSTRACT

Andrographis paniculata (Burm. f.) Nees is one of the high-demand medicinal plants and can mostly be found in Asian countries. The plant consists of a few active compounds, mainly andrographolide. This compound has various medicinal properties, including anti-cancer, anti-viral, anti-inflammatory, and anti-malaria. It has been used to treat fever, diabetes, and influenza. The latest research identified an anti-SARS-CoV-2 activity that can be used to treat Covid-19. However, this plant has a low germination rate, which affects its production yield. Thus, seed priming improved germination rate, seedling and plant growth. Osmopriming with polyethylene glycol (PEG) at -0.4 MPa, hormopriming with gibberellic acid at 100 ppm, and control were used in this study and evaluated their effect on plant growth and production of andrographolide compound. PEG treatment significantly produced the highest plant height, number of leaves and branches, leaf area, stem girth, fresh weight of shoot and root, and dry weight of shoot and root compared to the control. This study revealed that seed priming, especially osmopriming, has a high potential to enhance plant growth.

Keywords: *Andrographis paniculata*, *Hempedu Bumi*, seed germination, seed priming, seed treatment

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INTRODUCTION

The application of medicinal plants as an alternative medicine and natural health products is becoming popular. It increases the demand for medicinal plant-based raw materials. *Andrographis paniculata* (Burm. f.) Nees is a high-demand plant originating in South India and Sri Lanka. It is growing in Malaysia, China, Thailand, India, Pakistan

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and Indonesia (Anju et al., 2012; Kumar et al., 2012; Mishra et al., 2007). The plant is from a family of Acanthaceae. It is locally known as 'Hempedu' Bumi' in Malaysia, 'King of Bitter' in English, 'Kalmegh' in India and Bengali, 'Chuan Xin Lian' in China, and 'Quasabhuva' in Arabic (Hossain et al., 2014; Jarukamjorn & Nemoto, 2008). *A. paniculata* consists of a few active compounds, including andrographolide, 12-didehydroandrographolide (DDAG), 14-deoxyandrographolide (DAG), and 14-deoxy-11 that have various medicinal properties, including anti-inflammatory, antidiarrheal, anti-viral, anti-malaria, hepatoprotective, cardiovascular, and anti-cancer (Hossain et al., 2014; Kataky & Handique, 2010; Valdiani et al., 2012). The plant has been used to treat fever, respiratory infection, diabetes, high blood pressure, ulcers, leprosy, bronchitis, skin diseases and influenza (Joseph, 2014; Okhuarobo et al., 2014). Furthermore, the latest study showed an anti-SARS-CoV-2 activity in *the A. paniculata* plant, which has the potential to be used to treat COVID-19 (Sangiamsuntorn et al., 2021). Andrographolide is the main compound in *A. paniculata* with colourless and crystalline labdane diterpenoid lactone and has a very bitter taste (Tang & Eisenbrand, 1992). This compound shows an immunological activity against cancer and human immunodeficiency virus (HIV) (Mishra et al., 2015).

According to Valdiani et al. (2012), China, Thailand, Indonesia, Mauritius, and Malaysia have commercialised and intensively cultivated *A. paniculata* to fulfil the high demand. The plant can be grown between 30 to 100 cm (Figure 1). The stem is dark green, 2 to 6 mm in diameter, with several long divaricates branches (Hossain et al., 2014). The leaves are glossy, with dark green on the upper side and light green on the under (Krishnaswamy & Kushalappa, 2017). The plant can be grown on a variety of soil types but preferably on rich, loamy soil (Shalini & Narayanan, 2015). *A. paniculata* begins to flower 2 to 3 months after transplanting. An optimum time for harvesting is around 3 to 5 months after transplant or when 50% of the plant is flowering, which indicates a high concentration of andrographolide content (Ariffin et al., 2006).

However, this high value of *A. paniculata* has a low seed germination rate and thus can reduce raw material production (Saraswathy et al., 2004). Previously, plasma-treated seed showed a 50% germination percentage compared to the untreated seed with 37.3% on *A. paniculata* (Tong et al., 2020). Meanwhile, seed of *A. paniculata* treated with sodium hypochlorite resulted in 57.33% of seed germination and 22.67% in control (Promwee et al., 2023). Seed priming is an alternative method for reducing emergence time, increasing germination rate, promoting plant growth, and increasing crop yield. It is a simple process that partially hydrates the seed in a controlled environment before drying it without allowing the emergence of radicle (Paparella et al., 2015). Osmopriming is a seed priming technique that soaks the seeds in an osmotic solution with low water potentials, such as polyethene glycol (PEG), glycerol, or mannitol solutions (Kareem & Ismail, 2013). PEG is a water potential-reducing agent and did not have a toxicity effect on the embryo due to its large molecular size

(Thomas et al., 2000). At the same time, hormopriming treats the seeds in hormone solution, including gibberellins, ethylene, and abscisic acid (Ohri et al., 2015; Pirasteh-Anosheh & Hashemi, 2020). This study aims to evaluate the effect of seed priming on plant growth and development and production of andrographolide compound in *A. paniculata* plant.

MATERIALS AND METHODS

Seed Priming

A hundred healthy seeds were used in each treatment. The seeds were surface sterilised with 5% sodium hypochlorite ($NaHClO_3$) for 5 min and then washed with sterile distilled water for 5 m. The sterilised seed was osmoprimed in an aerated solution of PEG-8000 with an osmotic potential at -0.4 MPa. The osmotic potentials of PEG-8000 solutions were calculated using Michel's (1983) formula. Another treatment is hormopriming with 100 ppm of GA_3. All seeds were primed in the dark at 25°C for 24 hours. After priming, the seeds were dried for 24 hours in the dark at 25°C to reach their original moisture content prior to germination.

Seed Germination and Plant Growth

Both primed and non-primed seeds (control) were germinated on a petri dish containing wet Whatman filter paper No.1. The seeds were germinated in between the filter paper. The experiment was conducted in a growth chamber (25 ± 2°C, 65% relative humidity) for a month. Then, the germination seeds of -0.4 MPa of PEG-8000 (64%), 100 ppm of GA_3 (61%), and control (26.5%) were transferred to Jiffy-7 and grown under a rain shelter at the Department of crop science FPSM, UMT for approximately four weeks or until 3 to 4 true leaves emerged. Twenty-seven replicates of each treatment and control with true leaves were transplanted into a polybag (35.56 x 35.56 cm) containing soil mixture (3 topsoil: 2 organic matters: 1 sand) growing media. The seedlings were grown in a greenhouse with a 30 cm × 30 cm distance between polybags at the UMT campus in Bukit Kor, Marang. The plant was watered using a drip irrigation system for 10 minutes in the morning and evening, and 150 kg of compound fertiliser (15:15:15) was applied at a rate of 150 kg per hectare once a month (Abdallah, 2005).

Determination of Plant Growth Parameters

Plant growth parameters, including plant height, number of branches and leaves, and stem girth measurement, were measured at two-week intervals for ten weeks after transplanting (WAT). Plant height was obtained using a ruler from the base of the plant until the end of the plant shoot tip. Stem girt was measured using a Vernier calliper above the plant root collar region.

Plant Harvesting

The plants were harvested once they started flowering (approximately 10 WAT). They were uprooted, washed, and separated into shoots (leaves and stems) and roots. The root was washed carefully using tap water to remove soil and debris, followed by air drying at room temperature to remove access water. The fresh weight of the shoot and root was measured using an electronic weighing scale. The shoot and root were then placed in an oven at 50°C for 48 hours to dry until three times consistent weight was achieved. The dry weight of the shoot and root was measured using an electronic weighing scale.

Andrographolide Analysis

Plant Preparation and Extraction

Fresh leaves of *A. paniculata* were harvested from the UMT campus Bukit Kor Marang after they started flowering. They were carefully washed prior to drying for 24 hours in the dehydrator. The dried sample was ground into powder liquid nitrogen in the mortar and pestle. 10 g of powder leaf samples from each treatment were soaked in denatured absolute ethanol for two days before being filtered. After two days, the extracts were filtered, and the ethanol solvent in the samples was removed using a rotary evaporator. The crude samples were left in the oven at 40°C until all the solvents evaporated. The extracts were sealed and kept in a cold room at 4°C until further analysis.

Sample Preparation

Two (2) mg of ethanol extract was dissolved in 1 ml of 50% methanol. The sample was vortex until all dissolved in the solvent. Then, 1 ml of the sample was filtered into the vial using a syringe and polyvinylidene fluoride (PVDF) (0.25 µm) filter.

Standard Preparation

Two (2) mg of andrographolide standard were dissolved in 1 ml of 50% methanol to make a stock concentration of 2000 ppm. The stock solution was diluted into six concentrations: 1000, 500, 200, 100, 50 and 20 ppm. All the standard solutions were vortex-filtered until all the samples were dissolved in the solvent. Then, 1 ml of standard solution was filtered into the vial using a syringe and PVDF (0.25 µm) filter.

High-performance Liquid Chromatography-UV/VIS Analysis

The identification and quantification of the andrographolide compound were obtained using UFLC from Shidmadzu, Japan (CTO-10AS VP) with a column size of 4.6 × 150 mm, 5 µm (Agilent Zorbax Eclipse XDB-C18). Gradient flow for andrographolide was methanol: water (60:40). The chromatogram was monitored at 223 mm wavelength (andrographolide). The results were expressed in mg per g of dry weight.

Statistical Analysis

The data was analysed using the Statistical Analysis System (SAS) software (version 8.1). A one-way repeated analysis of variance (ANOVA) approach was used to discover significant differences in the means at the $p \leq 0.05$ level, and the means were subjected to the Turkey HSD All-Pairwise Comparisons test.

RESULTS

Seeds primed with -0.4 MPa of PEG-8000, 100 ppm of GA_3 and unprimed (control) were germinated and grown into matured plants for ten weeks (Figure 1). After ten weeks, treatment of -0.4 MPa PEG showed the highest plant height (65.3 cm), number of branches (78) and leaves (155) and a significant difference ($p \leq 0.05$) than GA_3 treatment and control (Figure 2). Meanwhile, no significant difference was observed in the size of stem girth between PEG and GA_3 treatment. On the other hand, all unprimed seeds significantly showed the lowest plant growth analysis at 10 WAT.

The plant height of primed seeds is larger than that of the control, so shoot and root weights are also significantly higher than the control's (Figure 3). Meanwhile, the main active compound, the andrographolide content, showed no significant difference between all treatments and the control (Figure 4).

Figure 1. *Andrographis paniculata* plant at 10 weeks after transplanting (WAT). (A) -0.4 MPa of PEG-8000 with 68 cm height; (B) control with 60 cm; (C) 100 ppm of GA_3 with 63 cm

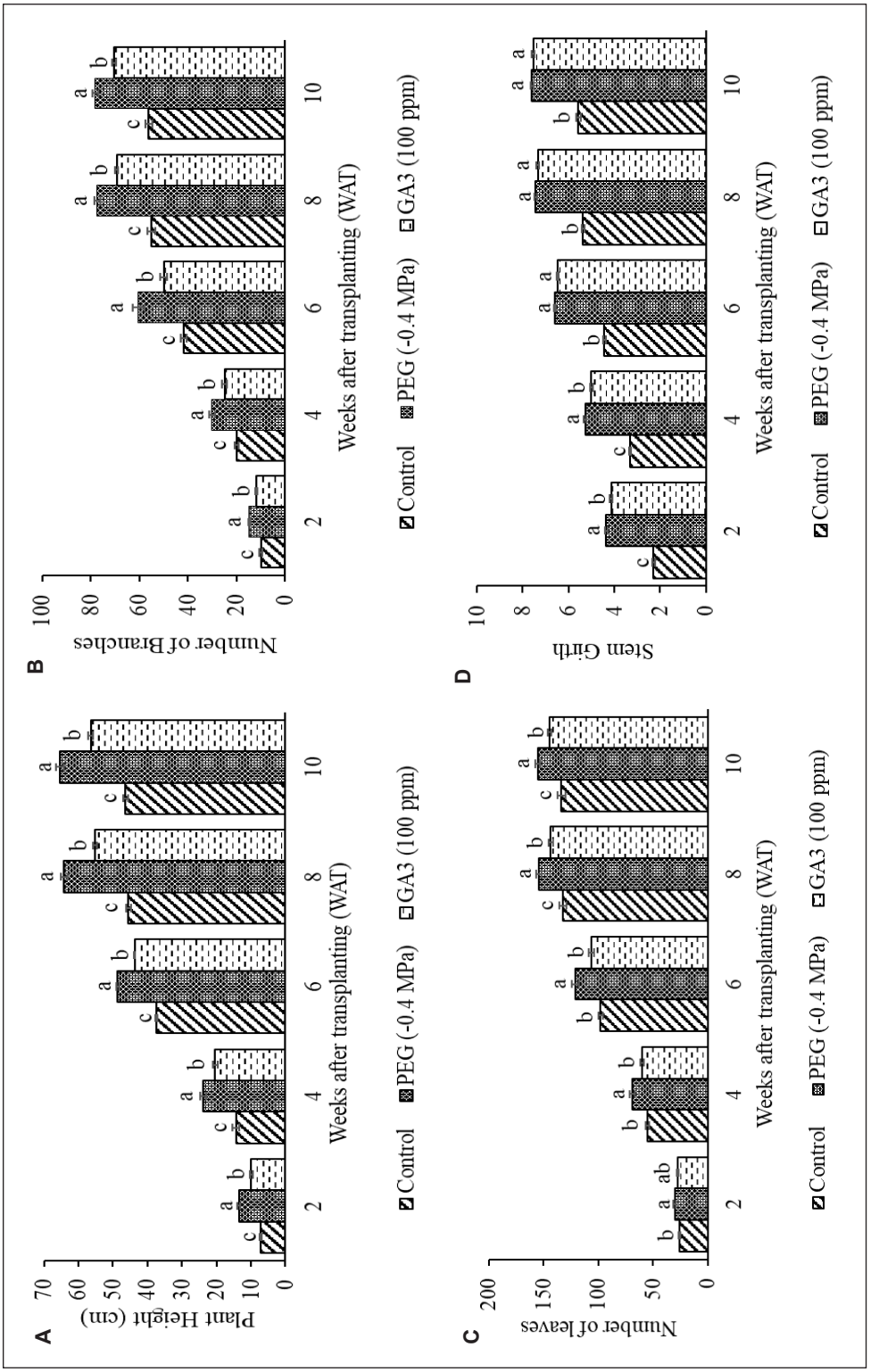

Figure 2. The effect of seed priming on (A) plant height; (B) number of branches; (C) number of leaves; and (D) stem girth of *Andrographis* within 10 weeks of plant growth. Different letters indicate statistically significant differences ($p \leq 0.05$)

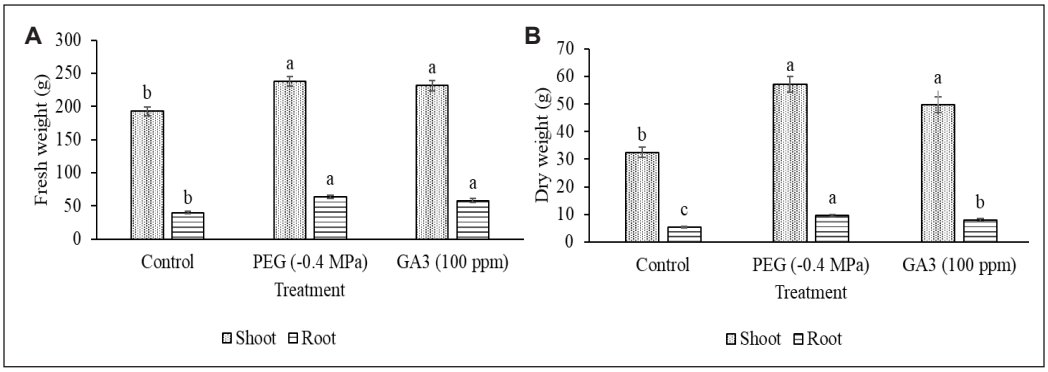

Figure 3. The effect of seed priming on (A) fresh weight of shoot and root and (B) dry weight of shoot and root of *Andrographis paniculata* at 10 weeks after transplanting (WAT). Different letters indicate statistically significant differences ($p \leq 0.05$)

DISCUSSION

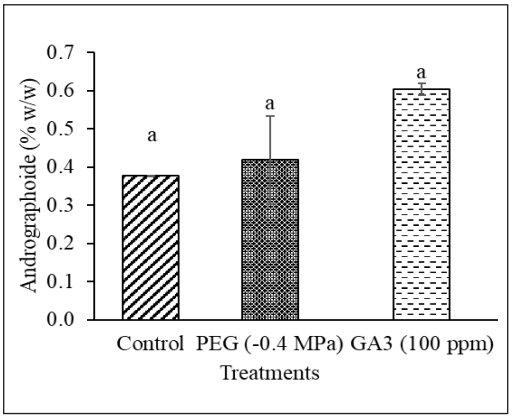

Figure 4. Percentage of andrographolide content (w/w) in *Andrographis paniculata* plant at 10 WAT in control, PEG, and GA_3

Seed priming is a low-cost and effective technique to enhance seed germination, plant growth, and yield. In this study, seeds primed with PEG-8000 at -0.4 MPa and GA_3 at 100 ppm were selected as an optimum treatment with high germination and seedling growth (high seedling vigour index, fresh weight, and length) based on the previous study (Abdullahi et al., 2021). High seedling growth might be because of PEG, which improves sugar accumulation and transpiration rate (Ahmad et al., 2020). As the plant grows, the plant size and length also increase due to the elongation of stem and vascular tissue (Falster & Westoby, 2003). The significant effect of PEG on plant growth may be due to the growing plant's seed structure, biochemistry, enzyme activities, and organic substances (Pradhan et al., 2014). PEG is a non-toxic, inert molecule that aids in enhancing the metabolic activities of seeds, resulting in faster plant growth and yield (Figoli et al., 2014).

As the plant height increased, the number of branches, leaves, and leaf area of *A. paniculata* increased as well, and this showed that the plant grows uniformly based on its age. A similar result was obtained by (Arif et al., 2014; Basra et al., 2003; Pradhan et al., 2014). Hence, results from this study confirmed that plants raised from osmopriming with PEG exhibited better plant height and increased the number of branches, leaves and leaf area compared to plants raised from non-primed seeds. Meanwhile, stem girth measurement

assesses the growth and width of the plant. Stem acts as the main reservoir of stored starch during plant growth. An increase in stem girth might result from initiating metabolic events in primed seeds (Scofield et al., 2009).

As the plant grows, the cell division within the apical meristem of the plant shoot and root increases and enhances the growth of the shoot and root (Farooq et al., 2006). Polyethylene glycol has low water potential that can enhance the hydrolysis of food reserves and thus enhance plant growth (Pradhan et al., 2014). When compared to other priming treatments, PEG has been shown to boost amylase activity for starch hydrolysis, which produces sugar, resulting in faster development, which might lead to heavier root fresh weight and root dry weight in the shoot and root (Zheng et al., 2015). This study is in accordance with the findings of Neamatollahi and Souhani (2010) on canola and Abbas et al. (2018) on wheat. Meanwhile, GA_3 hormone treatment is less effective compared to the PEG treatment on the seed of *A. paniculata.* It might be that most hormone treatments in seed priming technique are commonly used to improve seed germination in stress conditions (Jisha et al., 2013; Masood et al., 2012).

Meanwhile, Andrographolide is a main secondary metabolite in the *A. paniculata* plant. It is responsible for various medicinal properties, including antipyretic, anti-bacterial, anti-virus, anti-inflammatory, anti-angiogenic, and hepatoprotective, and it shows immunological benefits in cancer and HIV (Joselin & Jeeva, 2014). Generally, PEG is a non-toxic agent that can induce drought stress in plants, which later influences plant growth and development and the formation of secondary metabolites (Martinez-Santo et al., 2021; Turkan et al., 2005; Wu et al., 2005). Previously, PEG treatment in cell culture of *Scrophularia striata* significantly increased the total phenol content (Ahmadi-Sakha et al., 2022). Although in this study, no significant difference in andrographolide content was observed in the treated and control plants, PEG treatment significantly enhanced plant growth, including the size of root and shoot and the number of leaves and branches compared to the untreated seed.

CONCLUSION

PEG seed priming at -0.4 MPa significantly increased plant size, including height, number of leaves and branches, and stem girth. Higher plant growth and number of leaves indirectly increased the quantity of andrographolide compound.

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Mutagenesis Effect Using Gamma Ray Radiation on Morphological Changes, Productivity, and Genetic Variation in *Chloris gayana* cv. Callide

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ABSTRACT

Chloris gayana grass is a perennial tropical grass that is highly adaptable and resistant to climate change. As a cover crop, this grass can improve soil conditions. This grass has high palatability and potential to be improved through breeding to enhance the quality and productivity of ruminants' feed. This research was conducted to determine the effect of gamma ray radiation on the morphology, productivity and genetic variation of *C. gayana* cv. Callide. This research used 4 radiation doses of 0, 75, 150 and 225 Gy, which were given to *C. gayana* cv Callide plant seeds via a Gamma ^{60}Co irradiator chamber. A radiation dose of 150 Gy was proven to increase the development of culm diameter ($p < 0.05$). The best productivity was shown by radiation doses of 75 and 150 Gy, which increased the production of fresh, dry, and organic matter. The results of genetic variation analysis using Random Amplified Polymorphic Deoxyribonucleic Acid Polymerase Chain Reaction (RAPD PCR) showed that the polymorphism resulting from using the Organophosphate Degradation (OPD) 8 primer was 66.67%, and the OPD 11 primer was 62.50%. Overall, gamma ray radiation with 75 and 150 Gy doses on *C. gayana* cv. Callide has been proven to improve plant productivity.

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INTRODUCTION

Based on data from the Central Bureau of Statistics Indonesia, the population of beef and dairy cattle increased from 2020 to 2023, but the availability of forage decreased (Badan Pusat Statistik, 2023).

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Challenges in providing forage can come from several factors, including the type and quality of forage varieties, productivity, and ability to adapt to the growing environment. Climate change is also an issue regarding the stability of the current forage availability. Therefore, resilient grass with high adaptability and good nutrient content needs to be enhanced and reproduced; thus, it can meet the needs of ruminant livestock feed.

Chloris gayana grass is included in the cover crop and can grow in tropical and sub-tropical areas because it is highly adaptable to various environmental conditions. *Chloris gayana* is a perennial grass that can survive for years. That grass has a sturdy structure and strong stolon shape, is resistant to drought, and is able to reproduce quickly using seeds and vegetatively using stems. This plant can be found in grasslands, forests, savannas, riverbanks, lakes and swamps. *Chloris gayana* grass can survive in tropical climates with 6-month dry periods because it has a strong root system that can grow deep down into the soil for 4 meters (Rojas-Sandoval, 2020).

Chloris gayana grass is included in the C_4 plant group, which can carry out photosynthesis efficiently at hot environmental temperatures, making it suitable for use as a protective plant. The formation of grass biomass comes from utilizing soil moisture and absorbing sunlight by 80%. Planting under the shade will cause less optimal growth (Bilgin & Tansi, 2020). Planting *C. gayana* in pasture soil can increase soil organic matter (OM) content and water infiltration capacity, increase water holding capacity and reduce soil temperature during the dry season (Valenzuela & Smith, 2002). The deep root system and its ability to bind soil particles form stable soil aggregates so that the grass can prevent soil erosion (Mulualem et al., 2012).

Chloris gayana grass has good palatability in ruminants (Allah & Bello, 2019). In other studies, it had been reported that using that grass for sheep's feed showed lower palatability compared to *Brachiaria ruziziensis*. Palatability is influenced by the grass's crude fiber (CF) content. High CF will reduce the palatability of feed in ruminant livestock (Kenana et al., 2020). The dry matter (DM) content of *C. gayana* tends to be higher than other types of grass. It affects palatability, which is lower when used as ruminant animal feed. Therefore, the quality and productivity of *C. gayana* grass potentially improved through breeding to meet the needs of ruminant livestock feed.

A plant breeding program employs a structured and systematic approach to improving the genetic characteristics of plants and developing new cultivars with desirable traits. It is a cyclical process, with each cycle comprising three major phases: (1) creating genetic diversity (inducing mutation, making crosses, introducing exotic germplasm and using genetic engineering techniques), (2) assessing and screening to identify superior recombinant (utilizing marker-assisted selection, introducing quantitative trait loci (QTL), using high-throughput phenotyping platforms, resulting in the identification of potential cultivars), and (3) introducing, disseminating and embracing new cultivars. The application

of plant breeding has been proven to increase productivity by up to 50% (Ceccarelli, 2015). Plant breeding is carried out by changing the genetic composition of plants so that the desired breeding objectives are achieved (Koryati et al., 2022). This research marks the first step in a plant breeding program targeting the enhancement of genetic diversity in *C. gayana* grass. It used gamma ray radiation for mutagenesis in seeds of *C. gayana* cv. Callide.

Mutagenesis in plant breeding involves artificially increasing mutation frequency to induce genetic changes without genetic segregation or recombination (Raina et al., 2021). Gamma rays, a form of physical mutagen, are effective in altering the genetic structure of mutant plants due to their penetrative nature and ability to break down hydrogen bonds and sugar-phosphate groups within cells (Toker et al., 2007). Gamma-ray radiation causes a plant mutation, altering traits seen through morphological or cell structure changes. This radiation causes the radiolysis of water, producing reactive oxygen species (ROS) by splitting water molecules, leading to oxidative stress (Riviello-Flores et al., 2022).

High doses of gamma ray radiation cause oxidative stress in plants, resulting from an imbalance between ROS production and its enzymatic and non-enzymatic detoxification processes. Elevated ROS production due to high doses of radiation can result in photooxidative damage to Deoxyribonucleic Acid (DNA), proteins and lipids, ultimately resulting in cell death (Tripathy & Oelmüller, 2012). It can cause abnormal development of leaves, flowers, and seeds. Exposure to gamma-ray radiation can induce chromosomal aberrations in mitotic cells, causing chromosomes to become sticky, slow-moving and prone to breakage. Similarly, gamma ray radiation can affect meiotic cells, causing stickiness and disrupted polarity, which may reduce the germination ability of plant seeds (Nurmansyah et al., 2018). At low radiation doses, gamma rays can act as priming radiation, enhancing the germination process and improving cell proliferation, cell growth, stress resistance and productivity yield. The type of plant and the quantity of radiation dose also influence seed germination and seedling growth (Beyaz et al., 2020).

Gamma-ray radiation at doses of 100 to 150 Gy on seeds has been proven to increase germination, root length, the weight of seed yield, DM content and leaf chlorophyll content at the beginning of plant growth. A radiation dose of 50 Gy is known to increase the DM content and fresh weight of seeds compared to other doses (Beyaz et al., 2016). Low-dose radiation 25 Gy can also increase the secondary metabolite content in *Silybum marianum* L plant callus. The interaction between gamma rays and free radicals in plant cells will activate signal molecules in the plant defense system and synthesize plant secondary metabolites (Khalifa et al., 2022).

Gamma-ray radiation can speed up flowering time and produce better agronomic productivity in *Brachiaria*. Applying a dose of 40 Gy yielded the highest dry matter content in *Brachiaria*. Increasing doses of gamma-ray radiation also boosted the number of tillers, leaf-to-stem ratio, leaf length and DM content. However, grass treated with

gamma rays showed lower chlorophyll content compared to the control group. Damage to plant pigments due to radiation will cause the loss of the plant's photosynthetic ability. Morphological characteristics are linked to DM yield and plant nutrient quality (Hoka et al., 2019). Furthermore, applying gamma radiation on the nappies grass (*Pennisetum purpureum* Schumach.) increases their morphology and productivity. Furthermore, the impact of the gamma radiation has even produced a new cultivar, namely *P. purpureum* cultivar Gama Umami, whose growth and productivity are higher than its parent Napier grass and the grass produces good quality silage (Ananta et al., 2019; Fahmi et al., 2019; Mudhita et al., 2024; Respati et al., 2018; Umami et al., 2022, 2023).

The appropriate dose of gamma-ray radiation needs to be sought in the breeding process to improve the quality and productivity of *C. gayana* cv. Callide. Gamma-ray radiation at both low and high doses has never been applied in previous studies involving *C. gayana* cv. Callide. The optimal gamma-ray radiation dosage required to enhance the growth and productivity of *C. gayana* cv. Callide will be determined based on the findings of this study.

The effect of mutation on genetic diversity in *C. gayana* cv. Callide must be analyzed to determine the genetic alterations resulting from the gamma-ray radiation process. Random amplified polymorphic DNA (RAPD) analysis, a Polymerase Chain Reaction (PCR)- based method, can identify variations or polymorphism induced in plants by radiation. RAPD PCR analysis can reveal genetic changes in *C. gayana* cv. Callide is caused by gamma ray radiation, allowing the assessment of the relation between irradiated and non-irradiated grass. Anggereini (2008) stated that the RAPD analysis can quickly and effectively identify genetic markers to distinguish between closely related and morphologically indistinguishable species. The RAPD marker is used to create genetic maps identifying strains, species, populations and systems of various organisms. Therefore, an RAPD-PCR analysis was also conducted to identify the genetic variation that occurred. In this study, the effect of mutagenesis using gamma-ray radiation is determined through the morphological parameters, productivity and genetic variations observed.

MATERIALS AND METHODS

Material Preparation

This research was conducted from January to May 2023 in the greenhouse area of Forage and Pasture Laboratory, Faculty of Animal Science, Universitas Gadjah Mada, Sleman, Special Region of Yogyakarta Province, Indonesia. *Chloris gayana* cv. Callide seeds were purchased from Crop Mark Seed Company, New Zealand, without detailed harvest time and seed content information. Gamma ray radiation on *C. gayana* cv. Callide seeds was carried out using IRPASENA 4000 A gamma isotope Cobalt-60 chamber at the Research and Development Center Laboratory for Isotope and Radiation Technology, National Nuclear Energy Agency of Indonesia (BATAN). Seeds were given radiation with doses of

0 (P0), 75 (P1), 150 (P2) and 225 Gy (P3). Irradiated seeds are selected seeds that have good quality. Seeds are put in plastic and labeled with each dose of radiation number, then irradiated in a Gamma Chamber irradiator (IRPASENA BATAN, Indonesia) with a dose rate of 10Gy/87 seconds. The dose size is a function of time, and the dose rate of the Gamma Chamber is at that time.

Planting was carried out for 90 days at the greenhouse area of the Forage and Pasture Laboratory, Faculty of Animal Science, Universitas Gadjah Mada. Planting preparations included germination and preparation of planting media. Germination is carried out to ensure that the seeds planted are good quality seeds that grow successfully. The planting medium used in this research was a mixture of soil, manure, and bamboo humus in a ratio of 2:1:1 in polybag media. Maintenance, weeding and fertilization were carried out during the planting period. Plant maintenance included watering every morning and evening, as well as weeding to remove weeds. Fertilization is carried out on the 30th day after planting (1 kg/ha).

Experimental Design

This study used a completely randomized design with one primary factor of gamma irradiation, three levels of treatment and one control group. Plants were moved to polybag media two weeks after germination. One plant was planted in each polybag. Plants were grouped based on radiation doses of 0 (P0) or control, 75 (P1), 150 (P2) and 225 Gy (P3). The four groups of plants are evenly placed to receive the same environment in the greenhouse. Each group of plants had 20 replications, so the total number of polybags in this study was 80. Samples from each plant in each polybag were used to collect data on morphological and productivity parameters.

Data Collection of Morphological Parameters and Productivity

Morphological measurements were carried out at harvest, 90 days after planting. Morphological variables measured include plant height and length, number of leaves, leaf length and width, culm diameter growing and creeping on the ground and number of tillers. Plant height was measured starting from the ground surface to the tallest leaf. Plant length was measured starting from the soil surface to the longest leaf. The number of leaves was observed by counting the green leaves on each plant. The length of the leaves was observed by measuring the base to the tip of the leaf on each plant. Leaf width is the longest extension of any two points on the blade edge of the leaves. Creeping culm diameter is the size of the diameter of the culm that grows as a vine. Growing culm is culm that grows upwards. The number of tillers was calculated by counting each polybag's shoots.

Grass productivity is measured in fresh, DM, and OM production. Fresh production was determined by weighing all parts of the defoliated plant. Plant weight at harvest

(g/polybag) was converted into units of t/ha, then multiplied by the percentage of DM to determine the DM production. The results of DM production calculations (t/ha) are multiplied by the percentage of OM to get the total value of organic matter production. The DM and OM contents were determined using the method explained by Association of Official Agricultural Chemists (2005).

RAPD PCR ANALYSIS

RAPD PCR analysis was carried out using leaves by extracting, quantifying, and diluting the DNA. Leaves were washed with running water until clean and dried with tissue. DNA extraction using 0.1 gram of leaves concentration was then used for the PCR process. This analysis uses ten samples of *C. gayana* cv. Callide (Table 1). Quantification of DNA uses Gene Quant to determine the DNA concentration and DNA-RNA ratio, which was obtained by measuring light absorption at a wavelength of 260 nm. Next, DNA dilution was carried out by adding ddH_2O solution to obtain a suitable concentration for amplification.

Amplification of DNA was carried out using primers OPD 8 and OPD 11 (Table 2) with the nucleotide base sequences listed in Table 3. PCR reactions were carried out in a total volume of 10 µl for each PCR tube. Each PCR reaction consisted of 5 µl PCR mix Go Taq® Green (Promega, USA), 0.25 µl 100 µM primer (Sigma-Proligo, Germany), 2.5 µl DNA sample and 2.25 nuclease-free water. DNA amplification was carried out using the BOECO PCR System. First, heating was carried out at 94°C for 30 seconds, annealing at 37°C for 30 seconds, and elongation at 72°C for 1 minute 30 seconds, followed by final elongation at 72°C for 7 minutes. The DNA resulting from the PCR was then electrophoresed using 1.0% (w/v) agarose, which had been added with florosafe DNA stain as a dye, in TBE buffer (which consisted of 0.45 M Tris-HCl pH 8, 0.45 M Boric acid, 20 mM EDTA) with a voltage of 100 volts for 45 minutes. The amplification results are then visualized with UV light.

Table 1
List of Chloris gayana *cv. Callide samples for RAPD PCR analysis*

No	Sample name	Sample code
1.	P0A1	A1
2.	P1A1	A2
3.	P1A2	A3
4.	P1A3	A4
5.	P2A1	A5
6.	P2A2	A6
7.	P2A3	A7
8.	P3A1	A8
9.	P3A2	A9
10.	P3A3	A10

Table 2
Levels of polymorphism in each of the OPD 8 and OPD 11 primers

Primer	Amplified loci	Polimorphic loci	Polimorphic loci (%)
OPD 8	9	6	66.67
OPD 11	8	5	62.50

Table 3
List of primers used in DNA amplification

Primer	Sequence of nucleotide bases
OPD 8	GTGTGCCCCA
OPD 11	AGCGCCATTG

Statistical Analysis

Production yield and morphological parameters of *C. gayana* cv. Callide were analyzed using completely randomized designed One-Way Analysis of Variance (ANOVA). Mean comparisons were conducted using the Duncan Multiple Range Test at $p < 0.05$. Genetic diversity data was obtained by scoring the electrophoresis results for each individual at a certain size; if a band appeared, they were given a score of = 1, and if no band appeared, they were given a score of = 0. Binary data was then analyzed with Genalex 6.1 to determine the occurring polymorphism.

RESULTS AND DISCUSSION

Plant Morphology

The statistical analysis results in this study show plant height, number of leaves, culm diameter, and number of *C. gayana* cv. Callide tillers exhibited significant differences ($p < 0.05$) among treatments. The results for plant height, number of leaves and number of tillers indicate that grass without gamma-ray radiation yielded significantly higher results ($p < 0.05$) compared to grass treated with 75, 150 and 225 Gy radiation doses. The highest culm diameter was observed at a radiation dose of 150 Gy ($p < 0.05$). Plant length, leaf length and leaf width did not show any statistical differences ($p > 0.05$) among treatments. Data on growth measurement results are presented in Table 4.

Plant height and length are formed through cell division and elongation during the physiological phase. The activity of auxin influences the increase in plant height and length. Results of this study showed that the height of *C. gayana* cv. Callide without gamma-ray radiation was significantly ($p < 0.05$) higher than grass treated with radiation, reaching 72.13 cm. In contrast, the length of the grass *C. gayana* cv. Callide did not show a significant

Table 4
Morphological characteristic data of Chloris gayana *cv. Callide with different radiation doses*

Morphological characteristics	Gamma ray radiation dose (Gy)			
	0	75	150	225
Plant height (cm)	72.13 ± 2.88^{a}	52.36 ± 4.23^{b}	42.81 ± 3.62^{b}	52.38 ± 5.10^{b}
Plant length (cm)	170.35 ± 3.40	159.50 ± 6.64	168.51 ± 5.73	167.03 ± 8.21
Leaf number	183.05 ± 3.05^{a}	92.25 ± 5.99^{bc}	114.73 ± 14.63^{c}	75.44 ± 7.02^{c}
Leaf width (mm)	6.82 ± 0.33	7.77 ± 0.25	7.74 ± 0.30	7.07 ± 0.32
Leaf length (cm)	68.63 ± 3.13	87.38 ± 33.38	52.63 ± 1.46	56.56 ± 2.65
Creeping culm diameter (mm)	3.10 ± 0.05^{c}	4.16 ± 0.06^{b}	5.04 ± 0.02^{a}	4.24 ± 0.04^{b}
Growing culm diameter (mm)	2.18 ± 0.08^{c}	3.53 ± 0.06^{b}	4.04 ± 0.04^{a}	3.41 ± 0.04^{b}
Tiller number	102.00 ± 1.06^{a}	53.60 ± 6.41^{c}	76.78 ± 8.71^{b}	46.77 ± 6.45^{c}

Note. ^{a,b,c} Different superscripts in the same row indicate a different significance ($p < 0.05$)

difference ($p > 0.05$) in this study. According to Valenzuela & Smith (2002), the height of *C. gayana* grass can generally reach 50 to 200 cm. The research results of Jabessa et al. (2023) reported that planting *C. gayana* grass in the high and midlands in the Guji region, Ethiopia produced different plant heights. The height of *C. gayana* in the highlands was 106.8 cm, while in the lowlands, it reached 172.1 cm. Research conducted by Mohamed & Gebeyew (2018) on planting *C. gayana* showed that the plants matured on the 87th day, marked by flowering reaching 50% and a height of 139.10 cm. This site is reported to be significantly higher than buffalo grass and *Panicum maximum*.

The height of *C. gayana* grass planted on savanna land in Ethiopia at 8 weeks reached 100.7 to 121 cm. Based on research conducted by Daba et al. (2019), *C. gayana* harvested 75 days after planting showed plant height reaching 93 to 120 cm in each ILRI-7384 and ILRI-6633 varieties. Mganga et al. (2015) reported that rapid plant growth and development can result from a faster germination process. High doses of radiation will cause changes in the ratio of the auxin and cytokinin, leading to alteration in cell differentiation patterns. An increase in plant height can occur when radiation is given at doses of 10 and 30 Gy, while higher doses will reduce plant height. Increasing the radiation dose will damage the chromosome structure, thereby reducing plant height. Hartati et al. (2021) stated that physiological damage due to gamma-ray radiation may include cell death, inhibition of the cell division process and changes in plant reproductive characteristics. Gamma-ray radiation can increase plant height if administrated at the right dose because it induces hormonal changes as a result of increasing stress factors in plants (Singh et al., 2019). Taller plants are capable of producing greater biomass due to the stronger structure of their stems and shoots (Joshi et al., 2016).

All the leaves on each *C. gayana* cv. Callide were counted, and the results of this study were reported. The number of leaves in this study showed significant differences ($p < 0.05$) among treatments. *Chloris gayana* cv. Callide without seed radiation produced the highest number of leaves, 183.05. Meanwhile, the number of leaves resulting from the radiation of 75, 150 and 225 Gy were 92.25, 114.73 and 75.44, respectively (Table 4). These results are consistent with research on gamma-ray radiation given to chili plants. Treatment without radiation resulted in the highest number of leaves compared to 100, 200 and 300 Gy radiation (Tias et al., 2022). Increasing the radiation dose will prolong the exposure time to gamma rays, thereby increasing the cellular damage from the radiation energy (Makhziah et al., 2017).

The correct radiation dose can increase the number of leaves on plants due to increased tissue differentiation from the gamma ray radiation process. Gamma-ray radiation using Cesium-137 on soybean seeds can increase the number of leaves at a dose of 75 Gy, while lower and higher doses have been shown to reduce the number of leaves (Nuraeni et al., 2023). Gamma ray radiation using Cobalt-60 at a dose of 10 Gy was also proven to increase

the number of leaves compared to treatment without radiation in *Amorphophallus muelleri* plants (Santosa et al., 2014).

The activity of the auxin influences the growth of leaf width and length. This study's leaf width and length measurements showed no differences ($p > 0.05$) among treatments. The sizes of the leaf width resulting from gamma ray radiation with doses of 0, 75, 150 and 225 Gy, respectively, were 6.85, 7.77, 7.74 and 7.07 mm (Table 4). Meanwhile, the length of each leaf was 68.63, 87.38, 52.63 and 56.56 cm, respectively (Table 4). Leaf size is important for plants because it affects the amount of chlorophyll available for the plant's photosynthesis process. Non-optimal photosynthesis reactions can be influenced by a small amount of chlorophyll, which can cause plant growth to be hampered (Sarah et al., 2023). Gamma ray radiation with a dose of 20 Gy can produce the longest leaves compared to treatment without radiation and another dose on the ambon banana plant (*Musa paradisiaca* var. sapientum). Gamma ray radiation did not have a significant effect on leaf width (Due et al., 2019). Radiation doses that are too high are likely to damage the mobilization of nutrients from seeds to leaves (Santosa & Sugiyama, 2007). Longer leaf life is a desirable characteristic to maximize plant nutrient production. Cytokinin hormones influence leaf fall time, so damage to these hormones will result in less optimal plant production (Santosa et al., 2014).

The creeping culm of *C. gayana* is part of the vegetative reproductive organs; meanwhile, erect culm results from plant growth and is influenced by auxin activity. The Culm of the plant also determines the amount of biomass and crude fiber contained in the plant. Results of culm measurements on *C. gayana* cv. Callide from this research showed that gamma ray radiation has a significant effect on the diameter of creeping culm and growing culm ($p < 0.05$). A radiation dose of 150 Gy gave the biggest diameter, 4.04 mm in growing and 5.04 mm in creeping culms. Radiation of 75 and 225 Gy produced higher diameter sizes compared to treatment without radiation. The diameter of the growing stem from radiation results of 0, 75 and 225 Gy, respectively, were 2.18, 3.53 and 3.41 mm, while the respective creeping diameters were 3.10, 4.16 and 4.24 mm, respectively (Table 4). Each part of the organ in a plant provides a different biological function. The leaf part of the plant has the function of forming carbohydrates; the root function is to absorb water and nutrients, while the culm has the mechanical function of transporting nutrients and storing nutrient reserves for the plant (Poorter et al., 2012).

The development of tiller is influenced by the activity of auxin and cytokinin in plants. It is important to know tiller development because it affects the productivity and reproductive ability of the grass through the formation of flowers. The statistical analysis results in this study showed that gamma-ray radiation on *C. gayana* cv. Callide significantly reduced the number of tillers that developed ($p < 0.05$). Several tillers of *C. Gayana* cv. Callide without radiation were 102 segments, while radiation of 75, 150, and 225 Gy each produced 53.60, 76.78, and 46.77 tiller segments, respectively (Table 4).

Tillers determine the amount of plant biomass. The number of tillers per plant in *C. gayana* for 75 days of planting reached 5.4 to 9.4 segments, depending on the varieties (Daba et al., 2019). The bigger number of tillers will produce higher non-structural carbohydrate components because they increase the number of leaves. It is in accordance with the aim of grass breeding to increase the biomass content and quality of the grass. *Chloris gayana* is able to produce a high number of tillers so that it has better productivity and vegetative growth (Tadesse et al., 2022).

Warid et al. (2017) reported that higher radiation doses reduced the survival ability of soybean plants, especially at a dose of 400 Gy. Wiryosimin (1995) also reported that gamma ray Co-60 radiation could produce high energy, damaging the chemical bonds of a new compound when given to seeds, plant tillers, pollen, apical shoots, plant tissues and cells. Radiation of gamma ray Co-60 in seeds can cause the cell nucleus to experience genome mutations, chromosome mutations, gene mutations or mutations outside the nucleus, such as in the plastids and mitochondria. Genomic mutations will cause changes in the number of chromosomes. The addition or reduction of chromosome cells will result in changes in the characteristics and morphology of plants in the form of height, number of leaves and number of tillers. Harmini et al. (2021) stated that 50 Gy gamma ray radiation on *Pennisetum purpureum* cv Taiwan produced a lower number of tillers in 90% of the replications compared to the control. Higher radiation doses can reduce these plants' growth tiller, leaves and roots.

Gamma ray radiation provides random results on the influence of the metabolism of cells exposed to the radiation process. Gamma rays can influence meristem cell metabolism and protein synthesis during stem development. The radiation process causes the formation of free radicals, which react with organic molecules, thereby disrupting cell metabolic processes (Hartati et al., 2021).

Plant Productivity

This research shows that gamma ray radiation influenced fresh yield, DM yield and OM yield of *C. Gayana* cv. Callide ($p < 0.05$). The highest fresh yield and OM yield were obtained at a radiation dose of 150 Gy, while the highest DM yield was obtained at a radiation dose of 75 and 150 Gy. Production results of *C. gayana* cv. Callide treated with gamma ray radiation is provided in Table 5.

Production of fresh biomass from *C. gayana* cv. Callide in this study was proven to be significantly influenced by gamma ray radiation ($p < 0.05$). Radiation doses of 75 and 150 Gy gave the highest results (2.38 and 2.61 t/ha, respectively). Treatment without radiation gave a fresh yield of 1.97 t/ha, and the lowest production was produced by a radiation dose of 225 Gy (1.35 t/ha) (Table 5). Biomass allocation to plant morphological components gives varying results. Biomass calculations are often carried out on feed plants' leaf and

Table 5
Production of fresh yield, dry matter yield and organic matter yield of Chloris gayana *cv.* Callide *with different radiation doses*

Yield (t/ha)	Gamma ray radiation dose (Gy)			
	0	75	150	225
Fresh yield	1.97 ± 0,09[b]	2.38 ± 0,10[a]	2.61 ± 0,09[a]	1.36 ± 0,10[c]
Dry matter yield	0.31 ± 0,02[b]	0.38 ± 0,02[ab]	0.48 ± 0,02[a]	0.18 ± 0,01[c]
Organic matter yield	1.61 ± 0,08[b]	1.99 ± 0,08[a]	2.21 ± 0,08[a]	1.15 ± 0,09[c]

Note. [a,b,c] Different superscripts in the same row indicate a different significance ($p < 0.05$)

stem ratio (Poorter et al., 2012). Based on studies using allometric equations on biomass in plants' roots, stems and leaves, it was reported that increasing plant size causes biomass allocation to the stem to increase and the leaves to decrease (Liu et al., 2021). It is in accordance with the results of this study that the highest production at radiation doses of 75 and 150 Gy was due to the highest culm diameter obtained at these radiation doses (Table 4).

Kebede and Bobo (2023) reported that fresh biomass production in *C. gayana* was influenced by the type of variety and altitude of the planting area. *Chloris gayana* planted in the highlands produces lower fresh biomass production than when planted in the lowlands. Production of fresh biomass from *C. gayana* cv. Masaba and cv. ILRI-7384 in the highlands were 3.45 and 3.25 t/ha, while when planted in the lowlands, it produced 4.24 and 4.08 t/ha, respectively. Hidosa et al. (2018) reported that the fresh biomass production of Chloris Gayana grass on irrigated land was 53.56 t/ha/year. Giving gamma ray radiation will increase cell proliferation, but at high doses, it can damage cells and inhibit plant growth.

Gamma ray radiation in this study was proven to significantly influence the DM yield of *C. gayana* cv Callide ($p < 0.05$) among treatments. The radiation dose treatment of 150 and 75 Gy gave the highest results (0.48 and 0.38 t/ha, respectively), while the radiation dose treatment of 225 Gy produced the lowest DM yield (0.18 t/ha). Treatment without radiation gave results of 0.31 t/ha (Table 5). These results are lower than those Abera (2017) reported in that the DM production of *C. gayana* planted together with *Medicago sativa* could reach 3.90 to 4.44 t/ha. *Chloris gayana* is a perennial plant that can grow for up to 3 years with 2-week harvest interval. Jabessa et al. (2023) reported that planting *C. gayana* in the highlands resulted in a DM production content of 8.94 t/ha/year while planting in the midlands resulted in a DM production content of 13.34 t/ha/year. Increasing the dose of gamma ray radiation can cause dehydration or changes in the ratio of meristem cells and special plant cells, which include root hair cells, palisade, xylem, and phloem. It causes division in small cells and is detrimental to large vacuolated cells, which accumulate secondary metabolites so that DM production will increase. Each plant species has a different response to gamma ray radiation. Several studies related to gamma ray radiation show that giving a radiation dose of 40 Gy will cause a decrease in growth indicators in

plants (Ciocan et al., 2023). Furthermore, Respati et al. (2018) reported that *Brachiaria brizantha* cv. MG5, germinated from the seeds, radiated using gamma radiation at 100 Gy, resulting in the best growth and production at the regrowth phase 2.

Chloris gayana cv. Callide radiated by gamma ray in this study was proven to significantly influence the results of Organic Matter (OM) yield production ($p < 0.05$). Radiation doses of 75 and 150 Gy resulted in the highest OM production (1.99 and 2.21 t/ha, respectively). Meanwhile, a radiation dose of 225 Gy resulted in the lowest OM production (1.15 Gy) (Table 5). Delastra et al. (2021) stated that a gamma ray radiation dose of 300 Gy to *Sorghum sundanese* plants was proven to increase the DM and OM content.

Changes in plant OM production due to gamma ray radiation are caused by disturbances in cellular metabolism caused by oxidative stress, which reacts with all structural and functional organic molecules, including proteins, lipids and nucleic acids. This reaction causes lipid membrane peroxidation, thereby disrupting membrane stability and increasing permeability. It may cause cell damage and disruption of plant physiological functions, such as changes in plant physiology, increased respiration, increased ethylene production and changes in enzyme activity (Marcu et al., 2013).

Genetic Variation Using RAPD PCR

Amplification results from the RAPD PCR analysis on *C. gayana* cv. Callide, using primers OPD 8 and OPD 11 (Table 3), showed that all plant samples produced clear bands, which can be seen in Figures 1 and 2. The samples that amplified the most and thickest bands originated from plants irradiated with a dose of 150 Gy, specifically samples A5, A6, and A7. The appearance of the amplification band, which differs from that of the control group, indicates a genetic change resulting from the gamma ray radiation in this study.

Genetic diversity testing with RAPD PCR involves primer selection. Primers suitable for RAPD PCR analysis must meet certain criteria: they should generate polymorphic DNA band, yield clear and reproducible results, exhibit stable DNA band amplifiationc, be easy to interpret, and possess a G=C base pair content between 60-70% (Hartati et al., 2007). The success of the PCR test is also influenced by factors such as DNA concentration and template, annealing temperature, and concentration and quality of the primers and buffers used. A DNA template concentration that is too low will result in unclear or absent amplification of DNA bands (Setyawati & Zubaidah, 2021). The freshness level of the leaf samples could influence the differences in the thickness of the bands observed. Fresh samples produce thicker bands (Siregar & Diputra, 2013). Based on the result of the primer selection conducted in this study, only the OPD 8 and OPD 11 primers displayed DNA bands clearly. Therefore, these primers were chosen for RAPD PCR analysis.

Based on the analysis conducted in this research, it is evident that the OPD 8 primer produced 66.67% polymorphic loci, while the OPD 11 primer produced 62.50%

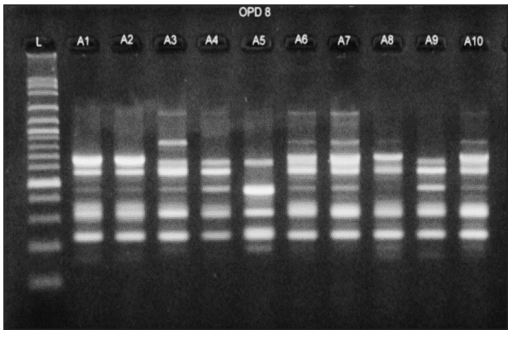

Figure 1. RAPD profiles amplified from 10 Chloris gayana cv. Callide plant using primer OPD-8
Note. A1=Control group plant; A2-A3=Seeds irradiated with 75 Gy; A4-A7=Seeds irradiated with 150 Gy; A8-A10=Seeds irradiated with 225 Gy

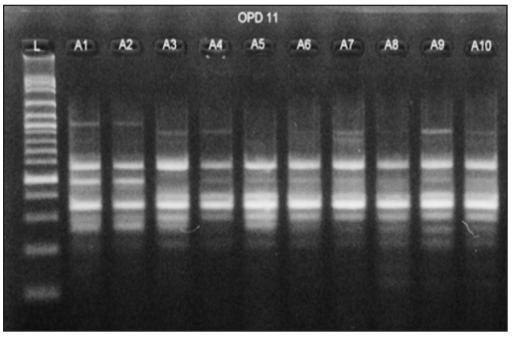

Figure 2. RAPD profiles amplified from 10 Chloris gayana cv. Callide plant using primer OPD-8
Note. A1=Control group plant; A2-A3=Seeds irradiated with 75 Gy; A4-A7=Seeds irradiated with 150 Gy; A8-A10=Seeds irradiated with 225 Gy

polymorphic loci, resulting in a total of 11 polymorphic loci. Sulistyawati and Widyatmoko (2017) stated that the higher number of polymorphic loci resulting from RAPD PCR analysis represents high heterozygosity and heterogeneity. This research indicates that gamma ray radiation induces mutant plants with a moderate level of diversity compared to the control group without radiation treatment. Diversity values are divided into three categories: low (0.1 to 0.4), moderate (0.5 to 0.7), and high (0.8 to 1.0) (Nei, 1987). Greater genetic diversity within a population leads to increased availability of germplasm for developing new varieties (Gusmiaty et al., 2016).

Gamma ray radiation applied to ginger plants at doses of 0, 5, 7, 9, 11 and 13 Gy resulted in a 98.29% polymorphism percentage. The high levels of polymorphism indicate a significant alteration in the DNA sequence of mutant plants resulting from radiation, as influenced by the primer binding used (Mohd Sharim & Shamsiah, 2021). The RAPD PCR analysis was conducted on *Oryza sativa* L. cv. Barak Cenana plants, using five primers, and subjected to gamma ray radiation doses of 100, 200, 400 and 800 Gy, produced six polymorphic bands. Changes in DNA structure, such as transposition, breaks or deletions, can lead to the emergence of new DNA bands as a consequence of induced mutations. The plants resulting from 100 Gy radiation showed the highest level of similarity to the plants without radiation, with a similarity index of 0.75. The lowest level of similarity was observed in the plants resulting from 400 Gy radiation, with a similarity index of 0.33 (Pujiyanti et al., 2021). Genetic diversity testing using RAPD analysis on four samples of ramie plants resulted in a polymorphism percentage ranging from 81% to 100% (Maryeni et al., 2019). The results reported in the literature are significantly higher than those obtained in this study.

Based on RAPD PCR analysis, gamma ray radiation was reported to increase genetic diversity and genetic distance values in *A. muelleri* plants. Gamma ray radiation

produced random results; therefore, the appearance of DNA bands in RAPD analysis is not concentrated at low or high radiation doses. Damage to the DNA double helix occurs due to chemical changes caused by radiation, leading to breaks in the DNA molecular chain. The amplification of DNA fragments in RAPD PCR analysis depends on the primer sequence in the DNA genome. The RAPD technique can detect changes in a single base in genomic DNA. Hence, a difference in one nucleotide will produce a distinct RAPD profile. (Poerba et al., 2009). The results of the RAPD PCR analysis in this study indicated a general increase in polymorphism in plants resulting from radiation, although it remained within the moderate category.

CONCLUSION

Based on this research, a 150 Gy gamma ray radiation dose in *C. gayana* cv. Callide showed the best results in the culm diameter, while 75 and 150 Gy gamma ray radiation doses produced the best yields of fresh, DM and OM. Analysis of RAPD PCR shows that plants resulting from 150 Gy radiation produce the best results that can be used for the further breeding process in *C. gayana*.

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Short Communication

First Attempt to Survey Montane Bird Species Using Camera Traps in Cameron Highlands, Peninsular Malaysia

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ABSTRACT

The use of camera traps to record tropical forest bird diversity is uncommon. In 2020, over 200 active camera trap days, ten camera traps were deployed at three selected forest reserves, namely Terla A, Bertam and Bukit Bujang Forest Reserves in Cameron Highlands, Peninsular Malaysia, to assess the potential of recording montane bird species at lower strata using camera traps. *Enicurus schistaceus*, *Arborophila campbelli* and *Rhipidura albicollis* were the most recorded camera trap images. This survey recorded 16 diurnal and one nocturnal bird species. These preliminary findings demonstrated the potential of using a non-invasive method to assess understory bird species in montane habitats, which may complement other survey methods.

Keywords: Activity patterns, biodiversity, birds, habitat change, photo trapping

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INTRODUCTION

At an elevation of more than 1,200 m, Malaysia's highlands comprise submontane and montane habitats that support a distinctive biotic community, especially montane specialists. In Peninsular Malaysia, Cameron Highlands, Fraser's Hill, and Maxwell Hill are popular hill stations in fragile and vulnerable forested highlands (Chan, 2019). Cameron Highlands hosts

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over 700 plant species, 145 endemic, including 32 orchid species (Chan, 2019). A total of 56 mammals, 199 birds, 58 reptiles and 14 amphibian species have also been recorded in Cameron Highlands (Chan, 2019). These mountainous highlands are home to various bird species specialising in montane habitats (Wikramanayake, 2002) with colder temperatures and relatively higher precipitation at higher altitudes. Montane species generally are less resilient to heavy human activities and land development (Soh et al., 2021), many of which, such as the Mountain Peacock-pheasant (*Polyplectron inopinatum*) and Malayan Whistling-thrush (*Myophonus robinsoni*), are listed in the International Union for Conservation of Nature (IUCN) Red List (Baharudin et al., 2023).

Camera-trapping is a non-invasive approach widely utilised in wildlife ecological studies for decades to record the presence and behaviour of wildlife, especially rare and elusive ones, without human intervention (Bondi et al., 2010; Smith & Coulson, 2012). Furthermore, photos and videos captured using a camera trap allow additional information pertaining to the phenotype appearance of animals, such as body size, colour, and condition, to be captured (Kühl & Burghardt, 2013). Camera trapping has been demonstrated to be one of the more cost-efficient sampling methods for faunal assessments, although it requires a large initial monetary investment (Bondi et al., 2010). Specifically, it is an exceptionally pragmatic method for monitoring mammals of medium-large body size (Rendall et al., 2014) because of its practicality in producing large amounts of information and recording the presence of target and non-target species.

In avian ecological research, camera traps have been progressively used. A study by Tanwar et al. (2018) in Germany demonstrated the effectiveness of different camera trap models on birds. At the Nanling National and Chebaling National Nature Reserves, Guangdong, China, based on the surveys made from 2011 to 2016, it was found that 47 bird species were recorded by camera traps, with only six species caught (Zhang et al., 2018). Elsewhere in Southeast Asia, camera trapping was carried out to survey birds in Indonesia (Brooks et al., 2018; Dinata et al., 2008; O'Brien & Kinnaird, 2008), Myanmar (Naing et al., 2015), Thailand (Pla-ard et al., 2021; Suwanrat et al., 2015), and Malaysia (Samejima et al., 2012; Jambari et al., 2015). A survey on terrestrial vertebrates using camera traps by Jambari et al. (2015) managed to capture 777 (9.30% out of the total) images of birds. Previous studies utilised camera traps targeting mostly mammals, inadvertently capturing images and videos of birds nearby. While most studies in the past were performed in lowland forests, this study assesses the potential of recording montane bird species at lower strata of a montane forest, namely Cameron Highlands, using camera traps.

MATERIALS AND METHODS

A survey was conducted at Cameron Highlands, comprising an area of 712.18 km^2 in the north-western of the Pahang state (Figure 1), namely in Terla A Forest Reserve (9.34 ha)

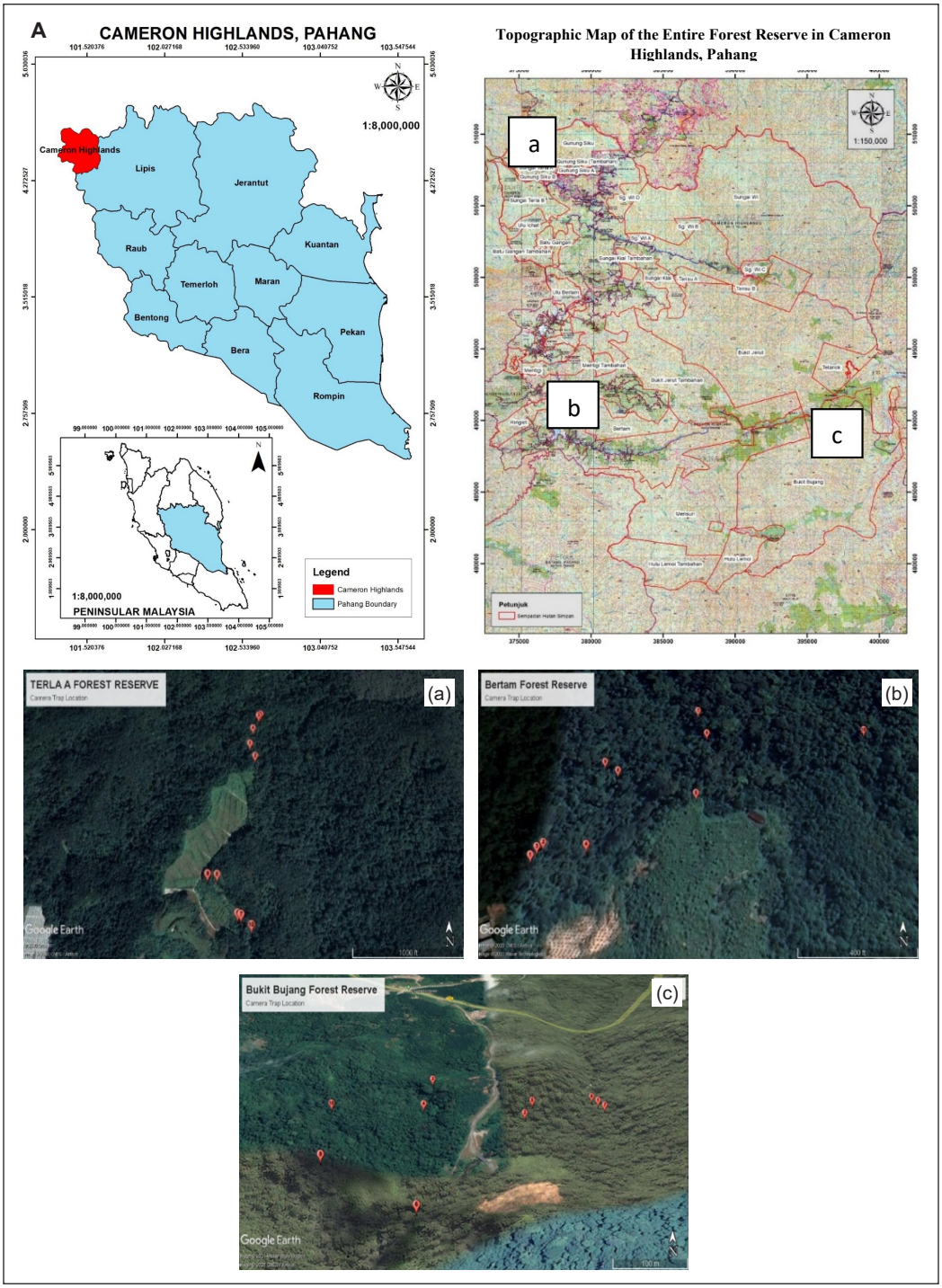

Figure 1. (A) The location of Cameron Highlands, Pahang. (Adapted from the Forestry Department of Peninsular Malaysia, 2020), and the distributions of camera traps at (a) Terla Forest Reserve, (b) Bertam Forest Reserve and (c) Bukit Bujang Forest Reserve

(04°35'36.6" N, 101°22'54.7" E) with an elevation of 1300–1500 m, Bertam Forest Reserve (15.4 ha) (04º25′ 15.0″ N, 101º26′ 41.4″ E) with an elevation of 1200-1300 m and Bukit Bujang Forest Reserve (0.50 ha) (04º24′ 07.06″ N, 101º35′37.28″ E) with an elevation of 400-500 m. The camera-trapping method was conducted for five days at each site based on two sampling phases between August 2020 and January 2021. These forests were chosen because there was minimal human interference.

At each site, ten units of camera traps were mounted over 40 cm from the ground on trees or poles (Jambari et al., 2015; Mohd Azlan et al., 2018) and marked using Global Positioning System (GPS). Bird images or videos captured on camera traps were identified in detail based on appearance and by consulting experts—other cues, such as movements and behaviour captured via videos, aided species identification. The activity time of birds was categorised into daytime (0601 hr to 1800 hr**)** and nighttime (1801 hr to 0600 hr). Rarefaction curves were derived from interpolating limited smaller samples and estimating species richness at each site.

RESULTS AND DISCUSSION

A total of 96 photos or videos of birds (17 genera,10 families, 17 species), most of which are montane specialists, were recorded at the three forest reserves. There is one species, *Pterorhinus mitratus,* recorded as Near Threatened (NT) and another species, Mountain Peacock-pheasant*,* as Vulnerable (VU) (IUCN, 2021). Most birds captured are classified as Totally Protected (TP) in Wildlife Conservation Act (WCA) (2010) except for *Pterorhinus mitratus, Anthipes solitaris,* and *Cyornis whitei*, which are not on the list. According to Soh et al. (2021), higher altitudinal specialists are also at risk of local extirpations in some mountain tops. In Peninsular Malaysia, such vulnerable species include the montane specialists such as the Mountain Peacock-pheasant, which deserves closer conservation attention. Generally, Terla A Forest Reserve caught the highest number of photos (43; Figure 2) on camera traps than Bertam and Bukit Bujang Forest Reserves that is 30 and 23 photos, respectively.

From Table 1, the highest number of photos were captured for *Enicurus schistaceus,* with 15 photos captured, while 14 photos were taken for *Alcippe peracensis*. *Myiomela leucura Niltava grandis* and Mountain Peacock-pheasant had the same number of photos captured that is three photos.

The activity time of birds was categorised into daytime (0601 hr to 1800 hr) and nighttime (1801 hr to 0600 hr), as shown in Table 2. Fifteen species were detected at 0700 hr to 1700 hr. Four species of diurnal birds, namely *Alcippe peracensis*, *Cyornis whitei*, *Myiomela leucura* and *Niltava grandis*, were detected to be active after dusk; they were likely on their way to roost. *Alcippe peracensis* were the most recorded species during the day as nine photos were captured, and only two photos of the species were captured in

the late evening when they returned to roost. This study demonstrated that camera traps can capture diurnal and nocturnal bird species (Daniel et al., 2016). Findings showed that cameras set near the rivers, streams, and waterlogged areas captured a higher number of bird species. It should be noted in the case of Bukit Bujang Forest Reserves, even though it is a

Table 1
List of bird species recorded at Terla A, Bertam and Bukit Bujang Forest Reserves in Cameron Highland using camera traps

Family	Conservation status (IUCN, 2021)	WCA (2010)	Numbers of photos (P) / video (V) captured			N
			Terla A	Bertam	Bukit Bujang	
Alcippeidae						
Alcippe peracensis (Sharpe,1887)	LC (IUCN, 2021)	TP	10(V)	1(V)	3(P)	14
Leiothrichidae						
Pterorhinus mitratus (S. Müller, 1836)	NT (IUCN, 2021)	-	-	5(P,V)	-	5
Muscicapidae						
Anthipes solitaris (S. Müller, 1836)	LC (IUCN, 2021)	-	1(V)	-	-	1
Cyornis whitei Harrington, 1908	LC (IUCN, 2021)	-	4(V)	-	-	4
Enicurus schistaceus (Hodgson, 1836)	LC (IUCN, 2021)	TP	-	6(V)	9(P,V)	15
Larvivora cyane (Pallas, 1776)	LC (IUCN, 2021)	TP	7(V)	-	-	7
Myiomela leucura (Hodgson, 1845)	LC (IUCN, 2021)	TP	2(V)	1(V)	-	3
Niltava grandis (Blyth, 1842)	LC (IUCN, 2021)	TP	1(V)	2(V)	-	3
Nectariniidae						
Aethopyga saturate (Hodgson, 1836)	LC (IUCN, 2021)	TP	1(V)	-	-	1
Pellorneidae						
Pellorneum tickelli (Blyth, 1859)	LC (IUCN, 2021)	TP	2(V)	-	-	2
Phasianidae						
Arborophila campbelli (Robinson, 1904)	LC (IUCN, 2021)	TP	5(V)	-	6(V)	11
Polyplectron inopinatum (Rothschild, 1903)	VU (IUCN, 2021)	TP	3(P)	-	-	3
Pnoepygidae						
Pnoepyga pusilla Hodgson, 1845	LC (IUCN, 2021)	TP	2(V)	-	-	2
Pycnonotidae						
Alophoixus ochraceus (Moore, 1854)	LC (IUCN, 2021)	TP	-	6(V)	-	6
Ixos mcclellandii (Horsfield, 1840)	LC (IUCN, 2021)	TP	-	6(V)	-	6
Rhipiduridae						
Rhipidura albicollis (Vieillot, 1818)	LC (IUCN, 2021)	TP	5(P)	2(V)	5(V)	12
Strigidae						
Otus spilocephalus (Blyth, 1846)	LC (IUCN, 2021)	TP	-	1(V)	-	1
Total number of photos captured (N)			43	30	23	96

Note. N=number of photos captured; - = Not Available; IUCN=International Union for Conservation of Nature; WCA=Wildlife Conservation Act; LC =Least Concern; NT=Near Threatened; VU=Vulnerable

hill dipterocarp forest, there were still montane species (for example, *Alcippe peracensis* and *Enicurus schistaceus*) that extend their range into this lower elevation and captured by our camera traps. Rarefaction of species accumulation curves obtained for the three sites was found to vary, probably due to differences in habitat quality. Nonetheless, Terla A and Bukit Bujang Forest Reserves seemed to have reached asymptote, but not Bertam Forest Reserve (Figure 3). However, due to low sampling effort, we propose a prolonged survey to better estimate species richness.

Table 2
The time during which bird species were recorded on camera trap

Species	Daytime	Nighttime	Photo/Video
	0601 hr to 1800 hr	1801 hr to 0600 hr	
Aethopyga saturata	1	-	V
Alcippe peracensis	9	2	V
Alophoixus ochraceus	6	-	V
Anthipes solitaris	1	-	V
Arborophila campbelli	2	3	V
Cyornis whitei	3	1	V
Enicurus schistaceus	6	-	V
Ixos mcclellandii	6	-	V
Larvivora cyane	7	-	V
Myiomela leucura	2	1	V
Niltava grandis	2	1	V
Otus spilocephalus	-	1	V
Pellorneum tickelli	2	-	V
Pnoepyga pusilla	-	2	V
Polyplectron inopinatum	3	-	P
Pterorhinus mitratus	5	-	2V, 3P
Rhipidura albicollis	7	-	4V, 3P

Note. - = Not Available

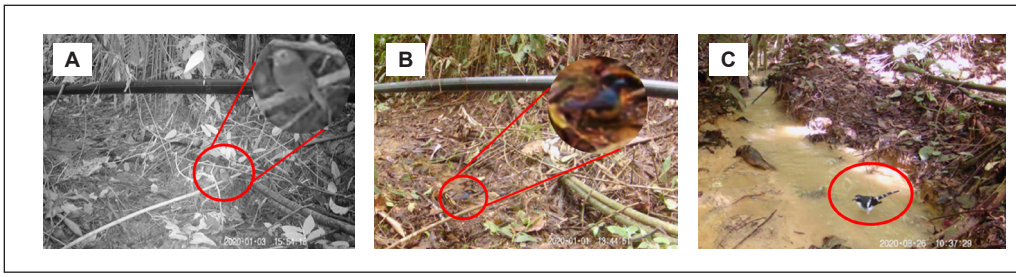

Figure 2. Seventeen species were caught by camera trapping in Cameron Highlands, Pahang. (A) *Anthipes solitaris* (Rufous-browed Flycatcher); (B) *Cyornis whitei* (Hill-blue Flycatcher); (C) *Enicurus schistaceus* (Slaty-backed Forktail)

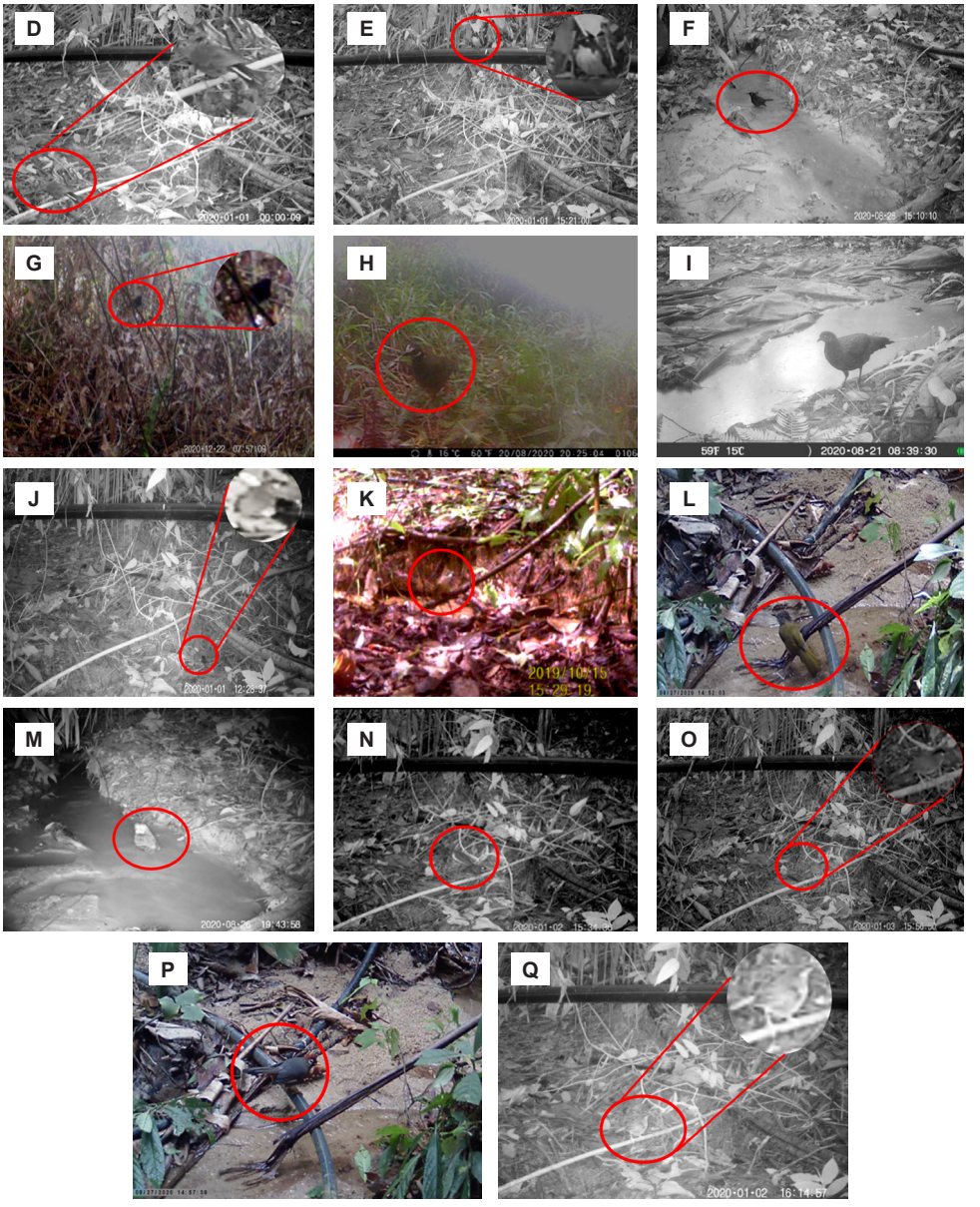

Figure 2 (continue). Seventeen species were caught by camera trapping in Cameron Highlands, Pahang. (D) *Myiomela leucura* (White-tailed Robin); (E) *Larvivora cyane* (Siberian Blue Robin); (F) *Niltava grandis* (Large Niltava); (G) *Aethophyyga saturate* (Black-throated Sunbird); (H) *Arborophila campbelli* (Malayan Patridge); (I) *Polyplectron inopinatum* (Mountain Peacock-Pheasant); (J) *Pnoepyga pusilla* (Pygmy Cunwing); (K) *Alophoixus ochraceus* (Ochraceous Bulbul); (L) *Ixos mcclellandii* (Mountain Bulbul); (M) *Otus spilocephalus* (Mountain Scops Owl); (N) *Rhipidura albicollis* (White-throated Fantail); (O) *Alcippe perancensis* (Mountain Fulvetta); (P) *Pterorhinus mitratus* (Chestnut-capped Laughingthrush); (Q) *Pellorneum tickelli* (Buff-breasted Babbler)

CONCLUSION

The study was the first attempt to survey bird species using camera traps conducted in Peninsular Malaysia. Our study showed that setting up camera traps close to water bodies may increase the capture rate and examine their behaviour or activity period. Besides terrestrial birds, many arboreal bird species were also detected at a lower stratum in the montane forest, while some extend their range into lower elevations. Although the number of species detected may be less than that of other sampling methods such as direct observation and mist-netting, camera traps may be complementary to detect elusive birds, especially during twilight and night.

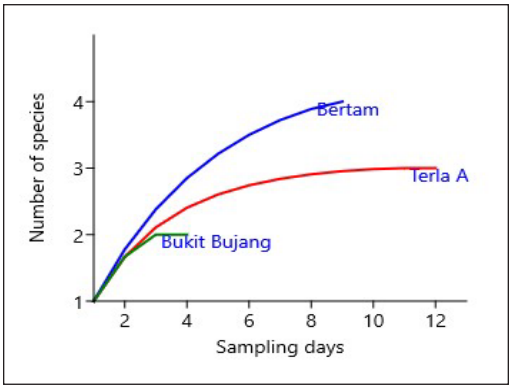

Figure 3. Rarefaction of bird species accumulation curves derived for the three forest reserves in Cameron Highlands

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Comparative Analysis of Antioxidant Properties in Water and Ethanolic Extracts of Propolis from Two Species of Indo-Malayan Stingless Bee

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ABSTRACT

Propolis, an organic material crafted by bees from a blend of their salivary secretions, beeswax, pollen, and resins, contains essential organic compounds. Phenolic and flavonoids, crucial for their antioxidant properties, are prominently present in this resinous substance. The antioxidant capabilities of propolis extract from *Geniotrigona thoracica* (G) and *Heterotrigona itama* (H), two species of Indo-Malayan stingless bees, were examined in this study by using different solvents (ethanol, E and water; W). The analysis involved four extracts of stingless bee propolis which included the ethanolic extract of *G. thoracica* and *H. itama* (GE and HE), and water extract of the two species (GW and HW). The Folin-Ciocalteu method evaluated total phenolic content (TPC), while colourimetric techniques were utilised for total flavonoid content (TFC) estimation. Additionally, antioxidant strength was assessed through ferric-reducing antioxidant power (FRAP) as well as IC_{50} values from the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. The findings confirmed that HE offered the most elevated values for both TPC and TFC, with 52.775 mg GAE g^{-1} and 467.37 mg QE g^{-1}, respectively. Meanwhile, the IC_{50} value was comparable among HE and GE at 0.351 and 4.536 mg/ml, respectively, compared to HW and GW (11.985 and 30.93 mg/ml). Consequently, both species are competitive natural sources of antioxidants under the same solvent. While ethanolic extract propolis demonstrates superior antioxidant properties, water extract demonstrates potential benefits, including being consumer-friendly and

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environmentally sustainable, making it a cost-effective alternative worth exploring. The extract holds promising potential for future applications, including in the cosmeceutical, functional food, and pharmaceutical industries.

Keywords: DPPH, flavonoid, FRAP, *Geniotrigona thoracica*, *Heterotrigona itama*, phenolic

INTRODUCTION

Stingless bees, characterized by reduced stinging ability (Michener, 2000), are globally represented by over 500 species in 32 genera, with over 100 species scarcely described (Abd Jalil et al., 2017; Avila et al., 2018). Stingless bees have a pivotal function in an ecosystem, with approximately 33 identified Malaysian species, notably *Geniotrigona thoracica* (*G. thoracica*) and *Heterotrigona itama* (*H. itama*) (Shamsudin et al., 2019). These species are frequently domesticated due to easy cultivation in suburban areas, and their log hives are possible to locate and collect in nature (Zullkiflee et al., 2022).

The chemical makeup of stingless bee species varies due to factors like collection timing, surrounding flora, and geographical positions (Bankova et al., 2000; Park et al., 2000). As essential pollinators, they contribute significantly to the ecosystem, propolis, wax, pollen, and honey (Bibi et al., 2008; Michener, 2012). These bee products, used in traditional medicinal practices, offer nutritional and health benefits (Maroof & Gan, 2022; Quezada-Euán, 2018).

Plant resins, pollen, beeswax, and certain essential and aromatic oils are the most common propolis compositions (Anjum et al., 2019). Abundant in phenolic compounds, esters, flavonoids, terpenes, and beta-sterols, propolis exhibits antioxidant properties primarily due to its phenolic and flavonoid constituents. Predominant phenolic compounds in stingless bee propolis encompass phenolic acids, catechins, flavonols, stilbenes, and tannins (Bonamigo et al., 2017; Cauich-Kumul & Segura Campos, 2019; Huang et al., 2014).

Propolis extracts exhibit antioxidant, antibacterial, and anti-inflammatory characteristics which can offer various health advantages (Ahmad et al.,2019; Berretta et al., 2020; Brodkiewicza et al., 2018; Junior et al., 2018; Siheri et al., 2016; Veloz et al., 2019; Vongsak et al., 2015). Widely applied in pharmaceuticals, cosmetics, and functional foods, propolis is recognized for its antioxidant potential and diverse chemical composition (da Silva et al., 2011; Santos et al., 2019; Vasilaki et al., 2019). Its antioxidant efficacy, attributed to hydroxyl groups in phenolic compounds, effectively neutralizes free radicals (Mihai et al., 2011). Similar to stingless bee honey (Mahmood et al., 2021), propolis constituents and their biological effects are contingent upon their botanical origin, geographical location, harvest season, bee species, and extraction methods (Ibrahim et al., 2016; Lim, Chua, & Soo, 2023; Shehata et al., 2020).

Simultaneously, an extraction process significantly affects the propolis molecular makeup (Kek et al., 2014; Przybylek & Karpinski, 2019). Other than maceration and Soxhlet (Rocha et al., 2023), techniques like ultrasound extraction are advantageous, with solvent choice affecting the extraction efficiency (Bankova et al., 2021; Silva-Beltran et al., 2021). Typically, a variety of solvents are used for this purpose, including water, ethanol, methanol, chloroform, dichloromethane, acetone, and ethyl ether (Martinotti & Ranzato, 2015; Wagh, 2013). Notably, Mello et al. (2010) found that the phenolic compounds are efficiently extracted when ethanol/water solvents are utilized. Additionally, propolis ethanol extracts have demonstrated superior antioxidant activity compared to aqueous extracts.

Nevertheless, Laskar et al. (2010) observed that water-extracted propolis demonstrated a greater phenolic content alongside improved reducing capability and scavenging activity in comparison to its ethanolic equivalent. It may be attributed to the efficacy of water in aiding the movement of extractable components, such as polyphenols, within plant tissue (Altiok et al., 2008; Borges et al., 2020). However, the dissolution of certain high molecular weight phenolic compounds like tannins in water may lead to the formation of colloids, a phenomenon discussed by Fraga-Corral et al. (2020) and Kusuma et al. (2022).

Despite extensive propolis research, studies on Malaysian stingless bee propolis remain limited (Lim, Chua, & Dawood, 2023). This research uses ethanol and water extraction to explores the properties of antioxidants from propolis found in *H. itama* and *G. thoracica*. Findings could potentially aid in determining the most suitable bee species and solvent for diverse applications in distinctive industries.

MATERIALS AND METHODS

Raw Materials Preparation

Propolis from *H. itama* and *G. thoracica* was harvested from Kuala Terengganu, Terengganu, from August until November and transported to the Postharvest Laboratory at Universiti Malaysia Terengganu (UMT). The raw propolis was meticulously purified upon collection to remove extraneous materials, including dead bees, wood fragments, and debris. Propolis was then frozen at -20ºC prior to analysis.

Extraction of Propolis

Propolis was extracted by adapting the methodology of Omar et al. (2020) with alteration. The powdered propolis weighed 10 g in total and was homogenised via two distinct solvents: water and 95% ethanol, using a 1:10 ratio. An ultrasonic bath was used to extract the propolis for 50 minutes at 50ºC. It was then agitated in an incubator shaker for 48 hours. Subsequent to this, the propolis mixture was strained using a cloth strainer and filter paper to eliminate solid particles and waxes. The resulting propolis extract was centrifuged at

9000 rpm for 15 min. A clarified solution was pooled and evaporated before storage at -20ºC using a rotary evaporator. The concentrated extract was dried using a vacuum oven to remove excess moisture. (Figure 1).

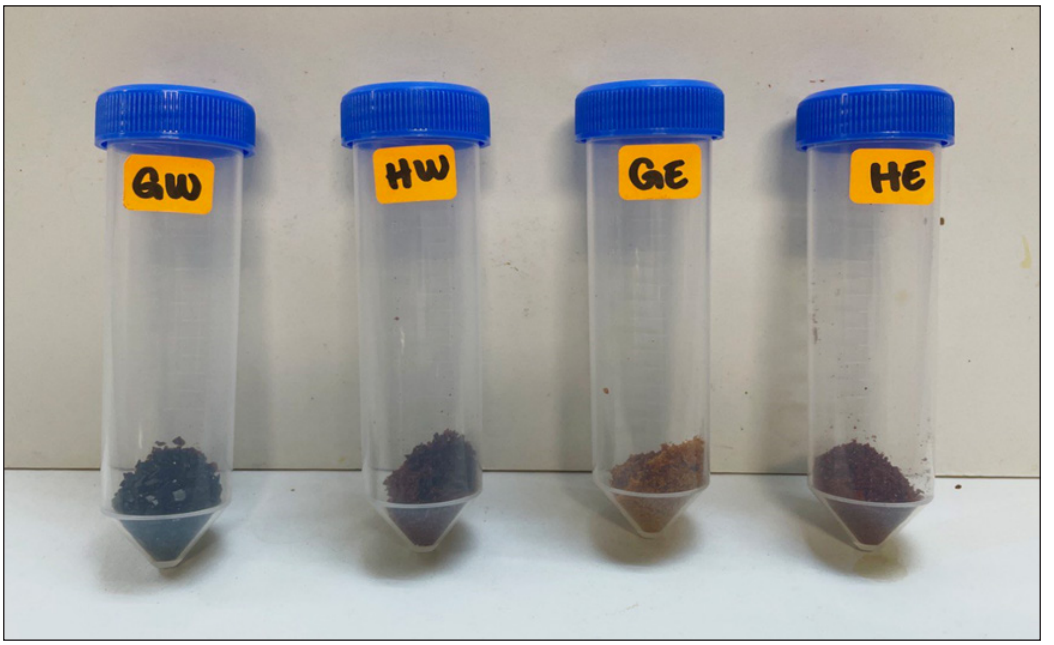

Figure 1. Propolis crude extract that were used for the analysis. From left to right: Water extract of *Geniotrigona thoracica* (GW), water extract of *Heterotrigona itama* (HW), ethanol extract of *G. thoracica* (GE) and ethanol extract of *H. itama* (HE)

Yield

The yield of extraction was recorded and calculated based on a formula by Pujirahayu et al. (2014):

$$\text{Yield} = (\frac{\text{Pe}}{\text{Pm}}) \times 100$$

where,
Pe = Propolis extract weight (g)
Pm = Raw propolis weight (g)

Phenolic Composition by HPLC

Identification of phenolic compounds of stingless bee propolis was implemented according to Sun et al. (2015) with slight modification. A diode ray detection (DAD) equipped HPLC

(Shimadzu, Japan) system was utilised for the study. A total of 10 µl of propolis extracts were injected into the HPLC coupled with Syncronis™ C18 column (250 × 46 mm, 5 µm) (Thermo Scientific™, USA). The mobile phase comprised 2% acetic acid in water (A) and 2% acetic acid in methanol (B). A constant flow rate was set at 0.75 ml/min with a 150 min gradient flow program. The gradient was set as follows: 0–25 min: 22%–36% B; 25–55 min: 36%–52% B; 55–90 min: 55%–63% B; 90–115 min: 63%–70% B; 115–135 min: 70%–75% B; and 135–150 min: 75%–80% B. Simultaneously, oven temperature was set at 35°C and the phenolic compounds of stingless bee propolis were identified based on previously reported publications.

Total Phenolic Content (TPC)

Total phenolic content (TPC) was implemented by the Folin-Ciocalteu colourimetric method (Syed Salleh et al., 2021). Initially, 1 ml of solution containing 5 mg crude propolis and the corresponding extraction solvent (water or 95% ethanol) was prepared. Next, 0.2 ml of sample solution was pipetted into a vial and diluted to 1 ml with 10% Folin-Ciocalteu reagent. After the solution was incubated at ambient temperature for five minutes, 1 ml of 8% sodium carbonate (Na_2CO_3) solution was introduced. Finally, a maximum volume of 3 ml was attained using 95% ethanol, and the mixture's absorbance was estimated at 725 nm via a UV-VIS spectrophotometer (Shimadzu, Japan). Total phenolic content was quantified and expressed in milligrams of gallic acid equivalent per gram of propolis (mg GAE g^{-1}).

Total Flavonoid Content (TFC)

The colourimetric technique involving aluminium chloride with minor adjustment was utilised to quantify the TFC within propolis (Sun et al., 2015). Crude propolis was first dissolved in its respective solvent to acquire a 5 mg/ml concentration. A volume of 0.5 ml of the prepared sample was homogenised with 0.3 ml of 5% sodium nitrite ($NaNO_2$), followed by an incubation period of 6 minutes. Subsequently, 0.3 ml of 10% aluminium nitrate ($Al[NO_3]_3$) was added to the mixture, which was incubated for another 6 minutes. Following this, 4 ml of 4.3 % sodium hydroxide (NaOH) was introduced, and the overall volume of the solution was fixed to 10 ml using the same extraction solvent. After incubation at ambient temperature for 15 minutes, a UV-VIS spectrophotometer (Shimadzu, Japan) was used to read the sample's absorbance at 510 nm. Total flavonoid content was estimated and presented as a milligram of quercetin equivalent per gram of propolis (mg QE g^{-1}).

DPPH Radical-scavenging Activity

Sun et al. (2015) outlined a technique to assess the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. A DPPH solution of 0.2 mM concentration was prepared in 95% ethanol. Several concentrations of crude propolis extract (0.2 to 10.0 mg/ml) were

prepared using the respective extraction solvent. The test is conducted by combining a volume of 200 µL of the propolis solution with 1.8 ml of the respective solvent and 2 ml DPPH solution. This mixture was then thoroughly vortexed and incubated for 20 minutes at room temperature in darkness. A UV-VIS spectrophotometer (Shimadzu, Japan) was employed to measure the absorbance of the solution at 517 nm and quantified using IC_{50} value, with ascorbic acid serving as the antioxidant standard.

Ferric Reducing Antioxidant Power (FRAP) Assay

A modified FRAP assay was performed as per the approach outlined by Wong et al. (2006). The FRAP reagent was prepared by combining 20 mM ferric chloride ($FeCl_3$), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ), and 300 mM sodium acetate buffer at a 10:1:1 ratio with a pH of 3.6. In the experimental procedure, a 100 µl sample was homogenised with 3 ml of FRAP reagent and incubated at 37 ºC for 4 minutes. At two time points, initially at 0 minutes (before incubation) and then at 4 minutes (after incubation), the measurement of absorbance was taken using a UV-VIS spectrophotometer (Shimadzu, Japan) at a wavelength of 593 nm. To prevent cloudiness in the solution, Tween 20 was added as a surfactant (Wojtunik-Kulesza, 2020). The FRAP value was evaluated in terms of mg Trolox equivalents antioxidant capacity per gram of propolis on a dry weight basis (mg TEAC g^{-1}).

Statistical Analysis

Every analysis was implemented in three replications to ensure accuracy. A Two-Way Analysis of Variance (ANOVA) was used on the resulting data, and subsequently, a post-hoc Tukey's test was run to verify its statistical significance. These analyses were completed utilising the Statistical Analysis System (SAS) program.

RESULTS AND DISCUSSION

Phenolic Composition by HPLC

The expected compounds in stingless bee propolis extract are shown in Table 1 and Figure 2. The presumption of compound availability was reported by comparing the retention time with the previous study at 280 nm since most phenolic compounds showed some degree of absorption at this wavelength (Gomez-Caravaca et al., 2015). However, the reported compounds still need to be attested with an external standard calibration curve to accurately identify and quantify them in future studies. Based on Figure 2, water extract propolis (GW and HW) most probably displayed a generally lower concentration of compounds when compared with ethanolic extract propolis (GE and HE). Despite having complex HPLC profiles, GW and HW had limited peaks, contrary to GE and HE.

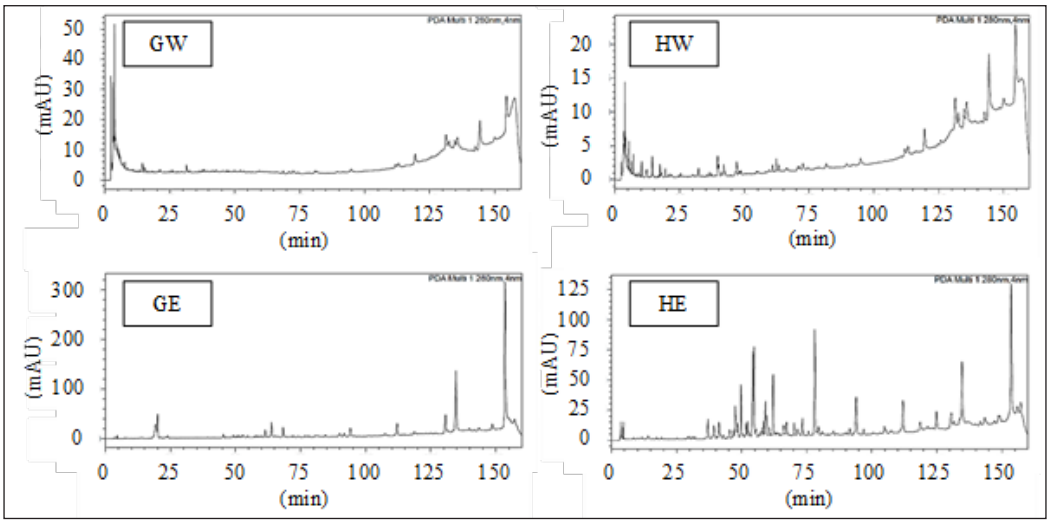

Figure 2. HPLC chromatograms of stingless bee propolis extracted with water and ethanol at 280 nm
Note. GW= *Geniotrigona thoracica* water extract; HW= *Heterotrigona itama* water extract; GE= *Geniotrigona thoracica* ethanol extract; HE= *Heterotrigona itama* ethanol extract

Table 1
Retention time (min) of the presence of the expected compounds in water and ethanolic extract propolis at 280 nm based on the compounds reported by Sun et al. (2015)

Retention time (min)	Expected compound	Propolis samples			
		GW	GE	HW	HE
7.00±1.00	3,4-Dihydroxybenzaldehyde	+	+	+	+
12.00±1.00	Caffeic acid	+	+	+	-
19.00±1.00	Ferulic acid	+	+	+	+
21.00±1.00	Isoferulic acid	+	+	+	-
24.00±1.00	Benzoic acid	-	+	+	+
33.00±1.00	Cinnamic acid	+	+	+	+
41.00±1.00	4-methoxycinnamic acid	+	-	+	+
46.00±1.00	5-methoxy pinobanksin	-	+	-	+
49.00±1.00	Pinobanksin	+	+	+	+
54.00±1.00	Quercetin	-	+	-	+
61.00±1.00	Alpinetin	-	+	-	+
65.00±1.00	Kaempferol	-	+	-	+
76.00±1.00	Pinocembrin	-	+	-	+
77.00±1.00	Isorhamnetin	-	+	-	-
80.00±1.00	Benzyl caffeate	-	+	-	-
82.00±1.00	Pinobanksin-3-O-acetate	-	+	-	+
96.00±1.00	Chrysin	-	-	-	+
97.00±1.00	Benzyl-p-coumarate	-	+	-	-
109.00±1.00	Pinostrobin	-	+	-	-
116.00±1.00	Tectochrysin	-	+	-	-

Note. + = Detected; - = Not detected

Nine compounds were expected to be identified in GW and HW, which include 3,4-Dihydroxybenzaldehyde, caffeic acid, ferulic acid, cinnamic acid, 4-methoxycinnamic acid, cinnamylideneacetic acid, pinobanksin, isoferulic acid, and benzoic acid. For GE and HE, 3,4-Dihydroxybenzaldehyde, caffeic acid, ferulic acid, isoferulic acid, benzoic acid, cinnamic acid, 4-methoxycinnamic acid, cinnamylideneacetic acid, 5-methoxy pinobanksin, pinobanksin, quercetin, alpinetin, kaempferol, isorhamnetin, pinocembrin, benzyl caffeate, pinobanksin-3-O-acetate, chrysin, benzyl-p-coumarate, pinostrobin, and tectochrysin were more inclined to be identified. Out of all, 3,4-Dihydroxybenzaldehyde, ferulic acid, cinnamic acid, and pinobanksin were found in all propolis samples, regardless of species and solvent used. The range of solvent polarity may cause differences in the variation of propolis's chemical profiles. More compounds tend to be characterised in GE and HE than in GW and HW, especially phenolic acids, which possess a notable antioxidant potential (Mahmad et al., 2023). The chemical makeup of propolis can also be affected by flowering plants' availability along with its resinous substance that eventually becomes the source of bee forages (Da Silva Araújo et al., 2016).

Yield

Based on Table 2, the present study indicated that propolis extracted with ethanol (GE and HE) has a markedly higher yield ($p<0.05$) by comparison to those extracted with water (GW and HW). GE exhibited the highest yield at 38.81%, followed by HE, GW, and HW at 23.29%, 10.36%, and 6.54%, respectively. This result is consistent with Kustiawan et al. (2022), who documented that methanolic propolis extract of *G. thoracica* produced a higher percentage of yield (33.96%) compared to *H. itama* (29.44%). Similar results were documented in earlier reports where propolis prepared with ethanol produced a higher yield than other tested solvents (Pujirahayu et al., 2017; Sambou et al., 2020). It was also mentioned that the difference was supposedly due to the organic solvent properties that can dissolve most propolis content. In addition, variations in yield and the chemical composition of each extract could be attributed to the hydroxyl groups in water, which render it a less effective solvent for many organic compounds. It suggests that solvent polarity is a significant factor influencing the differences observed in extraction yields, as Almeida et al. (2012) reported.

A study accomplished by Fikri et al. (2019) described that, on average, propolis extract prepared by ethanol had a significantly higher yield, enhanced antioxidant activity, and greater concentrations of total phenolics and flavonoids compared to those extracted with water. This finding aligns with the research on Malaysian propolis by Usman et al. (2016), which demonstrated that 70% ethanol was the most effective extraction yield, surpassing both 100% and 90% ethanol, as well as distilled water. Furthermore, the higher solubility of wax content in propolis extracted with ethanol compared to water, as noted by Fikri et

al. (2019), is likely a contributing factor to the higher yields observed in ethanolic propolis extracts.

Total Phenolic and Flavonoid Content

While achieving the highest yield, GE exhibited lower (p<0.05) phenolic and flavonoid content than HE, yet it was still more potent than both GW and HW. Based on Table 2, ethanolic extracts of propolis (GE and HE) contained significantly more phenolic and flavonoid compounds than water-based extracts (GW and HW), regardless of bee species. Value of TPC ranged from 11 to 52 mg GAE g^{-1}. This study revealed that HE had the highest TPC value (statistically significant with p<0.05) at 52.775 mg GAE g^{-1}, followed by GE, HW, and GW with values of 24.916, 11.247, and 11.022 mg GAE g^{-1}, respectively. The highest TFC was observed in HE at 467.37 mg QE g^{-1}, while the lowest was in GW at 12.664 mg QE g^{-1}, which shared a comparable value to HW at 13.450 mg QE g^{-1}.

Table 2
Comparison of the yield, total phenolic content (TPC), and total flavonoid content (TFC) of stingless bee propolis extract

Propolis	Yield (%)	TPC (mg GAE g^{-1})	TFC (mg QE g^{-1})
GW	10.36^{c}	11.022^{c}	12.664^{c}
GE	38.81^{a}	24.916^{b}	116.46^{b}
HW	6.54^{c}	11.248^{c}	13.450^{c}
HE	23.29^{b}	52.775^{a}	467.37^{a}

Note. Distinct letters within rows denote statistically significant differences (p<0.05)

As previously mentioned, the levels of both TPC and TFC across propolis samples were congruent with one another, exhibiting the highest levels in HE and gradually decreasing through GE, HW, and GW. This pattern aligns with the findings of Sun et al. (2015), which also indicated the highest TPC and TFC values in ethanolic propolis extracts, with the lowest in water extracts. Propolis extracts are known to contain a significant number of phenolic compounds. The differing chemical characteristics and polarities of antioxidant compounds impact their solubility in specific solvents, as Turkmen et al. (2006) noted. While water is a more economical and environmentally friendly solvent, as Lim et al. (2019) highlighted, polyphenols often dissolve more readily in less polar organic solvents like ethanol (Haminiuk et al., 2012).

Various aspects, such as the plant source choices and the extraction solvent, influence the TFC, as Abdullah et al. (2019) identified. In this study, propolis from *H. itama* species demonstrated superior TPC and TFC compared to *G. thoracica,* irrespective of the solvent used. This finding concurs with the discoveries made by Ibrahim et al. (2016), which

also indicated higher levels of phenolics and flavonoids in *H. itama* propolis than in *G. thoracica.* Furthermore, Asem et al. (2019) documented that samples characterised with higher polyphenols typically demonstrate stronger antioxidant activities. However, a study by Mohd Badiazaman et al. (2018) reported that TPC of methanolic extract of *G. thoracica* propolis collected from Besut, Dungun, Lundang, Tanah Merah, and Gua Musang in Terengganu and Kelantan were ranging from 9.23 to 23.43 mg GAE per gram of extract while TFC values ranged from 9.52 to 17.22 mg QE per gram of extract. Suggesting that GE has a comparable phenolic and higher flavonoid content with other local propolis.

Antioxidant Activities

An IC_{50} value of DPPH radical scavenging activity and FRAP assay were used for the assessment of propolis antioxidant capacity. The IC_{50} value represents the sample concentration essential for neutralising 50% DPPH free radicals. A lower IC_{50} value is indicative of an elevated antioxidant activity. Free radicals are reactive molecules produced by cells with the potential to cause oxidative harm to genetic information or cell membranes (Piantadosi, 2020). Table 3 shows HE and GE have the lowest (p<0.05) IC_{50} values, followed by HW and GW at 0.351, 4.536, 11.985, and 30.93 mg/ml, respectively. In other words, the ethanolic extract of stingless bee propolis (HE and GE) demonstrated a potent scavenging effect on the DPPH free radicals, as opposed to the propolis extracted with water.

Regarding the stingless bee species, *H. itama* showed better antioxidant activity than *G. thoracica*, irrespective of their extraction solvent. The outcomes of these analyses align with the observations regarding TPC and TFC results, suggesting a direct association between the elevation of phenolic and flavonoid levels and the antioxidant potential of the extract. Adli et al. (2022) reported a slightly different opinion, claiming that only TPC may be responsible for the antioxidant activity. TFC was not significantly correlated to the IC_{50} DPPH of propolis extract. Nonetheless, Barhe and Tchouya (2016) have previously observed that antioxidant activity is linked to the availability of flavonoids, phenolic compounds, and their distinct chemical structures. Additionally, research by Nafi et al. (2019) indicated that *H. itama* propolis exhibited the most potent antioxidant activity, evidenced by the lowest IC_{50} values when compared to propolis from *G. thoracica* and *Lepidiotrigona terminate* at 30, 40, and 128 µg/ml, respectively. This result also aligns with Abdullah et al. (2020), revealing that *H. itama* recorded the lowest IC_{50} than *G. thoracica* and *Tetrigona binghami* and eventually showed the highest total antioxidant capacity. Adli et al. (2022) revealed that the ethanolic extract of *G. thoracica* had the strongest antioxidant activity in the DPPH assay, with the lowest IC_{50} at 104.2 µg/ml.

The variance seen in antioxidant activity can be related to the variations in phenolic, flavonoid, or other components present in propolis that contribute to their potential

antioxidant properties (Nafi et al., 2019). Moreover, it has been observed that the chemical composition of the ethanolic extract of *H. itama* is more intricate when considering other species of stingless bees, primarily due to the abundance of chemical compounds it contains. As concluded by Mahmad et al. (2023), chemical compounds of *H. itama* are immense that includes tannins, betalains, peptides, nucleotides, phospholipids, indoles, coumarins, quinolines, cyanogenic glycosides, isoflavonoids, pyrroles, anthocyanins, saponins, carotenoids, amino acids, glycosides, xanthones, lignans, and quinones. Despite that, the crucial components contributing to the antioxidant potential of propolis extract are phenolics and flavonoids, in general (Puspitasari et al., 2022). Additionally, the diversity of chemical compounds identified in all propolis extracts may stem from the bees' preferences for specific plants or flowers during foraging (Nafi et al., 2019).

Ferric reducing antioxidant power assay involves the reaction of electron transfer from antioxidant (as electron donor) and ferric (Fe^{3+}) tripyridyl triazine complex (as electron acceptor) into ferrous (Fe^{2+}) form (Ismail et al., 2013). According to Table 3, HE demonstrated a notably higher FRAP value (statistically significant with $p<0.05$) of 0.299 mg TEAC g^{-1}, while HW and GE displayed similar results at 0.039 and 0.033 mg TEAC g^{-1}, respectively. GW showed the lowest FRAP value at 0.009 mg TEAC g^{-1}. In other words, HE reduced Fe^{3+} into Fe^{2+}, possibly owing to its higher antioxidant content than other extracts, as in Table 2. Sun et al. (2015) observed that 75% ethanolic propolis extract presented the highest FRAP value (200 µg Trolox/mg), nearly 15 times greater than a water extract, indicating a superior reducing capability. However, according to Asem et al. (2019), the antioxidant activity of the ethanolic extract obtained from *G. thoracica* was the most prominent, followed by *H. itama* and *Tetrigona apicallis*. The antioxidant activity of propolis presented in this study can be considered weaker when compared to stingless bee propolis from other locations like Hulu Bernam in Selangor at 104.2 to 332.7 ug/ml for both water and ethanolic extract of *G. thoracica* and *H. itama* propolis (Adli et al., 2022). Besides, Idris et al. (2023) reported lower IC_{50} values stretching from 27 to 122.7 µg/ml for ethanolic extract of *G. thoracica* collected from Serdang, Shah Alam, and Hulu Bernam, Selangor. Despite having a lower antioxidant capacity, the propolis extract from the present study can still be potentially utilised for antimicrobial activity due to its high content of flavonoids (Anjum et al., 2019; Sforcin, 2016; Wagh, 2013).

Table 3
Comparison of the DPPH and FRAP assay of G. thoracica *and* H. itama *propolis extract via water and ethanol solvents*

Propolis	DPPH IC_{50} (mg/ml)	FRAP (mg TEAC g^{-1})
GW	30.930[a]	0.009[c]
GE	4.536[c]	0.033[b]
HW	11.985[b]	0.040[b]
HE	0.351[c]	0.299[a]

Note. Distinct letters within rows denote statistically significant differences ($p<0.05$)

CONCLUSION

The antioxidant levels in propolis sourced from stingless bees fluctuate depending on the bee species and the type of extraction solvent employed. This investigation unveils a strong connection between the phenolic and flavonoid content and the ability to scavenge DPPH free radicals, suggesting a link between the antioxidant attributes and the efficacy of stingless bee propolis. However, a more detailed characterisation of stingless bee propolis profiles needs to be studied to identify the exact compound contributing to its antioxidant properties. Overall, ethanolic extracts exhibited elevated total phenolic and flavonoid content, along with heightened antioxidant potency in comparison to water extracts of propolis. Regarding the stingless bee propolis species, *H. itama* exhibited superior antioxidant properties and activity compared to *G. thoracica,* irrespective of the solvent used, with the order being HE>GE>HW>GW. Notably, research on Indo-Malayan stingless bees, especially in Malaysia, is limited. Therefore, this study is poised to add valuable knowledge to the relatively scarce information on stingless bee propolis in Malaysia, potentially aiding in future innovations related to stingless bee propolis.

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Meta-topolin Enhanced *In Vitro* Regeneration, Acclimatization, and Genetic Stability Assessment of Regenerated Watermelon (*Citrullus lanatus* Thunb.)

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ABSTRACT

The production of seedless watermelons, primarily through triploid varieties, has surged to meet the growing consumer demand, especially due to the convenience of eating. However, triploid watermelon production is time-consuming, and seed production is tedious. Hence, *in vitro* propagation has become an alternative, but it faces challenges such as low regeneration response, poor rooting, and low *ex vitro* establishment. These issues can be addressed by applying aromatic cytokinin *meta-topolin* (*m*T) in the regeneration system. Hence, this study aims to investigate the efficacy of *meta-topolin* (*m*T) compared to 6-benzyl adenine (BA) for in vitro regeneration and acclimatization of *Citrullus lanatus* (Thunb.). The effects of aromatic cytokinins BA and *m*T (0.5, 1.0, 1.5, 2.0, and 2.5 mg/L) on multiple shoot production from cotyledonary node explants of watermelon were evaluated. The highest shoot production (25.24 shoots/explant) was observed with 1.5 mg/L *m*T, while BA (1.0 mg/L) produced 11.36 shoots/explant. Rooting response in MS medium with indole-3-butyric acid (IBA 0.5 to 2.5 mg/L) showed the best results in IBA 1.0 mg/L with *m*T-derived shoots (1.5 mg/L), producing 13.33 roots per shoot, compared to 5.62 roots per shoot from BA-derived shoots (1.0 mg/L). After four weeks of acclimatization, *m*T-derived plants had a 97% survival, while BA-derived plants had 84%. Additionally, *m*T-derived plants had significantly higher photosynthetic pigments (chlorophyll a (9.2%), b (11.3%), and carotenoids (29.1%)). RAPD and SCoT markers confirmed the genetic

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stability of the regenerated plants. This research will aid in developing high-quality planting material to produce commercial triploid watermelon plants.

Keywords: Acclimatization, chlorophyll, *meta*-topolin, molecular markers, rhizogenesis, shoot induction

INTRODUCTION

Watermelon (*Citrullus lanatus*) is a widely cultivated fruit crop of significant economic and nutritional importance worldwide. Watermelon is an excellent functional food because it provides vital sources of essential nutrients, like vitamins, minerals, and antioxidants (Athanasiadis et al., 2023; Manivannan et al., 2020). The popularity of seedless watermelons has increased because of their convenience. Seedless watermelon is a hybrid of two inbreds, where the male parent is diploid, and the female parent is a tetraploid (Kaseb et al., 2023; Subasinghe Arachchige et al., 2022). However, the production of triploid watermelon is challenging and time-consuming compared to diploid cultivars (Phat et al., 2015; Solmaz et al., 2018). Hence, *in vitro* propagation, which can produce a large number of clones in a short duration, is an alternative solution to this issue. Although *in vitro* propagation in watermelon has been carried out earlier, several limitations prevent extensive use of this method. Previously, *in vitro* regeneration studies using triploid watermelon (var. Arka manik) gave low frequency of shoot production (Nasr et al., 2004; Thomas et al., 2000), poor rooting and low *ex vitro* establishment as the limiting factors (Shalaby et al., 2008; Vinoth & Ravindhran, 2016).

Several factors impact the establishment of *in vitro* regeneration in watermelon, including genotype, explant type, culture conditions, and hormonal treatments (Ameri et al., 2015; Vinoth & Ravindhran, 2016). Growth regulators play a significant role in the *in vitro* regeneration of watermelon, particularly cytokinins (Badr-Elden et al., 2012; Gnamien et al., 2013). However, different varieties or cultivars of watermelon react differently to the type and concentration of cytokinin; such different responses were observed among watermelon cultivars such as Arka Manik, Giza 1 and Giza 21 (Vinoth & Ravindhran, 2016; Zakaria et al., 2007). Thus far, the optimal shoot proliferation from watermelon explants was noted in a BA-supplemented medium. According to previous reports, the disadvantages of the application of 6-benzyladenine (BA) in plant multiplication through the *in vitro* method involve the production of unhealthy shoots, decreased root formation, and poor survival during acclimatization rate (Ameri et al., 2015; Badr-Elden et al., 2012; Vinoth & Ravindhran, 2016). These physiological disorders have the potential to negatively affect the quality of *in vitro* plant production. Hence, replacing BA with *m*T is an upcoming trend (Nowakowska & Pacholczak, 2019; Werbrouck et al., 2008) and is recommended for improving the regeneration frequency of watermelon.

The meta-topolin (*m*T) is a hydroxylated form of 6-benzylaminopurine and an innate aromatic cytokinin that was originally extracted from Poplar plant leaves (Strnad et al., 1997; Werbrouck et al., 1996). Topolins are currently used as substitutes for cytokinin, which is most potent in plant micropropagation. It has been observed that topolins increase shoot proliferation, maintain hormonal stability, and improve rooting efficiency in bananas (Aremu et al., 2012; Bairu et al., 2008). Using *meta*-topolin instead of BA usually produces a higher number of quality shoots with increased chlorophyll content and better rooting with an improved acclimatization rate (Koszeghi et al., 2014; Nowakowska et al., 2019). As far as we are aware, no study has been done on evaluating the impact of *m*T on *in vitro* propagation of watermelon (Arka manik), covering aspects from *in vitro* shoot induction, rooting and acclimatization. However, it is also necessary to ensure that side effects such as the somaclonal variation are not prevalent due to the use of *m*T.

Clonal propagation with increased cytokinin concentration resulted in somaclonal variations in strawberries and bananas (Biswas et al., 2009; Bidabadi & Jain, 2020). Changes in the plant genome during the regeneration of plants due to somaclonal variation may be hereditary or epigenetic (Duta-Cornescu et al., 2023). One of the contributing factors for somaclonal changes and mutations in the regenerated plantlets is the use of synthetic plant growth regulators, among others, such as the alterations in the nutrients supplied in the media, prolonged subculturing interval of plantlets in tissue culturing systems, and altered pH of the media (Duta-Cornescu et al., 2023). Since this study attempts to use *m*T for *the in vitro* production of watermelon, testing the *in vitro* clonally grown plants for genetic homogeneity is of utmost significance in producing tissue culture plants of elite genotype. It has long been advised to use molecular markers to examine the genetic homogeneity of *vitro*-cloned plants (Al-Khayri et al., 2022; Chirumamilla et al., 2021; Gantait et al., 2022; Koh et al., 2024). Molecular markers such as random amplified polymorphic DNA (Deoxyribonucleic acid; RAPD) and the start codon targeted (SCoT) polymorphism (Ajithan et al., 2020; Bisht et al., 2024; Elayaraja et al., 2019; Joshi et al., 2023; Sathish et al., 2022; Vasudevan et al., 2017) can be utilized as reliable tools to assess the genetic variations in regenerated plants. For establishing genetic fidelity via molecular marker-assisted analysis, start codon targeted (SCoT) polymorphism marker systems have been used as simple, reliable and highly reproducible tools (Bhattacharyya et al., 2023; Rai, 2023). Thus, two types of molecular markers were used in this study to screen the genetic stability of the *in vitro*-raised watermelon plantlets.

Hence, our current study aims to investigate the efficiency of *m*T in comparison with BA on *in vitro* shoot multiplication, rooting, and acclimatization and confirm the genetic homogeneity of plants using RAPD and SCoT molecular markers in regenerated watermelon.

MATERIALS AND METHODS

Explant Preparation and Media

Murashige and Skoog medium (Murashige & Skoog, 1962) containing 100 mg/L of myo inositol, and 3% sucrose was used as the culture medium for the present study. The pH of the medium was adjusted to 5.6 by using 1 N HCl and 1 N NaOH before adding 0.8% agar, and the medium was autoclaved for 15 min at 121°C. All the plant growth regulators were introduced into the media prior to autoclaving. All the required chemicals were purchased from HiMedia®, Mumbai, India. All the cultures were maintained at 25 ± 2°C under a 16-h photoperiod at a light intensity of 50 µmol m^{-2} s^{-1}.

The triploid watermelon seeds used in this study, namely Arka manik, were provided by the Indian Institute of Horticultural Research (IIHR) in Bengaluru, India. Surface sterilization of mature seeds was carried out using mercuric chloride (0.1%) for 3 minutes, after which the seeds were washed three times with distilled water. The sterilized seeds were inoculated into MS basal media (Murashige & Skoog, 1962) supplemented with agar (0.8% W/V) and 3% sucrose and kept in complete darkness for three days followed by in light (40 µmol m^{-2} s^{-1}) at 25±2°C for four days under a 16/8 h photoperiod (Figure 1a). Watermelon cotyledonary node explants (10–15 mm, Figure 1b) were prepared by removing cotyledons, apical meristems, and persistent hypocotyls from 7-day-old *in vitro*-grown plants.

Effect of Aromatic Cytokinins

Cotyledonary node explants were excised from 7-day-old *in vitro* seedlings and inoculated on MS medium (Murashige & Skoog, 1962) supplemented with various plant growth regulators. The medium for *in vitro* regeneration was standardized using various BA and *m*T concentrations. For multiple shoot production, MS basal medium supplemented with BA and *meta*-topolin (*m*T) at various concentrations (0.5, 1.0, 1.5, 2.0, 2.5 mg/L) was used individually. As a control, cytokinin-free basal MS media was employed. All cultures were transferred to a fresh medium every two weeks of incubation. After six weeks, the mean number of multiple shoots per explant, mean shoot length, and percentage of explants' response were recorded. The cultures were maintained at 25±2°C with a 16/8 hr photoperiod and 40 µmol m^{-2} s^{-1} light intensity.

Optimizing the BA and *m*T-Derived Shoots for Root Induction

BA- or *m*T-derived, healthy and well-elongated shoots (≥4 cm) with more than four fully expanded leaves were isolated separately in two groups (BA- and *m*T-derived shoots) from six-week-old cultures. After that, the shoots were transferred onto MS medium augmented with different doses of Indole-3-butyric acid (IBA) (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) to

evaluate the efficiency of the rooting response. A basal medium devoid of auxins was assigned as a control. After six weeks of culture, the percentage of explant response, the mean number of roots and root length were measured. The cultures were maintained at 25±2°C with a 16/8 h photoperiod and 40 µmol m^{-2} s^{-1} light intensity.

Acclimatization of Regenerated Plants

The rooted watermelon plantlets derived from *m*T (1.5 mg/L) and BA (1.0 mg/L) treated shoots were removed from the rooting media. The plants were thoroughly cleaned under running tap water to get rid of the gel residue that adhered to the roots. The rooted plantlets derived from BA and *m*T treatments were acclimatized in paper cups containing a pot mixture of sand and soil (1:1 v/v/v ratio). The acclimatized plants were covered with polyethylene bags for two weeks, and then the plantlets were transferred to the greenhouse condition. After four weeks of acclimatization, the percentage of plants that survived, the number of leaves per plant and shoot length were recorded.

Quantification of Photosynthetic Pigments

The leaves' from acclimatized watermelon plantlets derived from *m*T (1.5 mg/L) and BA (1.0 mg/L) treated shoots were collected for the quantification of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) at five different intervals (1, 7, 14, 21 and 28 days). Fresh leaves were harvested from 5 plants derived from *m*T (1.5 mg/L) treatments, approximately 100 mg from each plantlet was subjected to chlorophyll pigment extraction, and the experiments were carried out with three replicates. Similarly, fresh leaves were harvested from 5 plants derived from BA (1.0 mg/L) treatments, and approximately 100 mg from each plantlet was subjected to chlorophyll estimation. The analysis was carried out with methanolic extracts of leaves using a colorimetric method by Lichtenthaler (1987), with minor modifications as detailed by Aremu et al. (2012) and Vasudevan et al. (2017). The pigment concentrations were expressed as µg per gram of fresh weight.

Plant Total Genomic DNA Extraction and Genetic Fidelity Analysis

The acclimatized watermelon plantlets derived from *m*T (1.5 mg/L) and BA (1.0 mg/L) treated shoots were examined for genetic fidelity analysis. A genomic DNA extraction kit (Sigma–Aldrich, USA) was used for genomic DNA extraction from 10 plantlets (5 plants derived from *m*T (1.5 mg/L) and 5 plants derived from BA (1.0 mg/L) treatments, these plantlets were selected from a total of 50 plantlets for each treatment. The quantification of the genomic DNA was performed by using a NanoDrop spectrophotometer (Biodrop, UK). Analysis of clonal fidelity was performed using 10 RAPD markers and 15 SCoT markers.

A total of 10 RAPD primers (Table 4) and 15 SCoT primers (Table 5) were used for DNA amplification to evaluate the clonal fidelity of *in vitro* regenerated plants. Polymerase

Chain Reaction (PCR) amplification for RAPD was carried out according to the procedure followed by Vasudevan et al. (2017). PCR amplification was carried out by using Taq 2X master mix RED containing Tris HCl (pH 8.5), $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.2% Tween® 20, 0.4 mM of each dNTP, 0.2 units/µl of Taq DNA polymerase (AMPLIQON, Denmark), template DNA (100 ng) and 5 pmol of specific primers. A reaction mixture containing 25 µl of PCR mixture was prepared by adding 12.5 µl of 2X Taq PCR master mix, 7.5 µl of nuclease-free water, 2 µl of the required primer and 3 µl of a specific DNA sample. PCR was carried out in a PTC-100® thermal cycler (MJ Research Inc., USA) programmed with initial denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 1 min, annealing at 50°C (37°C for RAPD) for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. The PCR-amplified products were separated by electrophoresis in a 1.2% agarose gel and documented by a gel documentation system (UVITEC, France). For PCR-based marker studies, PCR was repeated at least three times with each primer, and only repetitive and scorable bands were used for genetic analyses of regenerated plants (Agarwal et al., 2015).

Statistical Analysis

All the experiments were replicated three times (except acclimatization, which had five replications). Each treatment consisted of 20 explants. The data were examined using IBM Statistical Package for the Social Sciences (SPSS) Statistics version 25. Mean values were compared using Duncan's multiple range test (DMRT) and one-way analysis of variance (ANOVA) at the 5% significance level. The results were expressed as the mean ± standard error (SE).

RESULTS AND DISCUSSION

Production of Multiple Shoots

Cotyledonary node explants derived from 7-day-old *in vitro*-grown seedlings (Figures 1a and b) were inoculated on MS medium containing two different cytokinins (*m*T and BA) individually at various concentrations ranging from (0.5–2.5 mg/L). After two weeks of incubation, multiple shoots were initiated from the cotyledonary node explants in both BA and *m*T treatments (Figures 1c and d). Among them, the media fortified with *m*T exhibited better results, irrespective of the concentration, than media supplemented with BA. The maximum number (25.24) of shoots were recorded in MS medium supplemented with 1.5 mg/L *m*T (Figure 1e, Table 1), followed by 2.0 mg/L *m*T (19.16) and 1 mg/L *m*T (13.06). In treatments with BA, the highest number of shoots was obtained in 1 mg/L BA (11.36 shoots per explant), followed by 1.5 mg/L BA (9.00 shoots) and 0.5 mg/L BA (8.00 shoots), the highest being only 50% compared with *m*T at 1.5 mg/L.

Table 1
Effect of mT and BA on shoot induction from cotyledonary node explants of watermelon in MS media after 6 weeks of culture

Cytokinins	Concentration (mg/L)	Percentage of response	No. of shoots per explant	Shoot length (cm)
Control	0.0	0.0	0.0	0.0
BA	0.5	35.33 ± 0.05 ^{h}	8.00 ± 0.32 ^{ef}	2.05 ± 0.03 ^{b}
	1.0	75.00 ± 1.45 ^{b}	11.36 ± 0.14 ^{cd}	2.45 ± 0.06 ^{b}
	1.5	57.66 ± 1.02 ^{e}	9.00 ± 0.80 ^{e}	2.21 ± 0.02 ^{b}
	2.0	45.00 ± 1.45 ^{g}	7.81 ± 0.12 ^{fg}	2.62 ± 0.04 ^{b}
	2.5	66.33 ± 1.45 ^{cd}	5.54 ± 0.64 ^{h}	2.54 ± 0.03 ^{b}
*m*T	0.5	42.66 ± 1.25 ^{g}	7.38 ± 0.14 ^{fg}	4.18 ± 0.04 ^{a}
	1.0	51.00 ± 1.16 ^{f}	13.06 ± 0.56 ^{c}	4.43 ± 0.06 ^{a}
	1.5	84.33 ± 0.37 ^{a}	25.24 ± 0.80 ^{a}	4.80 ± 0.03 ^{a}
	2.0	72.00 ± 1.02 ^{b}	19.16 ± 0.34 ^{b}	4.56 ± 0.04 ^{a}
	2.5	61.66 ± 1.15 ^{cd}	12.84 ± 0.12 ^{c}	4.12 ± 0.02 ^{a}

Note. Values represented mean ± SE. Means in each column followed by the same letters are not significantly different according to Duncan's multiple range test at $p = 0.05$; *m*T = *Meta*-topolin; BA = 6-benzyladenine

Table 2
Effect of auxin (IBA) on root induction from cytokinin-derived shoots of watermelon in MS medium after 6 weeks of culture

Cytokinin derived-shoots	IBA (mg/L)	Percentage of response	Number of roots per shoots	Length of roots (cm)
BA derived	0.5	54.66 ± 0.88 ^{f}	2.64 ± 0.07 ^{g}	1.53 ± 0.06 ^{c}
shoots	1.0	75.66 ± 1.33 ^{c}	5.62 ± 0.08 ^{e}	2.04 ± 0.02 ^{b}
	1.5	71.00 ± 1.20 ^{d}	3.46 ± 0.11 ^{f}	1.74 ± 0.06 ^{c}
	2.0	65.00 ± 1.15 ^{e}	3.41 ± 0.06 ^{f}	1.35 ± 0.04 ^{c}
	2.5	54.00 ± 1.00 ^{f}	1.49 ± 0.29 ^{h}	1.71 ± 0.03 ^{c}
*m*T derived	0.5	78.33 ± 1.33 ^{c}	8.28 ± 0.13 ^{c}	1.17 ± 0.03 ^{c}
shoots	1.0	96.66 ± 1.20 ^{a}	13.33 ± 0.27 ^{a}	3.61 ± 0.04 ^{a}
	1.5	87.66 ± 1.20 ^{b}	9.54 ± 0.08 ^{b}	2.23 ± 0.08 ^{b}
	2.0	76.00 ± 0.57 ^{c}	7.20 ± 0.16 ^{d}	2.80 ± 0.02 ^{b}
	2.5	64.66 ± 1.85 ^{e}	5.12 ± 0.24 ^{e}	2.47 ± 0.08 ^{b}

Note. Values represented mean ± SE. Means in each column followed by the same letters are not significantly different according to Duncan's multiple range test at $p = 0.05$; *m*T = *Meta*-topolin; BA = 6-benzyladenine; IBA = Indole-3-butyric acid

In the shoot multiplication stage, it was found that with an increase in the concentration of *m*T up to 1.5 mg/L, there was a gradual increase in the number of shoots (from 7 to 25 shoots). Our study showed that with an increase in concentration from 1.5 to 2.5 mg/L, a gradual decline in shoot number was observed, specifically, 12.84 shoot/explant in

comparison to 1.5 mg/L (25.24 shoots). Profuse growth and development of shoots were noticed in optimal doses of *m*T-fortified medium (Figure 1f). Likewise, in BA treatments, the number of shoots increased with an increase in BA concentration up to 1 mg/L, but thereafter, a gradual decrease in the shot number was recorded. Similar results were observed by Badr-Elden et al. (2012), whereby the use of a higher concentration of BA also showed reduced growth and development in watermelon *in vitro* regeneration. It appears that *m*T at 1.5 mg/L was the best for enhancing shoot production from cotyledonary node explants of watermelon. At the same time, *m*T applied at the same concentration was the most beneficial for shoot elongation (Figure 1g, Table 2).

As an alternative to commonly used cytokinins (BA), *m*T offers great potential for enhancing *vitro* regeneration and shows beneficial traits such as improved shoot production and increased shoot length in several crops (Gantait & Mitra, 2021). From the mechanism perspective, the presence of a hydroxy (-OH) group in the side chain of topolins over other cytokinins makes the absorption of *m*T easier, which allows the accumulation of O-glycosides (Krishna et al., 2021; Lalthafamkimi et al., 2021). These O-glucoside conjugates can convert into active free bases and supply cytokinins to the plants for a prolonged period, which leads to normal growth and development of plant cells and tissues (Erisen et al., 2020; Gentile et al., 2017). In the present study, a greater number of multiple shoots were produced in 1.5 mg/L *m*T-supplemented medium. These results clearly showed the use of *m*T as a potential alternative for BA in micropropagation of watermelon. In the case of watermelon, no such report has been available on the efficacy of *m*T in shoot proliferation.

Induction of Roots from *m*T- and BA-Derived Shoots

The *in vitro*-regenerated shoots (obtained from 1.5 mg/L *m*T and 1.0 mg/L BA) were inoculated in different concentrations of IBA (0.5 – 2.5 mg/L) individually, supplemented in MS medium along with control for assessing the efficiency of auxins on rooting traits. Among the treatments tested, the initial root induction was noticed from the *m*T-derived shoots after one week of incubation in a 1 mg/L IBA-supplemented medium (Figure 1h). After 6 weeks of culture, the maximum number (13.33) of roots was found to be induced in 1 mg/L IBA-supplemented medium.

In the case of BA-derived shoots, the highest number of roots (5.62 ± 0.08) and root length (2.04 ± 0.02) per shoot were recorded in 75% of cultures on MS medium augmented with 1 mg/L IBA. The efficiency of *in vitro* rooting of *m*T-derived shoots was high as compared to BA (Table 2). Similar to our studies, Ahmad and Anis (2019) observed that mT (2 mg/L) derived shoots were found to be more effective than BA-derived shoots for rhizogenesis in *Pterocarpus marsupium* (Roxb.) using cotyledonary node explants.

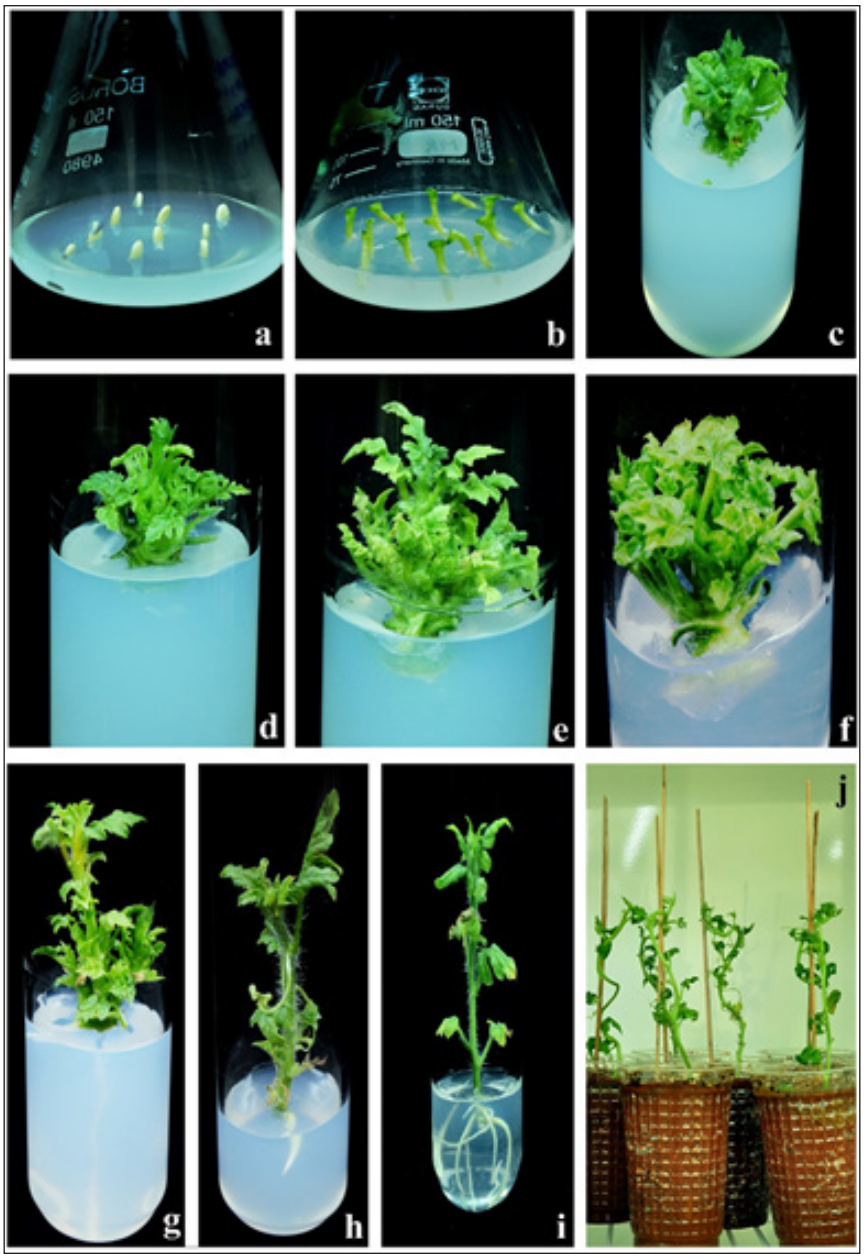

Figure 1. The effect of *m*T on the multiple shoot's induction and rooting efficiency of watermelon cv. Arka manik. (a) Seeds in MS medium, (b) Cotyledonary node explants, (c) Initiation of shoot buds in medium with BA (1 mg/L), after 2 weeks of initial culture, (d) Multiple shoot induction in medium with *m*T (1.5 mg/L), after 2 weeks, (e) Proliferation of shoot on MS medium containing *m*T (1.5 mg/L) after 4 weeks of culture, (f) shoot multiplication on medium containing *m*T (1.5 mg/L) after 6 weeks, (g) Elongated shoots on medium with *m*T (1.5 mg/L) after 6 weeks, (h) Elongated shoot on medium with IBA (1 mg/L) for rooting, (i) Shoots with profuse rooting on medium with IBA (1 mg/L), after 6 weeks of culture, (j) acclimatized plants

Acclimatization of BA- and *m*T-Derived plantlets

The *in vitro*-regenerated plantlets (obtained from 1.5 mg/L *m*T followed by 1 mg/L IBA-supplemented medium) were successfully acclimatized under *ex vitro* growing conditions for 4 weeks (Figure 1j). A more than 97% survival rate was recorded, confirming the effective root growth and development of acclimatized watermelon plantlets. The rooting efficiency of *m*T-derived plantlets was better, with a good survival rate of regenerated plantlets (97%) in *ex-vitro* conditions, compared to an 84% survival rate with BA-regenerated plantlets (Table 3). Some reports have documented that the type and dose of cytokinins have a profound effect on *ex vitro* rooting and acclimatization (Bairu et al., 2007; Hlophe et al., 2020). We have observed a moderate acclimatization success of BA-derived plantlets with a survival rate of 80% to 84%. Similarly, the negative effect of BA on root formation has been reported, resulting in deprived acclimatization rates in many plant species (Bairu et al., 2007; Werbrouck et al., 1996).

Table 3
Survival percentage of mT and BA-derived plants after 4 weeks of acclimatization

Cytokinin derived-shoots	IBA (mg/L)	Percentage of plantlets survived	Number of leaves per plantlet	Shoot length (cm)
BA derived shoots	0.5	81.66 ± 0.88 ^{d}	9.64 ± 0.07 ^{e}	9.53 ± 0.06 ^{e}
	1.0	84.00 ± 1.33 ^{c}	12.62 ± 0.08 ^{c}	10.04 ± 0.02 ^{d}
	1.5	82.33 ± 1.20 ^{d}	10.46 ± 0.11 ^{d}	9.74 ± 0.06 ^{e}
	2.0	81.66 ± 1.15 ^{d}	9.41 ± 0.06 ^{e}	9.35 ± 0.04 ^{e}
	2.5	80.00 ± 1.00 ^{d}	9.49 ± 0.29 ^{e}	8.71 ± 0.03 ^{f}
*m*T derived shoots	0.5	88.33 ± 1.33 ^{b}	12.28 ± 0.13 ^{c}	11.17 ± 0.03 ^{c}
	1.0	97.60 ± 0.20 ^{a}	15.33 ± 0.08 ^{a}	13.61 ± 0.08 ^{a}
	1.5	89.66 ± 0.34 ^{b}	13.64 ± 0.27 ^{b}	12.83 ± 0.04 ^{b}
	2.0	85.00 ± 0.57 ^{c}	12.20 ± 0.16 ^{c}	12.20 ± 0.02 ^{b}
	2.5	80.66 ± 1.85 ^{e}	12.06 ± 0.24 ^{c}	10.47 ± 0.08 ^{d}

Note. Values represented mean ± SE. Means in each column followed by the same letters are not significantly different according to Duncan's multiple range test at $p = 0.05$; *m*T = *Meta*-topolin; BA = 6-benzyladenine; IBA = Indole-3-butyric acid

Quantification of Photosynthetic Pigments

Overall, 97% of the plants (derived from *m*T) and 84% (derived from BA) that survived the acclimatization process were used to quantify photosynthetic pigments. During the acclimatization period, the concentrations of pigments were lower in the first week; later, they increased during the third and fourth weeks (Figure 2). When comparing the photosynthetic pigments (chlorophyll a, b and carotenoids) in the acclimatized plantlets after 4 weeks, there was a significant increase in the contents of photosynthetic pigments (chlorophyll a (9.2%), b (11.3%) and carotenoids (29.1%)) in *m*T-derived *in*

vitro regenerated plants (chlorophyll a, 178 µg/g; chlorophyll b, 98 µg/g; and carotenoid, 93 µg/g of FW) compared with BA-treated plants (chlorophyll a, 163 µg/g; chlorophyll b, 88 µg/g; and carotenoid, 72 µg/g of FW). The concentrations of all three assayed photosynthetic pigments increased and reached their maximum concentration in the fourth week of acclimatization (Figure 2). Similarly, Ahmad et al. (2018) reported that the carotenoid and chlorophyll concentrations increased during acclimatization and reached maximum during the fourth week in *Decalepis salicifolia*. This assessment strongly recommended that a stable increase in photosynthetic pigments is directly related to stress adaptation and proper physiological functions of acclimatized plantlets.

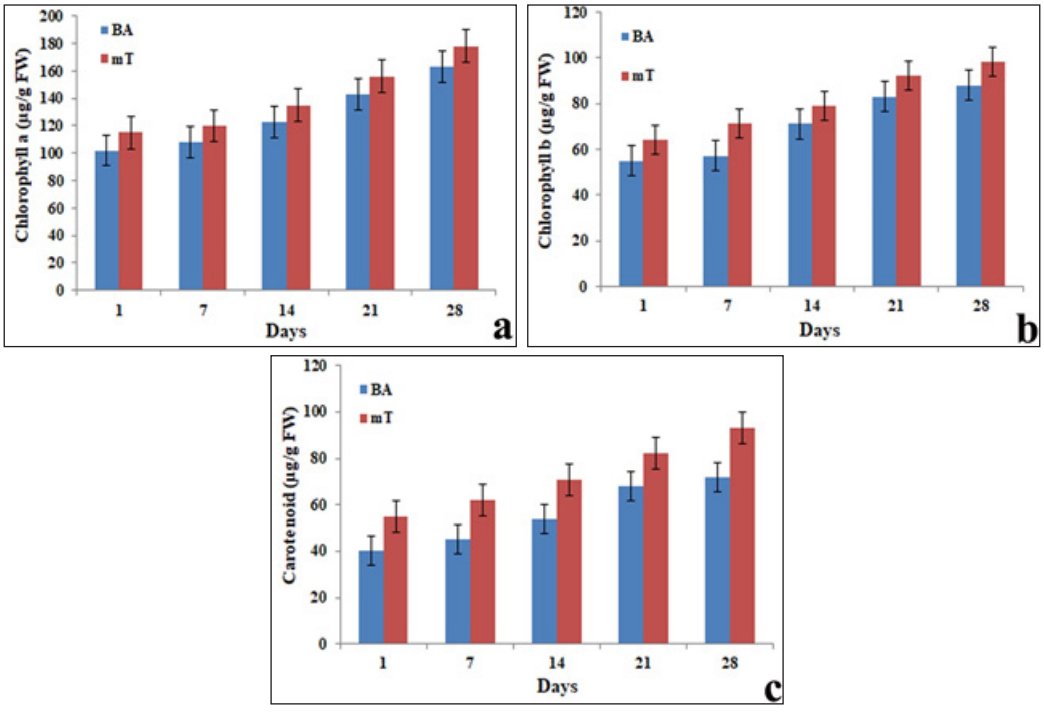

Figure 2. Effect of *m*T on photosynthetic pigments of *C. lanatus* (Arka Manik). a) Estimation of photosynthetic pigment (Chlorophyll a), b). Estimation of photosynthetic pigment (Chlorophyll b), c) Estimation of photosynthetic pigment (Carotenoid)

Interestingly, the carotenoid pigment level was drastically improved to 30% (93 µg/g of FW) in *m*T–derived plants compared to BA-treated plants (72 µg/g of FW) after four weeks of acclimatization. In accordance with our results, Jeon et al. (2005) confirmed that the carotenoids play a pivotal role in defending the photosynthetic apparatus from photo-oxidative injuries, and their elevation during acclimation was a result of the plants reacting to the stresses of acclimation, resulting in high survival of *in vitro* regenerated plantlets of *L. speciose*. In this study, the higher photosynthetic pigment accumulation in

the acclimatized plantlets implies that the *in vitro*-regenerated plantlets were successfully acclimatized to ensure proper growth. Our results are in accordance with a few studies where enhancement in chlorophyll and carotenoid content usually implies an increased rate of photosynthesis (Mahanta et al., 2023; Osorio et al., 2013). *Meta*-topolin regulates stress-associated problems and increases photosynthetic pigments, rooting, and acclimatization efficiency (Ahmad & Anis, 2019). Furthermore, they stated that *m*T lowers chlorophyllase activity and protects the photosynthetic pigments from environmental stresses during the acclimatization of plantlets.

Evaluation of Genetic Fidelity

The genetic fidelity of the *in vitro* regenerated watermelon plants with that of the mother plant was assessed by RAPD and SCoT markers. A monomorphic banding profile was obtained in all 10 RAPD and 15 SCoT primers. A total of 49 scorable and reproducible distinct bands of DNA were generated from 10 RAPD primers in the size range of 200 to 1800 base pairs (bp) (Table 4). A total of 49 scorable bands were obtained from all the RAPD primers with a maximum of 8 scorable bands per primer per sample observed in OPA1 primers (Figure 3a) with sizes ranging from 300-1800 bp wherein a minimum number of 2 bands were observed in OPA6 primer with size ranging from 400–1400 bp.

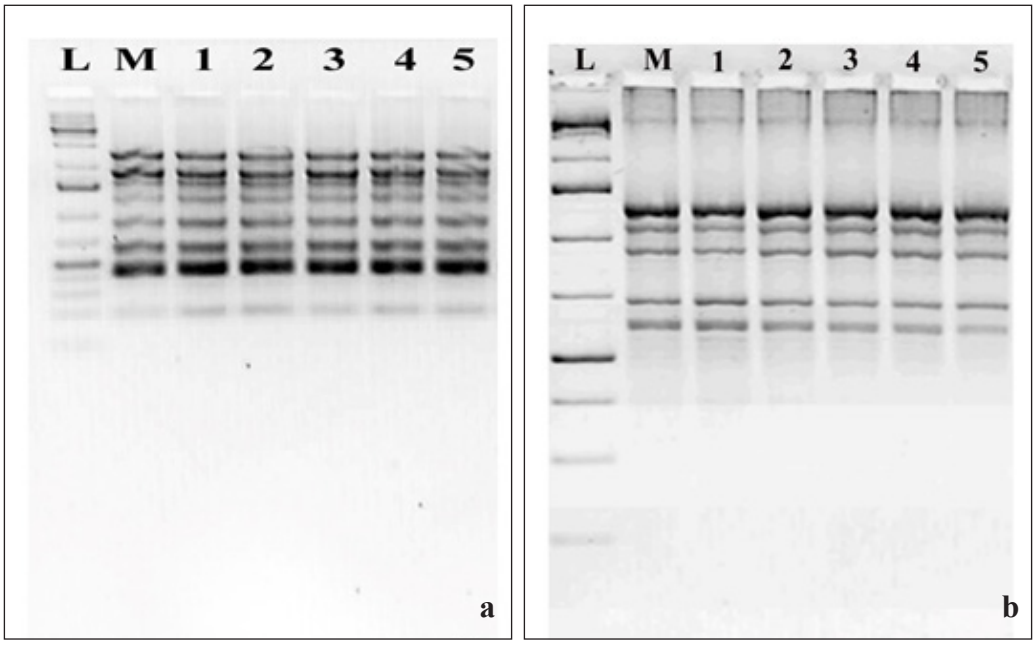

Figure 3. Genetic fidelity analysis of *in vitro* regenerated plants of watermelon. a) RAPD primer (OPA 01). Lane L-1 kb ladder, Lane M-Mother plant, lanes 1–5 Regenerated plants, b) SCoT primer (S16). Lane M-Mother plant, lanes 1–5 Regenerated plants. Lane L-1 kb ladder

Table 4
List of RAPD markers used for genetic fidelity analysis of regenerated watermelon

No.	Primer Name	Primer sequence (5′ – 3′)	Number of scorable monomorphic bands/primer	Size range of bands (bp)
1	OPA1	CAGGCCCTTC	8	300 - 1800
2	OPA2	TGCCGACCTG	5	300 - 1800
3	OPA6	GGTCCCTGAC	2	400 -1400
4	OPA7	GAAACGGGTG	5	500 - 1800
5	OPA8	GTGACGTAGG	5	200 - 1600
6	OPA11	CAATCGCCGT	6	400 - 900
7	OPA13	CAGCACCCAC	5	300 - 1200
8	OPA14	CTCGTGCTGG	4	200 - 1000
9	OPD13	GGGGTGACGA	4	400 -1200
10	OPD16	AGGGCGTAAG	5	200 -800
		Total	49	200 -1800

A total of 40 scorable and reproducible distinct bands of DNA were generated from 15 SCoT primers in the range of 300 to 2000 bp (Table 5). Among all the SCoT primers, the maximum number of 5 scorable bands were observed in S16 primers (Figure 3b) with sizes ranging from 600 to 1400 bp, while the minimum number of scorable bands was observed in S10, S17 and S26 primers with a molecular size range of 1700 bp, 1200bp and 1500 bp, respectively.

Table 5
List of SCoT markers used for genetic fidelity analysis of regenerated watermelon

No.	Primer name	Primer sequence (5′ –3′)	Number of monomorphic bands/primer	Size of bands (bp)
1	S1	CAACAATGGCTACCACCA	2	1200-1600
2	S2	CAACAATGGCTACCACCC	2	800-1000
3	S3	CAACAATGGCTACCACCG	4	500-2000
4	S4	CAACAATGGCTACCACCT	4	500-1500
5	S5	CAACAATGGCTACCACGA	3	300-1700
6	S6	CAACAATGGCTACCACGC	3	1000-1500
7	S7	CAACAATGGCTACCACGG	2	600-1500
8	S10	CAACAATGGCTACCAGCC	1	1700
9	S11	AAGCAATGGCTACCACCA	4	500-1200
10	S12	ACGACATGGCGACCAACG	2	300-1500
11	S16	ACCATGGCTACCACCGAC	5	600-1400
12	S17	ACCATGGCTACCACCGAG	1	1200
13	S25	ACCATGGCTACCACCGGG	2	1000-1600
14	S26	ACCATGGCTACCACCGTC	1	1500
15	S32	CCATGGCTACCACCGCAC	4	400-1400
		Total	40	300-2000

The banding pattern of all the 25 primers (10 RAPD and 15 SCoT primers) of *in vitro*-regenerated and subsequently acclimatized plantlets with the mother plant showed 100% monomorphism without any genetic variations. The DNA banding patterns of the RAPD and SCoT primers clearly revealed no discernible genetic variations, mutations, or polymorphisms among the regenerated watermelon plants. In many research reports, the use of the RAPD and SCoT marker method for evaluating the genetic fidelity of *in vitro*-regenerated plantlets has been effective (Ajithan et al., 2020; Bisht et al., 2024; Elayaraja et al., 2019; Joshi et al., 2023; Sathish et al., 2022; Vasudevan et al., 2017). Due to its affordability, simplicity, and repeatability, the RAPD and SCoT marker system combination is deemed effective.

CONCLUSION

This study highlights the positive effect of *m*T over the commonly used cytokinin (BA) in attaining double the number of multiple shoots from the cotyledonary node explants of watermelon. The potential of *m*T in improving shoot multiplication and subsequent rooting and its favorable impact on the acclimatization of regenerated plants were investigated for the first time in watermelon. With the aid of molecular markers, RAPD and SCoT, this study also confirmed that the plantlets produced using *meta*-topolin (1.5 mg/L *m*T followed by 1 mg/L IBA for rooting) was true to type. During acclimatization, the plantlets had higher success (97%) with increased photosynthetic pigment concentration (Chlorophyll a, b, and carotenoids) that supported vigorous plant growth and development.

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Review Article

Sambar Deer: A Review on Status, Distribution, Conservation, and Commercial Potential in Peninsular Malaysia

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ABSTRACT

Sambar deer (*Rusa unicolour*) are native to most parts of Asia, including Malaysia, Taiwan, Indonesia, and India. Listed as "vulnerable" by the International Union for Conservation of Nature's Red List, the animal has recently been introduced into the United States, Australia, and New Zealand. Although they can easily adapt to a wide range of habitats, the population of Sambar deer in the wild has dramatically declined, and this may be attributed to poaching, illegal wildlife trade and habitat loss when jungles are cleared for development. This article provides the status and distribution of Sambar deer in Peninsular Malaysia and protection efforts to conserve this species in the country. It also highlights the potential of Sambar deer commercial farming in Peninsular Malaysia as part of the conservation programme and meeting human demand.

Keywords: Conservation, genomics, Peninsular Malaysia, population, Sambar deer, wildlife

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INTRODUCTION

The geographical landscape of Peninsular Malaysia provides a suitable habitat

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reservoir for numerous types of flora and fauna, and the diversity is ranked among the highest in the world (Rintelen et al., 2017). The country's commitment to biodiversity conservation was demonstrated by the ratification of the Convention on Biological Diversity (CBD) in 1994 and the adoption of this concept in its development planning (Ministry of Natural Resources and Environment [NRES], 2006). However, maintaining and managing this huge biodiversity requires proper preservation and sustainable resource use. The dilemma occurred when the development and over-exploitation of resources for human needs impacted the survival of our flora and fauna. Development has reduced potential habitats for wildlife, which can be either for infrastructure, transportation, industry, new settlements, or agricultural purposes. Furthermore, exploitation of resources, either legally or illegally, like poaching, has drastically impacted the survival of protected animal species. For example, the Sumatran rhinoceros is now extinct in Malaysia, and the last rhino died in the captive facility in Sabah in 2019 (Edinur et al., 2022). While poor reproductive fitness and diseases can be linked to the extinction of the Sumatera rhino, as might also be expected for the Malayan tiger (Goossens et al., 2013; Nur-Farahiyah et al., 2021), the proximal risk factors for the extinction of Sumatera rhino might have arisen a few decades ago. These include deforestation and illegal poaching for their parts (i.e., horns; refer to Flynn and Abdullah (1984) for details).

One of the endangered animal species in Peninsular Malaysia is Sambar deer. They are essential in the ecosystem as prey for top predators like Malayan tigers (Kawanishi & Sunquist, 2004). They have high adaptability to diverse habitats and have proven success in captive breeding programs (Moriarty, 2004; Timmins et al., 2015). However, the population size of Sambar deer in Peninsular Malaysia shows a decreasing trend, and they are listed as vulnerable on the list of threatened species (International Union for Conservation of Nature, 2024). In this region, traditional hunting practices and consumption of wild deer venison have been culturally significant among indigenous communities (Bartholomew et al., 2021). Poaching of Sambar deer is also driven by the high value of their meat, which is considered a delicacy by Malaysians and for traditional medicine markets (Dai et al., 2011; Kawanishi et al., 2014). The following sections describe the status, distribution, conservation efforts, and commercial potential of Sambar deer, a native wildlife species in Peninsular Malaysia.

SAMBAR DEER: DISTRIBUTIONS, THREATS, LEGISLATION AND CONSERVATION EFFORTS

Sambar deer (*Rusa unicolour*) is one of the deer species native to Asia. They are classified into eight sub-species (i.e., *R. u. boninensis*, *R. u. swinhoei*, *R. u. unicolour*, *R. u. brookei*, *R. u. equina*, *R. u. cambojensis*, *R. u. dejeani*, and *R. u. hainan*), and their populations are region-specific. For example, *R. u. unicolour*, *R. u. cambojensis*, and *R. u. dejeani* are widely distributed in South Asia, Southeast Asia, and China, respectively (Ali et al., 2021).

In Peninsular Malaysia, Sambar deer are primarily distributed in the southern and northern parts of Peninsular Malaysia, particularly in protected areas such as Taman Negara, Endau-Rompin, and Belum-Temenggor Forest Complex; refer to Figure 1 and Ali et al. (2021) for range map of other deer species in Peninsular Malaysia. Sambar deer have also been widely introduced to various parts of the world, including Australia and New Zealand (Stafford, 1997; Watter et al., 2020). The animals are highly adaptive and can live from dipterocarp forests to steeper parts of forested hillsides, but they rarely roam far from water sources (Ali et al., 2021; Timmins et al., 2015).

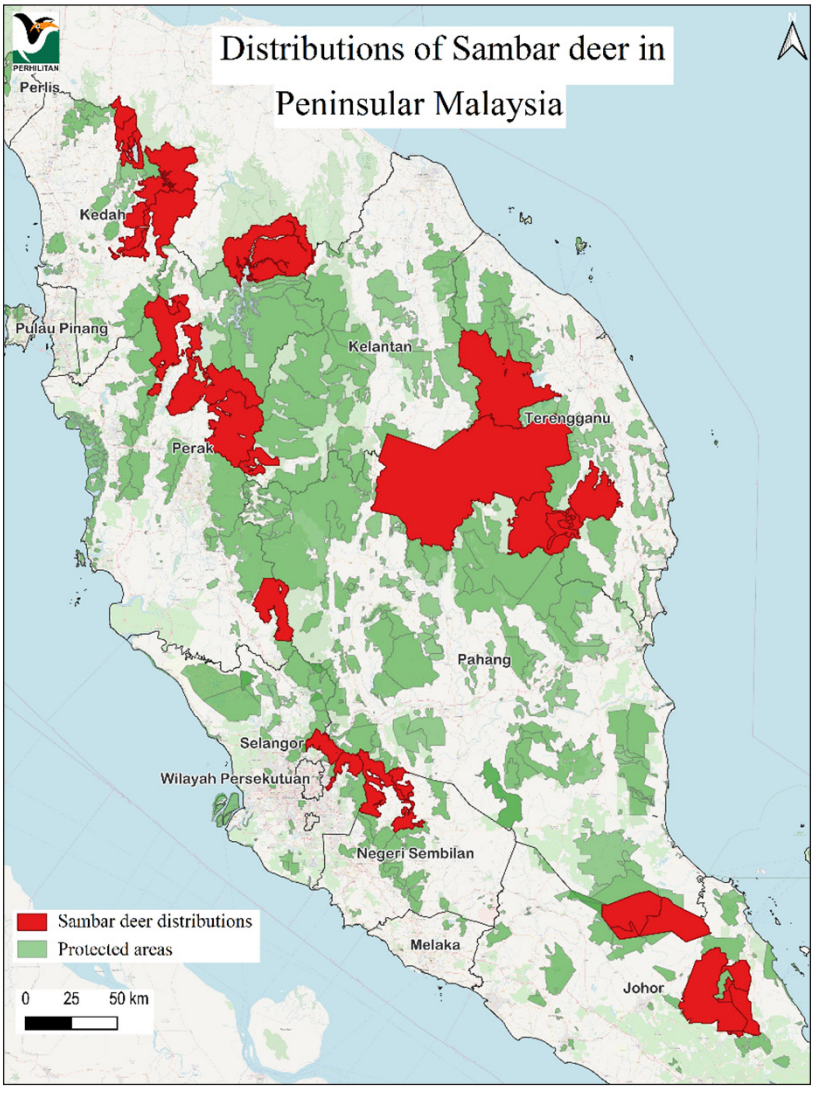

Figure 1. Sambar deer distributions in Peninsular Malaysia were mapped during the first National Tiger Survey (Department of Wildlife and National Parks, 2010)

In Peninsular Malaysia, habitats of the Sambar deer have dramatically shrunk from nearly 70,000 sq km in the 1980s to around 50,000 sq km in 2014 (Department of Wildlife and National Parks, 2017). Their population density ranges from around 0.10 to 0.20 individuals per square km (Ali et al., 2021). Even though deer are a natural prey for Malayan tigers, the real threats to their existence are poaching, unsustainable hunting and habitat destruction (Kawanishi et al., 2014). There is increasing demand for meat and antlers of Sambar deer in Southeast Asia, including in Peninsular Malaysia (Leslie Jr, 2011). Bioactive compounds in antlers have anti-infective agents and have been used to treat immune diseases (Dai et al., 2011). Thus, it is unsurprising that antlers are widely adopted in traditional Chinese and Korean medicine (Ali et al., 2021).

Peninsular Malaysia had become one of the hotspots for illegal wildlife trading within the Southeast Asia area, playing the role of a resource supply and transit country (United Nations Office on Drugs and Crime, 2024). Accordingly, a stricter law, the Wildlife Conservation Act 2010 (Act 716) (WCA, 2010), was later enforced in 2010 and replaced earlier laws, including the Wild Animals and Birds Protection Ordinance 1955 and the National Parks Act (PWA) 1972. However, Xin et al. (2022) reported that wildlife crime cases in Peninsular Malaysia showed an increasing and fluctuation pattern from 2012 to 2018, and cases involving Sambar deer were among the most frequently investigated by the Wildlife Forensic Unit, Department of Wildlife and National Parks, Peninsular Malaysia (Department of Wildlife and National Parks, 2018). Fortunately, Sambar deer were rarely involved in human-animal conflicts and as victims in roadkill, which may further reduce their population size. For example, anger and frustration among the farmers and community might result in revenge killing of wildlife, as reported for *Panthera onca* in Venezuela (Jedrzejewski et al., 2017).

In Peninsular Malaysia, Sambar deer are rarely involved in conflicts with humans and are less vulnerable to roadkill as compared with other wildlife species, such as *Macaca fascicularis*, *Sus scrofa*, and *Elephas maximus*; refer to Table S1 and Xin et al. (2024) for roadkill and human-wildlife conflict data in Peninsular Malaysia, respectively. A similar observation was reported elsewhere, including in India, Australia, and Thailand, where Sambar deer are less affected by vehicle collisions (Davies et al., 2019; Habib et al., 2020; Kummoo et al., 2020). There have been fewer than ten recorded road kills involving Sambar deer over the past five years, mainly occurring in forested areas intersecting with highways. As described earlier, the population size of Sambar deer in Peninsular Malaysia is small and is limited to highly protected forest reserves. These might explain the lower conflict and roadkill cases involving Sambar deer in Peninsular Malaysia. Nonetheless, the Malaysian Government spent billions to reduce animal roadkill, including constructing viaducts and bridges at roadkill hotspots (Ten et al., 2021a).

Table S1
Roadkill data for various species in Peninsular Malaysia from 2017 to 2021

Species	Local name	Number	Frequency
Varanus spp.	Biawak	555	0.2658
Macaca fascicularis	Kera	400	0.1916
Paradoxurus hermaphroditus	Musang	303	0.1451
Sus scrofa	Babi hutan	297	0.1422
Felis spp.	Kucing	116	0.0556
Python reticulatus	Ular sawa	111	0.0532
Tapirus indicus	Tapir	94	0.0450
Presbytis spp.	Lotong	35	0.0168
Naja kaouthia	Ular tedung senduk	16	0.0077
Lutra spp.	Memerang	16	0.0077
Other bird species	Lain-lain burung	14	0.0067
Other snake species	Lain-lain ular	14	0.0067
Callosciurus spp.	Tupai	13	0.0062
Arctictis binturong	Binturong	13	0.0062
Rattus spp.	Tikus	9	0.0043
Strigiformes spp.	Burung hantu	8	0.0038
Amaurornis phoenicurus	Burung ruak-ruak	8	0.0038
Helarctos malayanus	Beruang	8	0.0038
Gallus gallus	Ayam hutan	8	0.0038
Elephas maximus	Gajah	8	0.0038
Nycticebus coucang	Kongkang	7	0.0034
Hystrix brachyura	Landak	6	0.0029
Naja naja	Ular tedung selar	5	0.0024
Herpestes spp.	Cherpelai	4	0.0019
Calloselama rhodostoma	Ular kapak	4	0.0019
Panthera pardus	Harimau kumbang	4	0.0019
Macaca nemestrina	Beruk	3	0.0014
Rusa unicolor	Rusa sambar	3	0.0014
Manis javanica	Tenggiling	1	0.0005
Martes flavigula	Mengkira	1	0.0005
Buceros spp.	Burung enggang	1	0.0005
Tragulus kanchil	Pelanduk	1	0.0005
Panthera tigris jacksoni	Harimau dahan	1	0.0005
Tragulus napu	Napuh	1	0.0005
TOTAL		2,088	1.0000

Sources: Salman Saaban, personal communication, September 15, 2024. Nomenclature as per Malaysia Biodiversity Information System; https://www.mybis.gov.my

Conservation of the Sambar deer in Peninsular Malaysia involves either *in-situ* or *ex-situ* efforts, discussed by Abidin et al. (1991) and recently described by Ali et al. (2021). In Peninsular Malaysia, the Department of Wildlife and National Parks (DWNP) are actively running the conservation programmes under the Malaysian Energy and Natural Resources Ministry. *Ex-situ* conservation programmes (e.g., captive breeding and release) are primarily important as the deer population in the wild has not shown an increasing trend over the past 12 years (Kawanishi et al., 2014). Therefore, DWNP has taken the initiative to breed Sambar deer at its wildlife conservation centres in Sungkai, Perak, and Gua Musang, Kelantan (both located in Peninsular Malaysia) before introducing the animals back into their natural habitats (Abidin et al., 1991; Ten et al., 2021b). Sambar deer from these two centres were also used as a source of commercial captive breeding of wildlife species.

In 2021, there are 85 Sambar deer (8 fawns) and 101 (21 fawns) at the wildlife conservation centres in Gua Musang, Kelantan and Sungkai, Perak, respectively (Department of Wildlife and National Parks, 2021). From 2014 to 2020, a total of 86 Sambar deer (44 males and 42 females from these centres were released to the wild (i.e., Sungai Relau, Pahang National Park, Kuala Tahan, Pahang National Park, Amanjaya Forest Reserve, Perak, Tembat Forest Reserve, Terengganu, Terengganu National Park, Terengganu, and Gunong Basor, Jeli, Kelantan). These are protected areas, except for Tembat Forest Reserve and Terengganu. The latter is gazetted as a production forest reserve. The survival rate of the released Sambar Deer monitored using Very High Frequency (VHF)-radiotelemetry collars or Global Positioning System (GPS)-satellite collars is between 57 and 75% for the totally protected areas, as compared with 0% for the Tembat Forest Reserve, Terengganu. As might be expected, most Sambar deer released into Tembat Forest Reserve, Terengganu, were illegally hunted (Munisamy et al., 2022).

The population of Sambar deer has significantly declined due to illegal hunting and localised extinctions in several forested areas. This trend, coupled with habitat loss, has severely impacted the prey availability for tigers, which now number around 150 individuals. In areas where law enforcement is not consistently enforced, the Sambar deer population has vanished, leading to ecological imbalances and threatening the survival of apex predators like the Malayan tiger. According to the National Tiger Survey findings, the number of tigers in Malaysia is rapidly decreasing. This alarming decline correlates with the decreasing populations of their primary prey species, including Sambar deer and wild pigs (*Sus scrofa*). The survey revealed that Sambar deer and wild pigs are now rarely captured on camera traps, indicating a sharp decline in their populations. Local communities in tiger-range areas have reported increasing incidences of tigers preying on livestock, such as chickens, dogs, and cats. These behaviours suggest that tigers are venturing into human settlements in search of food due to the depletion of natural forest prey (Rahmat & Azhar, personal communication, September 15, 2024).

In response to this crisis, discussions arose about the potential reintroduction of Sambar deer into tiger habitats to replenish the prey base for Malayan tigers. The idea was initially met with scepticism, as the primary objective of wildlife conservation efforts, particularly those led by the DWNP, is to ensure the survival of species in their natural habitats. However, it became clear that tigers would continue to venture into villages without sufficient prey, posing a threat to both humans and livestock. The suggestion was made that releasing Sambar deer to boost the prey availability for tigers could help mitigate these human-wildlife conflicts (Rahmat & Azhar, personal communication, September 15, 2024).

One of the primary arguments favouring reintroducing Sambar deer was the necessity of sustaining tiger populations in the wild. Studies have shown that tigers require large territories with an abundant and diverse prey base to thrive (Kawanishi & Sunquist, 2004). The drastic reduction of Sambar deer and wild pigs, exacerbated by illegal hunting and habitat destruction, has forced tigers to seek alternative food sources. The depletion of prey species is widely recognised as one of the leading causes of the decline in tiger populations across their range (Karanth, 2010). Therefore, increasing prey availability by reintroducing Sambar deer is vital in supporting tiger conservation efforts. Opponents of the idea argued that the primary purpose of captive breeding and conservation centres is to preserve species like the Sambar deer and to restore their populations in the wild. Releasing Sambar deer into areas with high poaching risks might lead to their rapid demise, thus defeating the conservation purpose. However, a compelling case was made that reintroducing Sambar deer into protected forest reserves, where the risk of poaching can be mitigated, would serve both conservation and ecological objectives. The phrase "better to die in the wild than in the paddock" resonated with those involved, ultimately leading to an agreement that the reintroduction of Sambar deer to bolster the prey base for Malayan tigers was a necessary and noble endeavour (Rahmat & Azhar, personal communication, September 15, 2024).

Evidence from other tiger conservation efforts supports enhancing prey availability as a key to tiger recovery. In India, for example, efforts to restore prey populations through reintroductions and habitat restoration have increased tiger numbers in several reserves (Jhala et al., 2011). A similar approach could be effective in Malaysia, particularly in areas where Sambar deer and other prey species have been driven to local extinction due to overhunting and habitat fragmentation. Despite the potential benefits of Sambar deer reintroduction, several challenges must be addressed for the initiative to succeed. Strict anti-poaching measures must be in place, and effective law enforcement must be maintained to protect the reintroduced deer and the remaining tiger population. Additionally, long-term monitoring of reintroduced Sambar deer is necessary to assess their survival, reproductive success, and overall impact on the ecosystem (Rahmat & Azhar, personal communication, September 15, 2024).

PROSPECT OF SAMBAR DEER FARMING IN PENINSULAR MALAYSIA

High demand for wildlife products for consumption or medication will lead to over-exploitation of their sources in the natural habitat. Many endangered animal species are also facing poor reproductive rates and habitat loss. These issues, together with ineffective conservation programs, will lead to extinction. While specific data on Sambar deer venison consumption in Peninsular Malaysia is limited, demand for wild meat and antlers has been increasing and driven by traditional beliefs and culinary preferences (UNODC, 2024). Wild sambar deer was laundered as farmed *Rusa timorensis*, lower premium meat and surveys by a non-governmental organisation (Trade in Wild Species) showed that more than 8% of the inspected restaurants (i.e., 242) selling Sambar deer venison in Peninsular Malaysia (Kawanishi et al., 2014). One possible solution is wildlife farming to produce captive breed products to reduce wild harvesting and fulfil human needs demands (Ali et al., 2021; Kawanishi et al., 2014). This initiative has been a great success, including for Sambar deer. For example, Sambar deer were initially introduced and farmed in Australia, and the escape and release of Sambar deer have now become wild herds in the country (Moriarty, 2004).

In Peninsular Malaysia, commercial captive breeding of wildlife species requires a permit from DWNP, and from 2012 until 2018, only one permit for Sambar deer was issued for this purpose. It is far below captive breeding permits issued for other protected species, e.g., Malayan porcupine and Lesser mousedeer (Department of Wildlife and National Parks, 2013, 2014, 2015, 2016, 2017, 2018). The low number of permits issued for Sambar deer farming between 2012 and 2018 was primarily due to stringent permit regulations and the high costs associated with setting up and maintaining captive breeding facilities. The Wildlife Conservation Act 2010 (Act 716) imposes strict requirements on breeders, including habitat management standards, disease control measures, and ongoing monitoring by DWNP officials (Department of Wildlife and National Parks, 2018). Due to the complexity and financial burden of meeting these regulatory demands, it has deterred many potential breeders. Looking at the prospect of Sambar deer farming in other countries, a similar can also be done in Peninsular Malaysia. As described earlier, founder stocks of Sambar deer for commercial breeding can be readily obtained from the Sungkai Wildlife Conservation Centre and Gua Musang Wildlife Conservation Centre, which DWNP established. However, technical support (e.g., on diet and captive conditions) and continuous monitoring and support from DWNP are needed. They should be given to those setting up captive breeding facilities for Sambar deer. These are needed as DWNP is the only agency with experienced personnel on the Sambar deer breeding programme in Peninsular Malaysia.

GENETICS FOR WILDLIFE CONSERVATION

Genetic studies show that the major histocompatibility complex (MHC) and killer-cell immunoglobulin-like receptor (KIR) genes are the most polymorphic and dynamic in the human and animal genomes (de Groot et al., 2015; Parham & Moffett, 2013; Parham et al., 2012). It confirms the role of MHC and KIR in determining the ability of an organism to cope with disease outbreaks, growth processes, and reproduction. Many genetic studies and DNA bar-coding for species identification are currently using limited information in animal genomes, such as the *cytochrome oxidase subunit 1* genes (*CO1*) of mitochondrial DNA, variable number of tandem repeats (VNTRs), short tandem repeats (STRs), and single nucleotide polymorphisms (SNPs). It is important to note that evolutionary and adaptive processes within and between animal species can be accurately studied by scanning the entire animal genome, including coding and non-coding regions (Ramsay et al., 2019).

However, molecular biology and genomics advancements have limited application in attempts to understand animal biology and diversity and as an intelligent tool in conservation programs (Blanchong et al., 2016; de Groot et al., 2015). For example, wildlife genomic data obtained using next-generation sequencers may be used to inform the characterisation of conservation units (e.g., genes related to adaptive immunity), hybridisation between animal groups (comparative genomics) and drivers of divergence in wildlife (Supple & Shapiro, 2018). Therefore, genomic approaches to conserve Sambar deer and other protected species, especially in Peninsular Malaysia. Based on historical, anecdotal information (Sivananthan, Mohamad Tajuddin and Azhar, personal communication, September 15, 2024), the Sambar deer founders in the captive program established in late 1985 were likely acquired from Zoo Melaka and Sabah (another subspecies) via Singapore, as well as from Taiping Zoo (Kevin, personal communication, September 15, 2024). It would be worthwhile to conduct a genetic study to determine the presence of hybrids and assess the impact of genetic intermixing with the purebred populations from the Peninsular Malaysia subspecies.

FUTURE DIRECTIONS AND CONCLUDING REMARKS

Sambar deer are depleting in the wild due to habitat degradation and over-exploitation for food and medicinal purposes. Similar to other endangered animal species in Peninsular Malaysia, like sun bears, where illegal hunting for their bile products has caused a severe population decline (Edinur et al., 2022). The current framework of conservation and protection of Sambar deer, including greater enforcement of law in response to illegal poaching and trade, captive breeding and release, as well as habitat management and enrichment, should be expanded as their density has declined throughout Peninsular Malaysia (Ali et al., 2021). Here, using wildlife genomic data and Sambar deer farming could increase the number of Sambar deer in Peninsular Malaysia. Wildlife farming has been very successful elsewhere, including for musk deer (*Moschus* spp.), lion (*Panthera*

leo) and mink (*Neovison vison*); refer to Tensen (2016) for details). However, wildlife farming programmes for several other species (e.g., *Hystrix brachyura*, *Heosemys spinose*, *Manis* spp., and *Civettictis civetta*) have no positive impact on conservation, and this was largely due to high production cost, dependent on the wild population as founder stock and laundering of illegal products into captive breeding systems (Nogueira & Nogueira-Filh, 2011; Tensen, 2016). Therefore, attractive subsidy schemes, a systematic protocol for management and tagging of wildlife farming products and a good supply of founder stock (e.g., as currently set up at Sungkai, Perak and Gua Musang, Kelantan Wildlife Conservation Centres) are needed for Sambar deer farming in Peninsular Malaysia.

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Impact of Using Boiler Ash as Soil Ameliorant and Nitrogen Fertilizer on CO_2 Emission in Oil Palm Plantations on Peat Soil

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ABSTRACT

Peat soil has an important role in water and carbon storage. However, the utilization of peatland as an agricultural field requires drainage of water and the application of fertilizer or soil ameliorants to increase peat soil's fertility; this will increase greenhouse gas emissions, particularly CO_2, so the function of peat as a carbon sink turns into a source of greenhouse gas emissions. The research aims to determine the effect of using boiler ash as a soil ameliorant and nitrogen fertilizer on CO_2 emission and FFB yield in oil palm plantations on peat soil. The treatment consisted of three levels of boiler ash (0-ton/ha/yr, 1.5-ton/ha/yr and 3-ton/ha/yr) and three levels of nitrogen fertilizer (0 kg N/palm/yr, 0.45 kg N/palm/yr and 0.9 kg/palm/yr). CO_2 emission was measured using the closed chamber method. A PVC pipe with a length of 80 cm is the chamber. 60 cm of pipe was buried in the soil, and the other 20 cm was on the soil surface to catch CO_2 released into the air. The application of a high rate of N fertilizer significantly increased CO_2 emission from 0.56 g/m^2/hour to 0.67 gr/m^2/hour. Applying boiler ash at a low rate reduces CO_2 emission from 0.63 g/m^2/hour to 0.58 g/m^2/hour. The application of boiler ash as a soil ameliorant not only has an impact on CO_2 emissions but also improves peat soil chemical properties by significantly increasing soil pH, total phosphate, available phosphate, and exchangeable Calcium. The high boiler ash and low nitrogen fertilizer rates produce 29.13 t FFB/ha/yr with a CO_2 emission of 0.63 g/m^2/hour.

Keywords: Boiler ash, CO_2 emission, oil palm, peat

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INTRODUCTION

Tropical peat ecosystems have an ecological function, such as storing large amounts of carbon. However, peat ecosystems that are converted into agricultural and plantation areas result in increased greenhouse gas emissions and cause the peat ecosystem to change its function from storing carbon to

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being a source of carbon emissions (Astiani et al., 2022; Pratiwi & Yuwati, 2022). The increase in total greenhouse gas emissions has increased in the 2010–2019 period, and cumulatively, there has been an increase in CO_2 emissions since 1850 (IPCC, 2022). In the 2010–2019 decade, the average greenhouse gas emission reached 59$\pm$6.6 $GtCO_2$/yr. It was much higher compared to the previous decade. However, the growth rate between 2010 and 2019 was still lower than between 2000 and 2009. In 2019, around 34% of total GHG emissions, equivalent to 20 $GtCO_2$, came from the energy supply sector. The agricultural, burning, and other land use sectors contribute 22% of the total GHG emissions or 13 $GtCO_2$.

Peat swamp forest is a carbon-rich reservoir with 50–350 Gt stored carbon. However, peat swamp forests can become a source of carbon emissions, contributing to climate change (Lestari et al., 2022). Peat swamp forests release ~0.14 Gt of carbon into the atmosphere annually, equivalent to 1% of fossil fuel emissions (Loisel et al., 2021). Conversion of peatlands into agricultural and plantation areas requires changes in groundwater levels. A decrease in groundwater levels in peatlands will contribute significantly to increased carbon emissions caused by increased mineralization of organic matter and the risk of fire in the dry season (Taufik et al., 2020). It is different in the natural conditions of peat, where the decomposition of organic matter is inhibited by anaerobic conditions (Vernimmen et al., 2020).

The construction of drainage canals will cause a drastic decrease in the water level due to changes in the water level, which will eventually cause organic matter to decompose more easily and produce CO_2 emissions (Hayati et al., 2022). Conversion of tropical peat swamp forests into drainage-based agricultural areas can change greenhouse gas production (Cooper et al., 2020). Although the loss of carbon in peat soils caused by the decomposition of peatlands can be compensated for by plant growth, the conditions for plants in peat areas often tend to have low growth due to low nutrients in peat soils (Ojanen et al., 2019).

The fertility level of peat depends on the level of decomposition, the type of mineral layer beneath the organic layer and the depth of the peat itself (Permatasari et al., 2021). The environmental conditions of the peat, such as waterlogging, high acidity and oxygen deficiency, also worsen the fertility of peat soils, where many micro-nutrients become unavailable to plants (Sutarno & Mohamad, 2022). The low natural fertility of peat soil makes the oil palm plantation industry operate on peat-applied fertilizer in large quantities (Lestari et al., 2022).

Peat soil has a relatively high nutrient content even though it is unavailable to plants (Hartatik et al., 2011); nitrogen fertilization remains important for cultivating oil palm plants in peat soil. However, Long-term use of fertilizer on peatlands, especially on oil palm plantations, will increase the rate of peat soil decomposition and increase net soil CO_2 emissions by 0.85 g/m^2/hour or equivalent to 74.68-ton/ha/year at the immature location and 0.78 g/m^2/hour or the equivalent of 68.38-ton/ha/year at the mature palm location (Stephanie et al., 2021). Meanwhile, the use of ameliorants to improve the chemical properties of peat

soil has been widely studied. Amelioration has many benefits in improving the chemical properties of peat soil. The application of mineral soil as an ameliorant in peat soils can slow down the rate of decomposition of peat soils and reduce the rate of CO_2 emissions (Suratman et al., 2013).

This study aims to obtain accurate information regarding using nitrogen fertilizer doses and soil ameliorants to obtain high oil palm productivity and not give high CO_2 emissions to achieve sustainable plantation practices. This study used boiler ash from a palm oil mill as a soil ameliorant. Boiler ash has a pH > 12, which can reduce the negative effect of phenolic acids in peat soils (Ichriani et al., 2021)

MATERIALS AND METHODS

Research Sites

The study was conducted in Rokan Hilir District, Riau Province, Indonesia. In reference to USDA soil classification, the study site is classified into the soil group Typic Haplohemist. Based on Malaysian soil taxonomy, the site is classified as part of the Gondang series (Paramananthan, 2020). These soils have a high water table before being drained, and hemic materials make up the dominant material in the subsurface tier 950–100 cm depth). According to the Schmidt-Ferguson classification, this area has a flat topography with a slope of 0%–4%, annual rainfall of 2,182 mm, and a wet type B climate. The geological study area is a swamp deposit of sand, silt, clay mud and peat.

The research area is an oil palm plantation that has been managed since 1995. In 2013, it underwent replanting, so it is now an oil palm plantation that has undergone its second planting cycle. The research location has an area of 3 Ha. The boiler ash used in this research came from a palm oil mill at the research location. Tables 1 and 2 show the chemical properties of peat soil and boiler ash.

Table 1
Peat soil chemical properties

Parameter	Soil Peat Chemical Properties
pH	3.39
C-organic (%)	23.51
Total-N (%)	1.16
Total-P (ppm)	1,917.16
Available-P (ppm)	133.91
Exch-K (me/100g)	2.85
Exch-Ca (me/100g)	7.66
Exch-Mg (me/100g)	6.50
Exch-Na (me/100g)	0.08
CEC (me/100g)	90.50

Table 2
Boiler ash chemical properties

Parameter	Boiler Ash Chemical Properties
pH	10.95
C-organic (%)	5.61
Total-N (%)	0.02
Total-P (ppm)	3,339.12
Available-P (ppm)	119.28
K (%)	1.35
Ca (%)	2.32
Mg (%)	0.39
Si (%)	76.01
Fe (%)	19.10

Experimental Detail and Sampling

Experimental Design

The study was conducted using a factorial randomized block design with two factors: boiler ash (B) as soil ameliorant with three levels (0, 1.5-ton/ha/yr and 3-ton/ha/yr). The second factor is nitrogen fertilizer (F) with 3 levels (0, 0.45 kg N/palm/yr, and 0.9 kg N/palm/yr) with three replications. Each plot consisted of 16 oil palms, where four were used as sampling points and 12 were used as guard palms. Statistix software version 9.0 was used to generate experimental design, statistical analysis and regression model.

Boiler ash and nitrogen fertilizer are applied in palm circles and areas between palms, spreading evenly over the soil surface. The application of nitrogen fertilizer is carried out one week after the application of boiler ash to prevent the loss of nitrogen nutrients through volatilization due to the increase in soil pH after the application of boiler ash.

FFB Yield Recording

Yield data recording was conducted in one year to ensure the treatment did not affect the yield data starting one year after the first treatment application. Yield recording was carried out every harvesting round (9–10 days). Every bunch from the recorded palm was weighed, and the bunch number was used to calculate the FFB yield. This experiment was conducted on an oil palm field during the planting year 2013.

CO_2 Emission Measurement and Soil Sampling

Measurement of CO_2 emission in all plots uses closed chamber methods. Measurement will be carried out daily from 8 a.m. to 11 a.m. The measurement was conducted within 2 months after the first application of each treatment. An 80 cm pipe of PVC was used as the chamber. 60 cm of pipe was buried in the soil, and 20 cm was kept above the soil surface to catch the CO_2 released into the air. A portable Infrared CO_2 analyzer with the brand BIOEVOPEAK model CO_2A-3010E was used to measure the CO_2 emission.

The formula published by Sano et al. (2010) is used as a reference to calculate CO_2 emissions from peat soil.

$$F = \frac{V}{A} \times \frac{1}{22.4 \times \frac{273.15 + T}{273.15} \times 10^{-3}} \times \frac{dc}{dt}$$

With the symbol notation:

F = Emission CO_2 (µmol/m^2/s)

V = Volume of chamber (m^3)

22.4 = molar volume of gas at standard temperature and pressure that is 22.4 liters/mol or 0.0224 m^3/mole at 0°C (273° K) and 1 atm pressure

T = average temperature in the chamber (^{0}C)

dc/dt = Change in CO_2 concentration over the time (ppm / second)

Soil samples are taken using the disturbed soil method, namely soil samples that are no longer natural and have been disturbed by the external environment. Soil sampling using a soil auger. Soil samples are taken from depths 0–20 cm from the surface. Soil sampling for soil chemical analysis was conducted in the palm circle and interrow. The total number of soil samples from 1 plot is 4 for each location in a palm circle and inter-row. All soil samples are composited to represent each plot.

Water Depth Measurement

Groundwater depth measurements are carried out every time a CO_2 emission measurement is carried out. Groundwater measurements using piezometers were installed at 5 points at the research location. The average value of the measurements is used as groundwater depth data.

RESULTS AND DISCUSSION

The effect of boiler ash and nitrogen fertilizer on the FFB yield and its components is shown in Table 3. Application of boiler ash at rates of 1.5 and 3 tons/ha/yr significantly improved fresh fruit bunches (FFB) production by 28% and 40%, respectively, compared to the control plot. The boiler ash that has been used in this study contains 1.35% K_2O, so the application of boiler ash of 1.5 and 3-ton/ha is equivalent to 33.75 kg MOP/Ha/yr and 67.5 kg MOP/Ha/yr, respectively. The boiler ash content, which contains many macro and micronutrients, is expected to influence plant production and increase the soil's nutrient content and absorption. According to Haron et al. (2008), the utilization of boiler ash as an organic fertilizer, together with decanter cake, provides better nutrient absorption and growth of oil palm seedlings than inorganic fertilizer.

Application of boiler ash significantly increases FFB yield due to an increment in bunch weight. Potassium fertilizer application positively impacts oil palm plants in increasing FFB yields (Prabowo et al., 2023). According to Tohiruddin (2006), the application of KCL with 60% K_2O content increases oil palm FFB production by increasing bunches weight (Tohiruddin, 2006). The result of this study was similar to Arifin et al. (2022), where the application of potassium fertilizer significantly improves FFB yield through increments in a number of bunches. Subandi (2013) states that potassium is a macronutrient essential for the palm. The application of boiler ash, which is rich in potassium, increases the weight of

oil palm bunches; this is related to the role of potassium in enzymatic reactions, including carbohydrate and protein metabolism and also increases the quality of seeds and fruit.

Table 3
Main effect of treatment to CO_2 emission, FFB yield and its components on 9 years old oil palm

Treatment		CO_2 Emission ($g/m^2/hr$)	FFB Yield (t/ha/yr)	Number of Bunches/ha/yr	Average Bunch Weigh (kg)
Boiler ash (t/ha/yr)	0	0.63 a	16.42 b	1,417.3 a	11.59 b
	1.5	0.58 a	21.02 a	1,484.0 a	14.22 a
	3	0.62 a	23.07 a	1,556.0 a	15.07 a
Nitrogen Fertilizer (kg N/palm/yr)	0	0.56 b	15.46 b	1,157.3 b	13.52 a
	0.45	0.59 ab	23.39 a	1,629.3 a	14.32 a
	0.9	0.67 a	21.65 a	1,670.7 a	13.05 a
CV		16.66	16.08	10.96	13.48

Note. Numbers with the same lowercase letters do not show statistically significant differences in the LSD test at the 5% level

Application of nitrogen fertilizer (Ammonium Sulphate) at a dose of 0.45 kg nitrogen and 0.9 kg nitrogen increased yields by 7.94 tons/ha/year and 6.2 tons/ha/year, respectively, compared to control plots. Nitrogen fertilizer gave different results in increasing yield components compared to boiler ash treatment. Nitrogen fertilizer significantly increases the production of fresh fruit bunches by increasing the number of bunches. However, the application of nitrogen fertilizer did not significantly affect the bunch weight yield components.

The significant increment in the number of bunches per hectare due to the application of nitrogen fertilizers is also consistent with the results of research by Tohiruddin (2006) that the application of nitrogen fertilizers in experiments on oil palm fertilization in the long term in locations with volcanic soil parent material and high average rainfall in North Sumatra significantly increase the production of oil palm plants by increasing the number of bunches per hectare component.

Figure 1 shows the effect of each treatment combination on the value of CO_2 emission. During the CO_2 emission measurement period in April–June 2023, the highest CO_2 emission value is 1.70 g/m^2/hour or the equivalent of 148.92-ton CO_2/ha/year in plots with a combination of high rates of both boiler ash and nitrogen fertilizer. At the same time, the lowest emission value is 0.3 g/m^2/hour, equivalent to 26.28 tons/ha/year in the plot, with a low rate of both boiler ash and nitrogen fertilizer. The emission value in this study is bigger than the results obtained by Batubara et al. (2019), where CO_2 emissions in peat soil with a decomposition level of Saprik peat and a depth of 100–200 cm with 25-year-old oil palm stands can reach 39.3 ± 2.2-ton CO_2/ha/year. Different results were also reported

by Razak (2019) that CO_2 emissions in shallow peat soil with a depth of <175 cm and immature oil palm plantations range from 126.14-ton CO_2/ha/year up to 169.07-ton CO_2/ha/year, where this emission value is much bigger than the results obtained in this study.

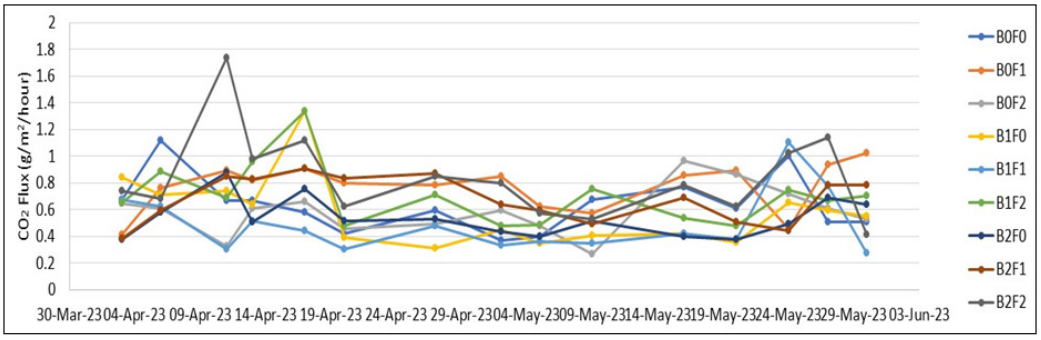

Figure 1. Fluctuation of CO_2 Emission in all plots

The trial was conducted from March to June 2023, which represents the wet season (April 2023 with rainfall > 200 mm) and the dry season (May–June 2023 with rainfall < 150 mm). Monthly rainfall data from January to June 2023 in the study area is presented in Figure 2. It is also to prevent the seasonal effects of climate change (rainfall and temperature) on CO_2 emissions. The effect of climate on CO_2 emission is shown in Figure 1; the CO_2 emission in April 2023 is higher in all treatment plots. Two weeks after treatment, in the last two weeks of April 2023, when the rainfall was still above 200 mm/month, the average value of CO_2 emission was only 0.58 g/m^2/hour. CO_2 emission increased in mid-May 2023 to the average value of 0.64 g/m^2/hour when rainfall dropped to only 101 mm/month. Ray et al. (2020) state that rainfall and soil temperature influence CO_2

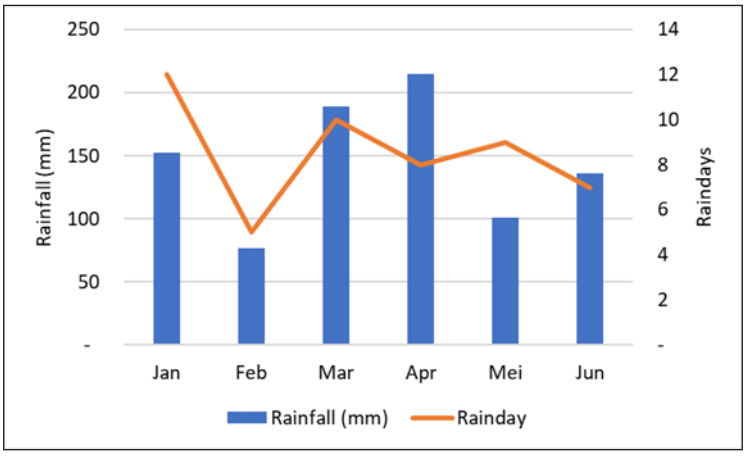

Figure 2. Rainfall distribution Jan–Jun 2023 in Manggala 3 estate (2.5 km from Trial Site)

emissions during the growing season. CO_2 emissions are relatively higher when air and soil temperatures are relatively warmer, and decreased rainfall increases soil temperature and affects groundwater conditions.

Nitrogen fertilizer treatment at a dose of 0.9 kg N/palm/year increased CO_2 emissions by 11% compared to control plots, which was 0.11 g/m^2/hour or equivalent to 9.6 tons CO_2/ha/year. The increase in CO_2 emissions by the application of N fertilizer in this study confirms several previous studies regarding the effect of fertilization on greenhouse gas emissions (Lin et al., 2021; Ojanen et al., 2019).

Nitrogen fertilizer increases CO2 emissions by increasing soil respiration and enzyme performance and the life of soil organisms. According to Babur et al. (2021), nitrogen fertilizer can increase microbial respiration by 97% compared to land that does not receive nitrogen fertilizer application. It is related to the needs of microorganisms for macronutrients such as nitrogen, phosphorus, and sulfur and basic elements such as hydrogen, carbon and oxygen. Nevertheless, according to Fitra et al. (2019) and Razak (2019), CO2 emissions in peat soil are not influenced by the combination of fertilization.

The correlation between CO_2 emissions and environmental conditions is shown in Figure 3. The results showed that the CO_2 emission was directly proportional to the depth of the peat water table. In other words, lowering the peat water level will further increase CO_2 emission on peat soil. These results confirm previous studies that also indicated a linear relationship between the groundwater table and CO_2 emission (Hoyt et al., 2019; Winarna et al., 2017)

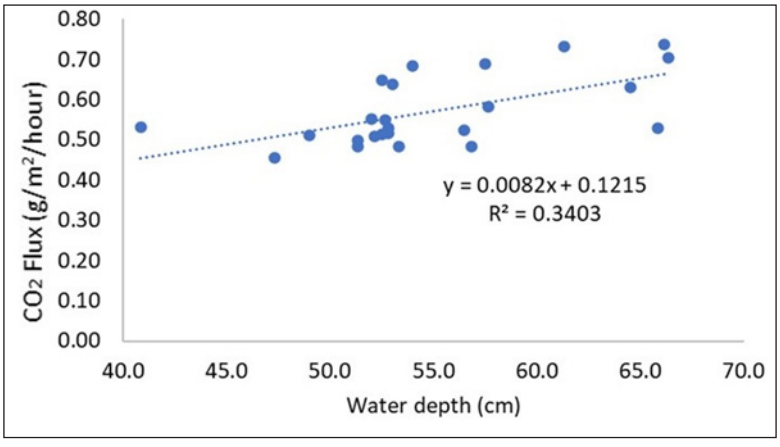

Figure 3. Correlation between CO_2 emissions with water depth (n=24; P-value <0.05)

Soil pH is a standard measurement of the level of acidity or alkalinity in a soil type, which significantly influences soil fertility and the availability of nutrients for plants. Low soil pH values in peat soils are primarily due to organic acids in the soil. Table 4 shows the

main effect of each treatment on peat soil chemical properties. Utilization of boiler ash, which has high pH characteristics, increased the pH of the peat soil at the study site by 25% relative to the control plot. This result confirms the previous study by Saputra and Sari (2021) that also reported ameliorants' positive effects on improving soil pH in peat soil.

Table 4
The main effect of treatment on peat soil chemical properties

Chemical Properties	Boiler ash (t/ha/yr)			Nitrogen Fertilizer (kg N/palm/yr)			CV
	0	1.5	3	0	0.45	0.9	
pH	3.05 b	3.82 a	3.84 a	3.58 a	3.59 a	3.53a	8.12
C-organic (%)	22.25 a	24.73 a	23.26 a	24.36 a	23.45 a	22.43 a	21.92
Total-N (%)	1.16 a	1.18 a	1.17 a	1.18 a	1.15 a	1.18 a	9.44
Total-P (ppm)	3,697 b	4,132 a	4,981 a	4,463 a	4,031 a	4,315 a	49.82
Available-P (ppm)	97.24 b	101.34 ab	130.86 a	111.37 a	110.05 a	108.01 a	29.96
Exch-K (me/100g)	1.80 a	1.96 a	1.98 a	1.914 a	2.09 a	1.73 a	40.78
Exch-Ca (me/100g)	5.40 b	5.98 ab	6.90 a	5.46 b	6.12 ab	6.71 a	18.92
Exch-Mg (me/100g)	3.88 a	2.74 a	3.28 a	2.97 a	2.88 a	4.05 a	42.18
Exch-Na (me/100g)	0.050 a	0.058 a	0.057 a	0.060 a	0.058 ab	0.047 b	23.32
CEC (me/100g)	94.36 a	91.24 a	110.42 a	94.13 a	98.84 a	103.06 a	23.05

Note. Numbers with the same lowercase letters do not show statistically significant differences in the LSD test at the 5% level

The increment of soil pH due to the addition of ameliorants causes a change in the nutrient content available to plants. Total soil P value increased by 34.73%, while available P increased by 34.02% respectively in plots with a high rate of boiler ash relative to the control plot. Furthermore, nitrogen fertilizer treatment did not significantly improve peat soil's chemical properties. The application of nitrogen fertilizers only significantly increased the exchangeable Ca cations and also significantly decreased the content of exchangeable Na cations. Furthermore, applying nitrogen fertilizers and boiler ash tends to increase the soil's cation exchange capacity. Ameliorants are not intended as the main source of nutrients for peat soils. However, the main function of ameliorants is to improve the chemical and biological properties of soil, which is the main aim of using ameliorants. Increasing soil pH will increase the nutrient content available to plants and increase soil microbiological activity.

Table 5 shows several treatment combinations tested in this study. The control plot only produced 12.47-ton FFB/ha/yr with a total CO_2 emission of 0.52 g/m^2/hour. The addition of 0.45 kg N not only improved plant nutrient status but also increased FFB production by 6.15 tons and increased CO_2 emissions by 0.18 g/m^2/hour. The combination of boiler ash and nitrogen fertilizer treatment with doses of 1.5-ton and 0.45 kg N per year, respectively, gives the lowest emission value, viz only 0.45 g/m^2/hour; however, this combination still increases FFB production by 3.82-ton FFB /year. Maximum FFB production was obtained with 3-ton boiler ash and 0.45 kg N, which produced 29.13-ton FFB/ha/yr. However, this combination increases CO_2 emission to 0.63 g/m^2/hour compared to the previous combination.

Table 5
Fitted crop data for specific treatment combination

Boiler ash (t/ha/yr)	Nitrogen Fertilizer (kg N/palm/yr)	FFB Yield (t/ha/yr)	CO_2 Emission (g/m^2/hour)	Leaf (% dry matter)				
				N	P	K	Mg	Ca
0	0	12.47	0.52	2.69	0.166	0.92	0.31	0.52
0	0.45	18.62	0.70	2.73	0.167	1.03	0.29	0.50
1.5	0.45	22.44	0.45	2.79	0.169	1.12	0.33	0.53
3	0.45	29.13	0.63	2.79	0.167	1.06	0.30	0.52

Table 5 shows that various treatment combinations have different values for FFB production, CO_2 emissions and leaf nutrient content. For this reason, the determination of the optimum combination is not only based on production value but also considers environmental conservation values, namely the CO_2 emission value, which affects the accumulation of greenhouse gases in the atmosphere.

CONCLUSION

The application of nitrogen fertilizer significantly increases FFB production by increasing the number of bunches as a production component. Meanwhile, the application of boiler ash significantly increases FFB production by increasing bunch weight. The application of nitrogen fertilizer significantly increases CO2 emissions, while the application of boiler ash does not significantly increase CO_2 emissions. The environmental factor that influences the value of CO_2 emissions is the height of the water level, where the deeper the water level, the more CO_2 emissions will increase and vice versa.

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