Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

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Pertanika Journal of Tropical Agricultural Science
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About the Journal

Overview

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

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

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

The journal is available world-wide.

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

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

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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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Operating and review process

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

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

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

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

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

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

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

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

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

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ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS

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Contents

Foreword

Welcome to the third issue of 2024 for the Pertanika Journal of Tropical Agricultural Science (PTAS)!

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 28 articles in which all are regular articles. The authors of these articles come from different countries namely Brunei Darussalam, India, Indonesia, Japan, Malaysia and Vietnam.

The regular article entitled "Identification and Quantification of Cucurbitacins B and E in Different Parts of Bitter Gourd Plants Derived from Different Planting Methods" determined the different levels of cucurbitacins B and E in the plants from two different planting methods, conventional and fertigation. Fruits, leaves, stems, and roots of bitter gourd plants from the two different planting methods were harvested for extraction using the sonication extraction method. The extract's cucurbitacins B and E content were identified and quantified using high-performance liquid chromatography. The outcomes concluded that plant parts and type of planting method can affect the cucurbitacin content in bitter gourd. Full information of this study is presented on page 843.

Suzan Benedick and her teammates from Universiti Malaysia Sabah examined better pollination techniques to achieve acceptable fruit quality for red-fleshed pitaya production under local climatic conditions. For this purpose, stingless bees (*Tetragonula laeviceps*), self-pollination, natural pollination, and hand pollination were used. Forty flowers were observed to obtain data on flowering phenology and fruit quality. They found out that the pollination by *T. laeviceps* generally resulted in better fruit quality than natural pollination and hand pollination of the non-native plant of red-fleshed pitaya, which indicates the integration of pitaya cultivation and stingless bees is likely to improve the yield and quality of the fruits on the farm. The detailed information of this article is available on page 955.

A selected article entitled "Identification of Phytochemicals and Mineral Nutrients of Selected Malaysian Plant Extracts and Its Effects on Seed Priming of Maize" identified the phytochemical compounds and quantify nutrients present in three plant extracts, namely *Euphorbia hirta*, *Polygonum minus*, and *Eleusine indica*, as well as to explore the effect on the growth of maize seedlings (*Zea mays* L.). Five concentrations of plant extracts, i.e., 5, 15, 25, 50, and 100%, were designed to evaluate seed germination and priming. The results showed that *E. hirta* and *E. indica* extracts exhibited inhibitory effects at higher concentrations, while *P. minu*s extract maintained a higher germination rate, indicating lower toxicity. Further details of this study are found on page 1003.

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

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

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

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

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Investigating the Effects of Bamboo Vinegar as An Organic Pesticide on Insect Pests and the Nutrient Content of Harumanis Mango (MA128), *Mangifera indica* L.

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ABSTRACT

Chemical pesticides have been widely used in plantations, and their effects have more disadvantages to the environment as well as to humans. Therefore, this study tries to implement organic pesticides using bamboo vinegar. Bamboo vinegar is one of the organic pesticides to control insect pests in plantation crops. The role of this organic pesticide on the insect pests of Harumanis mango is still unknown. Hence, this study aims to determine the impact of applying bamboo vinegar on the insect pests, quality, and nutrient content of Harumanis mango (MA128). Bamboo vinegar was applied in February 2021 during the flowering phase at the Harumanis plot in the Plantation Unit, Universiti Teknologi MARA Perlis. This study used a randomized complete block design with three treatments (spraying intervals) and three replications: T1 (3-day interval), T2 (5-day interval), and T3 (no bamboo vinegar). Sticky traps were installed to determine the population of insect pests in Harumanis mango. Matured fruits have been harvested, and the standard procedure of Harumanis mango postharvest handling has been followed. Then, the fruits were determined for total phenolic content (TPC), total flavonoid content (TFC), vitamin C, and sugar content. Bamboo vinegar significantly reduced the abundance of insects in Harumanis mango. Additionally, the highest TPC was observed from a 3-day interval of bamboo vinegar application. A

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similar trend was indicated for improving TFC with bamboo vinegar application. Similarly, there was a significantly higher vitamin C and sugar content after bamboo vinegar application compared to the control group. In conclusion, applying bamboo vinegar to Harumanis mango reduced insect pests and increased the quality and nutrient content of the Harumanis mango.

Keywords: Bamboo vinegar, flavonoid, nutrient content, sticky trap, vitamin C

INTRODUCTION

Demand for healthy fruit has promoted farmers to produce products free from any chemical synthetics such as fungicides, pesticides, and weedicides that may affect the environment and human health (Meena, 2015). The use of organic pesticides to replace synthetic chemicals is an initiative to minimize the use of chemical products in agriculture management (Al-Ani et al., 2019). Bamboo vinegar is an organic product with various functions that may help farmers control insect pests. It can also be used as an organic foliar fertilizer. The liquid form of bamboo vinegar is extensively utilized in organic farming, forestry, floristry, horticulture, animal husbandry, and human healthcare. It contains a variety of organic elements, including organic insecticides effective at preventing pest infestations, fostering plant growth, improving nutrient uptake, lowering fertilizer use, and encouraging compost generation (Alias et al., 2020).

Due to rising demand and bamboo's adaptability as a source of wood products, production has expanded today. In Malaysia, *Gigantochloa albociliata*, sometimes referred to as Buluh Madu, is a bamboo species that frequently reaches its highest size (Zhu et al., 2021). This bamboo is typically pyrolyzed to make charcoal, vinegar, tar, and other goods. This bamboo helps produce organic products such as vinegar for pest control and fertilizer (Yusoff et al., 2021). Bamboo vinegar is a by-product of condensed acidic bamboo carbon liquid obtained during the manufacturing of bamboo charcoal. It has a distinct smoky aroma and a pale yellow to brown tint. Bamboo vinegar contains over 200 chemical components, such as organic acids, phenolic, alkane, alcohol, and ester compounds (Mu et al., 2006).

Agriculture-related sectors are crucial for supplying food for human and animal consumption. In Malaysia, Harumanis mangoes (MA128), scientifically named *M. indica* L., is one of the most famous mango varieties due to its aromatic, sweetness, fragrance, and price. The Department of Agriculture Malaysia has registered this mango as an MA128 variety clone (Mahmood et al., 2011). Additionally, this crop has been commercialized in Perlis, Malaysia, where the geography is ideal for fruit production in a hot and dry climate. Uda et al. (2020) state that hot weather and precipitation will impact flowering patterns, fruit formation, and fruit development. Harumanis mango will only bear fruit once a year as a seasonal crop with proper planning

and maintenance. The skin is bright green with prominent light-yellow specks, and the fruits are still green, even though they are matured (Peng & Christian, 2005). It has thick yellow pulp with no fiber, is soft, and has high water content. The weight of Harumanis fruit can be found from 300 to 700 g, with Brix readings ranging from 14° to 18°Bx (Mahmood et al., 2011). The Harumanis tree is cultivated for its edible flesh fruit and is a good source of polyphenols, vitamin A, and vitamin C (Talib et al., 2020).

Aziz et al. (2020) reported that the fruit has a high total soluble solids (TSS) value based on the sugar solution in mango juices. The effects of bamboo vinegar on plant growth and phytochemical content were widely evaluated on other agricultural plants but lacking on mango, especially on Harumanis mango. Besides, according to Meena et al. (2015), organic products increase the nutritional value of vitamin and mineral content. Furthermore, the vitamin C content of tomatoes was enhanced after spraying with bamboo vinegar (Yao et al., 2012). In addition, the root length, plant height, living biomass, and chlorophyll content of tomatoes were increased with bamboo vinegar application compared to without spraying. Although research has indicated bamboo vinegar improves growing media properties and plant growth on crops, the effects of bamboo vinegar on the nutrient content of Harumanis mango have not been reported. Thus, this study was conducted to evaluate the effect of bamboo vinegar on insect pests, nutrient content, and quality of Harumanis mango (MA128).

MATERIALS AND METHODS

Sampling Procedures

This experiment was conducted at the Harumanis mango planting site at the Plantation Unit, Universiti Teknologi MARA, Perlis. Harumanis crop was planted in 2015 and is 6–7 years old. Bamboo vinegar treatment has been applied during the flowering season, from 14th February 2021 until 1st April 2021. This study used a randomized complete block design (RCBD) with three treatments (spraying intervals), three replications, and four experimental units. Harumanis mango trees were labeled with three different colors of tape to differentiate them according to their treatment: green (3-day intervals), red (5-day intervals), and yellow for control. The yellow sticky trap was installed a day before and after spraying to observe the insect abundance after being treated with bamboo vinegar. Yellow color has been chosen for this study due to the color preferences of insects (Bae et al., 2019). The population of insects has been identified up to order levels by referring to Borrow and Delong's book (Luqman et al., 2018).

Bamboo Vinegar Preparation and Application

The concentrated bamboo vinegar (100%) from Tadom Eco Living Sdn. Bhd. (Malaysia) has been used in this study. The 20% bamboo vinegar concentration was prepared by diluting it with water in a ratio of 1:5 for this experiment. A total of 200 ml bamboo vinegar concentration was

prepared for 10 L of water. The spraying process was conducted early morning using a knapsack sprayer, sprayed fully on Harumanis trees. The treatments have three different time intervals of bamboo vinegar application: 3 days (T1), 5 days (T2), and without applied bamboo vinegar (control). After treatment, the fruits were covered with carbon wrapping bags to protect them until harvest. After fruits fully matured, they were harvested and underwent postharvest treatment. Then, the fruits were analyzed for their nutrient content.

Harumanis Mango Extraction

The ripened Harumanis mango have been selected for nutrient analysis following Oviasogie's method (Rahman et al., 2007). The fruits were analyzed for TPC, TFC, vitamin C, and TSS. Mango fruit has been cleaned, peeled, and cut into slices for extraction to separate a particular chemical from a larger complex compound. The fruit samples of each treatment were homogenized using mortar and pastel, recorded weight, and fruit samples required to be macerated in methanol for 24 hr at room temperature. The extracts were filtered under vacuum conditions, and the residue was repeatedly extracted with the same solvent until colorless. A rotary evaporator was used to remove methanol from the extract solution.

Determination of TPC

The TPC of fruits was evaluated by drawing a calibration curve using gallic acid as the standard of reference. A test tube containing 9 ml of deionized water was filled with 1 ml of sample and 1 ml of a standard gallic acid solution (R&M Chemicals, Malaysia). Deionized water was also used to prepare the blank. The Folin-Ciocalteu reagent (R&M Chemicals, Malaysia) has been added to the solution and stirred with a value of 1 ml. The solution was then mixed with 10 ml of 7% sodium carbonate (R&M Chemicals, Malaysia) and incubated for 2 hr. A UV-visible spectrophotometer with a wavelength of 760 nm has been used to determine the absorbance of the TPC (Rahman et al., 2007).

Determination of TFC

TFCs were determined using a quercetin standard curve following the method of Mahmood et al. (2011). Flavonoid content has been determined using a UV-visible spectrophotometer. A sample with a value of 1 ml was added and mixed with 3 ml of 95% ethanol (R&M Chemicals, Malaysia) in a test tube. Then, 0.2 ml of 10% aluminum chloride (R&M Chemicals, Malaysia) was mixed and left for 5 min. Then, 0.2 ml of potassium acetate (R&M Chemicals, Malaysia) and 5.6 ml of deionized water were added and made up to 10 ml. A UV-visible spectrophotometer was used to measure the solution's absorbance at 415 nm after it had been incubated at room temperature for 30 min.

Determination of Vitamin C Content

All samples of fruits were properly washed with deionized water before the

extraction procedure to remove any cling impurities. Five (5) g of material was carefully weighed and pulverized in a mortar and pestle after being treated with 10 ml of 4% oxalic acid (R&M Chemicals, Malaysia). The mixture was then crushed and strained four times through muslin fabric. In a standard flask, the extract was prepared up to 25 ml with 4% oxalic acid (R&M Chemicals, Malaysia), and all the samples were processed similarly. The 2, 6-dichlorophenol indophenol (DCPIP) titration method calculated the ascorbic acid content of mangoes. In preparation for the standard solution, 5 ml of ascorbic acid (R&M Chemicals, Malaysia) working standard (500 µg/5 ml) and 10 ml of 4% oxalic acid (R&M Chemicals, Malaysia) were pipetted into a tube bottle. The contents of the tube bottle were titrated against the dye solution (DCPIP) V1 until a faint pink color appeared, which lasted a few minutes. Similarly, 5 ml of the test sample was titrated against the dye solution (DCPIP) V2 (Sani et al., 2018). The ascorbic acid content present in the test samples was determined using the formula:

$$\text{Amount of ascorbic content (mg/100 g)} = \frac{500 \text{ x } V2 \text{ x } 25 \text{ x } 100}{V1 \text{ x } 5 \text{ x } 5}$$

where, V1 = Amount of dye consumed by 500 µg of standard; V2 = Amount of dye consumed by 5 ml of a test sample; 25 = Total volume of the extract; 100 = Ascorbic acid content/100 g of the sample; 5 = Weight of sample taken; 5 = Volume of the test sample taken for titration.

Determination of Sugar Content

The TSS, or sugar content, measures the fruit's carbohydrates, organic acids, proteins, lipids, and minerals. The sugar content of Harumanis fruits was determined using Sani's method (Mahmood et al., 2011). It is determined by measuring the fruit's Brix degrees. A volume of 1 ml from homogenized fruits was determined for TSS value using a refractometer.

Statistical Analysis

The data were analyzed using a paired *t*-test to determine the significant effect of bamboo vinegar on the insect pests of Harumanis mango. Analysis of variance (ANOVA) and mean comparison Tukey's test at $P \leq 0.05$ were done to quantify the nutrient content of Harumanis mango after being treated with bamboo vinegar. All the analyses were done by using SPSS software (version 26).

RESULTS AND DISCUSSION

Insect Abundance

Figure 1 shows the total insect abundance before and after being treated with bamboo vinegar. A total of 508 individuals were collected in this study, comprising 5 orders and 11 families. Diptera was the highest insect order, with 229 individuals, and Orthoptera was the least, with 28 individuals. *Ceratitis cosyra* (Walker), *Ceratitis silvestrii*, *Ceratitis quinaria* (Bezzi), and *Bactrocera invadens* are four fruit fly species most commonly found in this study. Vayssieres et al. (2008) reported a comparable outcome with mango trees in

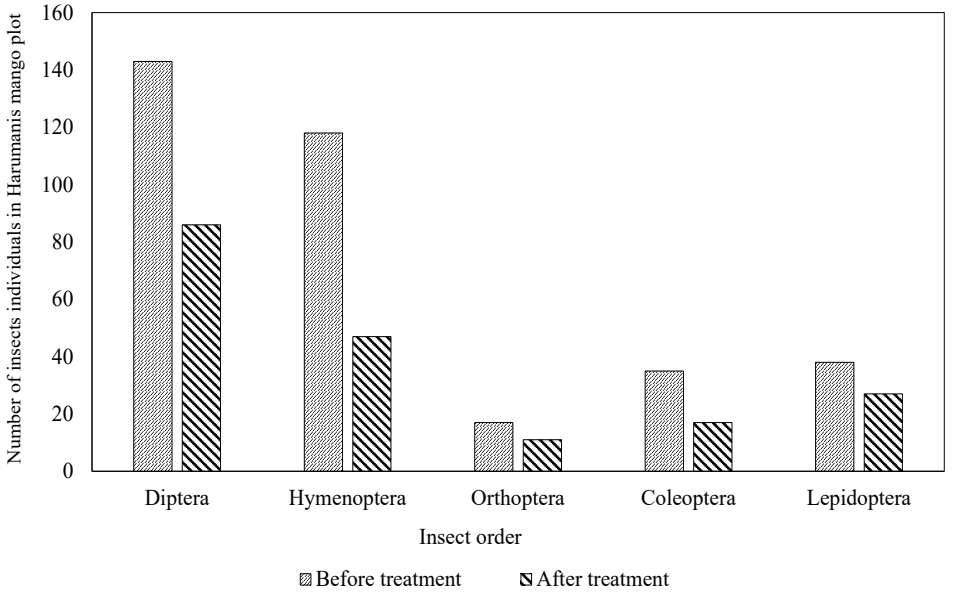

Figure 1. Number of insects collected before and after spraying with bamboo vinegar

Benin, which are affected by temperature, relative humidity, and rainfall. Among 508 individuals, 351 individuals were collected before bamboo vinegar treatment, while 188 individuals were after being sprayed with bamboo vinegar.

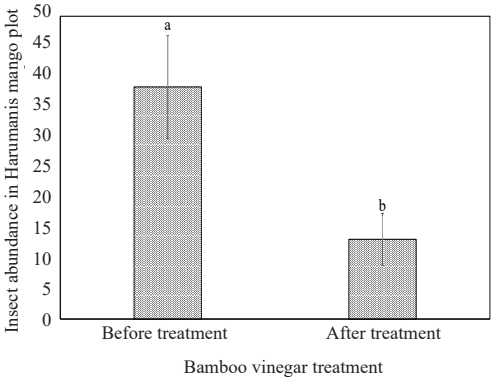

Figure 2. Insect abundance before and after spraying with bamboo vinegar

Note. The a and b letters represent the significant differences using a paired *t*-test at $p < 0.05$. Error bars indicate the standard error of means

Paired *t*-test results indicate that there was a significant difference between sprayed and unsprayed bamboo vinegar on insect abundance in the Harumanis mango plot ($t = 3.56$, $p < 0.05$) (Figure 2). Peng and Christian (2005) reported that organic treatment using organic insecticides can kill more fruit flies than biological treatment, which is the weaver ant treatment.

Spraying Intervals of Bamboo Vinegar

The 2-sample *t*-test result demonstrates that there was no significant relationship between spraying intervals of bamboo vinegar and insect abundance on Harumanis plot ($t = -1.50$, $p > 0.05$) (Figure 3). However, 3-day spraying intervals have lower insect abundance even though there was insignificant in which the bamboo vinegar had a better effect on shorter intervals compared to long intervals.

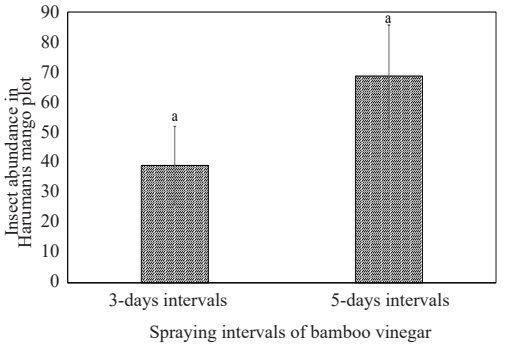

Figure 3. Insect abundance on 3- and 5-days spraying intervals

Note. The letters represent there is no significant difference using a paired *t*-test at $p > 0.05$. Error bars indicate the standard error of means

Nutrient Content of Harumanis Mango Fruit

Total Phenolic Content (TPC)

The Folin-Ciocalteu reagent assay method was used to determine the TPC, and that amount is equivalent to the calibration curve ($y = 0.1159x + 0.1232$, $R^2 = 0.95$). The TPC of Harumanis mango affected by bamboo vinegar application is shown in Figure 4. In this study, bamboo vinegar application at a 3-day interval (T1) shows significantly higher TPC in Harumanis mango as compared to other treatments. TPC was 13% higher with 3-day intervals (486.02 mg GAE/100 g) than without bamboo vinegar application (422.32 mg GAE/100 g).

The results of TPC obtained from this experiment are within the range (400-700 mg GAE/g) of the previous study conducted by Agatonovic-Kustrin et al. (2018). According to previous studies, the cultivar, crop, and ripening stage all influence the content and properties of phenolic acids (Burton-Freeman et al., 2017). Bamboo vinegar has a beneficial effect on the nutrient content in Harumanis mango fruits after showing improvement

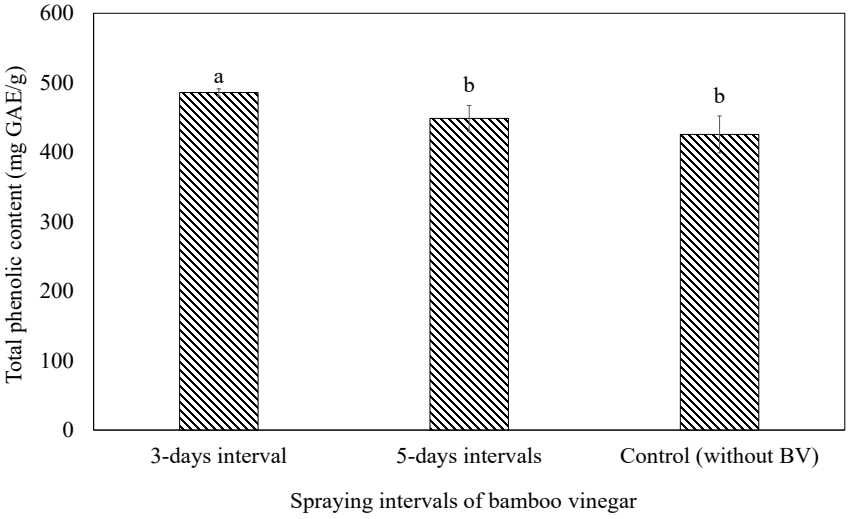

Figure 4. Total phenolic content of Harumanis mango as affected by bamboo vinegar application

Note. The different letters represent the significant differences using one-way ANOVA at $p < 0.05$. Error bars indicate the standard error of means

in TPC. In this study, Harumanis mango harvested from the tree without bamboo application shows significantly lower TPC because the pyroligneous acid (PA) in bamboo vinegar has been shown to increase crop growth, enhance soil quality, and lessen the impact of insect pests and plant diseases (Léchaudel & Joas, 2007). In addition, more than 200 chemical compounds have been found in bamboo vinegar, with organic acids, phenolic chemicals, carbon substances, alcohol, neutral materials, and base acidic substances (Oviasogie et al., 2009). This treatment may affect the growth of Harumanis mango trees and influence the amount of nutrient content in mangoes, where a tree treated with bamboo vinegar shows higher TPC than an untreated tree.

Antioxidants, structural polymers (lignin), attractants (carotenoids and flavonoids), UV filters (flavonoids), signal molecules (flavonoids and salicylic acid), and defensive response substances (tannins and phytoalexins) are all examples of plant phenolic compounds. Phenolics are plants' most prevalent secondary metabolites and the entire metabolic process. These phenolic substances are frequently connected to the defense mechanisms of plants. Increased pollination, color for camouflage, defense against herbivores, and antibacterial and antifungal properties are some additional phenolic metabolite-related functions (Mu et al., 2004).

TFC of Mangoes

The aluminum chloride colorimetric test method was used to calculate the TFC and the total flavonoid equivalent from the calibration curve ($y = 0.0069x + 0.0149$, $R^2 = 0.98$). As shown in this study, the application of bamboo vinegar has a positive effect on TFC (Figure 5). The highest amount of TPC (36.79 µg/mg QAE) was obtained from T1 at 3-day intervals after being treated with bamboo vinegar. However, there is no significant difference between the interval of bamboo vinegar application. As from Figure 5, T3 (without bamboo vinegar application) shows a significantly lower amount of TFC (25.37 µg/mg QAE) compared to other treatments. The total amount of flavonoid was higher by 31% from T1 (3-day interval) than without bamboo vinegar application.

Plant vinegar and wood vinegar are becoming more popular as organic products. These organic products share the same production process as bamboo vinegar. The previous study shows that wood vinegar may prevent stimulating pathogenic fungus growth (Chuaboon et al., 2016). The 2-phenyl-benzopyrene or flavan nucleus, made up of two benzene rings connected by a heterocyclic pyran ring, is flavonoids' most fundamental structural component. Antifungal activity is one of these substances' biological and pharmacological properties (Lashari et al., 2013). The effect of vinegar will enhance phytochemical change and simultaneously become an organic fungicide for plants. This change has affected flavonoid content to ensure plants have an excellent defense to prevent disease in crop growth.

Flavonoids are the polyphenols that are most prevalent in human diets. Flavonoids

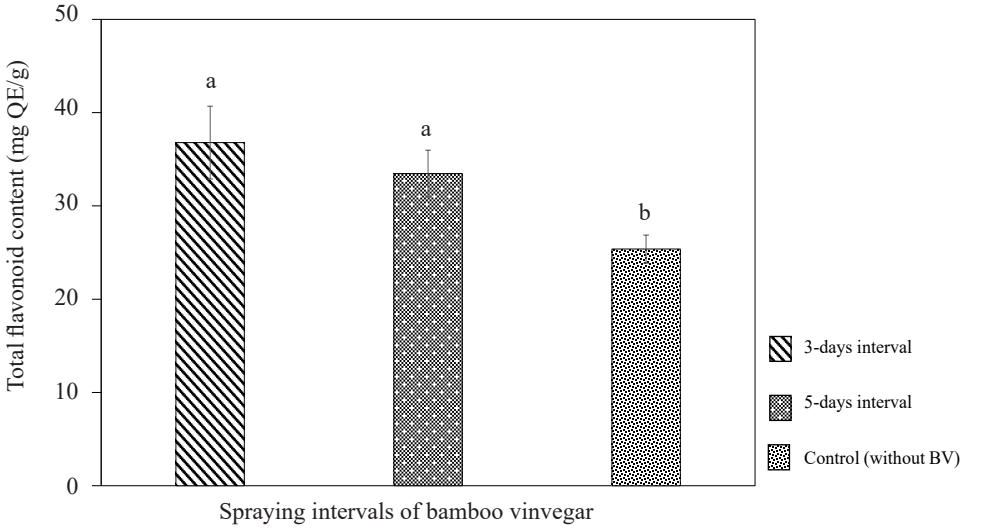

Figure 5. Total flavonoid content of Harumanis mango as affected by bamboo vinegar application

Note. BV = Bamboo vinegar; the different letters represent the significant differences using one-way ANOVA at $p < 0.05$. Error bars indicate the standard error of means

were once considered vitamins and were known by names like vitamin P and vitamin C. Many fruits and vegetables contain quercetin, the most prevalent flavanol in the human diet. Catechin, epicatechin, quercetin, isoquercetin (quercetin-3-glucoside), fisetin, and astragalin are all flavonoids present in mangoes. Quercetins and other flavonoids significantly influence the coloring of various fruits, flowers, and vegetables during ripening. Furthermore, mango peels have more flavonoids than mango pulps (Masibo & He, 2008).

Vitamin C Content

Applying bamboo vinegar increased the vitamin C content in Harumanis mango (Figure 6). A similar trend was observed with TFC, where there was a significantly higher vitamin C content for T1 (3-day interval) and T2 (5-day interval) as compared to control (without bamboo vinegar). The result shows that vitamin C content in Harumanis mango of T1 (53.33 mg/100 g) was increased by 17% as compared to without bamboo vinegar (45.55 mg/100 g). In addition, there is no significant difference between 3- and 5-day intervals of bamboo vinegar application on TFC in Harumanis mango.

The most significant dietary sources are fruits and vegetables because they contain phytochemicals, including phenol, vitamin C, and flavonoids, which can help maintain good health. These phytochemicals are necessary nutrients that are also found in fruits and vegetables. In this study, the amount of vitamin C in Harumanis mango increased after treatment with bamboo vinegar. This result is supported by a previous study where bamboo vinegar positively increases vitamin C content and

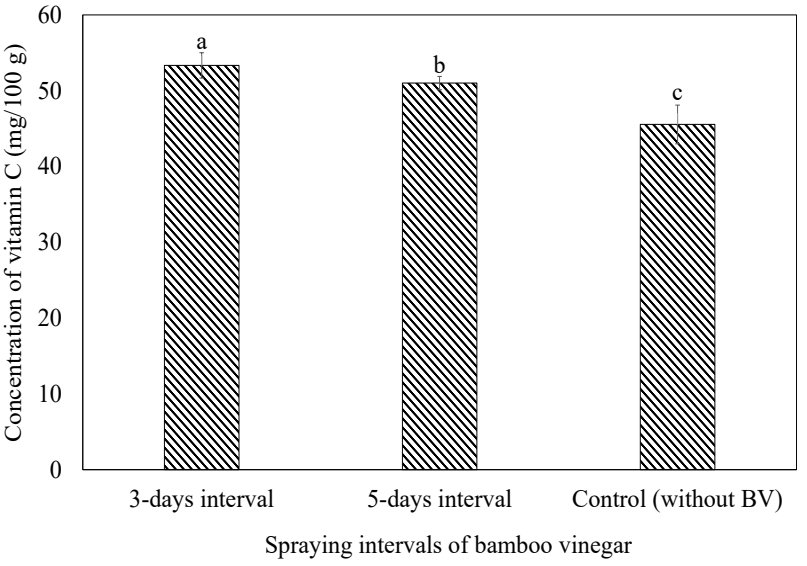

Figure 6. Vitamin C of Harumanis mango as affected by bamboo vinegar application

Note. BV = Bamboo vinegar; the different letters represent the significant differences using one-way ANOVA at $p < 0.05$. Error bars indicate the standard error of means

plant yield. The concentration of bamboo vinegar also plays an important factor, as higher concentrations provide a higher change value of vitamin C (Mun & Ku, 2010). It also stated that bamboo vinegar helps increase plant quality and yield. Plant variety, weather and climate conditions, cultivation technique, production factor, plant growth and health, maturation stage, fruits handled, and storage all influence the quantity of ascorbic acid in fruits (Dar et al., 2016). The benefit of bamboo vinegar is that it promotes plant growth and, at the same time, increases nutrients in Harumanis mango crops.

Sugar Content

The application of bamboo vinegar shows a positive effect on total soluble sugar in Harumanis mango (Figure 7). Sugar content from T1 (3-day interval) shows a significant difference ($p \leq 0.05$) compared to control (without bamboo vinegar). However, there is no significant difference between T2 (5-day interval) and control. The mean sugar content for Harumanis mango for T1 reaches up to 14.8%, T2 with a value of 14.3%, and T3 with a value of 13.1°Brix (Figure 7).

Mangoes are rich in carbohydrates, with 60% of the fruit containing sugar and acids. Mangoes have a major component that contributes to their sweetness and acidity. The quantity of carbohydrates trees provide is determined by the number of trees created by photosynthetic leaves (Lin et al., 2016). Bamboo vinegar has been used to develop crops, vegetables, and forest plants, and it has been examined. A healthy plant will

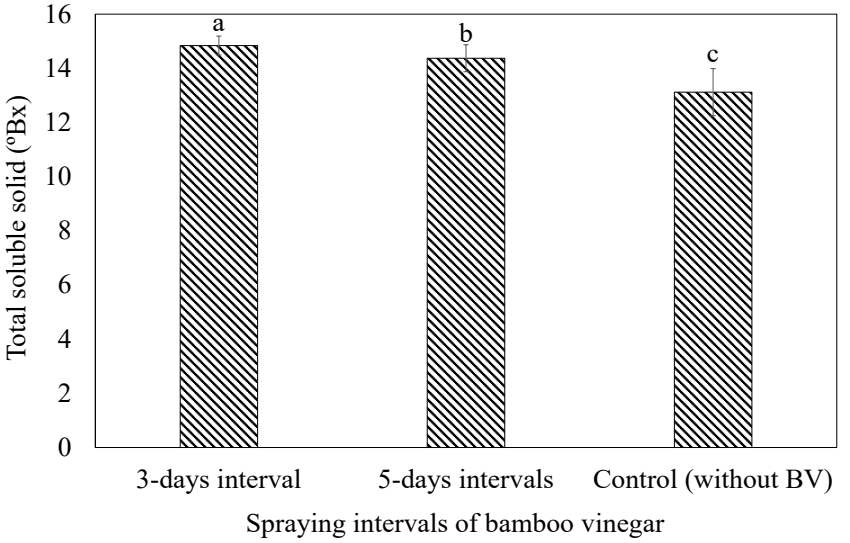

Figure 7. The sugar content of Harumanis mango as affected by bamboo vinegar application

Note. BV = Bamboo vinegar; the different letters represent the significant differences using one-way ANOVA at $p < 0.05$. Error bars indicate the standard error of means

provide a high process of photosynthesis to increase its mechanisms and, at the same time, can increase the sweetness of fruits.

An investigation has been done into the effects of wood vinegar only and a mixed combination of wood vinegar with different treatments, such as gibberellin (T1), sodium D-gluconate (T2), and melatonin (T3), on the rapeseed plant. As a result, the net rate of photosynthesis increased during the seedling and blooming periods. The net photosynthetic rates with treatment M, T1, T2, and T3 treatments at the seedling stage were 9.40%, 26.96%, 19.07%, and 14.92%, respectively, greater in two years than the control (water). Over two years, the blooming stages grew by an average of 12.81%, 21.29%, 19.07%, and 22.32% (Vayssieres et al., 2008).

CONCLUSION

In this study, the bamboo vinegar treatment with 3-day intervals (T1) is the most suitable treatment for reducing the insect abundance and enhancing the nutrient content and quality of Harumanis mango (MA128), *M. indica* L. Applying bamboo vinegar affected the quality and nutrient content of Harumanis mango positively. It has shown a positive effect on the nutrient content of Harumanis mango compared to other treatments. Bamboo vinegar application improved the TPC, TFC, vitamin C, and sugar content in Harumanis mango. According to several studies have shown the effectiveness of plant development after being treated with bamboo vinegar. The increasing mechanism could be that the major components in bamboo vinegar cause the plant to produce hormones in

trace amounts or increase photosynthesis in the leaves, which regulates the plant's development. Overall, the increased effect could be a synergistic effect of bamboo vinegar on plant growth.

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Evaluating the Performance of Alternate Wetting and Drying Irrigation Technology: An On-farm Rice Case Study in An Giang Province, the Mekong Delta of Vietnam

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ABSTRACT

Alternate wetting and drying irrigation (AWD) is a promising technique that has been tried across Southeast Asia to reduce water consumption and methane emissions in irrigated rice cultivation. The study conducted in the upper Vietnamese Mekong Delta compared the effectiveness of plant growth, yield components, and yield under three different water application regimes: the treatments of community AWD (AWD_C), household individually (AWD_H), and continuous flooding (CF) with the expectation to explore the ability to use water effectively in rice cultivation. The results showed no significant difference in water use between the three treatments. However, there was a considerable difference in coefficient of variation value (CV); the CV value of the water column in the AWD_C (1.32%) was a significant difference from that of AWD_H (0.87%) and CF (0.89%). The mean chlorophyll content, the yield, and the weight of 1,000 grains of the AWD_H treatment were significantly higher than that of the other two treatments. In another aspect, the water productivity of the AWD_H treatment was the highest (0.66 kg/m^3), a statistically significant difference compared to the AWD_C and CF (0.37; 0.33 kg/m^3). In conclusion, the AWD_H shows efficiency in leaf chlorophyll content, 1,000-grain weight, yield, and water productivity. The

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AWD_C is inferior to the AWD_H due to the large variation of field elevation. It is noted that field elevation is critical to the technique's success in being applied on a large scale.

Keywords: Alternate wetting and drying technology, continuous flooding, rice yield, the Mekong Delta, Vietnam, water productivity

INTRODUCTION

With the awareness of accelerating global climate change and ecological degradation, Southeast Asian states are grappling with worsening water insecurity, particularly in river delta regions, which form critical agricultural production and food security centres. The Mekong Delta of Vietnam provides more than 50% of the country's rice production and more than 90% of rice exports, making it crucial to the nation's economy. However, the delta faces multiple problems related to water resources insecurity, including worsening incidents of flooding, drought, and riverbank erosion driven by external/cross-border and local causes and processes (Boretti, 2020; World Bank [WB], 2022). One of the growing concerns is the increasing severity of water scarcity in the Delta, as surface water becomes more constrained and demand from agriculture and other sectors spirals basin-wide. In short, periods of drought are becoming more common in the Mekong region. Public irrigation providers and individual farmers find it harder to guarantee enough water for rice crop provision, especially in the dry season.

In this context, agencies responsible for water provision to rice farmers are increasingly keen to introduce techniques that might reduce irrigation water consumption and improve efficiency without hurting yields or farmer income. In theory, such water conservation efforts would free up more water supply for other downstream users and provide beneficial environmental flows (recognising the needs of the wider ecosystem and biodiversity in a river system, from both a water quantity and quality perspective) in the river system. Thus, water and agricultural research institutes across Asia have been testing a novel water-saving technique, AWD, which has shown some promise in lowering on-farm water use in irrigated rice cultivation, while reducing emissions of greenhouse gases (GHG) and not negatively affecting crop yields. Agriculture is the second largest contributor to global GHG emissions, accounting for 24% of total emissions (Adounkpe et al., 2021). In the agricultural sector, paddy rice cultivation is one of the most important sources of anthropogenic emissions of GHGs, thus making it a high priority to introduce methods to mitigate its significant impact (Arunrat et al., 2018).

A review of adoption trials across eight Asian countries (Lampayan et al., 2015) found that irrigation water usage had been reduced by up to 38% under AWD with no reduction in crop yield. Another study has suggested that AWD techniques helped to reduce freshwater use by 15–30%, methane emissions by about 30% compared to traditional flood irrigation techniques (Tivet & Boulakia, 2017) and, in some

cases, increased rice yields by 0.1–0.5 ton/ha (Nhẫn et al., 2013), and 0.7 ton/ha (Tin et al., 2015). AWD has been considered a "climate-smart" method, one of several potential tools to help rice farmers adapt to water shortages under more uncertain and extreme weather conditions (Allen & Sander, 2019). The benefits of AWD are reported to have been widely recognised by farmers in the Philippines: Palis et al. (2005) have claimed AWD saves water, time, and labour due to lesser expenditure, produces heavier 1,000-grain weight due to larger grains with good shape, and less pest problems. In An Giang Province, Vietnam, one study stated that AWD reduced water usage by 15–40% (Yamaguchi et al., 2016). As far as economic benefits go, based on a "with" or "without" AWD trial in the Mekong Delta, Lampayan et al. (2015) reported that farmers' incomes had increased by 17%, with decreased costs of water pumping. They concluded that, in general, and when applied correctly, AWD provided a high rate of return on investment both at the farm level and for research organisations experimenting with the technology, with an average benefit-to-cost ratio of 7:1.

Much research remains to be done to reliably measure the benefits of AWD and encourage adopting these practices at the scale needed, as at present, they have only been applied at a relatively small and localised scale, and not all risks or downsides have been identified. Previous studies have pointed to some potential drawbacks to applying AWD. The case in the paddy field of Padang Raja Kelantan, Malaysia, showed that it may be due to a lack of information, awareness, expertise, and successful experimental evidence (Ilahi et al., 2022). Farmers in the Philippines and Vietnam reported problems with rats when they used AWD (Quynh & Sander, 2015; Smedley, 2017) and weeds (Tirol-Padre et al., 2018). Furthermore, applying AWD on a relatively small scale to a few paddy fields within a larger irrigated block can make precise water management difficult, as there may be seepage from surrounding fields and coordination of water flows for the AWD practising farmers may be problematic.

These observed problems may be a limiting factor to the wider adoption of AWD in the Mekong Delta. Thus, to test the purported benefits of AWD for small-scale farmers within the Mekong Delta, a trial to measure the rice yield and water productivity of the technique over one dry season crop with two different treatments of AWD's applicability and control was arranged to understand how practical and productive this water saving technique would prove under actual farm-based conditions, and what were the limitations or obstacles to its application.

MATERIALS AND METHODS

Research Site and Experimental Design

The trial was carried out in Vinh Trung Commune, Tinh Bien district of An Giang province in the Upper Mekong Delta (Figure 1). This predominantly rural area is populated with Kinh and Khmer villagers. They practice farming as their main livelihood; the average farm size is approximately 1.3 ha/household.

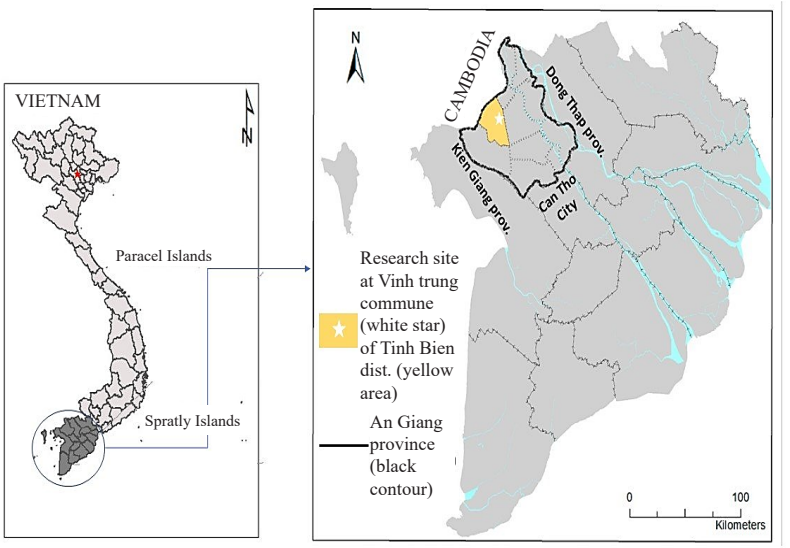

Figure 1. The map of Vietnam and the study area in Tinh Bien district, An Giang province

Note. Prov. = Province; Dist. = District

After consultation with the head of the Tinh Bien Agriculture and Rural Development office, the trial sites located in the complete dike area were agreed upon. It was in a deltaic floodplain area enclosed by full dikes that protect the land from seasonal floods and allow for triple rice cropping. The field experiment was implemented with the cooperation of three groups of households: Group 1 (the control) consisted of three farmers who use the standard local irrigation application regime of continuously flooded fields throughout the crop cycle (the control treatment), with their land occupying a total area of 3,240 m^2, hereafter called continuous flooding (CF, coordinates 10.556632°N, 105.024844° E); Group 2 consisted of three farmers applying AWD individually (the fields separately were irrigated by standard methods) with their land occupying a total area of 6,480 m^2, hereafter called AWD_H (coordinates 10.557766° N, 105.027514° E); and Group 3 was made up of four households with adjacent fields all cooperatively applying AWD, with a total area of 12,960 m^2, hereafter called AWD_C (coordinates 10.556018° N, 105.026574° E). All households grew rice variety OM18 (a high yield variety with a growth period of about 95–100 days, a hybrid combination of the variant OM 8017 and OM 5166), sowed on the same date, January 14, 2022, at a density of 150 kg of paddy seed/ha. It is a short-cycle rice variety from the Cuu Long Rice Research Institute of Vietnam. At this time of year, there is a low probability of rainfall. During the experimental period, it rained 13 times with an average rainfall of 10.08 mm, so crops almost entirely depend on the state-managed irrigation system.

Water Management In-field Trial Treatments

Irrigation water is provided by electric pumping stations that lift water from large secondary canals into a field network of tertiary and quaternary canals. The pumping station operates once every seven days to provide water for the whole area. Separate bunds and the farmers' surrounding rice fields decide the water height within each block of fields. In the trial, there were three types of water management as follows:

For the CF group, the field was flooded continuously from 7 to 76 days after sowing (DAS), according to local farmers' standard irrigation application regime.

Treatments AWD_H and AWD_C: The fields were flooded continuously from 7 to 17 DAS, then an irrigation regime of the alternate flood (three times at 40, 50, and 73 DAS) and drying periods were applied to harvest by blocking the inflow channel and diverting water to other fields. The water level in the field was monitored for these two treatments using a polyvinylchloride (PVC) pipe with a diameter of 20 cm, a height of 30 cm, and a perforated wall with multiple holes of 3 mm diameter to ensure that water was able to move freely through the pipes. Each participating household installed three plastic pipes in their field at different locations to monitor the water level and let farmers know when to reapply irrigation water. The pipes were installed into the ground at a depth of 20 cm from the ground level so the sub-surface water level could be easily observed and recorded, with details of the irrigation regime outlined in the section below.

Data Collection and Analysis

The following parameters were measured:

Soil Sample Analysis. The experimental area is on a soil type of Orthi Haplic Arenosols. Three soil samples were taken to represent each treatment and explore its capacity to store water related to the soil structure. Five sub-samples were collected within each treatment by travelling in a zig-zag pattern across the fields. Sub-samples were taken at a depth of 20 cm and then mixed well to make a combined 1 kg sample. The soil was analysed following these methods: Robinson method for soil texture (analysed at Can Tho University, Vietnam); total nitrogen (N), phosphate (P), and organic matter (OM) analysed at An Giang University, Vietnam by different methods, including the Kjeldahl method, colourimetric method, and atomic absorption spectroscopy (AAS).

Water Column Depth. A plastic ruler of 50 cm was used to measure the water column depth. It was measured once every three days while water was in the field. The water depth was measured from the ground level for the CF treatment. Treatments AWD_C and AWD_H were measured similarly to CF when standing water was in the fields, but when the water level was below ground level, the water column depth was measured inside the installed plastic pipes.

Growth, Yield, and Yield Components. Each treatment was tested using a square metre bamboo frame with three replicates held together by string. Five rice clusters were selected for growth and biological

characteristics data collection in each frame. The height of rice plants was measured weekly with a plastic ruler from the ground to the top of the tallest leaf. Chlorophyll content was determined by a soil plant analysis development (SPAD-502) meter, measuring the highest fully developed leaf at 3 points of its blade. From this, an average of the data taken at the top, middle, and bottom positions was calculated. At harvest time, all the shoots in the frame were measured for height and yield component parameters, including number of shoots, spikelet number/panicle, filled grain/panicle, and unfilled grain/panicle. The yield was calculated based on farmers' data at harvest time (checked the grain humidity from the provided samples).

SPSS 20 software was used to analyse one-way analysis of variance (ANOVA) treatment, and a Duncan's multiple range test at a 5% significance level was used to compare water management treatments.

RESULTS AND DISCUSSION

Soil Characteristics of the Study Area

The soil of the study area had a medium OM content of 6.36%. Total nitrogen, phosphorus, and exchangeable potassium were 0.20, 0.02, and 0.10%, respectively (Table 1). In terms of soil texture, it averaged 45.54% sand, 34.40% silt, and 20.06% clay. The soil type of the study area is classified as sandy loam due to an almost equal proportion of sand, silt, and/or clay in the samples. Soil texture composition determines the degree of water absorption and permeability, thus affecting the potential degree of control of water levels in clay soil, causing poor infiltration resulting in water logging, soil salinity, and reduced biological activity. On the other hand, sandy soil gives high infiltration, leading to low water-holding capacity and poor nutrient retention (Dhindsa et al., 2016). It implies that the soil of the study can absorb less water and easily lose water. That means that if AWD is applied in the research area, more irrigation water is expected; however, it depends on the quality of the bunds. In this study, the farmers took care of the bunds well, and the problem of water leaking was avoided.

Changes in Field Water Column Depth in Response to Irrigation Water Application

After initial sowing, all three comparative treatments were irrigated similarly,

Table 1
Soil characteristics of the study area

Sample no.	OM (%)	Soil chemicals			Soil texture			Type
		N_{total} (%)	P_{total} (%)	$K_{Exchangable}$ (meq/100 g)	Sand (%)	Silt (%)	Clay (%)	
1	6.00	0.19	0.03	0.08	48.90	38.44	12.66	-
2	7.21	0.24	0.02	0.16	32.09	34.16	33.75	-
3	5.86	0.17	0.02	0.07	55.64	30.60	13.76	-
Average	6.36 ± 0.30	0.20± 0.08	0.02± 0.03	0.10± 0.17	45.54± 1.73	34.40± 0.63	20.06± 3.34	Sandy loam

Note. OM = Organic matter; N = Total nitrogen; P = Total phosphate; $K_{Exchangable}$ = Exchangeable potassium

specifically at 10 and 17 days after sowing, as shown in Figure 2. From 22 days DAS to harvest, there were three irrigation applications for AWD_C and AWD_H (at 37, 52, and 70 DAS), while the CF treatment received six irrigation applications. In terms of water column height, all three treatments were identical during 17 DAS with a value of 9-10 cm water column height but varied considerably after that. From 22 DAS, the CF treatment ranged from 3 cm minimum to 15 cm maximum water depth. The AWD_C and AWD_H treatments followed similar water column depth value trends, with the maximum at about 5 cm and the minimum measured at about 20 cm below surface level. Controlling water depth depended on prevailing weather conditions, surrounding irrigation applications and soil properties (Tirol-Padre et al., 2018). The results of this study are quite different from the study of Tin et al. (2015), with seven watering times. The number of irrigation applications/crops for AWD depends on factors such as temperature (related to evapotranspiration), quantity of rainfall, and soil texture (determining the infiltration rate) (Huệ et al., 2016). Unfortunately, neither this study (Tin et al., 2015) recorded air temperature or humidity during the research period.

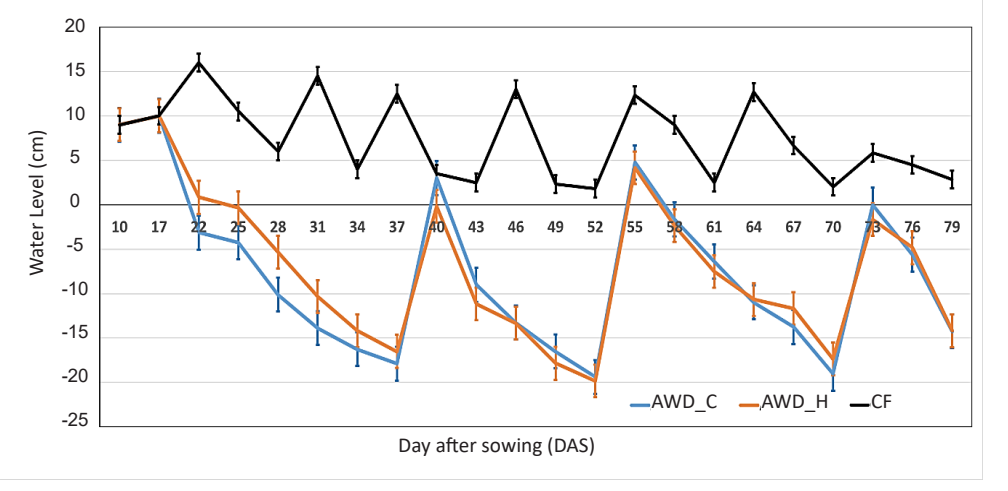

Figure 2. Changes in field water level over time to harvest

Note. AWD_C = Treatments of community; AWD_H = Household individually; CF = Continuous flooding

According to Kumar and Rajitha (2019), irrigation requirements vary from place to place, depending on local conditions, but are reported to be usually in the range of 900–2,250 mm per rice crop (equivalent to 9,000–22,500 m^3/ha). The volume of water applied in this trial varied from about 7,200 m^3/ha for AWD_C and AWD_H up to 7,500 m^3/ha for CF (Figure 3), which appears considerably lower than previous research

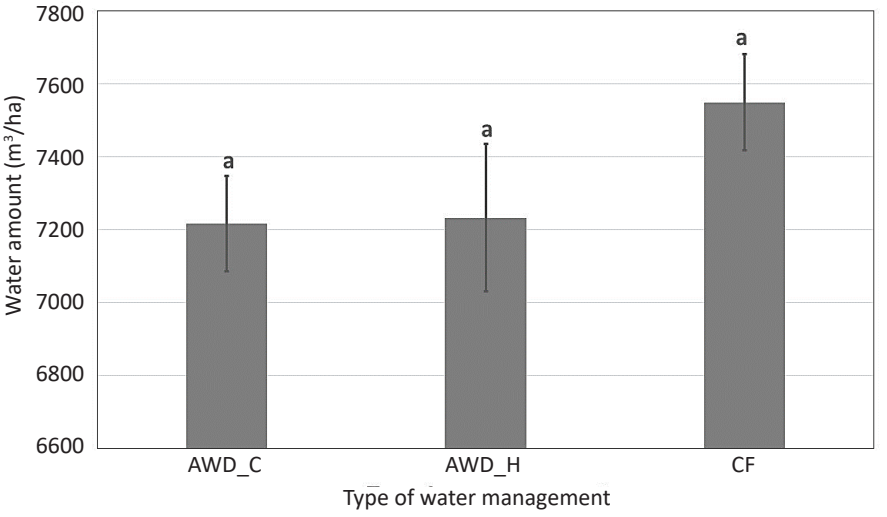

Figure 3. The water irrigation amount in each treatment

Note. AWD_C = Treatments of the community; AWD_H = Household individually; CF = Continuous flooding; The same letters indicate a similar between the treatments at a 5% confidence limit

results reported in the above research. Our research did not consider the initial water volume required for soil preparation, which Kumar and Rajitha (2019) report about 1,500 to 2,000 m^3/ha. No significant water use difference was found between the treatments in this study. However, there was a considerable difference in variation of water column between treatments, e.g., the AWD_C treatment showed a coefficient of variation (CV %) of water column height was up to 1.32%, which was significantly higher than that of the CF and AWD_H treatments with a CV% of 0.89 and 0.87%, respectively (Figure 4). According to actual observations, the water column height of the AWD_C treatment was not equal across the survey points.

This discrepancy is due to different field elevations between the survey sites, which may have affected the growth and yield of rice. A previous study demonstrated that decreased starch content in matured grains was explained that the shortening of grain filling stage under drought stress resulted in early plant senescence and decreased yield of rice under drought was more serious in susceptible variety (IR64) than tolerant genotype (N22) (Prathap et al., 2019). Elevation of the field level is an essential factor in applying AWD, particularly at the community level, to avoid a lack of water in the needed stages of growth.

Effects of Water Management on Growth, Yield Composition, and Yield of Rice

From 22 to 50 DAS, the rice plant height was similar across all three treatments (Table 2). However, at the flowering stage, the average height of rice plants in the CF treatment reached 78.61 cm, significantly

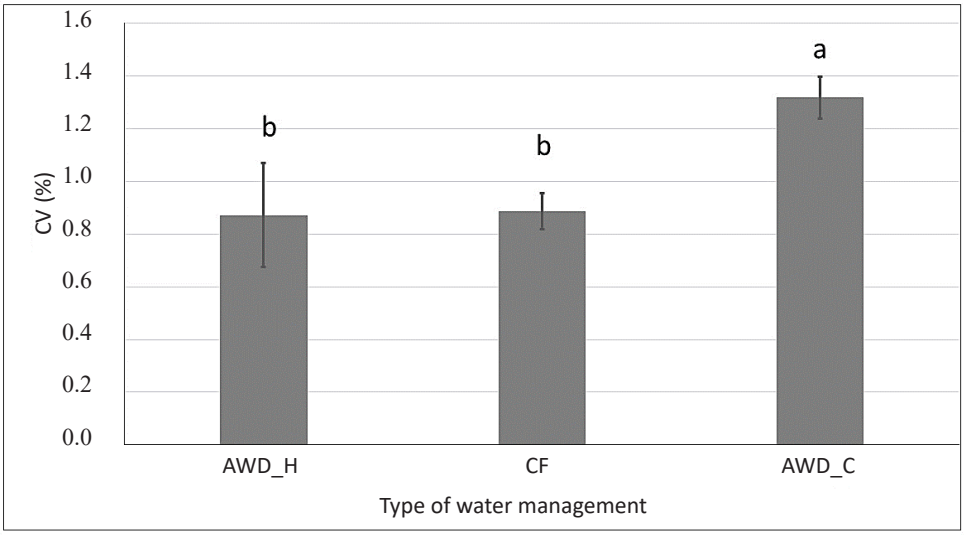

Figure 4. Variation of the water column in each treatment during measurement times

Note. AWD_C = Treatments of community; AWD_H = Household individually; CF = Continuous flooding; CV (%) = Coefficient of variation; Different letters indicate a significant difference between the treatments at a 5% confidence limit

taller than AWD_H at 5% confidence limit, but not significantly different from the AWD_C treatment. In the CF treatment, the water column in the field was always higher than that of the other two treatments (Figure 2), which could explain the greater height of rice plants at the panicle initiation stage.

The SPAD index is used as an indicator of the nitrogen concentration in the leaves of plants, including rice. Nitrogen in leaves is obtained by absorption from the soil. Previous research has shown that SPAD Value increased with increasing N level and growth stages to the flowering stage (Singh et al., 2020). Other research showed that there was a strong correlation between the nitrogen content of rice leaves and SPAD values at 45, 55, and 65 days after

Table 2
Effect of water management regime on plant height (cm)

Irrigation treatment	Day after sowing					
	22	29	36	43	50	57
AWD_C	31.83	47.44	51.06	52.06	58.50	72.06ab
AWD_H	36.33	44.50	52.11	55.06	58.94	69.44b
CF	36.67	44.56	56.44	61.78	64.50	78.61a
CV (%)	7.10	7.21	5.41	7.06	7.37	4.89
Sig.	ns	ns	ns	ns	ns	*

Note. AWD_C = Treatments of community; AWD_H = Household individually; CF = Continuous flooding; Sig. = Significance; ns = Non-significant; In the last column, different letters indicate a significant difference between treatments at 5% (*)

transplanting (Suresh et al., 2017), and rice leaves with a higher SPAD index (>35) indicated higher chlorophyll and nitrogen content (Islam et al., 2009).

In this study, when comparing the two AWD treatments, it was found that the AWD_H treatment had a considerably higher SPAD index than that of AWD_C at 22, 43, and 57 DAS. For the average SPAD index, the results showed that AWD_H gave the highest value (33.85±0.63), which was significantly higher than both AWD_C (31.84±0.25) and CF (31.63±0.73), although the amount of fertiliser applied was the same with each treatment. A difference in the SPAD index of rice between CF, AWD_C, and AWD_H treatments was measured, meaning that rice absorbed different nitrogen levels from the soil under different water regime conditions due to differences in uniformity between AWD_H and AWD_C (Figure 4). The results of previous studies claimed that the soil in AWD condition creates favourable conditions for the release of more nitrogen compared to the CF state, specifically in the period from sowing to 14 days for ammonium (NH_4^+) and 14–28 days DAS for nitrate (NO_3^-) being made available to plants during the drier periods (Đông et al., 2018).

Unfortunately, in our experiment, the total nitrogen of the investigated soil (Table 1) as baseline nitrogen was studied; nitrogen available during the experimental period should have been analysed to observe the relationship between nitrogen available and the SPAD index. It is the weakness of our study. The results from our study indicated that the SPAD value changed over time for each treatment, and the differences were found to be statistically significant between the treatments at 22, 29, 43, and 57 DAS (Table 3). The AWD_H treatment reached a SPAD index of 37.91 (at 22 DAS), 34.74 (at 29 DAS), and 34.63 (at 57 DAS), which were significantly higher values than that of CF.

According to an earlier study, rice yield under AWD conditions was significantly lower than CF (Chapagain & Yamaji, 2010) because drought stress at the flowering stage is recognised to have a strong influence on

Table 3
Effect of water management on soil plant analysis development index in rice leaves

Irrigation treatment	Day after sowing						Average
	22	29	36	43	50	57	
AWD_C	32.22±0.36b	35.56±1.39a	29.32±1.46	28.20±066b	33.36±1.49	32.35±1.06ab	31.84±0.25b
AWD_H	37.91±1.09a	34.74±0.10a	33.69±1.23	31.66±0.70a	30.44±1.78	34.63± 1.22a	33.85±0.63a
CF	33.93±1.35b	28.46±0.85b	31.15±1.62	31.94±1.09a	34.63±4.14	29.65±0.88b	31.63±0.73b
CV (%)	5.09	4.95	7.97	4.75	14.47	5.71	3.07
Sig.	*	**	ns	*	ns	*	*

Note. AWD_C = Treatments of community; AWD_H = Household individually; CF = Continuous flooding; CV (%) = Coefficient of variation; Sig. = Significance; ns = Non-significance; In the same column, different letters indicate significant difference between treatments at 5% (*) and 1% (**) confidence limits, respectively

rice physiological traits and yield (Yang et al., 2019). Another study showed a negative relationship between soil moisture and unfilled grains, with the lowest unfilled grains detected when soil moisture was at -30 kPa (Ullah & Datta, 2018). As noted earlier, using AWD under field conditions causes water levels to fall below surface level, which can provide favourable conditions for weeds to grow, which compete for soil nutrients and for rodents to eat the rice plants more easily. Researchers have tried to overcome these potential challenges, so more recent studies have shown some significantly positive results regarding rice yield under AWD compared to using CF (Yamaguchi et al., 2017).

The results of our study, shown in Table 4, indicate that the yield of the AWD_H treatment (4.72 tons/ha) was significantly higher than that of the AWD_C treatment (2.66 tons/ha) and CF (2.51 tons/ha). Most yield components, such as the number of panicles/m^2, number of grains per panicle, percentage of filled grain and percentage of unfilled grain, were not significantly different across the three treatments. However, Allen and Sander (2019) reported that rice under AWD conditions produces more tillers and has enhanced root depth and density compared with CF. It is thought to lead to better drought, disease and lodging resistance, plus increased nutrient and water uptake. One notable result from our research was the 1,000-grain weight measurements, which found a significant difference between the treatments. The AWD_H treatment produced the highest weight (24.60 g/1,000 grains), which was significantly higher than that of the other treatments, namely AWD_C (21.83 g/1,000 grains) and CF (21.82 g/ 1,000 grains). The yield of AWD_H treatment was significantly higher than others, which could be due to significant differences in 1,000-grain weight (Table 4).

Water Productivity of Water Management Methods

Two main objectives of AWD technology have been to save water and contribute towards climate change mitigation by

Table 4
Effects of the trial's treatment regime on rice yield and yield components

Irrigation treatment	Yield (ton/ha)	Panicle (s/m^2)	Spikelet number/ panicle	Filled grain/ panicle	Unfilled grain/ panicle	1,000-grain weight (g)	Filled grain rate (%)	Unfilled grain rate (%)
AWD_C	2.66b	367.67	51.57	41.10	10.47	21.83b	78.56	13.29
AWD_H	4.72a	457.33	55.23	48.70	6.53	24.60a	87.83	7.48
CF	2.51b	378.33	52.77	43.90	8.87	21.82b	83.49	10.81
CV (%)	16.24	18.50	20.90	23.33	25.75	2.54	5.56	28.77
Sig.	**	ns	ns	ns	ns	**	ns	Ns

Note. AWD_C = Treatments of community; AWD_H = Household individually; CF = Continuous flooding; CV (%) = Coefficient of variation; Sig. = Significance; ns = Non-significance; In the same column, different letters indicate significant differences between treatments at 1% (**) confidence limits

lowering water demand and emissions of greenhouse gases, most notably methane. Thus, many studies have focused on assessing water use efficiency or productivity, which measures how much water is required to produce 1 kg or a ton of rice. Bouman (2009) maintains that producing 1 kg of paddy rice requires from 800 to 5,000 L of fresh water, with 2,500 L needed on average. According to another calculation that considers the yield of rice per unit of water used, an average of 1.74 kg of rice/m^3 may be produced under AWD conditions (Chapagain & Yamaji, 2010). Under continuous flooding conditions, water productivity fluctuated in the range of 0.2–0.3 kg of paddy rice/m^3 of water, according to Kumar and Rajit (2019). In this study, it was found that the water productivity figures were not consistent with previous studies, where water productivity under the AWD_H treatment was the highest (0.66 kg/m^3), which was significantly higher than other treatments, namely AWD_C and CF with values of 0.37 and 0.33 kg/m^3, respectively (Figure 5). This difference is attributed to higher yields of the AWD_H treatment (Table 4), while there was no considerable difference in water consumed between treatments (Figure 3).

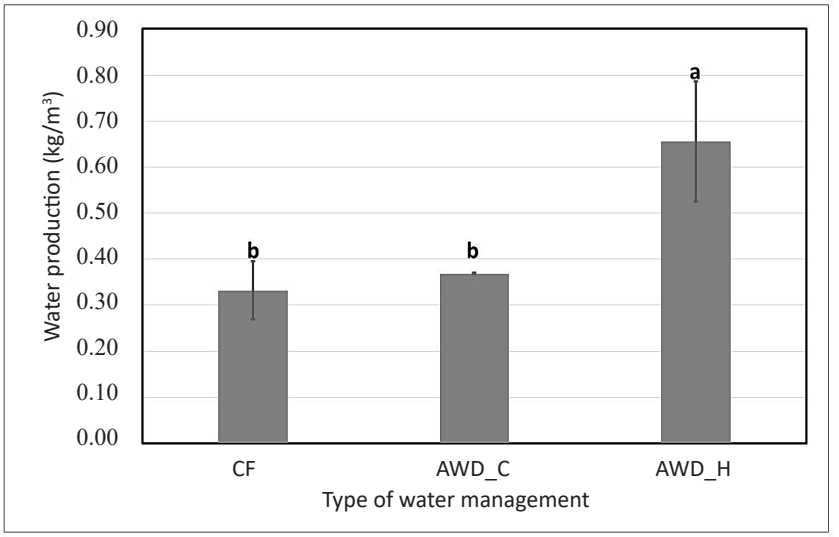

Figure 5. Evaluating water productivity in each treatment

Note. AWD_C = Treatments of community; AWD_H = Household individually; CF = Continuous flooding; Different letters indicate a significant difference between the treatments at a 5% confidence limit

The treatment that was expected to bring the highest rice yield and water productivity was the AWD_C treatment. After all, a larger area cultivated might have two advantages: (1) better water management due to the limitation of water loss, and (2) possibly less damage from weeds or pets because the rat population or weeds may be more dispersed. However, the results showed that AWD_H gave the highest water productivity

and higher rice yield, above that of AWD_H and CF. This result is consistent with previous research results, which indicated that AWD resulted in heavier and larger rice grains (Ilahi et al., 2022; Mboyerwa et al., 2021). The critical question is, why should AWD-H be more productive? Because AWD_C covered a relatively large area (from many households working together), where there were differences in land elevation levels between one field to another, leading to significant differences in water column depth across the field. The difference between the high and low fields is about 5–15 cm. The study also tested and compared the water column's coefficient of variation (CV %) between the three water management treatments over 22 measurement periods. The result shows that the CV % value of the water column under the AWD_C treatment was significantly higher than the other two treatments (Figure 4). Considering the SPAD index under AWD_H, it was significantly higher than both AWD_C and CF, which points to greater levels of photosynthesis, resulting in a greater yield.

CONCLUSION

The AWD_H treatment is more efficient than the AWD_C and CF treatments in terms of rice yield and water productivity due to the high 1,000-grain weight. The unequal field surface of the AWD_C treatment and lower SPAD index led to a lower grain weight than AWD_H. To improve benefits of AWD_C, checking the flat of field ground is necessary.

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Physical and Chemical Changes of Seven Selected Herbs Used as Herbal Bath Affected by Different Drying Methods

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ABSTRACT

The effect of oven drying (OD) at 50±5°C, sun drying (SD), and fresh leaves (control) of seven selected herbs used in herbal preparation as herbal baths were evaluated. Herbal baths (HB) involve mixing herbs into water and boiling them or simply immersing them in the mixture during a regular bath. The herbs selected were leaves of torch ginger, greater galangal, pandan, citronella grass, henna, betel leaves, and kaffir lime leaves and fruits. The herbs were planted in a plot at the Institute of Bioscience, Universiti Putra Malaysia, Malaysia. Physical changes such as HB herbs' colour, aroma, and chemical composition were evaluated. The hydro distillation method was used for the extraction process of HB herbs, where it produced essential oils (EO), essential water (EW), and boiling water (BW), and their chemical composition was determined by gas chromatography-mass spectrometry. As a result, the OD herbs possessed brighter and more attractive colours compared to the SD method, which was dull and pale. The colour of EO was yellow, colourless for EW, and reddish for BW. Additionally, OD herbs possessed 80% and only 50% of the scent strength of the SD herbs from extracting fresh herbs. The fresh and dried HB herbs contained chemical constituents mostly from the terpene group. Herbal preparation developed using all treatments retained an appreciable amount of chemical composition studied, thus having the potential for commercial purposes.

Keywords: Chemical compositions, drying method, essential oil, herbal bath, hydro distillation method

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INTRODUCTION

An herbal bath (HB) adds specific herbs to a hot bath with a specific goal. Many Malaysians believe HB could revitalise their bodies, improve blood circulation, and enhance their skin's beauty, radiance, and health. Most herbs used for HB are fragrant, common knowledge, and easily accessible (Tungsukruthai et al., 2018). Citronella grass, pandan, henna, betel leaf, torch ginger, greater galangal, and kaffir lime were the seven herbs employed in this investigation to produce this HB product (Zaman et al., 2007). Since most plant components can be used to extract or acquire volatile oils, the herb species listed here are also known as aromatic herbs (Solórzano-Santos & Miranda-Novales, 2012).

There are many different types of HB practises, including leaf baths, flower baths, and milk baths, that are dependent on ethnicity (Alsarhan et al., 2021; Hishamshah et al., 2010) as well as a variety of bathing techniques, including steam baths or saunas, regular baths with herbal water as the final wash, soaking the entire body in herbal water in the bathtub, mixing regular baths with herbal water, and gently massaging the entire body with EO (Mohamed & Hj. Bidin, 2012; Jamal et al., 2011; Li et al., 2006; Zaman et al., 2007). It requires 20 to 30 min to take a bath once or twice a day, three times a week (Panyaphu et al., 2011), so the nutrients from the herbs can be easily absorbed through the skin pores (Li et al., 2006). Although there has not been much scientific research on HBs, the chemical content of each herb convinces customers that HB practices are beneficial for enhancing health.

In HB practises, there are typically odd-numbered combinations of herbs, such as 5, 7, 9, or 11. The dosage of these many herbs varies depending on the practitioner and is not precise (Li et al., 2006; Zaman et al., 2007). Fresh herbs have a limited shelf life for commercial uses, and if they do not undergo processing immediately, they lose their stability. Therefore, dried herbs are also an alternative for producing HB because they are always accessible and usually cost less. However, they lack the aromatic qualities of fresh herbs because of the loss or oxidation of volatile oils through drying or other processing procedures (King, 2006).

To extend the shelf life of food products, people frequently dry foods, including meat, herbs, and fruits (Mashkani et al., 2018), to inhibit the activities of microbes and bacteria when there is no water present; this procedure employs air and heat to eliminate moisture from the product (Thamkaew et al., 2020). It also relies on the factors that affect drying, such as the climate, drying period, spread thickness, and relative humidity. Three categories of drying methods: (1) thermal, (2) chemical, and (3) special, can be used to dry leaves. Thermal drying refers to natural methods that use sunlight, shade, and wind. In contrast, special drying refers to artificial methods that use modern technologies like vacuum, microwave, dryer, and oven (Babu et al., 2018). The drying process will impact the product's chemical composition, colour, fragrance, and durability.

An optimised leaf drying process aims to maintain a high level of nutrients when the leaves are fresh while achieving the required end moisture content (Tasirin et al., 2014). So, choosing the correct drying method is crucial to guaranteeing the quality of the final product. This study used two different drying techniques: (1) oven drying (OD) and (2) sun drying (SD) to dry the plant leaves. In the OD technique, the sample is dried at a predetermined temperature using low-temperature convection air or thermos-gravimetric technology (Ahmed et al., 2013). Compared to the SD, this drying method is weather-independent, quicker, continuous, cleaner, and sanitary. However, the SD method approach is inexpensive and appropriate for bulk densities, particularly for commercial purposes (Özgüven et al., 2019).

According to several studies, dramatic drying alters the active components in fresh herbs' EO. However, the effect varies based on the plant species, drying temperature, and drying period. According to Mashkani et al. (2018), OD and vacuum obtained the highest EO content in *Thymus daenensis*. Additionally, according to Sefidkon et al. (2006), OD, shade drying, and SD increased the concentration of EO in *Satureja hortensis* L. leaves. Contrarily, Filho et al. (2018) claimed that the drying procedure did not impact the amount of EO in the dried parsley leaves.

The chemical composition in herbal leaves is believed to promote human health in several ways, including reviving the body, reducing stress, healing illnesses, and enhancing skin quality, even though the research on the impact of HB practises on human health is quite limited. Therefore, this experiment was conducted to determine the physical and chemical changes in HB herbs due to the impact of various drying processes to ensure and manage the herbs' quality. Additionally, this study will offer details on the results of using more than three herbs in a single product for potential applications in cosmetics.

MATERIALS AND METHODS

Description of the Study Area

The studied area for field and laboratory work was at the Institute of Bioscience, Universiti Putra Malaysia (IBS, UPM), Serdang, Selangor (30°00'56.3"N, 101°72'32.69"E). The climate of the location is mainly hot and damp; temperature ranges between 28–34°C with 65.5–87% relative humidity, received between 95.19–1,611.85 µmol of light density in the daytime and experiences rainfall annually about 2,000–2,500 mm means a month (Jabatan Meteorologi Malaysia [METMalaysia], 2020).

Collection and Preparation of Plant Materials

There were seven types of herbs used in the study: *Etlingera elatior* (torch ginger), *Alpinia galanga* (greater galangal), *Pandanus odours* (pandan), *Cymbopogon nardus* (citronella grass), *Lawsonia inermis* (henna), *Citrus hystrix* (kaffir lime), and *Piper betle* (betel leaves). The herbs were grown from cuttings at IBS, UPM. The

herbs were selected based on their long-term usage as HB in traditional Malaysian practices. Fresh herbal leaves and fruits (kaffir lime only) were collected between 8–10 a.m. (Chan et al., 2008; Mashkani et al., 2018). After collection, the leaves and fruits were repeatedly washed with tap and distilled water and air-dried for 72 hr at room temperature (Gebrehiwot et al., 2016). The herbs were cut into ±2.5 cm (1") or sliced into small parts (Jamal et al., 2011).

Drying Treatments

Two drying methods were used: oven-dried (OD) at 50±5°C and sun-dried (SD), with fresh samples as control. For the SD, the sample was spread on the cloth, while for OD, the sample was spread on the tray. The herbs were dried until the moisture content reached ±10% (Mashkani et al., 2018) or until they lost ±85% of their weight (Babu et al., 2018) or the dried leaves felt crunchy when crushed using a hand.

Preparation of HB Herbs

Two samples of HB herbs were prepared: fresh and dried samples. For the fresh herbs sample, 255 g of combined herbs (41 g of pandan leaves, 14 g of henna leaves, 22 g of kaffir lime leaves, 25 g of kaffir lime fruit, 40 g of betel leaves, 25 g of torch ginger leaves, 22 g of greater galangal leaves, and 66 g of citronella grass leaves) were prepared. The sample was air-dried in a closed room for one whole night before undergoing the extraction process using the hydro distillation method. For the dried herbs sample, the OD and SD herbs were weight 2 g for each herb (pandan, henna, kaffir lime leaf, kaffir lime fruit, betel leaf, torch ginger, and greater galangal) and 6 g of citronella grass. The samples were combined, wrapped and tied neatly in muslin cloth. One pack of combination dried herbs equal to 20 g. The samples were replicated four times for each sample (Mahanom Jr. et al., 1999; Tamboli & Bhong, 2018).

Physical Properties of HB Herbs and EO

Two physical properties measured manually are colour and scent, with fresh samples as a control. For colour analysis, the Munsell Colour Chart was used as standard. In contrast, for scent analysis, the strength was recorded in percentages between 0–100% using the fresh herbal leaf as control (Prof. Ir. Dr Yus Aniza Yusof, Department of Process and Food Engineering, Faculty of Engineering, UPM, personal communication in October 2018). Ten IBS UPM employees, who have actively produced these HB herbs for over three years, assessed fresh and dried HB herbs for this sensory investigation. Each sample was examined, felt, and smelled individually by the analyst manually in an air-circulated chamber.

Extraction of HB Herbs by Hydro Distillation Method

Fresh and dried HB herb samples weighing 255 and 80 g were put in a 20 L round bottom glass flask separately. Next, the flask was filled with 5 L of distilled water and mixed thoroughly. Then, the flask was fitted with Clevenger's apparatus, a glass

condenser, heated using a heating mantle, and hydro distilled at atmospheric pressure for 4 hr. The EO was isolated from the aqueous layer using 100 ml chloroform (Chemiz, Malaysia) and filtered using Whatman No. 1 filter paper. Finally, the extraction process products, EO, EW, and BW, were stored in the refrigerator at 4°C to keep them from being volatile. The chemical compositions of EO, EW, and BW were determined by gas chromatography-mass spectrometry (GC-MS).

$$\text{Yield (\%)} = \frac{\text{Volume of essential oil (ml)}}{\text{Weight of sample (g)}} \text{ X } 100\% \quad [1]$$

GC-MS Analysis of EO, EW, and BW of HB Herbs

EO, EW, and BW from fresh and dried HB herbs were analysed for their chemical compositions and quantity after dissolving in methanol using GC-MS by Shimadzu CG-17A (Shimadzu Corporation, Japan), which was directly coupled with QC-2010 high polar fused silica capillary column (Zebron™ ZB5-ms 30 m x 0.25 mm ID x 0.25 µm film thickness, Phenomenex Corporation, USA). The gas carrier was helium, delivered through split injection at a volume of 0.3 µl ratio, with a normal mode at a flow rate of 6.0 ml/min and head pressure of 49.7 kPa. The column temperature was held at 40°C for 5 min. The chromatographic condition was helium, 0.3 µl/min and 49.7 kPa; injector and interface temperatures were 220 and 250°C, respectively. Additionally, the column temperature was maintained at 40°C for 5 min. Methanol was used as the solvent to dissolve the oil.

EO, EW, and BW Identification and Quantification

The quality and quantity of EO, EW, and BW compounds that GC-MS detected were recorded in chromatograms. The quantitative data were determined from the peak total percentage areas of the analysed EO, EW, and BW extracted from fresh and dried HB herbs. The individual compounds were identified by comparing their relative retention indices, which were calculated using the *n*-alkanes formula and compared with the National Institute of Standards and Technology libraries (NIST).

Statistical Analysis

The results were statistically analysed using analysis of variance (ANOVA) in the statistical analysis system (SAS) programme (version 9.4). The data were expressed as means of four replications. Significant differences among the treatment mean at $p<0.05$ were determined by the least significant difference (LSD).

RESULTS AND DISCUSSION

Effect of Drying Method on Physical Properties of HB Herbs

The colour and scent of herb leaves are important for product marketing because HB herb is for external use. Therefore, product freshness and appearance are very important to meet consumers' demands.

Figure 1 shows the colour changes analysed in fresh and dried herbal preparations. The drying treatments caused significant colour changes for all herb leaves. Compared to fresh leaves, the herb's colour changes were reduced from slightly to significantly by the OD and SD methods.

The results showed that henna, torch ginger, betel leaves, and kaffir lime fruit did not significantly differ in colour between OD and SD methods but were darker than fresh samples. It indicated that OD and SD could not preserve the sample's green colouration. While there were significant colour differences between the OD and SD methods for greater galangal, kaffir lime leaves, citronella grass, and pandan leaves, in comparison to OD and fresh herbs, SD herbs exhibited a darker hue, indicating that OD herbs appeared more similar to the fresh samples.

The benefits of the OD perform over the SD method are supported by these results, which corroborated earlier reports in the literature on several types of herbs (Arslan & Özcan, 2010; Özgüven et al., 2019; Telfser & Galindo, 2019). The OD method at lower drying temperatures (<50°C) is particularly beneficial for herbs containing active compounds subjected to thermal degradation. Most nutritional and external properties are preserved since OD is performed at low temperatures and without oxygen (Laurence et al., 2019). In this study, OD herbs maintained a consistent and bright colour compared to SD herbs, which were dull and pale. It is a result of the colour degradation caused by the possible destruction of pigments like chlorophyll and anthocyanin during the drying process, showing that the SD approach has a bigger impact on colour degradation than the OD method (Thamkaew et al., 2020).

Figure 1. Herbal bath herbs colour changes before and after drying treatment: (A) fresh herbs, (B) oven-dried herbs and (C) sun-dried herbs

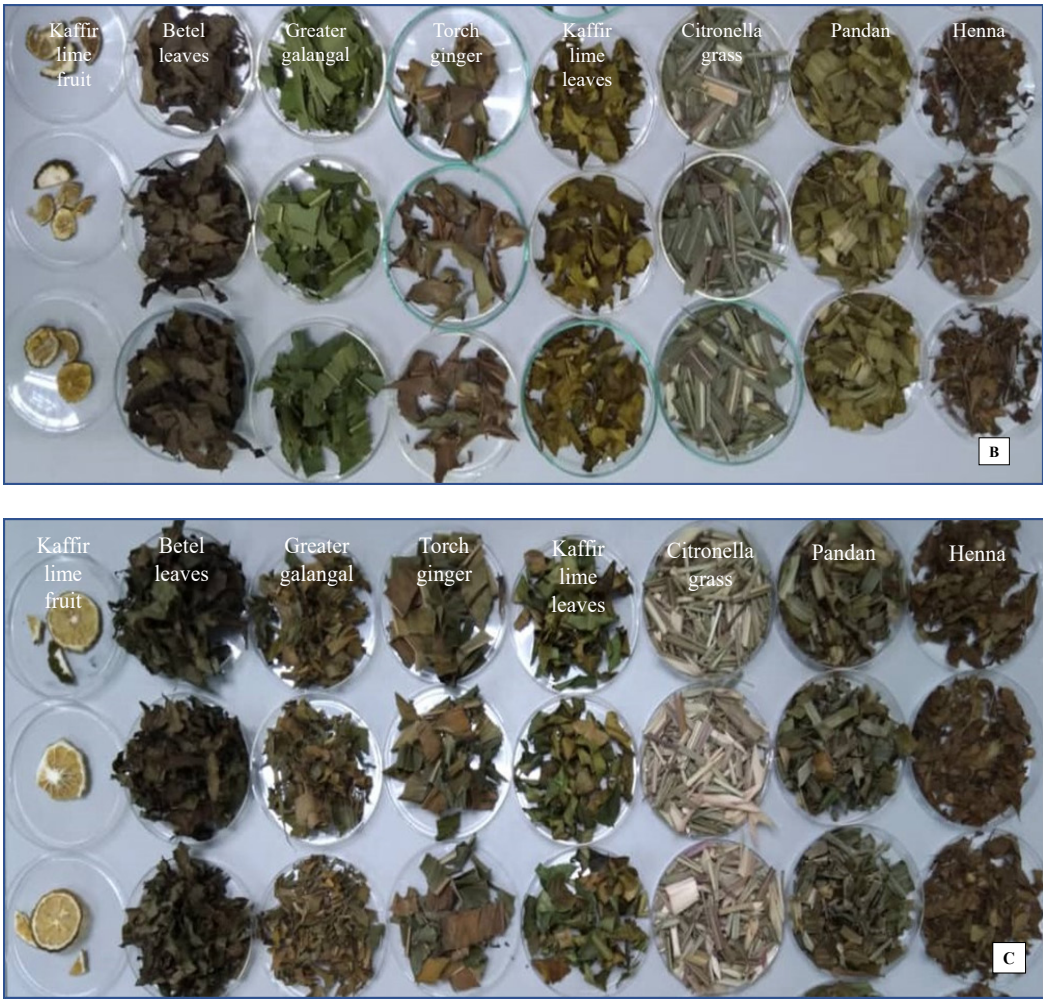

Figure 1. (Continue)

Table 1
Herbs' colour and scent changes before and after the drying process

No.	Analysis	Colour changes			Scent changes	
					Percentage (%) from the original smell of fresh leaves maintained	
	Drying process/ Herbal bath herbs	Fresh	OD	SD	OD	SD
1.	Kaffir lime fruit	5Y, 9/6	5Y, 7/10	5Y, 5/12	70–80	70
2.	Betel leaves	6GY,5/8	5YR, 1/4	5Y, 1/2		0

Table 1 *(Continue)*

No.	Analysis	Colour changes			Scent changes	
					Percentage (%) from the original smell of fresh leaves maintained	
	Drying process/ Herbal bath herbs	Fresh	OD	SD	OD	SD
3.	Greater galangal	6GY, 6/10	5GY, 6/10	5Y, 5/4		60
4.	Torch ginger	6GY, 7/10	5Y, 4/10	5Y, 3/6		60
5.	Kaffir lime leaves	6GY, 7/8	6GY,9/4	5GY, 3/8		60
6.	Citronella grass	6GY, 8/8	5GY, 7/6	5GY, 9/2		70
7.	Pandan	6GY, 8/12	5Y, 7/8	5GY, 6/4		30
8.	Henna leaves	6GY, 6/8	5YR, 4/8	5YR, 2/2		10

Note. Herbs colour analysis using Munsell Colour Chart for hue 5 yellow (5Y), 5 yellow-red (5YR), 5 green-yellow (5GY), and leaf hue 6 green-yellow (6GY), and scent analysis due to the effect of oven-dried (OD) and sun-dried (SD) methods

The colours of all dried herb leaves were compared using the Munsell Colour Chart for the hues 5Y, 5YR, 5GY, and leaf hue 6GY. The dried herbs were compared to the fresh herb leaves for the fragrance analysis, where they underwent slight to significant odour changes. Table 1 displays the colour data for fresh and dried HB herbs in terms of hue values and percentages of aroma before and after drying. According to this study, OD herbs retained their bright colour and had a strong, mild aromatic fragrance compared to SD herbs, which were dull and pale and had a less fragrant aroma. All dry herbs' leaves smelled, and compared to fresh herbs' leaves, the OD herbs had 70–80% of the scent of fresh herbs' leaves. Meanwhile, SD herbs also had a slightly smoky scent besides possessing 10–70% of the aromatic fragrance.

The aroma of herbs results from the chemical content found in EO. The higher the chemical content, the stronger the aroma of the resulting EO. The lack of EO aroma is caused by damage to the secretory cells, epidermal cells, or glandular trichomes that store the Eo in the plant (Thamkaew et al., 2020). Therefore, the result indicates that SD causes a higher damage effect than the OD method. Telfser and Galindo (2019) further claim that drying at low temperatures (40–60°C) is preferable for preserving volatile chemicals and effectively prevents the loss of scent. Thus, in their study, OD has been recommended as the optimum dehumidification and dehydration method for dry aromatic plant species by Özgüven et al. (2019).

Effect of Drying Method on the EO, EW, and BW of HB Herbs' Content

Hydro distillation is used in this study to extract EO, EW, and BW from HB herbs. Boiling HB herbs in a pot is a typical practice that produces EO and EW, but all these extracts are combined in BW. Therefore, boiling the HB herbs with most of the pot's top covered is recommended to trap the hot water vapour and ensure that it releases little heat into the atmosphere but returns to the BW instead. Figure 2 shows the colour of fresh and dried EO, EW, and BW of HB herbs obtained from the hydro-distillation extraction process. EO was yellowish, colourless for EW, and reddish for BW. The drying procedures did not produce significant colour changes when extracting HB herbs. The OD or SD methods did not significantly reduce the HB herb's colour changes in extraction compared to fresh leaves.

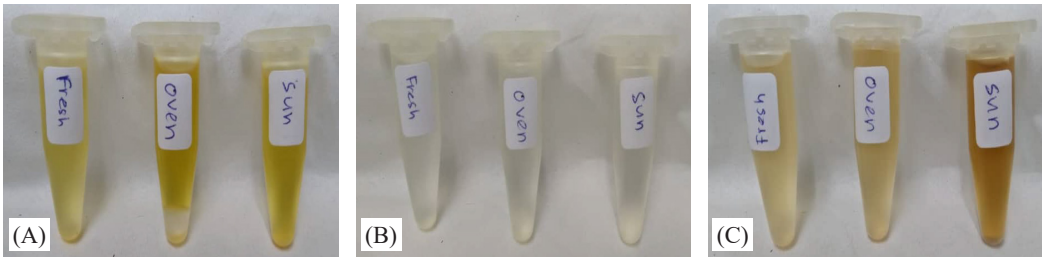

Figure 2. The extraction process of different types of drying of herbal bath herbs using the hydro distillation method produces (A) essential oil, (B) essential water, and (C) boiling water

The extraction was compared using the Munsell Colour Chart for hue 5Y. For the scent analysis, the HB herbs' extractions were compared to the fresh, where they went through slight to very significant odour changes. Table 2 compares the colours and scents of the extracted HB herbs. In contrast to SD herbs, which were darker, the EO and BW colours for fresh and OD herbs were the same. BW's reddish colour might be the result of the effects of henna and betel leaf. For all EW, there were no changes in colour. The BW possessed a strong aromatic fragrance of betel leaves, henna leaves, and a small amount of citronella grass. However, the EO and EW for all drying techniques possessed a mild aromatic, smell-like mixture of citronella grass and kaffir lime fruit. Although the EO and BW of SD herbs were darker, they had a scent similar to fresh and OD herbs but with different strengths.

OD herbs extraction possessed 80% scent strength, while SD herbs only had 50% scent strength from extracting fresh herbs. The drying treatments caused significantly different scents to strengthen for HB herbs' extraction. The OD and SD methods caused a slighter to a greater reduction in the HB herb's extraction of scent strength compared to fresh leaves. Table 3 shows the EO yields produced from the extraction of HB herbs using different drying methods.

Table 2
Comparison of herbal bath (HB) herbs extraction colour and scent

No.	Analysis	Colour changes			Scent changes		
					Percentage (%) of scent strength from fresh HB extract		
	HB extraction/ Drying process	EO	EW	BW	EO	EW	BW
1.	Fresh	5Y, 9/14	0	5YR, 8/6	100	100	100
2.	OD	5Y, 9/14	0	5YR, 8/6	80	80	80
3.	SD	5Y, 7/14	0	5YR, 6/10	50	50	50

Note. Essential oil (EO), essential water (EW), and boiling water (BW) colour analysis using Munsell Colour Chart for hue 5 yellow (5Y) and 5 yellow-red (5YR) and scent analysis due to the effect of oved dried and sun-dried methods, respectively

According to the result, the drying methods significantly affected EO yields. Fresh herbs produced a higher EO yield than OD and SD HB herbs. The lower production of EO yield was from the SD HB herbs. The yield of EO from the fresh and dried herbs was in the order of fresh herbs oil 88% (v/w) > OD herbs oil 63% (v/w) > SD HB herbs oil 392% (v/w).

Table 3
Effects of different drying methods on essential oil's yield from the herbal bath herbs' extract

Drying method	Yield (%)
Fresh (control)	88%[a]
OD	63%[b]
SD	39%[c]

Note. Essential oil yields from fresh, oven-dried (OD), and sun-dried (SD) herbal bath herbs because of drying methods. Means with the same letters are not significantly different at $p>0.05$ using the least significant difference

More oil content was present from fresh and dried HB herbs that could be extracted, but these compounds were lost or transformed during the processing of hydro distillation and evaporation (Aziz, 2015). So, to minimise EO losses and preserve the distinctive components, drying should be done immediately after harvest. Additionally, post-harvest activities, as well as agricultural practices, have an impact on oil content (Özgüven et al., 2019). The conclusion that the OD technique can boost the concentration of EO isolated by hydro distillation agrees with most prior investigations on various plants.

Additionally, according to Özgüven et al. (2019), different drying techniques result in varying concentrations of certain aromatic herbs, while Mashkani et al. (2018) found that the kind of tissue temperature duration and drying technique typically influenced variations in EO content after drying. Additionally, even if a plant is of the same species, its chemical composition varies due to internal and external factors such as the kind of media used for planting, the type and rate of fertilisation used, the timing of crop harvest, as well as topography and environmental conditions (Aziz, 2015; Tunjung et al., 2015).

In contrast to the OD approach, which employs hot air flows and merely dries the leaf surface, the SD method directly penetrates heat into the leaf cells, and prolonged exposure to it may damage nearly all plant cell walls (Thamkaew et al., 2020). The findings show that the lack of EO in SD herbs results from strong heat factors (>50°C) breaking the membrane cells of oil sacs in herbs. It suggests that, compared to the OD method, the SD method had a more significant impact on HB herbs.

Effect of Drying Method on the EO, EW, and BW of HB Herbs' Chemical Contents

The chemical contents of the plant can be determined by using EO, EW, and BW after they undergo determination techniques such as column chromatography, high-performance liquid chromatography, ultrafiltration, and many other techniques. In this study, the GC-MS technique was used to determine the chemical contents of HB herbs. The use of EO, also known as volatile oils, extracted or derived from most plant parts, including flowers, leaves, buds, fruits, twigs, bark, seeds, wood, and roots (Solórzano-Santos & Miranda-Novales, 2012) is quite common in aromatherapy (Jaradat et al., 2018). According to Patel and Patel (2016), phytochemicals are bioactive chemical compounds used as antimicrobial, antibacterial, and anticancer agents. Herbs are traditionally used for food or medicinal purposes and are known to be phytochemically rich (Guldiken et al., 2018).

The GC-MS analysis of the EO, EW, and BW in this study reveals 42 numbers (Table 4), 22 numbers (Table 5), and 12 numbers (Table 6) of identified compounds, respectively, by comparing their mass spectra with the National Institute of Standards and Technology (NIST) 2005 library of mass spectra and their retention (R) times. So, few unidentified components were present that either no mass spectra could be obtained or the spectrum was too weak to be interpreted. The drying process significantly affected EO compounds. The total oil of the EO chemical components (99.44%, 99.36%, and 99.15%) was isolated from fresh, OD, and SD plants (Table 4). Only 34 and 35 of the 42 identified chemical compounds from new extraction were discovered in OD and SD herbal leaves, respectively. Drying OD and SD herbs produced 8 and 7 newly discovered chemical compounds, respectively.

The highest compound percentage of HB's EO produced was 2,6-octadienal, 3,7-dimethyl-(E), another name is α-citral (fresh: 26.08%, OD: 35.79%, and SD: 24.32%), while the lowest percentage was contributed by tau-cardinal (fresh: 0.19%, OD: 0.31%, and SD: 0.14%). The fresh, OD, and SD HBs had different chemical compositions; while some compounds may be high in OD or SD plants, others have a high proportion in fresh herbs. For instance, OD and SD herbs have higher percentages of naphthalene,1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-[2R-(2alpha., 4a. alpha)] (9.86% and 7.91%, respectively) than fresh herbs

(3.01%). Additionally, certain substances, including 1,4-cyclohexadiene and 1-methyl-4-(1-methylethyl), remained the same in composition regardless of the drying process.

According to the results obtained, the five chemical contents that recorded the highest content composition in fresh HB herbs can be considered the main active ingredients of HB products, considering that the sample is a combination of seven types of herbs used as HB herbs. There were 2,6-octadienal,3,7-dimethyl-(E) (26.08%), copaene (14.52%), 1,6,10-dodecatrien-3-ol,3,7,11-trimethyl (7.71%), 2,6-Octadien-1-ol,3,7-dimethyl-acetate(E) (6.52%), and 1-naphthalenol,1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl) (5.48%). The most significant reduction in the composition of the main chemical content after the drying process was in 1,6,10-dodecatrien-3-ol,3,7,11-trimethyl, where only 0.91% and 0.74% were produced after undergoing the OD and SD methods, respectively.

Table 4
Percentage composition of the identified components in essential oil of herbal bath analysed by gas chromatography-mass spectroscopy

No.	Component	R. time[a]	Area (%)[b] / Types of drying		
			Fresh	Oven	Sun
1	β-pinene	6.300	0.74	0.68	0.49
2	Bicyclo[3.1.0]hexane,4-methylene-1-(1-methylethyl)-	6.857	0.36		0.27
3	B-myrcene	8.477	0.14	0.08	
4	D-Limonene	9.348	0.85	0.32	0.54
5	Eucalyptol	9.572		0.23	0.15
6	1,4-Cyclohexadiene,1-methyl-4-(1-methylethyl)	10.843	0.15	0.15	0.15
7	4-Nonanone	13.559	0.30	0.56	0.36
8	5-Hepten-2-one,6-methyl-	13.941		0.10	0.23
9	2H-Pyran,tetrahydro-4-methyl-2-(2-methyl-1-propenyl)	14.205			0.19
10	2-Furanmethanol,5-ethenyltetrahydro-.alpha.,.alpha.,5-trimethyl-,cis	16.751	0.53	0.52	0.37
11	Cyclohexanol,5-methyl-2-(1-methylethenyl)-	17.308		0.16	
12	2-Furanmethanol,5-ethenyltetrahydro-.alpha.,.alpha.,5-trimethyl-,trans	17.510	0.28	0.31	0.17

Table 4 *(Continue)*

No.	Compound	R. time[a]	Area (%)[b] / Types of drying		
			Fresh	Oven	Sun
13	Copaene	17.872	14.52	9.45	21.39
14	6-Octenal,3,7-dimethyl-,(R)-	18.250	0.30		
15	1-Cyclohexene-1-acetaldehyde, .alpha., 2-dimethyl	18.396	0.22		
16	Bicyclo[3.1.1]heptan-3-one,2,6,6-trimethyl-	19.215		0.14	
17	1,6-Octadien-3-ol,3,7-dimethyl	19.619	3.27	2.68	2.56
18	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1.alpha.,2.beta.,5.beta.)	19.738	0.85	0.63	1.37
19	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-,cis-	19.883			0.13
20	Cyclohexanol,5-methyl-2-(1-methylethenyl)-, [1R-(1.alpha.,2.beta.,5.alpha.)]-	20.018	1.27	1.15	1.09
21	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene	20.387	2.49	1.77	1.26
22	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R(1R*,4Z,9S*)]	20.579	0.17		0.38
23	Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-,[1S (1.alpha.,4. alpha.,7.alpha.)]-	20.798	2.08	3.23	3.33
24	1H-Cyclopenta[1,3]cyclopropa[1,2] benzene,octahydro-7-methyl-3-methylene-4-(1-methylethyl)-,[3aS	20.983		0.08	
25	Naphthalene,1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	22.058	0.51	0.26	0.36
26	Naphthalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,(1S-cis)	22.157		0.25	0.22
27	α-Caryophyllene	22.251	1.55	1.97	2.18
28	1,6,10-Dodecatriene,7,11-dimethyl-3-methylene-	22.554	0.36	0.12	
29	2,6-Octadienal,3,7-dimethyl-,(Z)	22.771	4.21	5.55	8.06
30	1,6-Cyclodecadiene,1-methyl-5-methylene-8-(1-methylethyl)-,[s-(E,E)]	23.054	1.83	2.32	1.96
31	Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-	23.404	0.41	0.21	
32	γ-elemene	23.552	0.25	0.25	0.18

Table 4 *(Continue)*

No.	Compound	R.time[a]	Area (%)[b] / Types of drying		
			Fresh	Oven	Sun
33	2,6-Octadienal, 3,7-dimethyl-,(E)	23.956	5.93	7.68	11.03
34	α-farnesene	24.147	1.37	1.88	1.20
35	2,6-Octadien-1-ol, 3,7-dimethyl-,acetate,(E)	24.431	6.52	2.62	1.76
36	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-,[2R-(2.alpha.,4a.alpha.,	24.674	3.01	9.86	7.91
37	2,6-Octadienal,3,7-dimethyl-,(Z)	25.369	0.16	0.36	0.22
38	2,6-Octadienal,3,7-dimethyl-,(E)	26.571	26.08	35.79	24.32
39	1-Dodecanol	28.559	1.21		
40	Caryophyllene oxide	28.909	0.87	0.97	0.55
41	1,6,10-Dodecatrien-3-ol,3,7,11-trimethyl	30.238	7.71	0.91	0.74
42	Cyclohexanemethanol,4-ethenyl-.alpha.,.alpha.,4-trimethyl-3-(1-methylethenyl)-,[1R-(1.alpha.,3.alpha	30.939	0.13	0.72	0.67
43	2-Naphthalenemethanol,decahydro-.alpha.,.alpha.,4a-trimethyl-8-methylene-,[2R-2.alpha.,4a.alpha.,8	31.408	0.19	0.29	0.75
44	1-Naphthalenol,decahydro-1,4a-dimethyl-7-(1-methylethylidene)-,[1R-(1.alpha.,4a.beta.,8a.alpha.)]-	31.755		0.18	0.25
45	tau.-Cadinol	32.555	0.19	0.31	0.14
46	2-Naphthalenemethanol,1,2,3,4,4a,5,6,7-octahydro-.alpha.,.alpha.,4a,8-tetramethyl-,(2R-cis)	32.663	0.35	0.68	0.35
47	1-Naphthalenol,1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)	33.156	5.48	3.29	1.26
48	α-cadinol	33.495	0.65	0.49	
49	trans-.alpha.-Bergamotene	35.730	0.60		0.11
50	2,6,10-Dodecatrien-1-ol,3,7,11-trimethyl,acetate,(E,E)-	36.192		0.16	0.10
51	5-(1-Bromo-1-methyl-ethyl)-2-methyl-cyclohexanol	36.516	0.15		0.24
52	Phytol	40.259	1.20		0.16
	Total (%) of identification of compound		99.44	99.36	99.15
	No. of components		42	42	42

Note. [a] Retention time; [b] Percentage of oil (%)

Table 5 shows the percentage composition of the identified components in EW of HB analysed by GC-MS. The chemical compounds of EW extracted from fresh, OD and SD herbs made up 99.71%, 96.87%, and 88.06% of the total essential water, respectively. Out of the 18 numbers of identified chemical compounds produced from fresh extraction, only 10 and 12 numbers of identified compounds were found in OD and SD herbs, respectively. The drying process produced another 4 and 3 new numbers of identified chemical compounds from OD and SD herbs, respectively. The drying process significantly affected on EWs compounds.

The highest compound percentage of HB's EW produced was phenol, 2-methoxy-3-(2-propenyl)- (33.30%) for fresh, 2-furanmethanol,5-ethenyltetrahydro-alpha, alpha,5-trimethyl-, trans (37.81%) for OD, and cyclohexanol,2-(2-hydroxy-2-propyl)-5-methyl- (31.36%) for SD. Meanwhile, the lowest compound percentage found in fresh (0.21%) and SD herbs (0.55%) was contributed by 5-hepten-2-one,6-methyl, and for OD herbs (0.36%) was phenol,2-methoxy-4-(2-propenyl)-acetate. The compound composition among the fresh, OD and SD of HB's EW was varied, and there was no obvious pattern like EO.

According to the results obtained, the three chemical contents that recorded the highest content composition in fresh HB herbs can be considered as the main active ingredients of HB products, considering that the sample is a combination of seven types of herbs used as HB herbs and there were: phenol, 2-methoxy-3-(2-propenyl)- (33.30%), trans-geraniol (12.31%), and 2-furanmethanol, 5-ethenyltetrahydro-.alpha.,.alpha.,5-trimethyl-, trans (9.87%). After the drying process, the most significant reduction in the composition of the main chemical content was 1,6,10-dodecatrien-3-ol, 3,7,11-trimethyl, where only 5.34% and 3.71% are produced after undergoing the OD and SD methods, respectively. For most of the chemical contents contained in fresh EW, it was found that there were no significant changes after undergoing the SD method; on the other hand, the significant changes were with the OD method.

Table 5
Percentage composition of the identified components in essential water of herbal bath analysed by gas chromatography-mass spectroscopy

No.	Component	R. time[a]	Area (%)[b] / Types of drying		
			Fresh	Oven	Sun
1	5-Hepten-2-one,6-methyl	13.758	0.21		0.55
2	2-Furanmethanol,5-ethenyltetrahydro-.alpha.,.alpha.,5-trimethyl-,trans	16.732	9.87	37.81	11.29
3	Acetic acid	17.363	8.10		12.65
4	β-linalool	19.968	0.88	2.52	0.56

Table 5 *(Continue)*

No	Component	R. time [a]	Area (%)[b] / Types of drying		
			Fresh	Oven	Sun
5	Cyclohexanol,5-methyl-2-(1-methylethenyl)-,[1R-(1.alpha.,2.beta.,5.alpha.)]	20.219	0.35	2.77	0.79
6	3-Cyclohexen-1-ol,4-methyl-1-(1-methylethyl)((-)-4-Terpineol)	21.126	1.10	5.79	2.10
7	2,6-Octadienal,3,7-dimethyl-	23.133	2.43		
8	3-Cyclohexene-1-methanol,.alpha.,.alpha.,4-trimethyl-,	23.275	1.15	3.68	2.00
9	Cyclohexene,3-acetoxy-4-(1-hydroxy-1-methylethyl)-1-methyl-	23.942	2.72		
10	β-citronellol	24.813	0.39	1.82	1.20
11	trans-Geraniol	26.519	12.31	19.08	12.46
12	Cyclohexanol,2-(2-hydroxy-2-propyl)-5-methyl-	31.458	9.17	11.17	31.36
13	Phenol,2-methoxy-4-(2-propenyl),acetate	32.726	8.25	0.36	1.71
14	Phenol,2-methoxy-3-(2-propenyl)-	33.188	33.30	5.34	3.71
15	Phenol,4-(2-propenyl)-	35.773	3.49		
16	5-(1-Bromo-1-methyl-ethyl)-2-methyl-cyclohexanol	36.058	0.86		
17	1,5,9-Decatriene,2,3,5,8-tetramethyl	36.843	3.59		
18	4-Allyl-1,2-diacetoxybenzene	38.718	1.54		
19	Eucalyptol			0.43	
20	α-citral			2.65	2.87
21	β-Citral			2.63	3.20
22	Furfural			0.82	1.61
	Total (%) of identification of compound		99.71	96.87	88.06
	No. of components		18	14	15

Note. [a] Retention time; [b] Percentage of water (%)

Table 6 shows the percentage composition of the identified components in BW of HB analysed by GC-MS. The chemical compounds of BW extracted from fresh, OD, and SD herbs made up 99.15%, 99.99%, and 99.38% of the total boiled water, respectively. The drying process significantly affected BW compounds. Out of the 12 identified chemical compounds produced from fresh extraction, only 7 and 6 identified compounds were found in OD and SD herbal leaves, respectively. The

drying process produced another identified chemical compound from SD herbs.

The highest compound percentage produced was acetic acid (fresh: 86.74%, OD: 92.52%, and SD: 93.25%), while the lowest percentage was contributed by 2-butanone, 3-hydroxy (fresh: 0.3%, OD: 0.39%, and SD: 0.41%). It was found that there were no significant changes in the chemical content composition in fresh BW after undergoing the OD and SD methods. The chemical content found in fresh herbs significantly reduces content, almost half after drying by the OD and SD methods. It indicates that the exposure of herb leaves to thermal factors significantly affects some chemical contents that cause EOs decomposition and destruction of storage cells.

According to the results, only one chemical content recorded the highest composition in BW. It can be considered the main active ingredient of HB products because of the dominance of the chemical content over other substances. Therefore, nine chemical contents that can be considered active ingredients of HB products were 2,6-octadienal, 3,7-dimethyl(E), copaene, 1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl, 2,6-Octadien-1-ol, 3,7-dimethyl-, acetate,(E),1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl), phenol, 2-methoxy-3-(2-propenyl)-, trans-Geraniol, 2-Furanmethanol, 5-ethenyltetrahydro-. alpha., alpha.,5-trimethyl-, trans and acetic acid.

Table 6
Percentage composition of the identified components in boiling waters of herbal bath analysed by gas chromatography-mass spectroscopy

No.	Component	R. time[a]	Area (%)[b] / Types of drying		
			Fresh	Oven	Sun
1	2-Butanone,3-hydroxy	12.908	0.30	0.39	0.41
2	2-Propanone,1-hydroxy	13.390	3.25	1.80	
3	Acetic acid	17.229	86.74	92.52	93.25
4	2(5H)-Furanone, 3-methyl	23.998	0.46		
5	Cyclohexanol, 2-(2-hydroxy-2-propyl)-5-methyl	31.538	2.76	3.49	1.26
6	2-Hydroxy-gamma-butyrolactone	32.995	0.62	0.58	0.66
7	Phenol, 2-methoxy-3-(2-propenyl)	33.282	0.83	0.43	
8	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	34.584	0.39	0.78	
9	Phenol, 4-(2-propenyl)	35.785	0.69		
10	Benzofuran, 2,3-dihydro	36.817	1.35		0.63
11	4-Allyl-1,2-diacetoxybenzene	38.693	1.76		
12	Acetol				3.17

Table 6 *(Continue)*

No.	Component	R. time[a]	Area (%)[b] / Types of drying		
			Fresh	Oven	Sun
	Total (%) of identification of compound		99.15	99.99	99.38
	No. of components		11	7	6

Note. [a]Retention time; [b]Percentage of water (%)

Table 7 shows the chemical class distribution in EO, EW, and BW of fresh, OD, and SD HB herbs. The chemical class distribution of EO revealed the dominance of oxygenated monoterpenes for drying methods (fresh: 43.1%, OD: 55.3%, and SD: 49.89%). Next was the sesquiterpene hydrocarbons class, while the lowest percentage of EO was the monoterpene hydrocarbons, fresh 2.24%, OD 1.23%, and SD 1.45%. The compound 2,6-octadienal,3,7-dimethyl-(E) was the substance that contributed the highest percentage in the oxygenated monoterpenes class, while for sesquiterpenes, hydrocarbons were copaene.

The chemical class distribution of EW revealed the dominance of oxygenated monoterpenes for drying methods (fresh: 81.53%, OD: 91.17%, and SD: 68.85%), and the second highest chemical class was the monoterpene hydrocarbons (fresh: 3.98%, OD: 4.88%, and SD: 4.40%). Unfortunately, no percentage of compound composition was found in the sesquiterpene hydrocarbons class. Substances that contributed the highest percentage in the oxygenated monoterpenes class varied for each drying method.

The chemical class distribution of BW revealed the dominance of the other groups compared to the terpene group for all types of drying (fresh: 92.06%, OD: 95.29%, and SD: 97.49%). Other group constituents were substances from terpene esters, terpene aldehydes, terpene alcohols, aliphatic acids, and ketone. Next were in oxygenated monoterpene (fresh 5.33%, OD: 4.7%, and SD: 1.89%). No percentage of compound composition was found in the monoterpene hydrocarbons and sesquiterpene hydrocarbons class.

The drying process did not significantly affect the chemical class distribution in EO, EW, and BW of fresh, OD, and SD HB herbs. After the HB herbs were dried, the chemical content quantity and type were not significantly different. The only aspect of the chemical content that changed significantly after drying was its composition. Drying processes significantly decreased monoterpene hydrocarbons and oxygenated sesquiterpenes while increasing the content of oxygenated monoterpenes and sesquiterpene hydrocarbons.

According to the results, the HB herb combinations contain chemical compounds, mostly from the terpene group. Terpenes are aromatic compounds found in many plants. Thus, the herbs are suitable to become HB because one of the functions of having HB is to get therapy from the herbs' aroma or "aromatherapy". This therapy relaxes the

Table 7
The chemical class distribution in essential oil (EO), essential water (EW), and boiling water (BW) of fresh, oven-dried, and sun-dried herbal bath herbs

Constituent	EO						EW						BW					
	Types of drying																	
	NC	Fresh	NC	Oven	NC	Sun	NC	Fresh	NC	Oven	NC	Sun	NC	Fresh	NC	Oven	NC	Sun
		% area		% area		% area		% area		% area		% area		% area		% area		% area
Terpenes																		
Monoterpene hydrocarbons	5	2.24	4	1.23	4	1.45	4	3.98	3	4.88	2	4.40	-	-	-	-	-	-
Oxygenated monoterpenes	9	43.10	11	55.30	11	49.89	12	81.53	10	91.17	10	68.85	4	5.33	3	4.70	2	1.89
Sesquiterpene hydrocarbons	13	29.15	13	31.65	12	40.48	-	-	-	-	-	-	-	-	-	-	-	-
Oxygenated sesquiterpene	8	15.57	9	7.84	8	4.71	1	1.54	-	-	-	-	1	1.76	-	-	-	-
Other	7	9.38	5	3.34	7	2.62	5	12.66	1	0.82	3	14.81	6	92.06	4	95.29	4	97.49
Total	42	99.44	42	99.36	42	99.15	22	99.71	14	96.87	15	88.06	11	99.15	7	99.99	6	99.38

Note. NC = Number of compounds of herbal bath herbs analysed by gas chromatography-mass spectroscopy

body, mind, and soul. Inhaling aromatic herbs can also significantly increase sleep quality, and massage can improve mood and relax people (Edris, 2007).

In this study, important chemical compounds from single herbs such as β-pinene, D-limonene, copaene, and 6-octenal, 3,7-dimethyl-(R) from kaffir lime (Warsito et al., 2017), β-pinene, caryophyllene oxide, β-farnesene, 1,1-dodecanediol diacetate, 1-dodecanol, dodecanoic acid, myrcene, camphene from torch ginger (Juwita et al., 2018), α-farnesene, α-bergamotene, β-pinene, and 1,6,10-dodecatriene,7,11-dimethyl-3-methylene from greater galangal (Subramanian & Nishan, 2015), and linalool, geraniol, myrcene, 2,6-octadienal, 3,7-dimethyl-(E), D-limonene from citronella grass (Muttalib et al., 2018) were still detectable even though the herbs went through an extraction process in combination form.

It indicates that drying and combinations did not significantly affect chemical compounds' important and highest content. The drying process also causes many chemical compound losses. However, the most important and highest content of volatile compounds and the highest intensity of most aroma attributes were still found (Nöfer et al., 2018). Ashafa et al. (2008) reported that different drying methods have no significant effect on Felicia muricata leaves' quality and chemical composition.

CONCLUSION

The proper method and technique for drying fresh herbal leaves and fruit are critical to maintaining their medical value and benefiting all parties. Our result revealed that an oven is the best method to dry herb species because the herbs are bright and attractive and possess mild to strong aromatic fragrances. It is recommended that HB herbs be produced using the OD method because the product is uniform, and the production process is hygienic and continuous (weather-independent). However, this study did not consider the cost of drying the herbs because it is important to retain the chemical compounds as much as possible during the dried herbal preparation. Other than that, from this study, it was observed that the hydro distillation method could be used to extract the herbal bath herbs and provide an aromatic essential oil with a better yield. Further study of combining many herbs in one product is required to determine the effect on human health, especially for those who consume it directly in the body. Therefore, the emphasis is on the antimicrobial activities of the combination herb product extraction.

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Microsporum canis and *Sporothrix schenckii*: Fungi Causing Skin Infections in Cats

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ABSTRACT

Companion animals such as cats help to reduce stress among people as they delight their owners in their ways. Good management and hygiene practices of pets help in keeping them in a healthy condition. Nevertheless, since fungal infection develops rapidly, there is a high tendency for them to get infected. The paucity of data regarding skin mycoses among cats in Malaysia leads to this study. Private veterinary clinics from the Central Region of Peninsular Malaysia were approached for participation in this study. Sampling was conducted for one year, collecting hair plucked, skin scrapings, and swabs from lesions of the cats with skin problems and inoculating onto Sabouraud Dextrose Agar media. Diagnosing the fungal colony was conducted through a direct examination method using lactophenol cotton blue stain and molecular identification of the isolates using polymerase chain reaction targeting the fungi species' internal transcribed spacer region and β-tubulin gene. Of the 127 cats, 93 were positively infected, mainly with *Microsporum canis* (n = 38) and *Sporothrix schenckii* (n = 26). Saprophytic fungi detected on cats were *Alternaria* sp., *Aspergillus* sp., *Candida* sp., *Chaetomium* sp., *Chrysosporium* sp., *Curvularia* sp., *Fusarium* sp., *Geotrichum* sp., *Penicillium* sp., *Talaromyces* sp., *Trichoderma* sp., *Trichosporon* sp., and *Xylaria* sp. This finding represents the number of cats infected with fungal dermatitis in Selangor, Negeri Sembilan, the Federal Territory of Kuala Lumpur, and Putrajaya.

Keywords: Cat, Central Region of Peninsular Malaysia, *Microsporum canis*, skin problems, *Sporothrix schenckii*

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INTRODUCTION

Cats are among the favourable companion animals due to their characteristic that a human easily attracts. Despite bringing joy to humans, the health management of pets should be taken seriously, as some animal diseases are zoonotic and can be transmitted to humans. The development of fungal infections among companion animals has greatly increased globally after bacterial and viral infections, which are known to be the major factors that trigger animal diseases (Seyedmousavi et al., 2018).

Malaysia is a small country divided into six main regions: the Northern Region, the Central Region, the Southern Region, Sabah, and Sarawak. The Central Region is known to be the nation's hub. It comprises Selangor, Negeri Sembilan, the Federal Territories of Kuala Lumpur (the capital of Malaysia), and Putrajaya (the administrative and judicial capital) (Lim, 2002). A survey conducted in Putrajaya revealed that 47% of the households kept animals as their pets, with most respondents (72%) from Precincts 9 and 5 having cats (Debra et al., 2019).

In Malaysia, sporotrichosis was detected by the Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) on five adult male crossbred cats that had ulcerated wounds mostly on their forelimbs, cheek, and nose because of fighting between them (Zamri-Saad et al., 1990). As sporotrichosis is zoonotic, four students who treated the infected cats were also infected with the same infection through the cats' bites and scratches (Zamri-Saad et al., 1990). The sporotrichosis among cats in Malaysia is reported to be caused by cats' fighting and contact with a contaminated environment (Kano et al., 2015). From 1990 to 2010, only 12 cases of feline sporotrichosis were reported, and the number has increased recently; the University Veterinary Hospital at UPM recorded 80 cases in a year related to feline sporotrichosis in Selangor from 2008 to 2012 (Azam et al., 2019).

Nevertheless, aside from sporotrichosis, few fungal infections in cats were reported in Malaysia. Since Malaysia is a tropical climate country, the environment is suitable for fungal growth. Thus, this study aims to indicate fungal infection among pet cats in the Central Region of Peninsular Malaysia. Perhaps this study will help the community and animal handlers gain knowledge and exposure regarding skin mycoses in companion animals, specifically cats.

MATERIAL AND METHODS

Sample Collection

The participating veterinary clinics around the Central Region of Peninsular Malaysia were provided with owners' consent forms, research location consent forms and questionnaires about their cats' management. The veterinary clinics also provided sampling kits comprised of sterile petri dishes, sterile swabs with Amies transport media and sterile scalpel blades for specimen collecting purposes. The clinicians sampled only cats with skin lesions suspected of skin fungal diseases such as ulcerated wounds, erythema, papules, alopecia, scaling, and their hair and skin crusting. No asymptomatic cats were sampled in this study as it is deemed that the spores are ubiquitously present in

the environment and do not cause lesions in healthy animals. Samples (s) collected can be either hair plucked and/or skin scraping for suspected dermatophytosis cases and/or swabs taken from ulcerated wounds for suspected sporotrichosis cases of the cats from the infected area. The sampled cats consisted of both sexes, different types of breeds and age groups. The sampling was held for a year, from November 2018 to November 2019. The total cats sampled were 127 (n), comprised of hair plucked (s = 80) from 55 cats, skin scraping (s = 37) from 29 cats, a swab from the wound (s = 49) from 36 cats, and the remaining seven cats had all the three types of samples collected from the infected area of their body. All the collected samples were then brought to the Veterinary Bacteriology Laboratory, UPM, for a fungal culture procedure.

Sample Inoculation

Sabouraud Dextrose Agar (SDA) media supplemented with 0.5% chloramphenicol (Merck, Germany) and cycloheximide (Merck, Germany) were used to inoculate all the samples. Each sample was inoculated in duplicate onto the SDA media. All the inoculated plates were then incubated at room temperature for 1–4 weeks with daily observation. It is recommended to incubate the plates at 25–27°C in an inverted position to avoid contamination (Moriello et al., 2017). The inoculation of the collected samples showed positive growth of fungal culture from hair plucked from 49 cats, skin scraping (n = 16 cats), swab of wound (n = 22 cats), and other six cats with all the three types of samples taken from the infected area of their body.

Fungal Culture Identification

The fungal cultures were identified through two measures: (1) the macroscopic and (2) the microscopic observations for the genus identification and molecular method by polymerase chain reaction (PCR) to determine the fungal species. The colony's structure, texture, and colour were observed for macroscopic identification before proceeding with microscopic observation using lactophenol cotton blue stain and glass slide. The morphological evaluations of the fungal culture were based on the key taxonomy (Ellis et al., 2007) and the references from the International Society for Human and Animal Mycology (ISHAM) database.

Subcultures were conducted after the species identification to obtain the pure colony of the culture before proceeding to PCR. The DNA of the fungal colonies was extracted using the DNeasy Ultraclean Microbial Kit (QIAGEN, Germany) following the manufacturer's protocol. Two sets of primer sequences used were internal transcribed spacer regions (*ITS1* and *ITS4*) (Ferrer et al., 2001) and *the β-tubulin* gene (*Bt2a* and *Bt2b*) (Gupta et al., 2000). A total of 25 µl per reaction was used with the amplification of PCR at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 60 s, followed by cycle at 72°C for 7 min completed in a thermal cycler (Eppendorf™, Germany). Gel electrophoresis was conducted using 1.5% agarose gel and visualised under UV light transilluminator light cabinet gel documentation. The PCR products were

then sent to the sequencing service, and the gene sequences were blasted in the National Centre of Biotechnology Information (NCBI) to determine the fungal species.

RESULTS

Identification of *M. canis* and *S. schenckii*

For *M. canis*, the colony is flat and spreading; the surface is white to cream coloured with a fluffy or cottony texture on the surface and bright yellow to brownish-yellow pigment on the periphery but sometimes non-pigmented (Figure 1), similar to reverse morphology (Figure 2) (Ellis et al., 2007). The microscopic observation showed septate hyphae with numerous macroconidia in spindle-shaped (5–15 cells) (Figure 3), verrucose thick-walled with a terminal knob. Only a few microconidia were presented in pyriform to clavate shape (Ellis et al., 2007).

For *S. schenckii*, the colony morphology at room temperature (25–27°C) is small and white with no hyphae. Since it is a growth culture, the colony slowly becomes moist after a week, wrinkled (Figures 4 and 5) may produce short aerial hyphae, and the pigmentation varies from white to cream, then darker to brownish or black (Ellis et al., 2007). The microscopic morphology of *S. schenckii* is septate hyphae and branching with many small conidia pear-shaped, where the arrangement sometimes forms a flower or 'rosette-like' (Figure 6) (Ellis et al., 2007). The conidia are elongated, one-celled, and smooth-walled (Ellis et al., 2007).

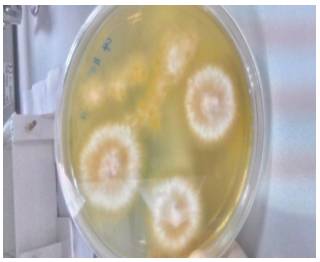

Figure 1. Microsporum canis colony morphology observed on Sabouraud Dextrose Agar media from the sample collected in this study

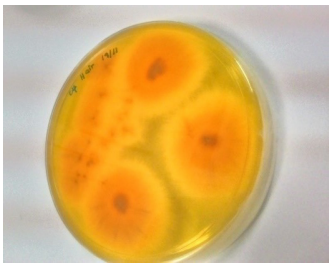

Figure 2. Microsporum canis reverse colony morphology on Sabouraud Dextrose Agar

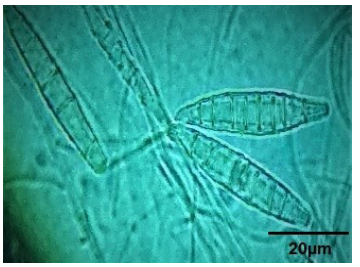

Figure 3. Photomicrograph of macroconidia of *Microsporum canis* stained with lactophenol cotton blue

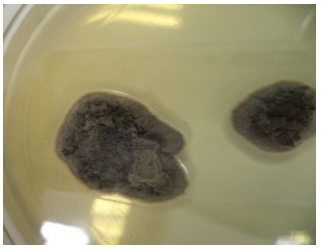

Figure 4. Sporothrix schenckii top colony morphology on Sabouraud Dextrose Agar

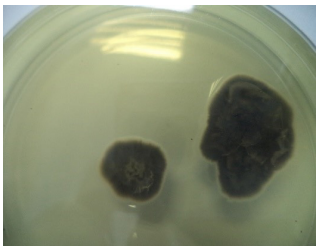

Figure 5. Sporothrix schenckii reverse colony morphology observed on Sabouraud Dextrose Agar media

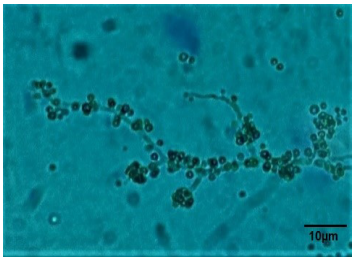

Figure 6. Photomicrograph of *Sporothrix schenckii* stained with lactophenol cotton blue

Fungal Species Identified from Collected Samples

Out of 127 cats, 93 cats were positive for skin fungal infections. Of the 93 infected cats, 35 were solely infected with *M. canis*; one was infected with *M. canis* and *S. schenckii* infections from the samples inoculated, and the remaining two were infected by saprophytic fungi, such as *Penicillium* sp. and *Fusarium* sp. simultaneously with *M. canis*. *Microsporum canis* were highly isolated from the hair-plucked samples (n = 30 cats) and skin scraping (n = 8 cats).

Out of 38 cats infected with *M. canis*, most cats infected were domestic breed (n = 20), followed by British Shorthair (n = 4), British Longhair (n = 1), Persian (n = 3), American Shorthair (n = 1), Balinese (n = 1), Munchkin cross (n = 1), Siamese breed (n = 1), and not stated (n = 6). Although long-haired breed cats, such as Persian cats, have a higher tendency to get infected with ringworms compared to other breeds (Bond, 2010), the condition of the environment also influences the growth of dermatophytes (Chermette et al., 2008). Animals can also carry the fungal spores without having any signs of infection on the animal's body (Chermette et al., 2008). This statement is echoed by Ilhan et al. (2016) and Nichita and Marcu (2010), where some cats were known to be a reservoir for *M. canis* as they did not show any visible lesions, thus becoming transient carrier cats.

However, *S. schenckii* infecting cats (n = 26) were mostly collected from swabs of ulcerated wounds. A total of 21 out of 26 cats were solely infected with *S. schenckii*. In comparison, the remaining five infected cats showed mixed infection with other fungal species, such as *Talaromyces* sp., *Penicillium* sp., *Curvularia* sp., and *Trichosporon* sp. The other remaining samples collected from 29 cats showed *Aspergillus* sp., *Chaetomium* sp., *Chrysosporium* sp., *Curvularia* sp., *Cladosporium* sp., *Fusarium* sp., *Geotrichum* sp., *Penicillium* sp., *Talaromyces* sp., and yeast.

Tables 1 and 2 present the fungal species found from the sampled cats in the Central Region of Peninsular Malaysia, identified using direct examination and molecular diagnosis.

Table 1
A single colony of fungal species from the sampled cats in the Central Region of Peninsular Malaysia

Fungal species	Number of cats (n)	Fungal species	Number of cats (n)
Alternaria sp.	1	*Curvularia* sp.	1
Aspergillus sp.	3	*Fusarium* sp.	1
Candida sp.	2	*Geotrichum* sp.	1
Chaetomium sp.	4	Microsporum canis	35
Chrysosporium sp.	1	*Penicillium* sp.	3
Cladosporium sp.	1	Sporothrix schenckii	21
Cryptococcus sp.	1		

Table 1 *(Continue)*

Fungal species	Number of cats (n)
Trichoderma sp.	1
Xenomyrothecium sp.	1
Xylaria sp.	1
Yeast	2

Note. List of fungal species identified by direct examination method and (n) indicates the number of infected cats in this study

DISCUSSION

Dermatophytosis is a skin disease caused by a superficial fungal infection invading the keratinised tissues of the hosts by zoophilic, geophilic, or anthropophilic fungal organisms, mainly *M. canis*, *Microsporum gypseum*, and *Trichophyton mentagrophytes* (Moriello, 2004; Moriello et al., 2017; Paryuni et al., 2020). The results revealed that *M. canis* is a common fungal species that infect cats easily. A study conducted by Nichita and Marcu (2010) found that *M. canis* is the most prevalent fungal species observed from skin lesions and fur collected from cats (26.7%) and dogs (16.8%). From the sample collected, hair plucked, and skin scraping from the infected cats found many *M. canis* when the samples were cultured on SDA media. The prevalence of dermatophytes in cats is usually higher by 20% compared to dogs (Nichita & Marcu, 2010). This fungal species grows after 4 to 7 days of inoculation. Chermette et al. (2008) stated that ringworm is one of the most occurring skin mycoses among pets and livestock. In addition, the infection is usually characterised by cutaneous lesions on the animals' skin, hair, and nails (Paixão et al., 2001).

Table 2
Mix fungal species on the sampled cats in the Central Region of Peninsular Malaysia

Multiple fungal species infecting the sampled cats	Number of cats (n)
Aspergillus sp. and *Curvularia* sp.	1
Chrysosporium sp. and *Cladosporium* sp.	1
Curvularia sp. and *Cladosporium* sp.	1
Microsporum canis and *Penicillium* sp.	1
Microsporum canis and *Sporothrix schenckii*	1
Microsporum canis, *Fusarium* sp., and *Penicillium* sp.	1
Penicillium sp. and *Aspergillus* sp.	1
Penicillium sp. and *Cladosporium* sp.	1
Sporothrix schenckii and *Curvularia* sp.	1
Sporothrix schenckii and *Penicillium* sp.	1
Sporothrix schenckii and *Talaromyces* sp.	2
Sporothrix schenckii and *Trichosporon* sp.	1

Note. List of fungal species identified by direct examination method and (n) indicates the number of infected cats in this study

Furthermore, this study found that 15 out of 38 infected cats were young cats under one year old. According to Paixão et al. (2001), animals that are below a year old are susceptible to dermatophytoses. This study also observed that the number of male and female cats infected was almost equal. This result is in line with the previous study by Paixão et al. (2001),

which claimed that gender does not influence fungal infection. Other studies stated that hair coat, sex, age, season of sampling, and geographical condition did not show any significant association with the prevalence of dermatophyte (Proverbio et al., 2014). Nevertheless, the transmission of dermatophytes can occur when cats are exposed to the environment and have contact with infected hair or fomites from clippers and brushes (Ilhan et al., 2016). In this study, the number of infected cats that frequently went for grooming was high, 24 out of 38.

Apart from dermatophytosis, *S. schenckii* recorded many fungal infections that caused dermatitis in the sampled cats. Twenty-six (26) cats were positive for *S. schenckii*, where 21 were pure culture, and the remaining four were a combination of *S. schenckii* with other saprophytic fungi. All cats sampled showed ulcerated lesions on their body parts. Siew (2017) stated that feline sporotrichosis has been reported in Malaysia since the 1990s. Thus, since then, he revealed that *S. schenckii sensu stricto* clinical clade D of single strain is Malaysia's most common cause of sporotrichosis (Siew, 2017). Before that, the *S. schenckii* clinical clade C and D were reported to be commonly found in human sporotrichosis in Asia (Kano et al., 2015).

Sporotrichosis is caused by the dimorphic fungus, *S. schenckii*, which can be present in two forms: (1) yeast form when invaded the host body (body temperature state) and (2) mycelial form at environmental temperature, thus capable of infecting dogs and cats and other mammalians species, including horses, camels, cattle, and swine (Lloret et al., 2013). According to Spickler (2017), sporotrichosis is contagious in cats as an abundance of yeasts can be found in skin lesions, and this organism can enter the body. However, there is only a tiny amount of damage or even undamaged on the skin. Cutaneous, lymphocutaneous and disseminated forms were the three clinical forms of sporotrichosis in cats (Lloret et al., 2013; T. M. P. Schubach et al., 2004). Cats suffer sporotrichosis in the cutaneous form usually have multiple, ulcerated and crusted nodules, draining tracts and abscesses or cellulitis on the common areas of bites and scratching during fights, which is the head, limbs and tail-base region (Lloret et al., 2013; Reis et al., 2012). In addition, most cases of cutaneous forms arise from hematogenous spread, while lymphatic form can be seen through histology can be either biopsies or necropsy samples (Crothers et al., 2009; Lloret et al., 2013; Welsh, 2003). Other than that, dissemination may occur via inoculation during grooming, where spores enter through cuts in the skin (Crothers et al., 2009; Leme et al., 2007; Lloret et al., 2013; T. M. P. Schubach et al., 2004). Nevertheless, systemic or disseminated sporotrichosis was rarely observed in humans and animals and was often only associated with host immunodeficiency status (Duangkaew et al., 2019; Rodrigues et al., 2013).

In this study, a cat got a wound after being hit by a car, and four cats had a history of sporotrichosis. Late treatment of a prolonged unhealing wound might also become the factor that causes the spreading

or complication of the infection. The irregularity of treatment and missing the follow-up could delay the recovery process and worsen the condition (Chaves et al., 2013). Several factors contribute to the low percentage of recovery in sporotrichosis among cats, such as irregular administration of medications, difficulty administering oral medication, and increased virulence of the fungal species (da Santos Silva et al., 2018). In addition, the recurrence might occur as some cats did not always respond to the treatment (Gremião et al., 2014). Furthermore, the chances for the reactivation of the lesions are also high despite the end of treatment (Chaves et al., 2013; Gremião et al., 2011, 2014; Pereira et al., 2010). Hirano et al. (2006) stated that medication should be continuously given for one to two months after the cat appeared clinically normal to avoid reappearing and failure of the treatment. Besides, the host's immune response is also important to avoid recurrent infection (Siew et al., 2017).

This study observed that 20 out of 26 cats were male. Based on other studies, diseases in cats, such as sporotrichosis, were reported mostly in free-roaming intact males (Lloret et al., 2013). From the data collected, 21 cats have free access outside their house. It might be the factor that causes the cats to get infected with sporotrichosis, as the fungus species can be found in contaminated environments, such as living and dead vegetation (Kano et al., 2015). In addition, *S. schenckii* can be isolated from skin lesions, claws, and nasal and oral cavities (Lloret et al., 2013; Spickler, 2017). The data in this study revealed that out of 26 infected cats, one got the infection from another cat suffering from sporotrichosis reared in the same house. Based on several studies, cats get the infection through scratches and bites transmitted from infected cats (Kano et al., 2015; Lloret et al., 2013; Siew, 2017; Spickler, 2017). In Malaysia, *S. schenckii* is the main species causing sporotrichosis in cats, while *S. globosa* is extensively reported in other Asian countries as the main cause (Moussa et al., 2017).

The hair of animals is a collector of environmental fungi where indoor pets are easily contaminated by air-borne fungi (Aho, 1983). Nevertheless, a study by Aho (1983) also stated that the spreading of fungal flora indoors or outdoors is not constant and varies sporadically. From this study, 37 sampled cats showed the growth of saprophytic fungi species from the inoculated samples, with 13 of the cats having mixed colonies of fungal species, with some showing a combination with *M. canis* and *S. schenckii*. The genera of the saprobe fungi were *Alternaria* sp., *Aspergillus* sp., *Byssochlamys* sp., *Chaetomium* sp., *Chrysosporium* sp., *Cladosporium* sp., *Curvularia* sp., *Fusarium* sp., *Geotrichum* sp., *Penicillium* sp., *Talaromyces* sp., *Trichoderma* sp., and *Xylaria* sp. This study also observed yeast genera infected five cats: *Candida* sp., *Trichosporon* sp., and two other unidentified yeast.

The genus of *Talaromyces* is introduced as the teleomorph of *Penicillium* sp. (Yilmaz et al., 2014). This genus was reported as endemic in most rural areas in Southeast Asian countries and commonly found in contaminated soil, causing pulmonary

infection in immunocompromised humans and bamboo rats (Kauffman, 2017). Some species within the *Talaromyces* genus, such as *Talaromyces marneffei*, are known to cause pneumonia in a dog from Southern Brazil (Headley et al., 2017), while *Talaromyces helices* cause granulomatous lymphadenitis in the dog (Tomlinson et al., 2011). Other than that, *Aspergillus* sp., *Penicillium* sp., *Alternaria* sp., *Mucor* sp., and *Fusarium* sp. are the common saprobe fungi frequently found isolated on the skin of cats and dogs with dermatophytes infections caused by contact with contaminated soil, air, and plants (Paixão et al., 2001; Stojanov et al., 2009). Besides, *Aspergillus* sp., *Penicillium* sp., and *Talaromyces* sp. were recognised as indoor microbiota as they can be found in dust samples in houses worldwide (Visagie et al., 2014) while *Cladosporium* sp. are common fungi that can be found worldwide, in outdoor and indoor air and frequently encountered as secondary invaders contaminants (Bensch et al., 2018; Sandoval-Denis et al., 2015). *Fusarium* sp. has been reported to cause mycetoma, keratomycosis, and onychomycosis in humans (Kano et al., 2002). In contrast, *Curvularia lunata* causes diseases in plants, animals, and humans, and the severity level varies among patients, thus making it a public health concern (Bengyella et al., 2017).

From this study, the cats with the saprophytic fungi species growing together with dermatophyte and *S. schenckii* from their inoculated samples might be because of the contaminated environment around them, as 78 of the total sampled cats stayed indoors. Other than that, there were two cats infected with *Chrysosporium* sp. Although *Chrysosporium* sp. is often considered a contaminant since it is saprophytic fungi from soil, the clinical symptoms were similar to dermatophytes infection, causing misdiagnosis of the species (Dokuzeylul et al., 2013).

As dermatophytosis and sporotrichosis are zoonotic (Seyedmousavi et al., 2015, 2018), it can easily be transmitted from animals to humans naturally since cats and dogs have a close relationship with human beings as both animals are known to be the most popular among pets. Therefore, zoonotic fungal infections have become a public health concern (Seyedmousavi et al., 2018). A study conducted in Italy stated that cats and dogs should be considered the primary source of pathogenic dermatophytes for humans. However, they do not present any clinical signs of dermatophytosis, where the risk of transmission of *M. canis* to humans is higher in asymptomatic cats compared to asymptomatic dogs (Cafarchia et al., 2006; Mancianti et al., 2003). Besides, the household environment can be contaminated with *M. canis* through either asymptomatic *M. canis* carriers or symptomatic animals, which is risky to human health (Mancianti et al., 2003). Since *M. canis* known as a worldwide zoophilic and zoonotic dermatophyte, these isolates that are commonly in cats and dogs can easily be pathogenic and highly contagious to humans (Müştak et al., 2019; Šubelj et al., 2014). In addition, this zoophilic species lives in close association with animals other than humans, and the

transmission to humans usually occurs through the reservoirs, where dermatophyte fungi can occur either symptomatically or asymptomatically in the fur of animal hosts. It can become an epidemic (de Hoog et al., 2017). Another author also mentioned that *M. canis* from cats, dogs, horses, and all other mammals give high zoonotic risk to humans (Hubka et al., 2018).

Sporotrichosis also has become an important dimorphic fungal infection as it is zoonotic, and humans can acquire it via scratches and bites from infected cats (A. Schubach et al., 2008; Gremião et al., 2017; Siew, 2017; Tang et al., 2012). Although there were only 12 cases of zoonotic sporotrichosis were reported from 1990 to 2010 (Tang et al., 2012; Zamri-Saad et al., 1990), unpublished data by Chan and Selvarajah (2013) recorded that feline sporotrichosis cases showed an increased trend from 2008 to 2012 with 80 feline sporotrichosis cases per year in the state of Selangor. It could be speculated that the increase in feline sporotrichosis cases possibly led to increase human cases from cat scratches and bites (Azam et al., 2019).

Despite the devastating fungal infection, especially seen in sporotrichosis, most fungal contaminants are known to be harmless to healthy humans and animals. However, if the infection becomes invasive in a condition that reduces the body's resistance to fight against infections, it can become pathogenic to the host (Aho, 1983; Paixão et al., 2001). Thus, when involved with saprobe fungi in handling mycoses in cats, it is crucial to differentiate the pathogenic fungi contributing to the infections.

CONCLUSION

Fungal infections can affect cats through direct contact with the contaminated environment or contact with the infected cats. The infected cats should be treated right away after being diagnosed. For future studies, the questionnaire regarding the management of the cats should be more precise so that the risk factors can be analysed. Besides, the sampling area can be widened to measure the prevalence of skin mycoses among cats in Malaysia.

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A Review of Plant Growth Promoting Rhizobacteria and Their Characteristics as Potential Biofertilizer

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ABSTRACT

The growing demand for agricultural products for food requirements caused the use of excessive inorganic chemical fertilisers, insecticides, fungicides, and pesticides for a quick and simple way to maximise and boost crop yield. This practice harmed food safety and caused the degradation of environmental, physical, and biological conditions. It has become alarming, and now is the time for a greener approach to increase agricultural output while minimising the use of inorganic chemical fertilisers. It was proven through many previous studies that using environmentally friendly biofertilisers has managed to increase crop yield while reducing the usage of chemical fertilisers. Plant growth-promoting rhizobacteria (PGPR) are mostly used in biofertiliser production because these types of microbes will enhance plant growth and yield by mobilising the available nutrients through several biological mechanisms, including fixation of atmospheric nitrogen, solubilisation, and mobilisation of phosphate and potassium, phytohormones production, disease suppression, and stress protection. Understanding their characteristics, biological mechanisms of action, and the nutritional and physical requirements for growth is important for successfully formulating and applying PGPR as a biofertiliser. The selection of the right PGPR with the desired characteristics, the ability to adapt to the environment, and the ideal formulation of the biofertiliser are the main criteria that should be emphasised when determining the success of biofertiliser. Knowledge and awareness regarding the

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use, benefits, and production of PGPR as a potential biofertiliser are important and should be explored to fulfil the crop's nutritional requirements more economically and sustainably.

Keywords: Biofertilizer, microbes, nutrient, PGPR, plant, soil

INTRODUCTION

Agriculture is one of the most powerful tools in the country's economic development. Its activities are important in achieving rapid economic growth, poverty reduction, and structural transformation, thus playing an important role in food security to feed the growing population. A tremendous increase in the world population has led to the increase of high demand and production of agricultural products year by year. However, the pandemic, economic instability, and climate variability have threatened agricultural growth and put food security at risk. Unfortunately, in achieving the goal of feeding the expanding population, the use of intensive off-farm inputs such as chemical fertilisers and pesticides to increase crop productivity was also increased. The excessive and indiscriminate use of these chemical inputs for enhancing agricultural production has caused a lot of negative impacts on humans, the environment and biodiversity and risks to food security.

Amidst the current situation, there is a growing awareness of mitigating the agricultural sector and improving agricultural sustainability, which suggests regenerative methods that make the best use of naturally occurring processes and locally available resources. Biofertilizer is an organic fertiliser formulated using beneficial microorganisms such as the plant growth-promoting rhizobacteria, better known as PGPR. This microbial inoculant can be applied to plants and soil to enhance plant growth and yield by mobilising the available nutrients through a biological process. PGPR, through its several mechanisms, such as the synthesis of antibiotics, enzymes, and siderophores, can also be exploited as a successful strategy for protecting plants against the deleterious effects caused by biotic and abiotic stresses (Govindasamy et al., 2008). The application of biofertiliser on seed, plant surfaces, or soil caused the beneficial microorganisms to colonise the plant's rhizosphere or the interior to promote plant growth by increasing the availability of nutrients to the host plant (Fasusi et al., 2021). It also helps to build up the micro-flora biological activity and enhance soil fertility (Fasusi et al., 2021).

The rising awareness of the hazardous effects and increasing cost of chemical fertilisers have given momentum to the use of biofertiliser. Moreover, the production cost of biofertiliser is lower, with tremendous potential as an additional, sustainable, and green source of plant nutrients. Biofertilisers have now become an important component of integrated nutrient management (INM) and integrated plant nutrition systems (IPNS) (Sangeeth & Suseela Bhai, 2015). A wide range of PGPR, either in single species or in combinations, are used to

supply different kinds of nutrients to the soil with different modes of action. It produces a higher yield while being safe for both the environment and people, which promotes more sustainable economic growth for farmers, agriculture, and the nation.

Compared to chemical fertiliser, biofertiliser will ensure constant and sustainable nutrient supplies and prevent nutrient leaching through the microorganism's activities. However, in certain cases, biofertilisers sometimes require longer to show their real effects. This mostly occurs in newly applied areas or problem areas that have long been used for agricultural or other purposes. It also requires frequent application of biofertilisers for the beneficial microbes to dominate a place and be effectively functional due to adaptation factors and competition with other microorganisms in that applied area (A. Sharma & Chetani, 2017). Nevertheless, the right timing and frequent application of biofertiliser can partially substitute, enhance the function, and then subdue the application quantities of chemical fertilisers and still maintain the same yield for the application of cash or other types of crops (Lyu et al., 2023; Mustapha et al., 2017).

Studies have demonstrated that PGPR inoculation on the soil/plant ecosystem can enhance soil health, soil quality, crop development, yield, and quality. PGPR is frequently and widely used in organic farming and natural agriculture since it helps to solve issues related to the usage of chemical pesticides and fertilisers. Biofertilisers have improved and increased the number of beneficial microbes in the soil, thereby promoting a healthy environment for plants. Numerous field and greenhouse trials indicate the benefits of PGPR as a biofertiliser in crop production. The application of PGPR was proven to enhance crop growth and yield, giving crops protection and, at the same time, conserving natural resources for ultimately sustainable agriculture and environmental systems (García-Fraile et al., 2012). The prospects for improved agriculture using PGPR are particularly impressive because they have lower costs, give better yield, and reduce dependence on chemical substances. The role of PGPR as a biofertiliser is an added dimension that, if used properly, can enhance and optimise the best soil and crop management practices.

PLANT GROWTH-PROMOTING RHIZOBACTERIA (PGPR) AND ITS FUNCTIONAL CHARACTERISTICS

Most beneficial or effective microbes (EM) in biofertilisers have a close relationship with plant roots. *Rhizobium* has symbiotic interaction with legume roots, while plant growth-promoting rhizobacteria (PGPR) refers to any beneficial bacteria that colonise the region under the influence of the plant's roots, known as the rhizosphere. These beneficial soil bacteria flourish in the plant's rhizosphere by growing in, on or around plant tissues and stimulate plant growth via direct or indirect means. Numerous species of PGPR have been studied, and among them are strains from genera such as *Bacillus*,

Pseudomonas, *Rhizobium*, *Burkholderia*, and *Enterobacter* (Khandelval et al., 2023).

Generally, PGPR functions in three different ways to enhance plant growth. As stated above, for biofertiliser, PGPR can synthesise compounds for plants, such as hormones and enzymes. They are also responsible for lessening or preventing plants from diseases and facilitating the uptake of certain nutrients from the soil. Plant growth promotion and development by PGPR are carried out by both direct and indirect mechanisms (Figure 1). Symbiotic and non-symbiotic PGPR showed direct plant growth promotion through nitrogen fixation, solubilisation of minerals such as phosphate and potassium, and production of plant hormones have been reported for several bacterial genera (Ashraf et al., 2004). Indirect plant growth promotion includes preventing the deleterious effects of phytopathogenic organisms by producing siderophores, antibiotics, and enzymes (S. B. Sharma et al., 2013).

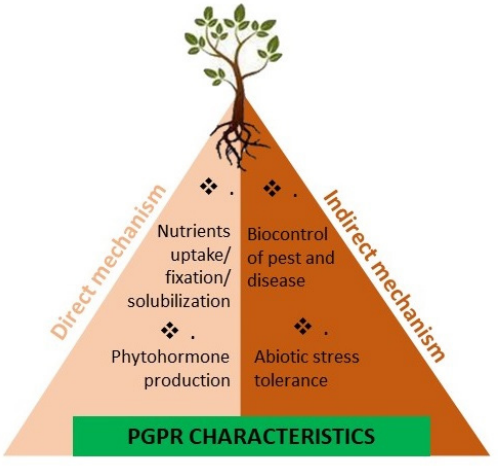

Figure 1. Plant growth-promoting rhizobacteria (PGPR) characteristics as biofertiliser

Biological nitrogen fixation (BNF), phosphate-solubilisation, potassium solubilisation, and phytohormone production are frequently cited as the main mechanisms of PGPR in enhancing crop growth and production. The inoculation of single or multiple strains of PGPR, which have multiple beneficial characteristics, is critical because this technique will reduce the amount of chemical fertiliser inputs while increasing crop growth and production. Thus, using PGPR in biofertiliser production is the current area of interest in developing sustainable agriculture. It is emphasised with the intention of obtaining further cumulative effects from the specific strains in the prepared inoculum without having any negative effects on the environment or plants.

Nitrogen Fixing Bacteria

Nitrogen is the most important and commonly considered one of the foremost restrictive nutrients for plant growth. Nitrogen in the biosphere is available in the form of atmospheric nitrogen (N_2), which cannot be utilised by plants (Mustapha et al., 2018). The natural process of biological nitrogen fixation (BNF) is to make the unavailable form of nitrogen from the atmosphere accessible to plants. The process has been regarded as the main plant growth-promotion effect by soil microorganisms. It involves a specific enzyme called nitrogenase to convert nitrogen to an accessible form of ammonia (NH_3). The BNF process is only mediated in nature by bacteria and certain species of actinomycetes through

symbiotic or non-symbiotic relationships with plants (Soumare et al., 2020). The *Rhizobium*, which has a high degree of host specificity when infecting the roots of leguminous plants, is the best illustration of the symbiotic relationship between nitrogen-fixing microbes and plants. Whereas only a few groups of microorganisms, including free-living bacteria and blue-green algae, can fix nitrogen without symbiotic relationships (Soumare et al., 2020).

The inorganic chemical fertilizer N, such as urea, is widely used by farmers because of its immediate effect in supplying nitrogen to plants. However, many studies have shown that the application and increment of chemical fertilizer N only give a marginal yield increment on plants. Due to the very low only 30% nutrient uptake efficiency by plants, the remaining 70% of the applied fertiliser is typically lost through a variety of processes, including leaching, evaporation, and surface runoff to the natural water supply (Anas et al., 2020). This process will eventually cause the problem of eutrophication and result in the emission of nitrous oxide (N_2O), carbon dioxide (CO_2), and greenhouse gas (GHG) that are harmful to the atmosphere (Kusin et al., 2015). Moreover, the application of chemical fertiliser might lead to a decline in the community of beneficial soil microorganisms and soil fertility (Zainuddin et al., 2022).

Therefore, to be utilised as a biofertiliser on plants, the selection of PGPR with N_2-fixation capability is essential and necessary (Bakar & Othman, 2022). The use of N-fixing PGPR in biofertiliser is significant in reducing the use of synthetic nitrogen fertilisers. It could also increase the nitrogen uptake efficiency of the crops, thus conserving the environment. Biofertilisers with N-fixing bacteria are formulated because of their successful ability to fix free atmospheric nitrogen into the soil and enter the plant roots. The use of N-fixing biofertiliser has been proven effective in reducing the use of chemical fertilisers, thus reducing the harmful effects on soil and environmental health.

Phosphate Solubilising Bacteria

Phosphorus is the second most important macronutrient required by plants after nitrogen. Phosphorus is widely distributed in nature, both in organic and inorganic forms, in a bound state that is not readily available to plants. This element is still one of the major plant-limiting nutrients due to its availability and low solubility in the soil. It mostly remains in insoluble phosphates of iron, aluminium, and calcium in the soil (S. B. Sharma et al., 2013). The main problem with the application of mineral or organic phosphates fertiliser is the fact that a large portion of P-fertilizer is unavailable to plants because it is bound to the soil, creating a pool of residual P, or is lost via leaching, runoff, and/or erosion to the surface water creating eutrophication (Conijn et al., 2018). Thus, the important aspect of increasing soil phosphorus availability is the release of insoluble and fixed forms of phosphorus into the form accessible to plants.

Many PGPR communities known as phosphate solubilising bacteria (PSB) were identified, especially from the genus *Bacillus* and *Pseudomonas* (Illmer & Schinner, 1992; Wani et al., 2007). These groups of microorganisms are capable of hydrolysing organic and inorganic phosphorus compounds from insoluble compounds. The use of PSB can optimise crop production by increasing P uptake by the plant and minimise P losses from soils by various approaches, including lowering the soil pH, chelation, and mineralisation to make phosphorus accessible for plants to absorb (Ismail et al., 2016; Kalayu, 2019). PSB will produce organic acids or releases of protons that lower the soil pH (Kaur, 2019). It was proven in the P-solubilization test that a strong positive correlation had been reported between the solubilisation index and organic acids produced. The hydroxyl and carboxyl groups from the organic and inorganic acids produced by PSB will chelate the cations bound to phosphate, thereby converting them into soluble forms. Production of phosphatases enzyme by PSB will mineralise the soil organic P by hydrolysing organic forms of phosphate compounds, thus releasing inorganic phosphorus that will be immobilised by plants (Kalayu, 2019).

Potassium Solubilising Bacteria

In soils, potassium can be found in four main forms: water-soluble, mineral, exchangeable, and non-exchangeable (Kaur, 2019). These forms are not uniformly distributed throughout soils, but they are all in a state of dynamic equilibrium with one another and are often governed by the physicochemical characteristics of the soil. The readily available K in soil is usually very low, at 1–2% of total K, and exists in soluble and exchangeable forms (Lalitha & Dhakshinamoorthy, 2014). Most soil mineral potassium can be found in silicate minerals, including mica and K-feldspar, even though they make up more than 90 to 98% and are unavailable for direct plant uptake (Goldstein, 1994). Release of non-exchangeable K to the exchangeable form occurred when levels of exchangeable and soluble decreased due to crop uptake or leaching and perhaps by the increase in microbial activity (Sparks, 1999).

The potassium solubilising bacteria (KSB) can make up approximately 1–10% of available soil potassium, which contributes significantly to plant uptake (Memon et al., 1988). A few mechanisms involved in the potassium solubilising process by KSB include the secretion of organic acids and inorganic acids and polysaccharides, acidolysis, complexolysis, chelation, and exchange responses (Meena et al., 2015). Since there is abundant insoluble K in the soil, converting them into a form of K that plants can absorb may be more economically feasible. Studies have shown that a variety of KSBs can cause soluble K to be released from K-bearing minerals such as mica, illite, and K-feldspar by producing organic acid that will dissolve rock and chelate silicon ions to release K ions into the soil, which could uptake by plants (Zhang & Khong, 2014). PGPR such as *Bacillus mucilaginosus*, *Bacillus edaphicus*, and

Bacillus circulans have been explained as effective K solubilisers, while other PGPR such as *Burkholderia*, *Acidithiobacillus ferrooxidans*, and *Enterobacter hormaechei* have been described to effectively solubilise the silicate rocks to produce an available K for plant uptake (Etesami et al., 2017; Meena et al., 2015).

Potassium is usually added as an inorganic fertiliser source to optimise crop yield. However, intensive application of inorganic fertiliser has several negative impacts on the environment as not all fertilisers will be absorbed by plants. One possible alternative could be to exploit the reservoir of K in the soil fully. The use of K-solubilizing microbes to increase the concentration of available K ions in the soil may mitigate K deficiency. Thus, the potassium solubilisation ability of PGPR is one of the crucial characteristics that promote plant growth and development. The application of KSB as a biofertiliser could support sustainable crop production by improving agriculture development by reducing the use of inorganic chemical fertilisers or other agrochemicals.

Phytohormone Production

Plant cells typically communicate using chemical signals secreted from the sending cell and released to the neighbouring cells. The plant growth and development process is majorly impacted by the availability and communication of transporting mineral nutrients, hormones, and other secreting metabolites in the plant cells. In this case, PGPR has various characteristics and functions in influencing plant growth and development, including the production of plant growth regulators, also known as phytohormones, such as auxin, gibberellin, cytokinin, salicylic acid, and ethylene. Almost all communication in plant cells is brought by plant hormones produced by plant cells or by rhizobacteria (Maheshwari et al., 2015). The synergistic effect of hormone secretion is one of the main criteria of PGPR as the attraction to engage with the plant cells.

The most prevalent auxin phytohormone, indole acetic acid (IAA), is produced in the shoot apical meristem of plants and can be found across the body of the plant. IAA production was believed to be one of the bacterial colonisation strategies on plants other than phytostimulation of the basal plant defence mechanisms (Spaepan et al., 2009). IAA secretion by soil microorganisms was believed to be an important factor for plant growth and development. It encourages the growth of more and longer root hairs, increasing the surface area of the roots for better water and nutrient absorption (Vessey, 2003). Furthermore, optimal root growth boosts root vitality, safeguarding the plant, particularly from soil-borne pests and disease infections (Vessey, 2003). Many PGPRs, such as *Bacillus*, *Acetobacter*, and *Herbaspirillum*, are isolated from various rhizosphere crops that can produce IAA. It was also reported that IAA production by PGPR has significantly promoted rooting and growth in many crops such as rice, wheat, maise, kiwifruit, and oil palm (Biswas et al., 2000; Erturk et al., 2010; Om et al., 2009; Spaepan et al., 2009).

Plants that receive PGPR treatment frequently develop persistent, broad-spectrum systemic resistance to a variety of phytopathogenic bacteria and fungi. This situation develops through an induced resistance mechanism response via two forms, including induced systemic resistance (ISR) and systemic acquired resistance (SAR) (Heil & Bostock, 2002). Certain PGPR that affect plant cells will produce salicylic acid as their exogenous metabolite that can induce the resistance mechanism response in plants (Pieterse et al., 2014). The induction of ISR and SAR is generally associated with salicylic acid signalling and the production of volatile organic compounds such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which decreases the plant ethylene levels, thus inhibiting the functioning of several phytopathogens (del Carmen Orozco-Mosqueda et al., 2023). The ACC deaminase will regulate the endogenous production of ethylene by PGPR, which is also helpful in sustaining plant growth and development under stress conditions (Shaharoona et al., 2011). Salicylic acid is the plant growth regulatory phenolic phytohormone that also serves as an intermediate precursor in pyochelin siderophores biosynthesis (Ankenbauer & Cox, 1988). According to Baldwin et al. (1997), salicylic acid application to plants has been found to inhibit the synthesis of jasmonic acid as an ISR response against pathogens' infection. In addition to the involvement of salicylic acid in SAR, this hormone is involved in the mitigation of various plant biotic and abiotic stresses, including both high and low temperatures, high levels of salt and toxic organic chemicals (del Carmen Orozco-Mosqueda et al., 2023).

Plant growth and performance are significantly influenced by the soil bacteria's synthesis of phytohormones. Different types of PGPR produce different levels of phytohormone, and one type may produce more than one type of phytohormone. PGPR, such as *Bacillus amyloliquefaciens*, was proven to produce gibberellins, auxin, and salicylic acids (Miljaković et al., 2020; Shahzad et al., 2016). Since then, it has also been noted in other bacterial species, including *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae* (Bastián et al., 1998), and *Bacillus* spp. (Gutiérrez-Mañero et al., 2001). Gibberelic acid (GA) was initially described in *Rhizobium meliloti* (Atzorn et al., 1988). GA causes early flowering and budding, breaks seed dormancy, and delays plant senescence. Naturally occurring cytokinins, such as zeatin and adenine, have specific functions in cell division, leaf growth, and the induction of seed germination (Mok, 1994). Different bacteria from the genera *Proteus*, *Klebsiella*, *Bacillllus*, and *Pseudomonas* have been reported to have the ability to produce cytokinins.

Siderophores Production

Iron (Fe) is an essential nutrient for soil microorganism's metabolism. It is contradictory to plant that needs only a trace amount of iron. The availability of iron in the soil is always limited because of the low

iron concentration and very low solubility of the ferric ion (Fe^{3+}) (Siddiqui, 2005). Iron in the soil builds up in typical mineral phases such as iron oxides and hydroxides, the minerals that give the soil its red and yellow hues, and these minerals cannot be readily used by organisms. Soil microorganisms release siderophores to scavenge iron from its solid phases, resulting in soluble iron (Fe^{3+}) that plants can absorb.

Under conditions of low iron stress, some bacteria and fungi produce siderophores, which are ferric-ion-specific chelating agents (Ngamau et al., 2014) with a molecular weight of below 1000 Da. Studies have shown that one crucial mechanism for biological control is the siderophore-mediated competition for iron between PGPR and soil-borne pathogens. Most plants can obtain iron from the soil via bacterial iron siderophore complexes. The implications of this condition have reduced phytopathogens' ability to compete for root colonisation (Ren et al., 2005).

Pseudomonas aeruginosa FP6 is the siderophores producer that was isolated from the rhizospheric soil and was found to significantly reduce the growth of *Rhizoctonia solani* and *Colletotrichum gloeosporiodes* that cause diseases in chilli (Sasirekha & Srividya, 2016). Siderophores production by *Chryseobacterium* C138 has significantly increased iron, chlorophyll content, and yield of the iron-starved tomato plants, indicating that siderophores are effective in providing iron to the plant (Radzki et al., 2013). A study found that the production of the siderophore by *Pantoea* sp. strain (EA106) has increased the ability of roots to absorb iron and promotes the development of a more oxidative environment in the rice rhizosphere (Lakshmanan et al., 2015). The inoculation with siderophores-producing microbes can change the levels of both arsenic and iron in rice, indicating that the bacterial strain may potentially improve rice quality by lowering the buildup of toxic arsenic species in the plant's aerial parts.

NUTRITIONAL REQUIREMENTS FOR PGPR GROWTH

It is well known that environmental factors can impact how bacteria adapt, proliferate, and produce secondary metabolites. Two requirements for microbial growth are the nutritional and physical factors that vary greatly between species (Cappucino & Sherman, 2004). The formulation and production of biofertilisers, as well as the effective growth of microorganisms in the laboratory, depend on an understanding of these requirements. Moreover, bacterial fermentation must compete favourably with chemical synthesis in the biofertiliser market. It is essential since many potential microbiological uses that have been considered for developing biofertilisers depend on whether they can be generated economically. This is because the fermentation medium can reduce the cost of microbial fermentation by up to 30%, which is critical in the commercial industry (Hofvendahl & Hahn-Hagerdal, 2000). Complex media commonly employed for bacterial growth in the laboratory are unsuitable for commercial production and are not economically attractive due to their

high amount of expensive nutrients such as yeast extract, salts, and peptone (Batish et al., 1990).

All bacteria require certain basic nutrients for life sustenance, and the requirements vary greatly among species. Nutritional needs are supplied through a variety of media that have various essential nutrients for bacterial growth, such as carbon, nitrogen, metals and non-metals elements, vitamins, and water (Cappucino & Sherman, 2004). Many bacteria can be grown in laboratories in the nutrient medium, which are designed to provide all the essential nutrients needed by bacteria for their growth. It is one of the several non-selective media useful in the routine cultivation of microorganisms. Nutrient agar/broth is a general-purpose nutrient medium supporting the growth of a wide range of non-fastidious organisms. This medium contains many nutrients needed for bacterial growth and can grow various species of bacteria and fungi.

Before being used in the industry, microbes were typically cultivated in a nutritional medium of the necessary quantity. Bacterial cultivation uses a variety of carbon sources, including glucose, fructose, and lactose. However, using such pure or mixed media on an industrial scale would be quite expensive (Michailides et al., 2015). Industrial applications of microbes need to use a more economical carbon source. Thus, molasses is an important agro-industrial by-product containing high sugar (48-50%) (Quan et al., 2005). It can be utilised as a more affordable source of nutrients for microbial development compared to other biological or chemical mediums in the market. According to Curtin (1983), the chemical composition of molasses was almost similar and seemed to be a standard one. It was then proven by Sutigoolabud et al. (2004) that the composition of molasses produced in Thailand and Okinawa was almost similar, with high concentrations of total sugars and reduced sugars, as shown in Table 1.

Molasses is the basic raw material used for a lot of microbiological processes (Quan et al., 2005). The dark brown thick syrup remained as the residue of inverted sugar crystallisation. Molasses is one of the other organic materials used as carbon and nitrogen sources for bacterial growth. Due to its many benefits, molasses is preferred as a medium for microbial growth over chemical substances. It has higher biodegradability and is effective at extreme temperatures or pH values, and most importantly, it has low toxicity (Rodrigues et al., 2006). High values of caramelised and inverted sugar in high concentrations of molasses could usually cause cell toxicities (Baei et al., 2009). Usually, less than 10% of molasses is used in a bacterial fermentation medium, and the percentage depends on the purpose of the fermentation. Therefore, the precise amount of molasses to be utilised as a medium for bacterial growth must be measured accordingly to achieve optimum bacterial growth.

There are many types of molasses, but the one that has gained much attention is sugar cane molasses. This molasses has been reported to be used as the growth

medium in many fermentation processes of several bacteria and other microorganisms. The by-product of producing sucrose from sugarcane, which comprises more than 46% of inverted total sugar, is cane molasses (Curtin, 1983), which has a high concentration of total sugar (38.8%), which is made up of glucose (3.8%), fructose (7.9%), sucrose (27.7%), and reducing sugar (23.5%) (Aslan et al., 1997). Molasses has been shown to have other additional minerals other than the sources of carbon and nitrogen, including manganese, iron, calcium, potassium, magnesium, succinic acid, malic acid, citric acid, vitamin B6, and selenium (Aslan et al., 1997; El-Enshasy et al., 2008; Sutigoolabud et al., 2004).

Table 1
The chemical and physical properties of molasses, as adopted from Sutigoolabud et al. (2004)

	Molasses	
Component	Produced in Thai	Produced in Okinawa
Brix (%) (1:100)	5.1 ± 0.0	1.2 ± 0.0
Moisture (%)	24.6 ± 1.3	21.6 ± 1.3
Ash (%)	9.5 ± 1.3	16.8 ± 0.0
Total sugar (%)	38.8 ± 2.9	35.3 ± 1.6
Reducing sugar (%)	23.5 ± 1.9	24.9 ± 4.7
Glucose (%)	3.8 ± 2.2	4.1 ± 1.1
Fructose (%)	7.9 ± 1.9	10.9 ± 3.8
Sucrose (%)	27.7 ± 4.4	24.4 ± 4.6
Citric acid (mg/kg)	1 179 ± 81	1 002 ± 462
Malic acid (mg/kg)	410 ± 90	603 ± 32
Succinic acid (mg/kg)	2 134 ± 60	3 218 ± 179

Note. All the values are mean of triplicate analysis on a wet weight basis

ENVIRONMENTAL FACTORS ON PGPR GROWTH

As biofertiliser, the microbial inoculants will be introduced to the soil, seeds, or plant itself. The introduced bacteria must adjust to the soil environment upon inoculation to achieve successful and effective colonisation and be vigorous enough to compete with local microorganisms. Bacterial growth and survival depend directly on several environmental factors, and the requirements differ among species (Figure 2). These specialised requirements show how bacteria have adapted to their surroundings. Environmental factors such as pH, temperature, available water, nutrient level, oxygen levels, and competition with other microbes and toxins could influence bacterial growth rate and activity. Bacteria have optimal growth conditions under which they flourish. However, the stress can result in reduced or stalled growth outside their required condition and environment. Some PGPR, such as *Bacillus* species,

may be dormant by the formation of spores to protect themselves. In more serious conditions, morphological changes could happen in the cell, or the emergence of resistance to the same stress factor or other types of stress factors or even death (Jones & Lennon, 2010; Święciło & Zych-Wężyk, 2013).

PGPR has various mechanisms, including the production of antibiotics, enzymes, metabolites, and scavenging of nutrients to protect themselves from biotic and abiotic stress. Other than protecting themselves, these mechanisms could also influence different physiological activities and induce systemic resistance, thus protecting plants from the biotic stress caused by other pathogenic infections and abiotic environmental stress factors (Shameer & Prasad, 2018). Studies on the effect of pH, temperature and salinity on bacterial growth and extracellular polymeric substances (EPS) and extracellular enzymes have been reported for various strains of microorganisms. A study found that the growth of *Rhizobium meliloti* was increased when the 10% molasses medium pH was increased from 6 to 8 at a constant temperature of 28°C (Singh et al., 2011). They added that at pH 7 in a 10% molasses medium, the growth of *R. meliloti* growth was higher in high temperatures (28–30°C) compared to lower temperatures (26–27°C), and the optimum temperature for the highest bacterial growth was at 28°C.

Other than the effects on bacterial growth, environmental conditions could also affect bacterial functions. The pH, temperature, nitrogen source, carbon source, organic acid, and iron concentration influence the production of siderophores by the *Bacillus* sp. strain VITVK5 and *Enterobacter* sp. strain VITVK6 isolated from the iron-enriched soil sample (Kumar et al., 2017). In other cases, the cultural conditions such as pH and temperature and media components, for example, carbon and nitrogen source as well as tryptophan concentration, have effects on IAA production of *Bacillus* and *Lactobacillus* species isolated from the rhizosphere soil of banana, cotton, and maise (Mohite, 2013).

Knowledge and understanding of the factors affecting microbial growth are very important in predictive microbiology approaches to recognise the level of bacterial response and its efficacy when used in different soil conditions. As in crop production, the use of chemical fertiliser will increase soil salinity. However, a combination of biofertiliser and chemical fertiliser has been reported to increase various crops' yield and quality. Thus, a comprehensive study needs to be done to determine the type of PGPR used as the inoculant in biofertiliser production. The ability of a microorganism to survive and react in other extreme environmental conditions, such as in too low or high salinity, high pH, or high temperature, is also an important characteristic to be emphasised.

CONCLUSION AND FUTURE PERSPECTIVES

The demands for agricultural products and the supply of foods had caused robust

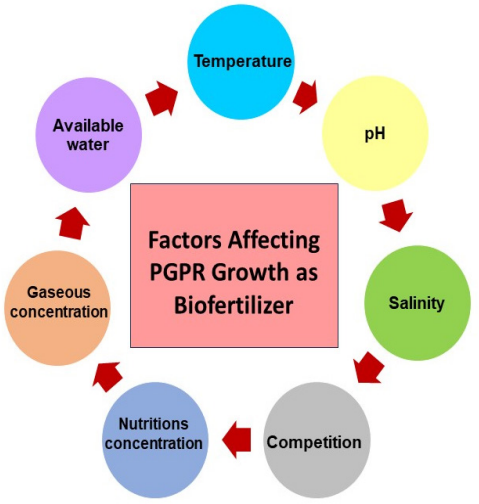

Figure 2. Environmental factors affecting plant growth-promoting rhizobacteria (PGPR) growth as biofertiliser

development in agriculture. This situation caused the use of many chemical fertilisers, which are expensive and harmful to the environment, soil health, and food safety. The use of biofertilisers is highly recommended and considered as an alternative to solve this chemical fertiliser issue. Biofertilizer is the organic fertiliser prepared by living microbial cells such as PGPR to activate the various natural processes and enhance plant growth and yield through various mechanisms. Biofertilisers are a promising tool for crop production and agricultural ecosystems as a supplementary, renewable, and eco-friendly source of plant nutrients. The application of biofertilisers was hoped to be a key element in maintaining crop productivity and soil fertility at a sufficiently high level and vital to achieving sustainable agricultural goals.

The changing approach to more sustainable agricultural practices makes biofertilisers a crucial part of crop production in this century. A number of rhizosphere microorganisms, especially PGPR, were identified to exert multifunction plant growth-promoting activities. The selection of the right PGPR with the desired characteristics and ability to adapt to the environment, as well as the ideal formulation of the biofertiliser, is the main criteria that should be emphasised and very important in determining the success of biofertiliser. Some PGPR is root-inhibiting, while others are free-living diazotrophs in soil and are believed to develop mechanisms for survival in the competitive soil environment. Survival PGPR are protected from competition with other soil microbes and adverse soil environmental conditions by producing secondary metabolites, scavenging nutrients, and becoming root endophytes.

Several research projects conducted worldwide have demonstrated the PGPR's contributions as a biofertiliser to improve agricultural productivity and quality and preserve soil and environmental health. Despite demonstrating their potential, biofertilisers are not widely used to replace chemical fertilisers. Therefore, there is an indispensable need to encourage individuals, farmers, and industry participants to explore the use of PGPR as biofertilisers to achieve the goal of higher agricultural sustainability. Farmers and the authorities need to jointly play a role in making this plan a success and should be educated and aware of the use and advantages of biofertilisers. Farmers should have easy access to biofertilisers, and government officials should start offering

rigorous training and capacity building for agricultural or industrial workers regarding biofertiliser use, production, maintenance, and quality control. In addition, it would be very significant if the authorities could provide subsidies on biofertilisers to farmers and encourage them to accelerate the use of biofertilisers for the time being.

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The Effect of UV-B And UV-C Radiation on Contamination Rate and Shoot Proliferation of Tamban Pineapple Crown Explants (*Ananas comosus* L. Merr.)

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ABSTRACT

This study examines the effect of ultraviolet-B (UV-B) and ultraviolet-C (UV-C) radiation on contamination rate and shoot proliferation of Tamban pineapple crown explant. The experimental design was nested and completely randomized with a separate control. The first factor was the type of UV light, namely UV-B and UV-C. The second factor was the duration of UV light exposure, namely 10, 20, 30, and 40 min. This study was carried out from March to June 2023 at the Plant Tissue Culture Laboratory, Faculty of Agriculture, Lambung Mangkurat University, South Kalimantan, Indonesia. Observations were made on the contamination percentage, survival percentage, time of first shoot formation, percentage of explants able to regenerate shoots, and number of shoots. The results showed that UV light treatment decreased the contamination rate. Increasing the duration of UV light exposure decreased the contamination rate, delayed the formation of the first shoot, and affected the number of shoots. UV-B light exposure produced a higher number of shoots than UV-C light. These results suggest that UV-B and UV-C radiation have the potential to optimize surface sterilization protocol and promote somaclonal variation.

Keywords: Fruit, plant tissue culture, radiation, somaclonal variation

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INTRODUCTION

Tamban pineapple is a local superior variety of pineapple from Barito Kuala Regency, South Kalimantan, Indonesia. It has the advantage of being quite tolerant of low soil acidity under 4.0. Besides that, it is known to tolerate high levels of iron (Fe) and aluminum (Al) (Balai Penelitian Pertanian Lahan Rawa [Balittra], n.d.). The pineapple

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is shade tolerant and is usually planted as an intercrop in oil palm, coconut, and rubber plantations (Cahyana & Destina, 2013).

According to the Badan Pusat Statistik (BPS) (2024), the three largest pineapple-producing regions in Indonesia in 2021 were Lampung (705,883 tons), South Sumatera (476,074 tons), and Riau (354,878 tons). Meanwhile, pineapple production in South Kalimantan in the same year was only 13,788 tons. It showed that the production of Tamban variety pineapples in South Kalimantan is still quite far from pineapple production in other regions. Additionally, the quality of Tamban pineapples (shelf life and vitamin C content) is still lower than other pineapple varieties, such as the MD2 variety, which is one of the superior and popular pineapple varieties on the international market. Thus, both the quantity and quality of Tamban pineapples need to be improved.

Ultraviolet (UV) radiation is a component of non-ionizing radiation in the electromagnetic spectrum, where 8-9% of total solar radiation is UV radiation (Hollósy, 2002). Rai and Agrawal (2017) mentioned that UV radiation is divided into three based on its wavelength range, namely UV-A (315-400 nm), UV-B (280-315 nm), and UV-C (200-280 nm). UV-A radiation has been observed to have a smaller effect than UV-B radiation on the morphology of *Ocimum basilicum* (Qian et al., 2023). Similarly, Sarghein et al. (2011) reported that red pepper (*Capsicum longum* A.DC.) was more sensitive to UV-C radiation than UV-A radiation. Furthermore, UV-A radiation has been proven to have an indirect impact on DNA because it is not easily absorbed by DNA (Mohamed et al., 2016). However, both UV-C and UV-B have sufficient energy to destroy chemical bonds, causing photochemical reactions, which are the main cause of the biological effects on plants (Kovács & Keresztes, 2002).

Several authors have reported the effects of UV-B and UV-C radiation on various plants. Metwally et al. (2019) reported that *Spathiphyllum cannifolium* plantlets treated with 45 min of UV-B radiation had the highest values on growth parameters, such as shootlet length, number of shoots, root length, and survival rate (%) when compared to other treatments (0, 15, and 30 min). In addition, Sadeghianfar et al. (2019) revealed that exposure to 12 hr UV-C light caused a significant increase in the germination rate and radicle length of maize (*Zea maize* L.) seeds. Meanwhile, UV-C light exposure on Persian violet shoots in vitro with low intensity (30 $\mu W/cm^2$) for 4 hours showed the maximum increase in the number of roots, root length, plantlet height, and number of shoots compared to other treatments (including control group) (Phanomchai et al., 2021).

According to several previous research, UV-B and UV-C radiation affected plants' morphological changes. Therefore, this research was conducted to investigate (a) the difference between the effect of control and UV light exposure on the morphological development of Tamban pineapple crown explants, (b) the effect of UV-B and UV-C light on the morphological development of Tamban pineapple crown explants, and (c) the effect of the duration of UV light

exposure nested in a type of UV light on the morphological development of Tamban pineapple crown explants.

MATERIALS AND METHODS

Plant Materials and Sterilization of the Explant Surface

Crowns of Tamban pineapples were obtained from a single source in Tamban Village, Mekarsari District, Barito Kuala Regency, South Kalimantan, Indonesia. The crown leaves were removed, and the crowns were washed under running tap water. The crowns were sterilized with 0.3% bactericide solution (Agrept®, Indonesia) for 15 min and 0.5% fungicide solution (Bendas, Indonesia) for 30 min. The crowns were then rinsed with sterile distilled water. Next, the crowns were transferred to a laminar airflow cabinet. The explants were later soaked in 70% alcohol (OneMed, Indonesia) for 1 min and 0.1% mercuric chloride ($HgCl_2$) solution (Merck, Germany) with several drops of Tween 20 (Merck, Germany) for 5 min. The crowns were later rinsed three times in sterile distilled water. The sterilized crown explants were split into two parts and then treated with UV light. The explants were then cultured on Murashige and Skoog (MS) media with 2 mg/L 6-benzylaminopurine (BAP, Glentham Life Sciences, United Kingdom).

Experimental Design and Statistical Analysis

This study used a nested, completely randomized design with a separate control. The treatment factor consisted of two factors. The first factor was the type of UV light, which consisted of 2 levels: UV-B light and UV-C light. Artificial UV-B light comes from 2 UV-B lamps (each with a power of 18 W), while UV-C light comes from a 30 W UV-C lamp. The distance between the lamp and the explants is ± 20 cm. The second factor was the duration of UV light exposure, which consisted of 4 levels, namely 10, 20, 30, and 40 min. The nested arrangement is in the form of the time of UV light exposure nested in the type of UV light. Besides that, the control group was non-treated crown explants.

Data were collected and expressed as the mean of the cultures of three replicates, each containing five explants. The effect of the control and UV light treatment was analyzed using orthogonal comparison. Meanwhile, the effect of the type of UV light and the duration of UV exposure were analyzed using variance analysis.

RESULTS AND DISCUSSION

Effect of UV-B and UV-C Exposure on the Percentage of Contamination and Survival Explants

Based on Table 1, the recorded contamination rate of the control group was 33.33% at 4 WAP (weeks after planting) and 60.00% at 8 WAP. Furthermore, the UV light treatment resulted in a significantly reduced contamination rate of 7.50% at 4 WAP and 38.33% at 8 WAP. The same pattern was observed in the sterilization protocol of bear's garlic (*Allium ursinum*) explants,

where the combination of UV-C exposure for 40 min and sodium hypochlorite solution (ACE, United Kingdom) for 10 min showed a high sterilization efficiency with a contamination rate of 10% (Tomaszewska-Sowa et al., 2015). Apart from that, Sriana et al. (2022) found that no significant difference was observed between UV light exposure and various sterilization agents on the contamination rate of Talas banana (*Musa paradisiaca* L. var. *sapientum*) corm explants. They concluded that sterilization of Talas banana corm explants could be carried out using only UV light exposure treatment. Overall, the reduction of contamination rate due to UV light exposure indicates UV light potential to enhance the surface sterilization protocol.

Takada et al. (2017) found a bactericidal effect from UV-B light exposure, with a decrease in the percentage of living bacteria as the duration of UV-B light exposure increased. Abdelrahman et al. (2018) reported that UV-C light exposure for 118 J/cm^2 was sufficient to decrease the fungal contamination of *Penicilliun commune* and *Chaetomium globosum*. Other studies reported that 15 to 60 min of UV-C light exposure is effective for fungal decontamination of *Aspergillus niger* and *Aspergillus flavus* (Jhahan et al., 2022).

Table 1
Mean contamination rate (%) and mean survival rate (%) of Tamban pineapple crown explants after control and ultraviolet (UV) light treatment

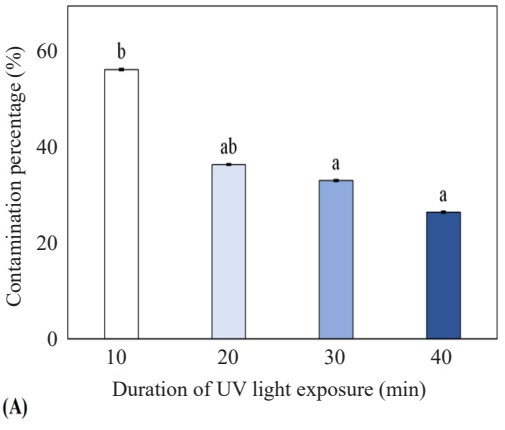
Treatment	Mean percentage of contamination (%)		Mean percentage of survival (%)*
	4 WAP	8 WAP	
Control	33.33b	60.00b	100.00
UV light	7.50a	38.33a	100.00

Note. Means followed by the same letter(s) within each column are not significantly different ($p \leq 0.05$) using orthogonal comparison; WAP = Weeks after planting; * = Data were collected after 8 weeks

The mechanism of the germicidal effect of UV light is related to the absorption of UV light by the nucleic acid components of a microorganism (Gurzadyan et al., 1995). As a result, damage to a microorganism's DNA occurs due to the dimerization of thymine molecules, which subsequently produces cyclobutane pyrimidine dimers (CPDs). The production of these CPDs makes it difficult for nucleic acids to replicate and causes cell death. Even when replication occurs, defects frequently prevent the microorganism from surviving (Dai et al., 2012).

According to the result of the variance analysis, no significant difference was detected between UV-B and UV-C in response to the contamination rate. However, the different durations of UV light exposure showed significant differences. As for the contamination rate in different durations of UV light exposure (Figure 1A), the results show values ranging between 26.67% (40 min UV light exposure) and 56.67% (10 min UV light exposure). Thus, increasing the duration of UV light exposure from 10 to 40 min decreased the contamination rate. A

similar result has been reported by Ferreira et al. (2021) that a longer UV-C exposure of 3 hr increased the efficiency of reducing the fungal colonies and mycotoxin levels compared to 1 hr exposure. Abdelrahman et al. (2018) also found that the fungicidal effect of UV radiation depends on the concentration of fungal spores and the dose of UV radiation. Similarly, Mengmeng et al. (2022) reported that the dose of UV radiation positively correlates with the bactericidal rate.



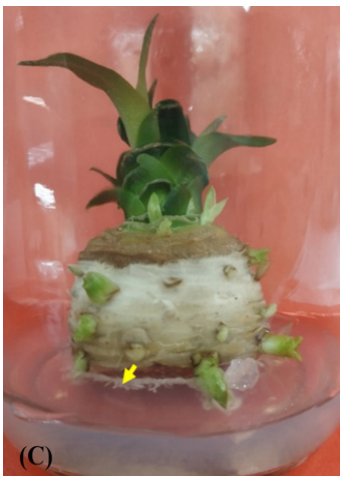


Figure 1. (A) Contamination rate of Tamban pineapple crown explants at 8 weeks after planting (WAP) after ultraviolet light treatment. Different letters indicate statistically significant differences between factors (Duncan's multiple range test at $p \leq 0.05$); (B) (C) Contamination on Tamban pineapple crown explant at 8 WAP. The blue arrow shows fungal contamination, and the yellow arrow shows bacterial contamination

Note. UV = Ultraviolet

Effect of UV-B and UV-C Exposure on Shoot Proliferation

The first shoot formation in UV light treatment was significantly slower than the control group, with the time of first shoot formation respectively of 14.82 days after planting (DAP) and 10.67 DAP (Table 2). In terms of the percentage of explants able to regenerate shoots, UV light treatment was significantly lower than the control at 2 WAP. All explants of the control group were able to regenerate shoots at 2 WAP, while UV light treatment resulted in 100% of explants being able to regenerate shoots at 4 WAP. These results indicated that UV light treatment on Tamban pineapple explants significantly slowed down the formation of the first shoot.

Table 2
Shoot proliferation of Tamban pineapple crown explants after control and ultraviolet light treatment

Treatment	Time of first shoot formation (DAP)	Mean percentage of explants able to regenerate shoots (%)		Mean number of shoots per explant			
		2 WAP	4 WAP	2 WAP	4 WAP	6 WAP	8 WAP
Control	10.67a	100.00b	100.00	2.80b	5.73b	7.27	8.47
UV light	14.82b	60.83a	100.00	1.93a	4.94a	7.05	8.60

Note. Means followed by the same letter(s) within each column are not significantly different ($p \leq 0.05$) using orthogonal comparison; DAP = Days after planting; WAP = Weeks after planting

The effect of UV-B and UV-C was not significantly different in terms of the time of first shoot formation and the percentage of explants able to regenerate shoots (Table 3). Based on Figure 2, the time of first shoot formation in the different durations of UV light exposure ranged from 13.30 DAP (10 min UV light exposure) to 16.40 DAP (40 minutes UV light exposure). This result indicated that increasing the duration of UV-B and UV-C exposure delayed the time of first shoot formation. In addition, increasing the duration of UV light exposure also reduced the percentage of explants able to regenerate shoots at 2 WAP (Figure 2).

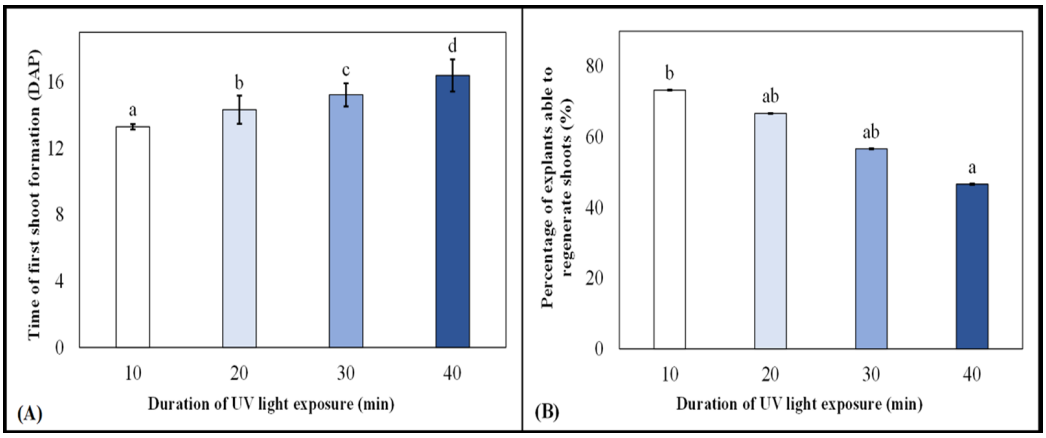


Figure 2. (A) Time of first shoot formation on Tamban pineapple crown explants after different durations of ultraviolet (UV) light exposure; (B) Percentage of explants able to regenerate shoots after different durations of UV light exposure

Note. Different letters indicate statistically significant differences between factors (Duncan's multiple range test at $p \leq 0.05$); DAP = Days after planting

Table 3
Effect of the type of ultraviolet (UV) light on shoot proliferation

Type of UV light	Time of first shoot formation (DAP)	Mean percentage of explants able to regenerate shoots (%)		Mean number of shoots/explant			
		2 WAP	4 WAP	2 WAP	4 WAP	6 WAP	8 WAP
UV-B	14.28	65.00	100.00	2.35b	5.70b	8.20b	10.03b
UV-C	15.35	56.67	100.00	1.50a	4.18a	5.90a	7.17a

Note. Means followed by the same letter(s) within each column are not significantly different ($p \leq 0.05$) using analysis of variance; DAP = Days after planting; WAP = Weeks after planting

Pineapple crown explants have dormant axillary buds located beneath every leaf axil of the crown leaves (Agogbua Josephine & Osuji Julian, 2011). These dormant axillary buds have the potential to grow into shoots (Py et al., 1987). The slowing down of first shoot formation from UV light treatment may be related to the increased dormancy period of axillary buds on Tamban pineapple crown explants.

The dormancy and development of axillary buds can be influenced by reactive oxygen species (ROS) content. Chen et al. (2016) stated that the hydrogen peroxide (H_2O_2) compounds contribute to the inhibition of the axillary bud outgrowth in tomato plants (*Solanum lycopersicum* L. cv Aisla Craig and M2). Similarly, Porcher et al. (2020) reported that increased H_2O_2 caused the axillary buds on rose plants (*Rosa* sp.) to remain dormant. Contrariwise, when the content of H_2O_2 decreases, axillary buds on rose plants begin to form. In addition, Li et al. (2022) also mentioned that the low level of ROS leads to dormancy release.

UV radiation can have an indirect biological impact by increasing ROS production (Tan et al., 2023). Metabolic disturbances from UV-B radiation, such as impaired electron transfer and other quinone components in photosystem II, can induce ROS production in photosystem I (Renger et al., 1989; Vass et al., 1996). UV-B radiation can turn H_2O_2 into highly reactive hydroxyl radicals through photo-conversion, which can lead to oxidative damage (Czégény et al., 2014). Xue et al. (2022) reported that the H_2O_2 content was increased in *Neoporphyra haitanensis* after UV-B light exposure. They also added that the intensity and duration of UV-B light exposure were positively correlated with the H_2O_2 content. A similar result was also recorded in tomato plants in which 20- and 40-min UV-C exposure significantly increased the ROS contents (O_2^-, OH^-, and H_2O_2) than without UV-C exposure (Dawood et al., 2022).

The hormones that play an important role in releasing bud dormancy are gibberellin (GA) and abscisic acid (ABA) (Yue et al., 2018). Meyer et al. (2021) mentioned that UV-B radiation can affect increasing ABA, reducing GA, and increasing ROS. The same pattern was also observed by Pascual et al. (2017), where *Pinus radiata*

plants irradiated with UV-C light showed a decrease in GA and an increase in ABA. Changes in GA and ABA content and increased ROS may be associated with the delayed release of axillary buds' dormancy in Tamban pineapple crown explants after UV-B and UV-C light treatment. It resulted in the highest duration of UV light exposure with the latest time of first shoot formation and the lowest percentage of explants able to regenerate shoots. The shoots formed on the Tamban pineapple crown explants can be seen in Figure 3.

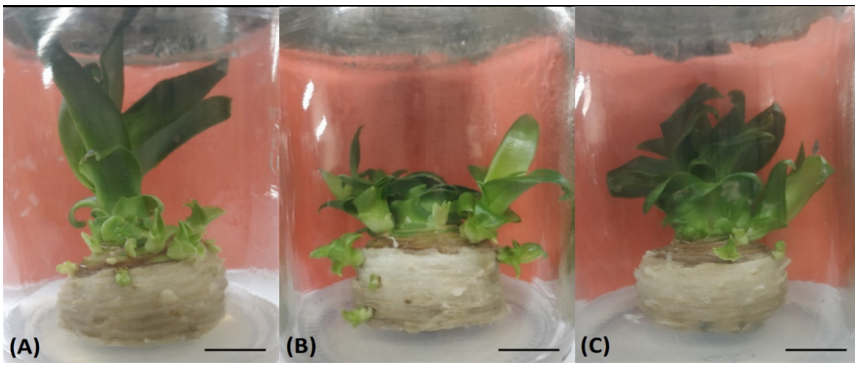


Figure 3. Shoot formation on the Tamban pineapple crown explants as the effect of UV light exposure after 8 weeks of culture. (A) control; (B) Ultraviolet-B (UV-B) light; and (C) UV-C light

Note. Bar = 1.0 cm

The UV light treatment was significantly different from the non-treated explants in terms of the mean number of shoots at 2 and 4 WAP (Table 2). UV-B light treatment produced a higher mean number of shoots than UV-C light treatment at 2-8 WAP (Table 3). Stapleton (1992) mentioned that DNA has maximum absorption in the electromagnetic spectrum range of UV-C light (260 nm). Therefore, the most energetic radiation is UV-C, but as a result, it often causes damage to plants, although at lower doses (Vanhaelewyn et al., 2020).

Increasing the duration of UV-B light exposure from 10 to 30 min increased the number of shoots per explant to 11.20 shoots at 8 WAP, and yet when the duration of UV-B light exposure was further increased to 40 min, the number of shoots per explant decreased to 10.13 shoots (Figure 4A). Meanwhile, increasing the duration of UV-C light exposure from 10 to 40 min increased the number of shoots from 6.40 to 8.27 (Figure 4B). These results were in accordance with the previous study that found an increment in the number of shoots in *S. cannifolium* after UV-B radiation (Metwally et al., 2019). Similar results were also observed in *Amsonia orientalis*, where UV-C light treatment produced a higher number of shoots than without treatment (Acemi et al., 2018).

Mallet et al. (2022) stated that the growth of axillary buds is controlled by a

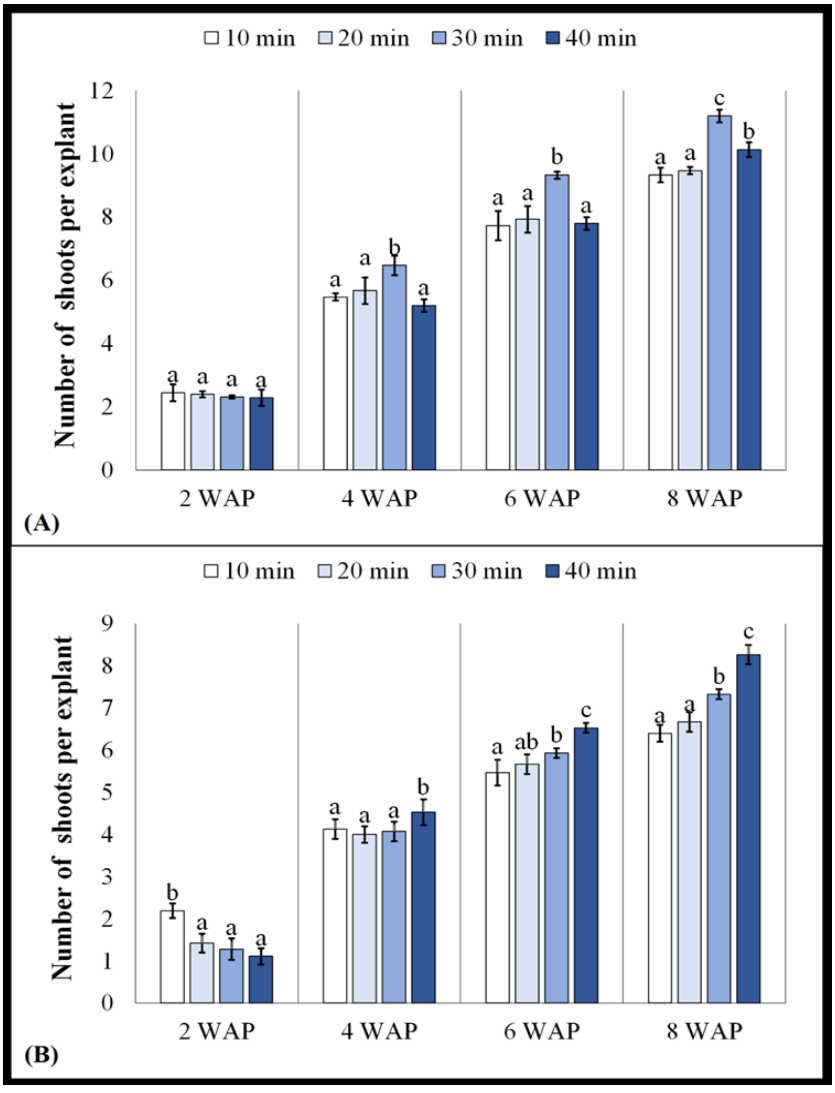


Figure 4. Number of shoots on Tamban pineapple crown explant at 2, 4, 6, and 8 weeks after planting (WAP) after different durations of ultraviolet (UV) light exposure: (A) UV-B exposure; (B) UV-C exposure

Note. Different letters indicate statistically significant differences between factors (Duncan's multiple range test at $p \leq 0.05$)

complex interaction between several main factors, such as hormones, nutrients (sugar and nitrogen), and ROS. The hormones that regulate the induction of axillary shoot growth are auxin (IAA), cytokinin (CK), and strigolactone (SL) (Barbier et al., 2021; Domagalska & Leyser, 2011). In the interaction of these three hormones, CK acts as a promoter of axillary shoot growth, while auxin and SL act as inhibitors of axillary

shoot growth (Gomez-Roldan et al., 2008; Leyser, 2009; Umehara et al., 2008). Auxin inhibits axillary shoot growth indirectly by inhibiting CK synthesis and promoting SL synthesis and signaling (Mallet et al., 2022).

UV light catalyzes the photodestruction of auxin (Ros & Tevini, 1995). UV-B radiation can cause the oxidative degradation of IAA, which begins with a decarboxylation process involving peroxidase on the side chain or oxidation of the indole ring (Berli et al., 2013; Normanly, 2010). Additionally, Hayes et al. (2014) revealed that UV-B radiation leads to the degradation of the PIF4 and PIF5 proteins, which can further inhibit the regulation of auxin biosynthesis.

As a result of UV-B radiation, the auxin content in rice leaf lamina decreased by 17.3% compared to natural light treatment. Increasing the dose of UV-B radiation from 2.5 to 5 kJ/m^2 also decreased the auxin content (Ling et al., 2022). IBA decreased significantly in the second leaves of *Cucumis sativus* L. irradiated with UV-B light (Qian et al., 2021). According to Katerova and Todorova (2011), exposure to low doses of UV-C light (0.1 kJ/m^2.d) on pea plants (*Pisum sativum* L.) caused the IAA content in the second leaf to be higher than the control. Meanwhile, the lowest IAA content was found in plants exposed to high doses of UV-C light (0.3 kJ/m^2.d). Therefore, the response of the different durations of UV-B and UV-C light exposure to a number of shoots may be due to the reducing auxin synthesis.

CONCLUSION

UV light treatment showed better results than the non-treated explants in terms of the contamination rate. However, UV light treatment delayed the formation of the first shoot and reduced the percentage of explants able to regenerate shoots at 2 WAP. UV-B light treatment produced a greater number of shoots than UV-C light treatment. Increasing the duration of UV light exposure reduced the contamination rate, delayed the formation of the first shoot, and reduced the percentage of explants able to regenerate shoots at 2 WAP. Increasing the duration of UV-B exposure to 30 min significantly increased the number of shoots at 8 WAP, but the number decreased at 40 min. Meanwhile, increasing the duration of UV-C exposure to 40 min significantly increased the number of shoots. As ROS and hormone relating were not tested during this study period, future studies should consider that.

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The Post-weaning Growth of Lambs from Crossbreeding Between Garut Ewes with Dorper Rams

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ABSTRACT

This study investigates the performance of post-weaning growth of Garut lambs and the results of crossing Garut ewes with Dorper rams. This research phase occurred in the post-weaning phase period of 3–7 months using 168 lambs consisting of 85 Garut lambs and 83 crossed Dorper lambs in the post-weaning phase with an average body weight of 15.15±3.23 kg. The lambs were assigned to a factorial completely randomized design (2 x 2 factorial experiment). They were then divided into two groups based on breed (Garut and Crossed Dorper) and two sex groups (male and female). Data observed included feed consumption, as fed feed consumption, dry matter (DM) consumption, DM consumption per body weight, crude protein (CP) consumption, total digestible nutrient (TDN) consumption, average daily gain (ADG), and feed conversion. Results showed that consumption of as fed, DM, CP, TDN, and ADG were higher (P<0.05) in crossed Dorper lambs than in Garut lambs; a total ADG of crossed Dorper, Garut, male, and female lambs were 106.92±11.68, 79.25±10.02, 102.49±17.54, and 86.79±14.48 g/day, respectively. Male lambs showed higher results (P<0.05) in as-fed feed consumption, DM, DM consumption per body weight, CP, TDN, and ADG than female lambs. The feed conversion of crossed Dorper lamb and male lamb was lower (P<0.05) than that of Garut and female lamb. There was an interaction between male breed and sex at seven months ADG and feed conversion at 4 and 7 months. Sheep from Garut ewes crossed with Dorper

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rams improved the growth performance of their offspring.

Keywords: Average daily gain, crossbreeding, Dorper cross, Garut sheep, sheep performance

INTRODUCTION

Sheep farming has a high economic benefit and potentially effectively achieves national food security. According to Badan Pusat Statistik (BPS) (2023), the sheep population in Indonesia in the last five years has decreased by 11.40%, from 17.61 million in 2018 to 15.62 million in 2022. On the other hand, there is an ongoing demand for sheep and sheep meat for religious rituals, large restaurants or small traders, and export demand. Consumer trends, interests, and preferences favor sheep under one year old because they have a more tender meat quality for consumption (Athifa et al., 2022). These consumer preferences demand that sheep have a high weight at a relatively young age. Thus, in responding to these customer needs, an improvement in sheep productivity is essential.

The weight of the sheep reflects productivity improvement and is consistent with the growth performance rate as seen from feed consumption and feed conversion ratio (FCR) (Budisatria et al., 2021). To improve sheep productivity regarding the growth rate as an essential trait that determines lamb production. Thus, indigenous and local sheep's productivity must be improved to boost output and profitability (Ayichew, 2019). Sheep crossbreeding is one way to improve and increase genetic combinations to provide favorable opportunities to increase production efficiency by exploiting breed diversities, heterosis, and complementary breeds (Getahun et al., 2019). Heterosis or hybrid vigor is the combined performance of the genetic average of the two sheep of origin, so this heterosis effect is used to increase the offspring's output. Therefore, the combination of genetic breeds can merge the advantages of each breed as seen from the crossbred offspring (Panjono et al., 2022).

One of the superior sheep growing in Indonesia is the Garut sheep. Garut sheep are a popular germplasm cultivated as meat producers. This sheep is a crossbreed of Fat Tail sheep (Gibas), Parahyangan Local sheep, and Merino sheep. According to the Badan Standardisasi Nasional (BSN) (2015), the body weight of males and females Garut sheep aged 8-12 months is 23 and 22 kg, respectively. Garut sheep are local Indonesian sheep adaptable to tropical climates and resistant to disease (Wijaya et al., 2019). However, indigenous and local sheep have a relatively small body size, slow maturity, and low carcass yield (Udo & Budisatria, 2011). The weaning weight of Garut sheep is 11.55±2.15 kg, with ADG post-weaning until eight months of age is 45.85±8.15 g/head/day (Praja et al., 2020). Therefore, it is considered necessary to cross with superior breeds to increase body weight and improve the productivity of local sheep. Dorper is one of the sheep that is excellent in growth.

Dorper sheep is a superior meat composite sheep produced in 1930 that was originally from South Africa due to crossing Black-headed Persian and Dorset Horn (Gavojdian et al., 2013). Dorper has excellent performance in weaning weight and post-weaning growth rate with good feed efficiency (Castillo-Hernández et al., 2023; Wanjala et al., 2023). After weaning, Dorper weighs 18.2 kg and can grow 0.23 kg/day (Cloete et al., 2000). Hence, it can reach a weight of 46.1 kg at eight months and an adult weight of 60–120 kg (Gavojdian et al., 2013). Dorper is used as a terminal-sires to improve the performance of crossbred lambs. In Ethiopia, body weight at different ages was significantly higher in 50% of Dorper crosses than in indigenous sheep breeds (Ayichew, 2019).

The initiative to introduce Dorper sheep (imported) to enhance the productivity of local sheep in Indonesia through crossbreeding has been widely practiced. Athifa et al. (2022) reported that the productivity of Garut sheep mated with Dorper rams was significantly better than that of Garut rams. Meanwhile, crossbred lambs (F1) also have better pre-weaning growth than Garut lambs. Birth weight, weaning weight, and ADG of Garut and Dorper crossbred lambs were 2.20±0.54 and 2.60±0.71 kg; 14.91±3.57 and 16.27±3.69 kg; 90.21±32.11 and 119.12±42.64 g/day, respectively. Following this previous study, the crossbred lambs' productivity during the weaning period must be continuously measured. Furthermore, this research was conducted to determine the productivity of the offspring resulting from the crossbreeding between Garut ewes and Dorper rams during the post-weaning period.

MATERIALS AND METHODS

Ethical Clearance

The design of this experiment has been approved by the Ethical Clearance Commission, the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Indonesia (No: 0037/EC-FKH/EKs/2020).

Research Location and Animals

This study was conducted at PT Agro Investama Sheep Farm, Malangbong, Garut Regency, West Java, Indonesia. This study was completed for 150 days, from August 2020 to January 2021. The research employed lambs from crossbreeding between Garut ewes with Garut rams and Dorper rams. Mating was carried out naturally in the colony pens (8 x 5 m^2) with 11–15 heads per colony equipped with feed and water bunks. The lamb's parents, the selected Garut ewes used, were above parity one or over sixteen months old with a live weight of 20 kg or above, then randomized mated with the Garut and Dorper ram. The rams were over two years old, with a live weight of 80–100 kg for Dorper and 60–70 kg for Garut ram. The mating duration was a month; the ewes were checked for with the *Draminski* ultrasound scanner probe transducer 5 MHz. The non-pregnant ewes were mated again, and those pregnant ewes were kept until the delivery. Routine maintenance of ewes, rams, and lambs

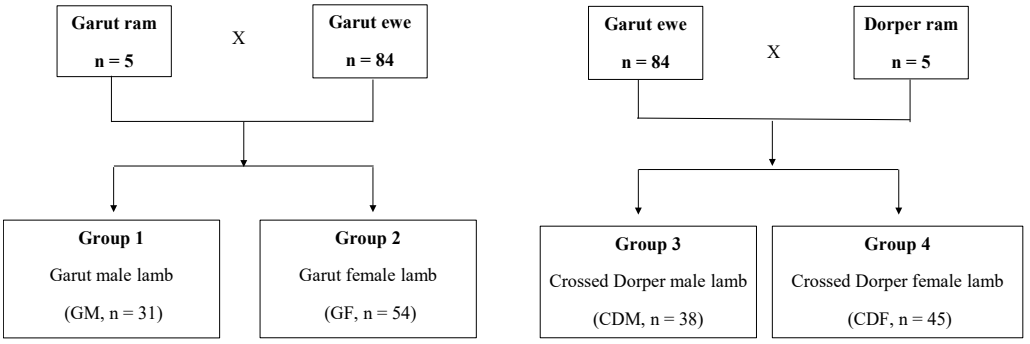

Figure 1. Diagram of the mating between Garut and Dorper ram with Garut ewe

included cleaning the pens every morning, bathing the sheep once a week, and shearing and trimming the long nails. The mating scheme is presented in Figure 1.

Lambs from Garut ewes mated with Garut rams are then referred to as Garut lambs, and lambs from Garut ewes mated with Dorper rams are then referred to as crossed Dorper lambs. The blood composition of crossed Dorper lambs is 50% Garut and 50% Dorper. The total sheep used in this study were 168 lambs consisting of 85 Garut lambs (31 male and 54 female) and 83 crossed Dorper lambs (38 male and 45 female) aged between 60 to 90 days or in the post-weaning phase with an average body weight of 15.15±3.23 kg. This research occurred in the post-weaning period of 3–7 months. The lambs were assigned to a factorial completely randomized design (2 x 2 factorial experiment) and then divided into two groups based on breed (Garut lamb and Crossed Dorper lamb) and two sex groups (male lamb and female lamb).

The pens are wooden stilt cages of 8 x 5 m^2 containing 10–12 sheep equipped with separate feed and water containers. Feeding is given twice daily at 08.00 a.m. and 04.00 p.m. Drinking water is supplied *ad libitum*. Feed is provided according to the needs of DM consumption, which is about 2.5% to 5% of the total body weight (National Research Council [NRC], 2010). Sheep in the growth phase with a live weight of 20 kg and a daily weight gain of 100 g require 410 g of DM or 3.5% of their weight (Kearl, 1982). The feed requirement calculation was based on body weight at the beginning of the research and updated after monthly weighing. The feed given is a complete feed, a mixture of concentrate and forage. The nutritional content of the complete feed included DM at 44.80%, CP at 15.14%, crude fat at 3.85%, crude fiber at 14.26%, ash at 9.40%, and the calculation of TDN was 64.15%.

Measurement Variables and Data Collection

The variables observed in this study were the growth performance of Garut lambs and crossed Dorper lambs, including feed consumption, ADG, and feed conversion. The calculation of feed consumption was

determined by weighing the feed and leftovers for three consecutive days in each month in each cage. The consumption calculation consists of as fed feed consumption, DM consumption, CP, and TDN. DM consumption consists of DM consumption per day (g/day) and DM consumption per body weight (%). The following is the formula for estimating feed consumption:

As Fed Feed Consumption

As-fed feed consumption is a form of as fed feed consumed by lambs with the following formula (Budisatria et al., 2021):

AF consumption = [AG - AL]

where,
AF = As fed feed consumption (kg/head)
AG = As fed feed given (kg/head)
AL = As fed feed leftover (kg/head)

DM Consumption

The amount of DM consumed with the following formula (Budisatria et al., 2021):

DMC = [FC x DM]

where,
DMC = Dry matter consumption (g/head)
FC = Feed consumption (g/head)
DM = Dry matter content in feed (%)

DM Consumption per Body Weight

The amount of DM consumed is divided by body weight with the following formula (Budisatria et al., 2021):

CBW = [DMC / BW] x 100%

where,
CBW = Consumption per body weight (%)
DMC = Dry matter consumption per day (g/head)
BW = Body weight (kg)

CP Consumption

The amount of CP consumed is calculated according to the following formula (Budisatria et al., 2021):

CPC = [DMC x CP]

where,
CPC = Crude protein consumption (g)
DMC = Dry matter consumption (g/head)
CP = Crude protein content in feed (%)

TDN Consumption

TDN is calculated based on the following formula (Budisatria et al., 2021):

TDNC = [DMC x TDN]

where,
TDNC = Total digestible nutrient consumption (g)
DMC = Dry matter consumption (g/head)
TDN = Total digestible nutrient content in feed (%)

Average Daily Gain

Lambs were weighed every month at the age of 4 to 7 months, and initial body weight was used as a covariate to analyze body weight

gain. ADG was calculated according to the following formula (Budisatria et al., 2021):

ADG = [FBW-IBW]/T

where,
ADG = Average daily gain (g/day)
FBW = Final body weight (g)
IBW = Initial body weight (g)
T = Duration of observation (days)

Feed Conversion

The calculation of feed conversion is made by counting the ratio or difference between the amount of feed consumed by the lambs and the resulting body weight gain as calculated according to the following formula (Budisatria et al., 2021):

FCR = AF/ADG

where,
FCR = Feed conversion ratio
AF = As fed feed consumption (g/head)
ADG = Average Daily Gain (g/day)

Differences in feed consumption, ADG, and feed conversion between sheep breed and sheep sex groups were analyzed using a two-way analysis of variance (ANOVA). Initial body weight was used as a covariate for analyzing body weight gain. Statistical analyses were conducted using SPSS (version 21).

RESULTS AND DISCUSSION

Feed consumption of crossed Dorper lambs was higher (P<0.05) than Garut lambs. Male lambs' as-fed consumption was higher P<0.05 than female lambs. However, there was no interaction between breed and sex on the consumption of as fed feed (Table 1). Crossed Dorper lambs have a better appetite than Garut lambs. It can be seen in the DM consumption (Table 1), which indicates that crossed Dorper lambs have higher DM consumption than Garut lambs (P<0.05). Table 1 also reveals that the DM consumption of male lambs is higher than females (P<0.05).

As fed feed consumption of crossed Dorper lambs was higher than that of Garut lambs. Garut lambs left much more stubble, while crossed Dorper lambs ate it. This condition is in line with the opinion of Ocak et al. (2016), who stated that Dorper is a sheep that is not selectively feeding. Male lambs have a better appetite than females. Table 1 shows that male lambs consume more as fed feed than female lambs. The male lambs produce hormones, such as androgen, in the testicular glands. However, the ovaries produce little. This hormone can stimulate masculine traits, making the males more aggressive when consuming large amounts of feed during the post-weaning period to get a mature weight (Lewis & Emmans, 2010). DM consumption is directly related to as fed feed consumption. Higher as fed feed consumption influences higher DM consumption. Consequently, the DM consumption of crossed Dorper lambs is higher than that of Garut lambs. Similarly, male lambs consumed more DM compared to female lambs. Data in Table 1 presents a significant difference in as fed and DM consumption of lambs in months 4, 5, 6,

Table 1
As fed and nutrients consumption of Garut lambs and Crossed Dorper lambs[1] during the post-weaning period

Variable	Month	Breed		Sex		Significancy		
		Garut (n = 85)	Crossed Dorper (n = 83)	Male (n = 69)	Female (n = 99)	B[2]	S[3]	B x S
As fed consumption (kg/day)								
	4	1.20±0.07	1.50±0.05	1.40±0.15	1.30±0.18	*	*	ns
	5	1.30±0.13	1.64±0.13	1.59±0.19	1.35±0.18	*	*	ns
	6	1.49±0.10	1.85±0.35	1.72±0.17	1.62±0.22	*	*	ns
	7	1.91±0.18	2.22±0.07	2.13±0.17	1.99±0.17	*	*	ns
	Total	1.47±0.29	1.80±0.29	1.71±0.32	1.56±0.33	*	*	ns
Dry matter consumption (g/day)								
	4	515.66±59.17	621.59±18.86	587.68±53.72	549.63±62.84	*	*	ns
	5	564.78±46.61	684.49±45.55	666.57±65.05	582.69±66.28	*	*	ns
	6	644.52±34.73	771.84±11.68	726.03±60.96	690.34±81.17	*	*	ns
	7	837.39±29.71	949.17±25.27	91779±60.13	868.77±62.94	*	*	ns
	Total	640.61±129.43	756.77±128.56	724.52±136.55	672.86±142.41	*	*	ns
Dry matter consumption per body weight (%)								
	4	3.12±0.33	2.95±0.07	3.05±0.06	3.03±0.14	ns	ns	ns
	5	2.88±0.03	3.03±0.02	2.97±0.07	2.94±0.10	ns	*	ns
	6	2.88±0.08	3.09±0.05	3.00±0.14	2.97±0.12	*	*	ns
	7	3.14±0.03	3.27±0.02	3.22±0.09	3.20±0.06	*	*	ns
	Total	2.96±0.12	3.13±0.10	3.06±0.13	3.03±0.15	*	*	ns
Crude protein consumption (g/day)								
	4	81.39±4.91	101.74±3.27	95.17±10.41	87.96±11,98	*	*	ns
	5	88.18±8.86	111.05±8.83	107.66±12.51	91.57±12,59	*	*	ns
	6	101.15±6.70	125.10±2.26	116.54±11.46	109.71±15,33	*	*	ns
	7	129.25±5.51	150.35±4.89	144.43±11.49	135.17±11,74	*	*	ns
	Total	99.99±6.49	122.06±4.80	115.95±11.47	106.10±12.91	*	*	ns
Total digestible nutrient consumption (g/day)								
	4	344.87±20.81	431.09±13.85	403.26±44.11	372.69±50,78	*	*	ns
	5	373.61±37.56	470.55±37.40	456.17±53.04	387.98±53,33	*	*	ns
	6	428.58±28.39	530.08±9.58	493.81±48.57	464.85±64,94	*	*	ns
	7	547.64±23.36	637.05±20.75	611.96±48.72	572.73±49.76	*	*	ns
	Total	423.67±27.53	517.19±20.39	515.06±47.11	449.56±54.70	*	*	ns

Note.
*$P<0.05$; ns = Non-significant
[1]Crossbreeding between Dorper ram and Garut ewe
[2]Breed
[3]Sex
B x S = Interaction between breed and sex

and 7. However, there was no interaction between breed and sex on feed consumption. Rodríguez et al. (2008) also reported no interaction between breed and sex on feed consumption in Assaf sheep.

DM consumption per body weight in this study ranged from 2.87-3.49% of body weight, as presented in Table 1. Similar to fed feed and DM consumption, DM consumption per body weight in the crossed Dorper lambs had a higher percentage (P<0.05) than Garut. Similarly, male lambs had a higher percentage (P<0.05) than female lambs. CP consumption in crossed Dorper lambs was higher than in Garut lambs (P<0.05). In the same way, male lambs had a higher CP consumption than female lambs (P<0.05).

The results of this study indicated that DM consumption per body weight in all groups ranged from 2.87% to 3.49% of body weight, as presented in Table 1. DM consumption of both breeds or groups is still in the normal range. According to NRC (2010), DM consumption of sheep is between 2.5–5% of body weight; DM consumption will determine the feed consumed by livestock. Significant differences in crossed Dorper lambs in months 6 and 7 were higher (P<0.05) than Garut, as well as DM consumption per body weight of male lambs in months 5, 6, and 7 were higher (P<0.05) than that of female lambs. The difference in DM consumption between the lambs is due to the ability of digestibility and digesta rate of feed between different animals (McDonald et al., 2011).

Table 1 demonstrates that CP consumption in Garut and crossed Dorper lambs varies between 81.35 and 150.35 g. The TDN of male lambs is higher (P<0.05) than female lambs (P<0.05). TDN in male lambs was higher (P<0.05) than female lambs (P<0.05). Similarly, TDN in crossed Dorper lambs was higher (P<0.05) than Garut lambs. TDN consumption presented in Table 1 indicates that TDN consumption is 326.62–655.98 g (63.34–69.18%). This TDN consumption is still in the normal range of 60–70% (Hasanah et al., 2021). However, there was no interaction between breed and sex on DM consumption per body weight, CP consumption, and TDN consumption.

There was a significant difference (P<0.05) in CP consumption between crossed Dorper lambs and Garut lambs, with higher CP consumption in crossbred lambs in months 4, 5, 6, and 7. The same applies to the CP consumption of male lambs compared to female lambs (P<0.05). It can be concluded that breed and sex significantly affect CP consumption (Table 1). Tao et al. (2022) reported that the amount of genetic variance affects protein consumption, and different hormones between males and females also have an influence that causes the protein needs of male livestock to be different from females. Different protein requirements between sexes and breeds relate to metabolic energy consumption (Abbasi et al., 2014). Metabolic energy is readily utilized by livestock for physical activity, reproduction, production, metabolism, and tissue formation (McDonald et al., 2011). In

addition, it is known that CP consumption is in the normal range. According to NRC (2010), sheep require 127–167 g CP.

TDN consumption determines nutrient consumption for energy sources, which consists of digestible components of protein, crude fiber, ether extract, anddigestible N-free extract. The TDN required for local sheep body weight in Indonesia is 400–800 g (Jayanegara et al., 2017). Kearl (1982) noted that to meet the TDN requirement of 15–25 kg, it is 310–410 g/head/day. Jayanegara et al. (2017) also add that the TDN requirement of sheep is 80%. The value of TDN consumption in this study ranged from 326–637 g/head/day, indicating appropriate TDN consumption. The results also showed an increase in TDN consumption from the 4th to 7th month, which is in line with the increase in body weight. Ngadiyono et al. (2019) stated that energy consumption will result in a faster growth rate and increase the size of livestock.

TDN consumption in this study indicated crossed Dorper lambs had higher TDN consumption (P<0.05) than Garut lambs, and male lambs had higher TDN consumption (P<0.05) than female lambs. It is attributed to the fact that feed consumption tends to be higher. In addition, larger animals certainly consume more feed in the form of DM to meet their needs (Table 1). Ngadiyono et al. (2019) explained that TDN consumption is determined by the percentage of DM consumed, the content of minerals and fat digested in DM, and the coefficient of DM digestibility. Another factor affecting DM consumption is hormones in male livestock, which support higher feed consumption than in female livestock (Purbowati et al., 2015). In addition, the genetic origin of the sheep also affects the feed consumption of the sheep itself. Sheep with larger genetic weights require higher consumption (Knapik, 2017). It can be seen from the crossed Dorper and Garut lamb consumption levels.

The ADG of crossed Dorper lambs was higher (P<0.05) than Garut lambs (Table 2). The difference in body weight between Garut lambs and crossed Dorper lambs is presented in Figure 2. The ADG of male lambs was higher than that of females (P<0.05). It is consistent with the feed consumption of males, which is more than that of females, as presented in Table 1—the interaction of male breed and sex on average at seven months of age.

The different body weights between Garut lambs and crossed Dorper lambs indicate that the crossbreeding of Garut ewes with Dorper rams affects the crossing outcome in the form of higher body weight of crossed Dorper lambs (P<0.05), as shown in Figure 2. The superior body weight will affect the ADG values. The difference in the breed of the parents causes the crossed Dorper lambs to have higher body weights and ADG. Sauza et al. (2013) reported that Dorper was used as a ram in several crossbreeding to produce fast-growing sheep post-weaning. Getahun et al. (2019) added that the advantage in the growth of crossbred sheep results from heterosis or complementary effects between breeds.

Table 2
Average daily gain and feed conversion of Garut lambs and crossed Dorper lambs[1] during the post-weaning period

Variable	Month	Breed		Sex		Significancy		
		Garut (n = 85)	Crossed Dorper (n = 83)	Male (n = 69)	Female (n = 99)	B[2]	S[3]	B x S
Average daily gain (g/day)								
	4	79.66±13.12	115.64±21.81	109.20±27.65	89.97±20.44	*	*	ns
	5	79.40±14.10	105.00±16.23	101.87±18.60	85.65±17.96	*	*	ns
	6	77.67±15.33	103.94±15.62	98.72±21.83	85.58±17.12	*	*	ns
	7	80.28±13.30	103.11±19.08	100.18±17.35	85.95±19.87	*	*	*
	Total	79.25±10.02	106.92±11.68	102.49±17.54	86.79±14.48	*	*	*
Feed convertion ratio								
	4	6.35±0.26	5.37±0.41	5.56±0.62	6.15±0.47	*	*	*
	5	6.93±0.20	6.48±0.67	6.59±0.18	6.82±0.32	*	*	*
	6	8.18±0.28	7.41±0.52	7.50±0.62	8.09±0.31	*	*	*
	7	10.25±0.50	9.18±0.52	9.26±0.60	10.17±0.59	*	*	ns
	Total	7.93±1.56	7.11±1.48	7.23±1.48	7.81±1.61	*	*	ns

Note.
*$P<0.05$; ns = Non-significant
[1]Crossbreeding between Dorper ram and Garut ewe
[2]Breed
[3]Sex
B x S = Interaction between breed and sex

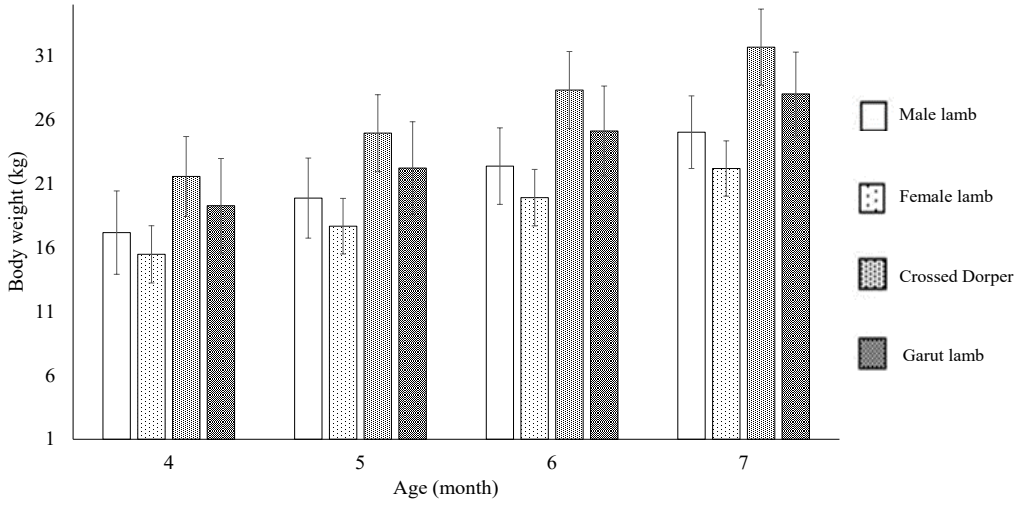

Figure 2. Body weight of Garut lambs and crossed Dorper lambs during the post-weaning period

The higher ADG (P<0.05) in male lambs indicates that sex affects body weight gain, as seen from the consumption of as fed feed or more DM consumption. Gemiyo et al. (2017), as well as Lewis and Emmans (2010), reported that high aggressiveness in consuming feed causes males to grow faster, resulting in higher body weight gain than females. Gemiyo et al. (2014) added that sexual dimorphism and hormonal differences between males and females affect growth differently. Mohammadi et al. (2010) explained that estrogen has a low effect on female growth in the endocrine system.

In this study, the ADG of Garut and crossed Dorper lambs ranged from 79–106 g/day. ADG in this study is under the normal range. Several studies reported the ADG of local and exotic lambs during post-weaning: Fetherstone et al. (2022) reported that New Zealand lamb have ADG of 247–274 g/day, Waheed et al. (2022) reported that Thalli lambs have ADG of 102 g/day, and Maulana et al. (2021) reported that Garut lamb have ADG of 104–195 g/day. Crossed Dorper lambs grow faster than Garut lambs, as depicted in Figure 3. The male lambs grow faster than the female lambs. However, the growth is not as significant as the crossed Dorper. Sheep body weight gain is influenced by genetics and sex (Ayele & Urge, 2019). Dorper belongs to a fast-growing sheep breed (Cloete et al., 2000). Freitas et al. (2020) reported in their research that there was an interaction between sex and breed significantly on ADG of crossbreeding between Dorper and Santa Inez in post-weaning rearing (P<0.05). There was interaction between breed and sex on ADG during the post-weaning period. This result follows the study of Nugroho et al. (2023), which reported the interaction factor between genetic factors (the buck breed and litter size) and environmental factors (birth season) in post-weaning

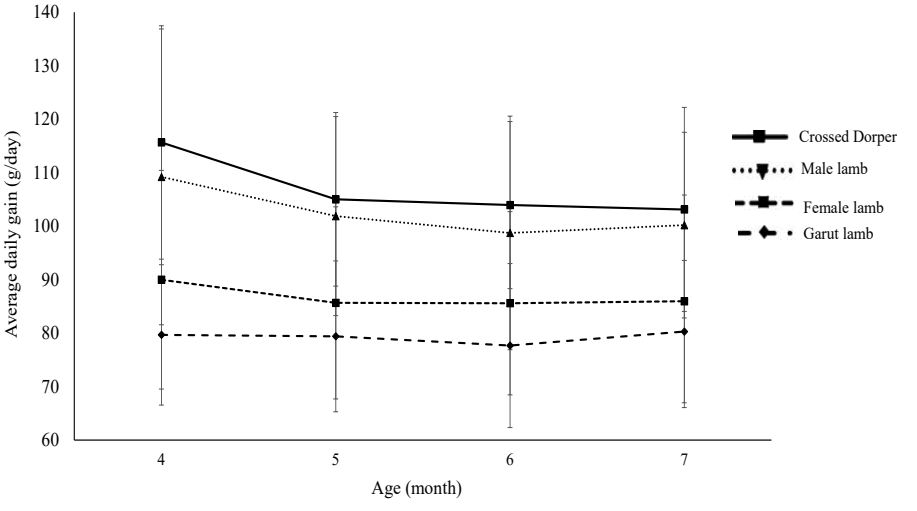

Figure 3. Average daily gain of Garut lambs and crossed Dorper lambs during the post-weaning period

growth of Boer and Boer Cross goats. Growth traits are important indicators of meat-type livestock performance, given their direct association with economic value. It is widely recognized that genetic factors, environmental conditions, and the interaction between genetics and the environment influence growth performance.

It was found that the FCR were significant differences (P<0.05) in crossed Dorper and Garut lambs, and male lambs and female lambs presented in Table 2. The highest ADG was in crossed Dorper lambs (Figure 3). FCR (Table 2) suggests that crossed Dorper lambs have lower FCR than Garut lambs (P<0.05). FCR of male lambs was more efficient than female lambs (P<0.05). There was an interaction between breed and sex in terms of feed conversion during the post-weaning period in 4–6 months. In this study, the feed conversion of crossed Dorper lambs ranged from 5.45 to 10.79.

FCR in this study is still in the normal range. FCR of sheep weighing 10–20 kg is 2.5–4 (NRC, 2010). The standard score of FCR for sheep is 4 in subtropical areas, which tends to be higher in Indonesia as it has a tropical climate (NRC, 2010). Purbowati et al. (2022) stated that the FCR of sheep in Indonesia ranges from 9–13, meaning that 9–13 kg of feed (in DM) produces 1 kg of body weight gain. There were significant differences in crossed Dorper lambs with Garut lambs and male lambs and female lambs, as shown in Table 2 (P<0.05). The crossed Dorper lamb is more efficient in consuming feed to produce body weight gain. The crossed Dorper lambs were more efficient than Garut lambs. Male lambs were more efficient than female lambs for producing body weight gain.

Crossed Dorper lambs are more efficient in converting feed into meat. It can be seen from the cross Dorper lambs that have a faster growth with 3% DM consumption per body weight (Table 1). Gavojdian et al. (2013) reported that Dorper is a sheep breed that is quite good in feed efficiency after weaning. Ocak et al. (2016) stated that Dorper is a productive sheep breed. It can increase FCR by 20% than other crossbred sheep. Dorper FCR is 4, meaning that 4 kg of feed consumption (in DM) produced 1 kg of body weight gain. Male lambs in this study are more efficient in converting feed into meat. Freitas et al. (2020) explained that feed consumption of male sheep results in a better quantity of muscle tissue and muscle distribution than females, and the accumulation of male muscle is more significant due to differential hormonal effects on growth. Sjaastad et al. (2010) also noted that testosterone, which appears in male livestock, has anabolic effects. Testosterone increases the growth of muscle tissue and bone in males by stimulating the synthesis and inhibiting protein breakdown.

Crossbreeding is one of the strategies used to address the interaction between genetics and the environment. This approach aims to develop superior genetic qualities that adapt to specific environmental conditions. Crossbreeding between exotic breeds and local Indonesian sheep breeds has been implemented in Indonesia.

This measure was primarily undertaken because most Indonesian local sheep have smaller body sizes. These crossbreeds are expected to be advantageous due to their larger body size and adaptability to the Indonesian environment. However, as crossbreeds, they may possess diverse genetic potentials, leading to varying responses to environmental stimuli. The result of this study would be beneficial for evaluating the crossbreeding program of Indonesian local sheep crossed with exotic sheep before it can be implemented widely by the farmers.

CONCLUSION

The lambs resulting from the crossbreeding between Garut ewes and Dorper rams have more excellent productivity, as seen from the performance of the crossed Dorper lambs in as fed feed consumption, DM, protein, TDN, ADG, and FCR that are better than Garut lambs. Crossed Dorper lamb has a 25% higher ADG, namely 106.92 g/day, compared to Garut lamb, which is 79.25 g/day, and Crossed Dorper lamb has an FCR 0.82 higher than Garut lamb. Male lambs also have better post-weaning performance than female lambs.

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Evaluation of Anti-tyrosinase, Anti-collagenase, and *In Vitro* Sun Protection Factor (SPF) of Ajwa Date Fruit (*Phoenix dactylifera* L.)

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Abstract

This research explores the potential of Ajwa date fruit extract as a bioactive component in cosmeceutical formulations, with particular emphasis on its anti-tyrosinase, anti-collagenase, and ultraviolet (UV) protection capabilities. The antioxidant activity was determined using the design of response surface methodology, considering the factor of sample-to-water-as-solvent ratio, extraction time, and size of the sample. This factor possesses the capacity to exert an influence on the production of antioxidants and enhance the efficacy of the extraction procedure. The Ajwa date fruit extract was obtained using the Soxhlet method. The extract showed notable inhibition percentages of 67.77 and 49.12 for anti-tyrosinase and anti-collagenase activities, respectively. Additionally, it revealed a sun protection factor value of 17.09. Previous research has indicated that Ajwa dates exhibit significant inhibitory properties against tyrosinase and collagenase enzymes, making them potentially valuable in cosmetic applications. Therefore, research has demonstrated this study's promise in skin pigmentation, elasticity, and UV protection. The study places significant importance on exploring natural alternatives in cosmetics. It highlights the encouraging outcomes obtained from using Ajwa date fruit extract, emphasising its potential for future advancements in cosmeceuticals. The

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present study offers a valuable opportunity to produce skincare formulas that are both safer and more effective.

Keywords: Ajwa date fruit extract, anti-collagenase, anti-tyrosinase, sun protection factor

INTRODUCTION

The cosmetics industry has witnessed substantial expansion and is presently flourishing worldwide. Cosmetic products are used daily by a great number of people, no matter their gender or age. The increasing significance of self-care among consumers globally has resulted in a notable growth of over 15% in the beauty sector market compared to the previous year (Petruzzi, 2024). The cosmetics market, known for its ever-changing nature, has witnessed a significant increase in interest towards novel products targeting skin pigmentation and collagen enhancement.

Advanced formulations have been developed to address hyperpigmentation, providing consumers with efficient remedies for achieving a glowing and uniform complexion. Tyrosinase is a copper-containing monooxygenase enzyme that is essential in the production of melanin. Tyrosinase transforms L-tyrosinase to I-3,4-dihydroxyphenylalanine (L-DOPA), which is then oxidised to generate dopachrome, which causes melanin pigment synthesis (Zengin et al., 2015). Melanin protects human skin from harmful UV rays, removes reactive oxygen species (ROS), and scavenges dangerous medicines. However, excessive melanin production can lead to larger pigmented areas on the skin, melanogenesis, and neurological illnesses, which are adverse to human favour, especially in the Asian continent (Momtaz et al., 2008; Vujanović et al., 2020). Because of their potential to decrease cutaneous melanin synthesis, tyrosinase inhibitors like kojic acid have been employed as whitening agents in human cosmetic goods.

Collagen is a very prevalent structural protein in mammals and is a type of protein found in abundance. A structural protein is a protein that helps to build the structure or framework of cells and tissues. It was discovered in the skin, bones, muscles, and tendons. Collagenase is an enzyme that aids in the breakdown of collagen. Collagen is the most important component, accounting for 70% to 80% of total skin weight (Utami et al., 2018). The skin is composed of three distinct layers: the epidermis, dermis, and subcutaneous tissue. These three skin components go through degenerative changes because of the ageing process, with alterations to the dermis being the most noticeable of these. The dermis mostly comprises the extracellular matrix (ECM) and fibroblasts. Collagen, a significant component of ECM, becomes fragmented and coarsely dispersed, reducing its total amount. It mostly results from increased matrix metalloproteinase (MMP) activity and decreased transforming growth factor-β-signalling caused by ROS produced during ageing (J.-W. Shin et al., 2019; Vijayakumar et al., 2017). This occurrence leads to the fragmentation of collagen molecules and a decrease in the production of new collagen.

Ultraviolet A (UVA) and ultraviolet B (UVB) radiation have the potential to induce several adverse effects on the skin, including sunburn, photoaging, erythema, and inflammation. Sunscreens offer protection to the human skin against damage caused by UV radiation by including active substances, categorised as either organic or inorganic, referred to as UV filters. These UV filters possess unique action modes responsible for the protective effects they confer. The necessity of applying sunscreen to human skin arises from the need to minimise skin exposure and safeguard it against harmful UV radiation. The date fruit is known to possess phenolic and flavonoid components, which have inherent antioxidant properties. According to Alharbi et al. (2021), those substances possess the capacity to penetrate both the superficial layer of the skin (epidermis) and the underlying layer (dermis), thus protecting against the harmful consequences of UV-induced oxidation and premature ageing of the skin.

The date palm is the primary agricultural crop in regions such as the Arabian Peninsula, North Africa, the Middle East, and Southwest Asia (Alharbi et al., 2021). There exists a vast assortment of about 2,000 distinct varieties of dates (Al-Shahib & Marshall, 2003). The Ajwa variety is recognised for being rich in nutrients and possessing healing properties, making it known as "super date" (Alharbi et al., 2021). Ajwa dates contain various antioxidants, including flavonoids and phenolic acid compounds. Natural compounds from plants exhibit significant potential as relatively unexplored opportunities for safe application in the beauty industry. Consumer demand for ecologically friendly products has drawn attention to using plant extracts in skin care products (Ribeiro et al., 2015). Antioxidants can complement the effectiveness of sunscreen. While sunscreens primarily act as physical or chemical barriers to UV rays, antioxidants can provide an additional layer of defence by neutralising free radicals that may escape the sunscreen's protection. Moreover, antioxidants help protect collagen by neutralising free radicals contributing to collagen degradation. Some antioxidants also stimulate collagen synthesis, promoting the maintenance of skin elasticity and firmness.

While prior research has extensively explored the antioxidant capabilities of Ajwa dates, a significant gap in our understanding exists concerning their potential functional applications. Considering the acknowledged lack of knowledge in this area, our research aims to explore new ground by examining the anti-elastase, anti-collagenase properties, and *in vitro* sun protection factor (SPF) of Ajwa dates extract. The objective is to gain fresh insights into the potential incorporation of Ajwa dates extract into cosmetic and medicinal products, specifically those with whitening and anti-ageing properties. In contrast to previous research that primarily focused on their antioxidant properties, this study pioneers an examination into the anti-elastase, anti-collagenase activities, and *in vitro* SPF of Ajwa dates extract.

Furthermore, the phytochemical screening was systematically conducted using gas chromatography-mass spectrometry (GC-MS) and ultra-high-performance liquid chromatography-quadrupole time-of-flight (UHPLC-QTOF). This screening is an analytical technique to identify compounds in Ajwa dates extract. GC-MS is well-suited for volatile and semi-volatile compounds and is widely applied in various industries. At the same time, UHPLC-QTOF is advantageous for analysing a broad spectrum of compounds in liquid samples, providing high-resolution and accurate mass measurements for precise identification. It was conducted to prove and elucidate the diverse chemical constituents in the Ajwa dates extract, aiming to pinpoint the specific functional component responsible for the observed biological activity.

MATERIALS AND METHODS

Sample Preparation and Chemicals

Fruits of *Phoenix dactylifera* were purchased from Madinah, Saudi Arabia, through a local supplier. The fruits and pits were manually separated and dried in a laboratory oven at 60°C for 2 weeks. Following the drying process, the flesh of the samples was crushed into smaller sizes, sieved into several sizes, and kept in air-tight bottles for subsequent processes. All chemicals were of analytical grade. Collagenase Kit and Tyrosinase Activity Kit were purchased from BiTA Lifesciences Sdn. Bhd. (Malaysia). The other solvent and apparatus, such as gallic acid and microplate, were purchased from Life Science of BioVision Inc. (USA).

The extraction of dates was performed using the Soxhlet extraction method, with distilled water employed as the solvent. This process is carried out repeatedly. Accordingly, the response surface methodology (RSM) design controlled the sample-to-water-as-solvent ratio, extraction time, and sample size. The proportion of a sample to the amount of water used as a solvent (ratio), extraction time (hr), and size of the sample (mm) in experimental produce were 1:10, 1:20, 1:30; 1.00, 2.87, 4.75, and 3.00, 4.50, 6.00. Next, the collected aqueous extraction sample was subjected to drying using a freeze dryer. The crude extract was collected and stored in air-tight bottles at -4°C until further experimentation. The present study utilised the antioxidant qualities of crude extract to determine the anti-tyrosinase, anti-collagenase, and *in vitro* SPF characteristics of Ajwa date fruit.

Determination of Anti-collagenase Inhibition

Collagenase inhibition was measured using a collagenase activity colourimeter assay kit and the technique outlined in the calorimeter (ab196999) #K792-100 (BioVision Inc., USA). Prior to usage, it is necessary to ensure that all materials and produced reagents are brought to room temperature. Reagent background wells, inhibitor test sample wells, positive control wells, inhibitor control wells, and solvent control wells are all set up in reaction wells. The sample extract (2 µl inhibitor test and 98 µl assay buffer) was added to the well. The positive control contained

10 µl collagenase and 90 µl assay buffer. The inhibitor control was prepared with 10 µl collagenase, 2 µl inhibitor control, and 88 µl assay buffer, while the solvent consisted of 10 µl collagenase and 88 µl assay buffer. One hundred (100) µl of the reaction mix consisting of 60 µl collagenase assay buffer and 40 µl collagenase substrate was poured into each well, and the activity was measured immediately. At 345 nm, the absorbance was measured on a microplate reader in kinetic mode for at least 5–15 min at 37°C protected from light.

The calculation of inhibition is as follows:

$$\% \text{ Inhibition} = \text{Activity}_{(\text{enzyme})} - \text{Activity}_{(\text{inhibitor})} / \text{Activity}_{(\text{enzyme})} \times 100\%$$

Determination of Anti-tyrosinase Inhibition

Anti-tyrosinase was driven using the procedure described by (Haliloglu et al., 2017) with minor modifications. The inhibition of mushroom tyrosinase by the tested sample, using L-DOPA as a substrate, formed the foundation for this approach. The experiments were carried out on a 96-well microplate, and the absorbance at 475 nm was measured using a microplate reader. Wells designed and labelled A, B, C, and D, each containing a reaction mixture (160 µl) for each concentration of the sample solution. The wells are labelled as follows: (A) 120 µl of a 0.1 M phosphate buffer (pH 6.8) and 40 µl of mushroom tyrosinase (33.3 units/ml); (B) 160 µl of the same buffer as blank; (C) 80 µl of the same buffer, 40 µl of tyrosinase (33.3 units/ml), 40 µl of the sample-buffer solution containing dimethyl sulfoxide (DMSO), (D) 120 µl of the same buffer, 40 µl sample solution containing DMSO. The content of each well was mixed and preincubated at 23°C for 10 min. Subsequently, 40 µl of L-DOPA (2.5 mM) was added. The well-contained reaction mixture was measured after incubation at 23°C for 15 min. The quantification of dopachrome in each reaction mixture was achieved by measuring the disparity in optical density before and after the incubation period. Kojic acid (Sigma-Aldrich, Germany) was employed as a positive control. The percentage of inhibition of tyrosinase activity was obtained using the equation:

$$\% \text{ Inhibition activity} = [(A - B) - (C - D)]/(A - B) \times 100\%$$

Dates extract anti-tyrosinase activity was reported as kojic acid equivalent (KAE) in mg/g of date extract. Kojic acid dilutions in methanol were made to obtain a calibration curve. Calculation was performed using the $y = -0.0066x + 0.0892$ ($r^2 = 0.9784$). The experiments were conducted repeatedly, presenting the outcomes as mean KAE values.

Determination of *In vitro* SPF

The methodology outlined by de Oliveira et al. (2007) was employed to determine SPF *in vitro*. The ethanol solution diluted the date extract, resulting in 50, 100, 500, and 1,000 g/ml concentrations.

Subsequently, the ethanolic extracts undergo spectrophotometric scanning over a wavelength range of 260 to 400 nm at intervals of 5 nm. The measurements were conducted using a quartz cell with a length of 1 cm, and ethanol was utilised as the blank solution. The SPF was determined using the mathematical equation provided as follows:

$$\text{PF spectrophotometric} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

where, EE (λ) = erythermal effect spectrum; I (λ) = solar intensity spectrum; Abs (λ) = absorbance of sunscreen extract; and CF = correction factor (=10). The value of EE x I is constant. Table 1 shows the standardised extract function that was used to calculate SPF.

Table 1
Standardised extract function used in the calculation of sun protection factor (SPF)

Wavelength (nm)	EE x I (normalised)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180

Note. It is the standardised formula used in the calculation of SPF, EE (λ) = Erythermal effect spectrum, and I (λ) = Solar intensity spectrum

RESULTS AND DISCUSSION

Anti-collagenase Activity

The anti-collagenase activity was assessed using a standard date extract concentration at 1,000 µg/ml, which exhibited a notable collagenase inhibition percentage of 49.12%. In contrast, the solution of ascorbic acid in its conventional form exhibited a measured value of 77.20%, as depicted in Figure 1. The findings indicated that the extract derived from dates exhibits essential inhibitory effects on collagenase enzymes compared to the standard ascorbic acid. It suggests that dates may serve as a promising source of anti-ageing agents.

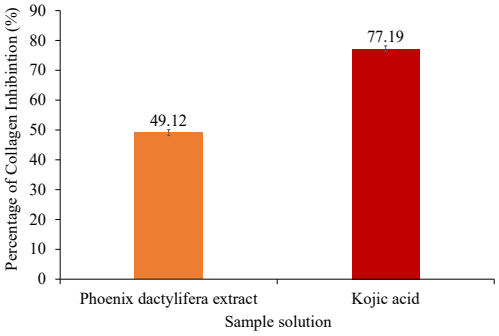

Figure 1. The percentages of collagenase inhibition on kojic acid and *Phoenix dactylifera* fruit extract

The production of ROS during the ageing process increases MMP expression while simultaneously inhibiting transforming growth factor beta (TGF-β) signalling, which results in collagen fragmentation and decreased collagen manufacturing. MMPs are a class of proteinases that contain zinc (Zn). MMP-1, or matrix metalloproteinase-1, is an enzymatic protein responsible for initiating degradation processes targeting Types I, II, and III collagens. These collagens are widely distributed throughout the dermis, making them the predominant interstitial collagens in this tissue. Matrix metalloproteinase-2

(MMP-2) is responsible for the degradation of Type I-III, IV, and VII collagens, with the latter two being prominently present in the dermal-epidermal junction (Vijayakumar et al., 2017).

Collagen production declines as people become older, whereas collagenase levels rise. However, undesired ageing can be delayed by using antioxidants to scavenge free radicals and suppress collagenase activity. The findings revealed that date extract can inhibit collagenase, implying that it could be used as an anti-inflammatory medication. Hydroxyl groups in the polyphenol compounds present in date extracts may interact with the collagenase backbone or other functional group side chains. The alteration in collagenase conformation, induced by hydrophobic contact with the benzene ring of polyphenol, reduces the enzyme's catalytic efficiency (Vijayakumar et al., 2017).

Another plausible consequence is the involvement of the Zn ion active site in collagenase. The involvement of a structural Zn ion in the active region of collagenase is crucial for enabling interaction with an inhibitor (Bigg et al., 1994). Hence, it is plausible that polyphenol compounds have the capability to bind to the active site of the Zn ion, thereby obstructing the substrate's ability to undergo enzymatic digestion. This mechanism potentially plays a role in date flesh extracts observed collagenase inhibitory function.

Anti-tyrosinase Activity

The anti-tyrosinase assay relies on suppressing the tyrosinase enzyme by applying mushroom extract. Tyrosinase is commonly acknowledged as a pivotal enzyme involved in the synthesis of melanin and in the pathogenesis of dermatological disorders characterised by abnormal melanin deposition. Melanin is the principal pigment accountable for human skin pigmentation. In recent years, the significance of tyrosinase inhibitors has expanded considerably due to their clinical efficacy in treating skin conditions and their widespread use in the cosmetic industry for skin whitening and depigmentation.

Tyrosinase is pivotal in melanin formation and dermatological conditions characterised by excessive melanin accumulation. The anti-tyrosinase activity was assessed using kojic acid as the reference standard. The results indicated that the extract derived from P. dactylifera with the highest concentration of 1,000 µg/ml demonstrated a moderate inhibition percentage of 67.76% against tyrosinase. In comparison, the standard solution of kojic acid exhibited a higher inhibition percentage of 76.03%. These findings are visually represented in Figure 2.

A strong correlation exists between the biological activities exhibited by plants and the presence of phytochemicals in their extracts (Vijayakumar et al., 2017). The significant inhibition of tyrosinase seen in the Ajwa dates extract could potentially be primarily attributed to the presence of many bioactive substances, including but not limited to vitamin C, B-complex, antioxidants, and other bioactive constituents (Alharbi et al., 2021; Al-Shahib & Marshall, 2003; Ribeiro et al., 2015). The research

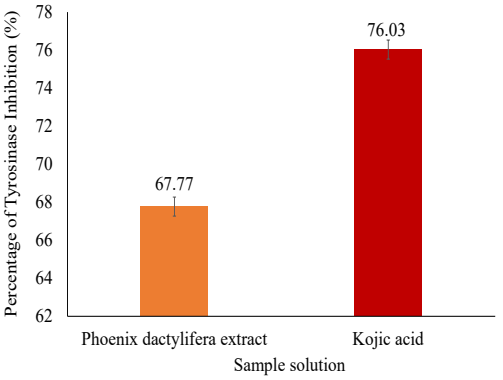

Figure 2. The percentages of tyrosinase inhibition on Kojic acid and *P. dactylifera* fruit extract

performed by Meer et al. (2017) showed that incorporating *Phoenix dactylifera* extract into the formulation of a topical cream reduced skin melanin levels, exhibiting whitening effects. This discovery has demonstrated that the extract derived from date fruit exhibits promising potential as a lightening agent.

The Value of SPF

Sunscreen contains a combination of active components that can absorb, reflect, and disperse solar radiation. The efficacy of sunscreen can be evaluated by utilising the SPF. Sunscreen products need to possess a standardised SPF suitable for human skin, considering its inherent characteristics and prevailing weather conditions, to mitigate the harmful effects of UV rays that pose a significant risk and can cause harm to the skin. Consistent utilisation of products containing SPF has the potential to mitigate and safeguard against the detrimental consequences of UV radiation exposure. The UV electromagnetic spectrum can be categorised into three distinct sections, namely ultraviolet A (UVA, 320 to 400 nm), ultraviolet B (UVB, 290 to 320 nm), and ultraviolet C (UVC, 200 to 290 nm).

The sunscreen activity assessment involves measuring the transmission spectrum of the *P. dactylifera* extract within the wavelength range of 200 to 400 nm. The absorbance acquired from the spectrum is utilised to compute SPF. Figure 3 illustrates the absorption spectrum of UVA, UVB, and UVC for both date extract and other commercially available UV sunscreen active components. The provided figure depicts the potential of the samples as UV sunscreen agents, as determined by the highest intensity peak observed in the UV zone. The current study observed that the transmission spectrum of date extract displayed notable photoprotective activity. The figure in the study indicates the presence of distinct spectrometric absorption peaks in the UVC and UVB regions, which implies a potential for photo-protection.

The *P. dactylifera* fruit extract has an absorption peak wavelength (λ_{max}) of 210 nm in the UVC region and 290 nm in the UVB region. UVC radiation is efficiently absorbed by the ozone layer located in the stratosphere. However, UVA and UVB radiation can penetrate the Earth's atmosphere, directly impacting human populations and ecosystems. The penetration of UVA and UVB rays into the skin can result in many effects, including sunburn, pigmentation changes, erythema, and inflammation. In contrast, the effects of UVA radiation become apparent only

after a longer period of sun exposure, regardless of the dosage levels (Fonseca & Rafaela, 2013). The outcomes of this study suggest that the extraction of dates can mitigate the occurrence of sunburns and pigmentation, which are predominantly attributed to exposure to UVB radiation (Fonseca & Rafaela, 2013). The detrimental consequences of sun exposure caused by UVB rays are more closely associated with immediate skin damage, such as induced erythema, as opposed to the detrimental consequences of sun exposure caused by UVA rays, which only become evident following an extended duration of exposure (Herrling et al., 2006).

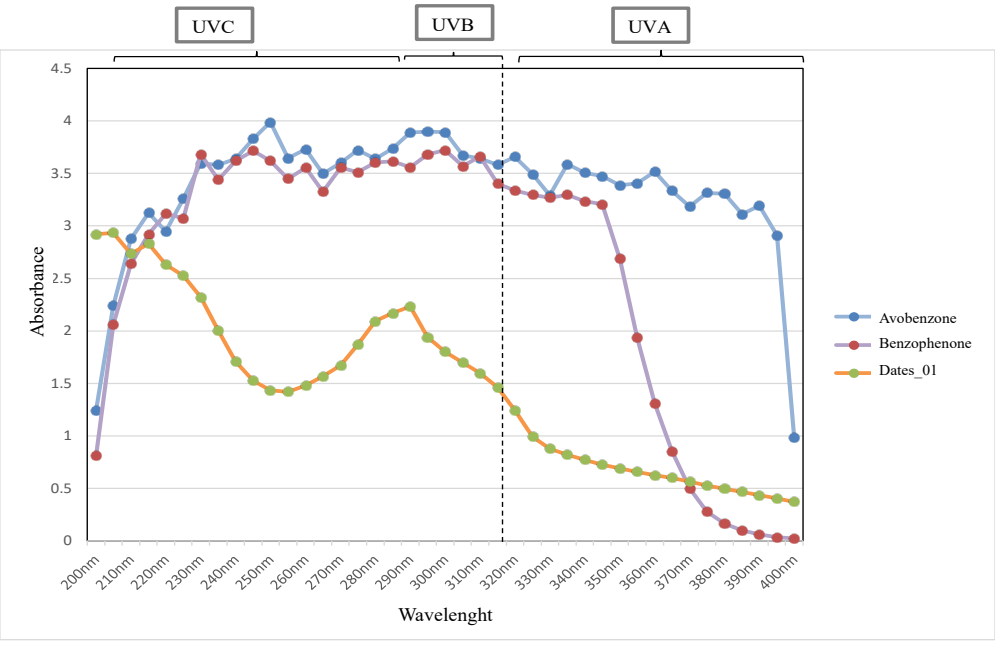

Figure 3. Spectrophotometric absorption profile of fruit extract of *Phoenix dactylifera*

Note. UVA = Ultraviolet A; UVB = Ultraviolet B; UVC = Ultraviolet C

Table 2 displays the calculations to determine the SPF value of *P. dactylifera* extract compared with other commercial UV sunscreen active ingredients. The level of UVB protection offered by a product can be quantified through its SPF, indicating that goods with higher SPF values offer greater defence against the detrimental consequences of solar radiation compared to those with lower SPF values. The table shows that date extract showed an SPF value of 17.09, compared to avobenzone and benzophenone as standard, with values of 37.45 and 36.21 at a concentration of 1 mg/ml, respectively. It is undeniable that this date extract has potential as a sunblock. According to European Union guidelines, products with SPF values of 6 and 10 are classified as low, medium (SPF 15, 20, 25), high (SPF 30, 50), and very

Table 2
SPF value of the extract and standards

No.	Wavelength (nm)	Erythema affect value	Dates extract	Avobenzone	Benzophenone
1	290	0.0150	0.3349	0.5838	0.5339
2	295	0.0817	1.5853	3.1843	3.0062
3	300	0.2874	5.1714	11.1919	10.6959
4	305	0.3278	5.5673	12.0411	11.7008
5	310	0.1864	2.9777	6.7874	6.8220
6	315	0.8390	1.2260	3.0058	2.8540
7	320	0.0180	0.2236	0.6583	0.6006
	Sun protection factor		17.0865	37.4526	36.2134

Note. The sun protection factor (SPF) value for the Ajwa date extract is 17.0865 compared to the other standards of avobenzone and benzonphenone, which are 37.4526 and 36.2134, respectively

high (SPF 50+). Based on Suva (2014), any substance with an SPF value greater than two is considered to have good sunscreen activity. The value of marketed sunscreen lotion with concentration (200 µg/ml) is approximately 10.66 ± 0.006 (Suva, 2014). The level of SPF directly correlates with the degree of protection a sunscreen offers against UV radiation. Therefore, the extract of *P. dactylifera* was discovered to fit the range of the best sunscreen agents.

The presence of antioxidants significantly supports the potential of the aqueous extraction of *P. dactylifera* in the absorbing UV region. In addition, the effectiveness of including antioxidant chemicals in sunscreens is believed to be higher when compared to using a conventional UV filter alone. Indeed, the combination received recognition within the realms of pharmacological and cosmetic scientific literature, as well as in commercially available products. Due to the obvious presence of phenolic chromophores, these antioxidants can provide photoprotective action (Morocho-Jácome et al., 2021). Among these phenolics, the literature indicates that antioxidant constituents such as flavonoids are the primary factor protecting plants from ultraviolet radiation. Flavonoids are polyphenols with two aromatic rings (two chromophores) that absorb at 240 to 285 nm and 300 to 550 nm.

Natural ingredients from various natural sources are frequently employed in formulating natural or vegan cosmetics. Furthermore, the cosmetic industry is witnessing a notable surge in introducing innovative, environmentally friendly products. Plants have met many human needs because their presence of various primary or secondary metabolites makes them active ingredients in cosmetics and medicines. Furthermore, diosmin compound high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis supports the function. According to Morocho-Jácome

et al. (2021), the maximum absorbance peaks of diosmin (in an alkaline medium) are at 268 and 285 nm. Consequently, the aqueous extract derived from *P. dactylifera* exhibits potential as an active ingredient for human protection through its ability to absorb, scatter, and reflect radiation, namely UVB rays.

Alharbi et al. (2021) have identified many phenolic acids, namely vanillic acid, ferulic acid, protocatechuic acid, caffeic acid, cinnamic acid, and catechin acid, produced from date fruit that exhibit cosmetic qualities. Vanillic acid and ferulic acid are present in fruit and seed of date. The bioactivity of vanillic acid has been shown to have potential benefits in the context of skin lightening and the reduction of skin pigmentation (Hong et al., 2006; Willcox et al., 2004). Moreover, the utilisation of ferulic acid has been extensively observed within the cosmetic sector. Ferulic acid plays a protective role in the primary structures of the skin, including keratinocytes, fibroblasts, collagen, and elastin. The substance hinders the process of melanogenesis, promotes the formation of new blood vessels (angiogenesis), and speeds up wound healing. The compound is commonly utilised in skincare formulations for its photoprotective properties, ability to postpone skin photoaging processes, and contribution to skin brightening (Zduńska et al., 2018).

Furthermore, protocatechuic acid has been found to contribute to attenuating the skin ageing process. A study examining the functional properties of protocatechuic acid (PCA) through experimentation on an *ex vivo* model of human skin has demonstrated that this particular phenolic molecule has the ability to enhance collagen formation (S. Shin et al., 2020). The results of this study indicate that PCA may have the ability to produce an anti-wrinkle effect in human clinical trials. At the same time, catechins enhance collagen organisation and function as a binding agent. The stabilisation of collagen by the plant polyphenol catechin has been shown through experimental and computational research. These studies suggest that hydrogen bonding and hydrophobic interactions play a significant role in this stabilisation process (Hong et al., 2006).

CONCLUSION

The results of the present study demonstrate that the fruit of *P. dactylifera* displays significant potential in the fields of cosmeceuticals, focusing on skin pigmentation and collagen enhancement. Using botanical constituents in cosmetic formulations presents a superior alternative to chemical alternatives. The natural origin of antioxidants derived from Ajwa dates is that they are rich, safe, and well-tolerated by the skin compared to synthetic chemicals that may raise concerns about potential long-term effects and interactions with the skin. Moreover, the proposed method presents a potentially superior option to using artificial skin anti-ageing chemicals in various industries, as it offers enhanced safety, cost-effectiveness, and efficiency. The aqueous extract of *P. dactylifera* has

promising potential as a cosmeceutical ingredient owing to its anti-tyrosinase and anti-collagenase activities, as well as its ability to provide substantial protection against UV radiation, thereby sheltering the skin against the harmful effects of sunshine.

In summary, antioxidant-rich date extract offers a natural and holistic approach to skin ageing, potentially providing various benefits through its diverse bioactive compounds.

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Evaluation of Foliar Application of *Elusine indica* Extract on Growth, Photosynthesis, and Osmoprotectant Contents in Maize under Drought Stress

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ABSTRACT

To investigate the effects of foliar application of different concentrations of *Elusine indica* extract (EIE) on growth, photosynthesis, and osmoprotectant contents in maize under drought stress. The weed powder was extracted using methanol, followed by a solid-liquid extraction procedure. Plants were sprayed with three different concentrations of EIE at 1, 3, and 5 g/L and morphological parameters, chlorophyll, relative water content (RWC), soluble sugar, proline, protein, glutathione (GSH), and malondialdehyde (MDA) contents were determined. The results showed that drought stress led to a decline in morphological characteristics, RWC and soluble sugar and increased proline, protein, GSH, and MDA contents. However, foliar application of EIE significantly improved plant height, fresh and dry weight, chlorophyll content, RWC, soluble sugar, and GSH, while the proline level was diminished compared to drought treatment. Soluble sugar showed a significant positive correlation with fresh and dry weight (r = 0.742 and 0.783, $p < 0.01$) and a strong negative correlation with MDA (r = -0.459, $p < 0.05$). Therefore, this result indicated that EIE can be used as an inexpensive and environmentally friendly biostimulant to help plants enhance tolerance to drought.

Keywords: Biostimulant, drought tolerance, maize, plant physiology, weed extract

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INTRODUCTION

Plants depend on their root systems to absorb water from the soil for sustaining growth, while drought stress, as a major abiotic stress factor, severely affects the growth and development of crops and reduces the yield (Gupta et al., 2020; Mohammadi Alagoz, Zahra, et al., 2023). With the exacerbation of the greenhouse effect, drought presents multi-region characteristics, is multi-frequency and unpredictable, causes economic losses, and threatens global food security (Trenberth et al., 2014). The growing population further exacerbates the challenge of meeting food demands in agriculture. Agricultural drought mainly depends on the evapotranspiration of plants, and only sufficient soil moisture can meet the water needs of plants (Goñi et al., 2018). Consequently, in arid regions, irrigation is the most direct and effective strategy to address crop water deficits. However, using irrigation can also give rise to issues such as high costs and soil salinisation. Additionally, the improper application of fertilisers and pesticides can further exacerbate the situation.

Drought induces plant apparatus such as chloroplasts and mitochondria to produce excessive reactive oxygen species (ROS), and the elevated ROS levels subsequently result in physiological disorders, reduced stomatal conductance, and degraded pigment, which negatively affect growth and development (Batra et al., 2014). Besides, studies have shown that drought stress increased the accumulation of proline, soluble sugars, and electrolyte leakage as well as decreased growth indices, relative water content, and triticale yield (Mohammadi Alagoz, Hadi, et al., 2023). Plants have developed intrinsic regulatory mechanisms, such as regulation of stomatal closure antioxidant enzyme activity and overexpression of genes, to improve drought stress tolerance, but these endogenous regulations are often inadequate (Hossain et al., 2017). Biostimulants have received widespread attention due to their positive effects in promoting plant growth, improving nutrient uptake efficiency and plant stress resistance (Goñi et al., 2018; Pourghasemian et al., 2020; Taha et al., 2020). Biostimulants are defined as "materials that contain substance(s) and/or microorganisms, whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and/or crop quality, independent of its nutrient content" (European Biostimulants Industry Council [EBIC], n.d.). Studies have shown that cypress leaf extract improves photosynthesis and antioxidative defence in zucchini seedlings under salt stress (ElSayed et al., 2022), and Wang et al. (2022) reported that pig blood-derived protein hydrolysate alleviates drought stress in tomato plants.

Maize (*Zea mays* L.) is a major economically important crop cultivated extensively worldwide. The maize yield is particularly sensitive to water scarcity during critical growth stages, such as the tasselling stage, because water deficit leads to a decline in pollen vigour, which further causes the reduction of corn kernels

and ultimately causes yield losses (Song & Jin, 2020). Thus, water deficit is the predominant factor limiting maize yield worldwide (Shemi et al., 2021). Studies have been reported to evaluate the effects of biostimulants on maize growth. Chen et al. (2023) reported that applying glycine betaine improves yield and water use efficiency (WUE) in maize under water deficit. Moreover, Tadros et al. (2019) illustrated that treated sweet corn using PERFECTOS® exhibited positive effects on agronomic performance.

Elusine indica is a common annual weed widely distributed in Asia, Africa, and South America and is extremely difficult to control because of its extreme stress resistance (Adoho et al., 2021). In addition, it is also considered one of the five most harmful invasive weeds, which usually grow in fields and compete with crops for nutrients, seriously affecting crop growth and yield (Okokon et al., 2010). Previous studies have illustrated that *E. indica* has anti-inflammatory, antimicrobial, and antioxidant activity (Iqbal & Gnanaraj, 2012). Based on the above content, the scientific and effective utilisation of weed resources can alleviate the detrimental impact of weeds on crops. Furthermore, extracts from weeds may serve as an economical biostimulant to promote plant growth. Thus, the hypothesis of this study is that EIE possesses antioxidant activity and has the potential to enhance drought tolerance, thereby stabilising plant growth. However, no study has been carried out to determine the effects of EIE on mitigating the negative impacts of drought stress on maize. To the best of our knowledge, the objectives of this study were to (1) quantify chemical constituents of EIE, (2) evaluate the effect of foliar spraying EIE on the growth, chlorophyll, and osmoprotectant contents of maize under drought stress, and (3) determine the reasonable concentration for foliar application of EIE. The findings of this study will provide a theoretical basis for the efficient utilisation of weed resources and the production of cheap biostimulants.

MATERIALS AND METHODS

Preparation and Analysis of EIE

All the chemicals were purchased from Chemiz (Malaysia). The *E. indica* weed was collected from a local crop field. The materials were extracted using the detailed method of Taha et al. (2020) with modifications. Each 10 g of materials was mixed with 80% methanol using an optimum ratio of 1/8 (w/v) sample weight to solvent volume. The mixtures were continuously shaken at 150 rpm for 24 hr via an orbital shaker. The extraction samples were filtered with Whatman No.1 filter paper (Cytiva, United Kingdom), and the alcohol and excess water were evaporated under vacuum at 30°C using a rotary evaporator (CCA-111, EYELA, Japan). The EIE obtained from 10 g materials was made as an extract at a concentration of 1.0 g/L by dissolving in 10 L distilled water. The extracts were kept in the refrigerator at 4°C until use.

The EIE was analysed, and its chemical constituents (on a dry weight basis) are shown in Table 1.

Table 1
Chemical constituents of Elusine indica *extract (on a dry weight basis)*

Component	Unit	*Elusine indica* extract
Total carbon (C)	%	46.10
Total nitrogen (N)	%	3.69
Total phenolic	mg GAE/g	13.51
Total flavonoid	mg Lutin/g	26.66
Free proline	mg/g	9.97
Soluble sugars	mg/g	13.23
Protein	mg/g	19.15
Glutathione (GSH)	mg/g	24.01
Phosphorus (P)	g/kg	0.32
Potassium (K)	g/kg	62.08
Calcium (Ca)	g/kg	0.39
Magnesium (Mg)	g/kg	1.31
Iron (Fe)	mg/kg	52.00
Copper (Cu)	mg/kg	4.29
Zinc (Zn)	mg/kg	61.82
Manganese (Mn)	mg/kg	6.72
Boron (B)	mg/kg	3.20

Plant Material and Growing Conditions

This experiment was conducted from March to June 2023 in a glasshouse located at the Faculty of Agriculture, Universiti Putra Malaysia, Selangor, Malaysia, with coordinates approximately 2° 98' N and 101° 73' E. Maize seeds (*Zea mays* L., hybrid F1 316, Malaysia) were procured from a local supplier (Agroniche Sdn. Bhd., Malaysia). Four seeds were sown in plastic pots with a diameter of 24 cm and a depth of 28 cm. Each pot was filled with sandy soil totalling 14 kg; the ratio of sand and soil is 1 to 4. The basic properties of soil are total carbon 1.55 g/kg, total nitrogen 2.10 g/kg, total phosphorus 3.87 g/kg, available phosphorus 0.53 g/kg, total potassium 3.40 g/kg, and CEC 4.51 cmol/kg. A completely randomised design (CRD) was designed, and an equal amount of water was added to each pot until drought stress was imposed. After two weeks, the healthiest seedlings were retained, while the others were removed. A total of 15 g compound fertiliser (N: P: K, 15:15:15) was applied to each pot: 5 g at sowing, 5 g after 28 days, and 5 g after 56 days, respectively. The average net greenhouse temperature during the day was 38.80±2.60°C, while during the night, it was 27.52±1.39°C. Additionally, the average net relative humidity during the day was 40.14±4.45%; at night, it was 82.00±4.42%.

EIE Treatments and Experimental Design

The plants grew under normal water conditions until tasselling (56 days). Then they were divided into five treatments: CK (well-watered, 75% soil field capacity), drought (35% soil field capacity), EIE1 (drought with 1 g/L EIE), EIE3 (drought with 3 g/L EIE), and EIE5 (drought with 5 g/L EIE). The concentrations of 1 and 5 g/L were selected based on previous studies by Pourghasemian et al. (2020) and Taha et al. (2020), and an intermediate concentration of 3 g/L was also designed. Each treatment was performed in 4 repetitions. EIE was applied by foliar spraying once a week, totalling four applications. Care was taken to ensure that each application covered all the leaves evenly.

A soil moisture meter monitored each pot's moisture daily, and appropriate volumes of water were added to maintain the desired soil water conditions. After the final spray (84 days), the top leaves of maize were collected and stored at -80°C until they were ready to analyse plant physiology and biochemistry.

Growth Characteristics and RWC

Plant height was measured using a rule. The plant's fresh weight (FW) was measured immediately, and the dry weight was measured after the sample was placed in the oven to constant weight.

Leaf RWC was assessed using the methods described by Wang et al. (2022). Briefly, the leaves were measured to FW and immersed in distilled water for 24 hr to record the turgid weight (TW). Subsequently, the leaves were subjected to oven drying to determine their dry weight (DW). The RWC was calculated using the following formula:

$$RWC\ (\%) = [(FW-DW) / (TW-DW)] \times 100\%$$

Determination of Chlorophyll Content

The contents of chlorophylls and carotenoids were assessed and then calculated, as detailed in Pourghasemian (2020). Samples of 0.3 g fresh squash leaves were extracted in 20 ml 80% acetone and left in the dark. After 24 hr of incubation, absorbance (A) was recorded at wavelengths of 646.8, 663.2, and 470 nm using an Absorbance Microplate Reader (Tecan Trading AG, Switzerland).

Determination of Osmoprotectant

Soluble sugar content was determined using the anthrone colourimetric method (Dubois et al., 1951). Briefly, 0.3 g fresh maize samples were homogenised with 10 ml 80% ethanol and then centrifuged at 10,000 × *g* at 4°C for 10 min using a centrifuge (D-78532, Hettich, Germany). The resulting extract was mixed with 5ml anthrone reagent and then heated at 100°C in a water bath for 10 min, and the absorbance was read at 630 nm after cooling to room temperature. Proline quantification was determined following the method of Bates et al. (1973), and the absorbance was read at 520 nm. The protein content was measured according to Bradford (1976) using bovine serum albumin as the standard.

Determination of GSH and Lipid Peroxidation

GSH contents were quantified using the method described by Griffith (1980). Briefly, 0.3 g fresh leaves were extracted with 10 ml 5% trichloroacetic acid (TCA) and centrifuged at 4,000 × *g* for 15 min. The 2 ml of extract was mixed with 0.4 ml of 5, 5'-dithiobis (2-nitrobenzoate) (DTNB); the absorbance was read at 412 nm.

MDA indicated lipid peroxidation following De Vos et al. (1991). The 2 ml of extract (the same as GSH) was mixed with 2 ml of 0.6% 2-thiobarbituric acid (TBA). The mixture was heated at 95°C for 15 min and cooled immediately in an ice bath. The absorbance of the supernatant was read at 532 nm after centrifugation at 4,000 × *g* for 10 min.

Statistical Analysis

Data were analysed using the SPSS (ver. 25.0). Significant differences between treatments were calculated by one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test at $p < 0.05$.

RESULTS

Morphological Characteristics

Table 2 indicates that fresh and dry weight and number of leaves were ($P < 0.01$) influenced significantly by drought and EIE treatments. Compared to the CK treatment, drought stress decreased plant height, fresh and dry weight, and number of leaves by 3.6, 42.2, 48.5, and 13.6%, respectively. In addition, the application of EIE exhibited a positive effect on all assessed morphological characteristics under drought conditions. Among them, EIE treatments increased plant height by 2.9–15.1%, fresh weight by 51.6–64.6%, dry weight by 11.8–71.2%, and number of leaves by 7.1–11.9%.

Table 2
Effect of drought stress and foliar application on growth of maize

Treatment	Plant height (cm)	Fresh weight (g)	Dry weight (g)	No. of leaves
CK	165.15±11.62ab	165.18±7.51a	64.47±4.49a	12.15±0.50a
Drought	159.15±10.34b	95.53±7.58c	33.23±2.31b	10.50±0.58b
EIE1	183.13±14.76a	144.79±10.67b	37.67±2.81b	11.25±0.50b
EIE3	163.87±14.84ab	147.29±9.47b	53.03±2.62ab	11.75±0.50ab
EIE5	171.70±11.80ab	157.28±11.80ab	56.88±7.36ab	11.50±0.58ab
		Source of variation		
Drought	-0.3	-0.983**	-0.981**	-0.882**
EIE	0.452	0.937**	0.916**	0.655**

Note. Different lowercase letters indicate significant differences between the treatments ($p < 0.05$); Analysis of variance results of the main effects and their interaction effect on the growth of maize are shown as ** as significantly as $p < 0.01$, respectively; CK = Well-watered; EIE1 = Drought with 1 g/L EIE; EIE3 = Drought with 3 g/L EIE; EIE5 = Drought with 5 g/L EIE; EIE = *Elusine indica* extract

Chlorophyll Content and RWC

Table 3 illustrates that drought and application of EIE affected chlorophyll content and RWC responses of maize. Drought treatment increased chlorophyll *a*, chlorophyll *b*, and carotenoid content by 48.5, 3.5, and 13.1%, respectively, and decreased RWC by 11.6%, indicating that less water resulted in a high chlorophyll content. EIE1 treatment exhibited the highest content of chlorophyll *a*, chlorophyll *b*, and carotenoid with increases of 57.4 and 5.9%; 36.5 and 31.8%; and 44.1 and 27.3% compared to the CK and drought treatments. Besides, applying EHE improved RWC under drought conditions, in which the EHE5 treatment exhibited the highest RWC and was significantly higher than all other treatments ($p < 0.05$).

Table 3
Effect of drought stress and foliar application on chlorophyll and relative water content (RWC) of maize

Treatment	Chl *a* (mg/g)	Chl *b* (mg/g)	Carotenoid (mg/g)	RWC (%)
CK	2.04±0.11c	2.55±0.15b	2.36±0.21c	60.08±5.63b
Drought	3.03±0.24ab	2.64±0.13b	2.67±0.14bc	53.12±4.69b
EIE1	3.21±0.19a	3.48±0.35a	3.40±0.34a	58.17±9.14b
EIE3	2.85±0.26b	2.62±0.06b	2.78±0.28b	57.92±2.90b
EIE5	3.02±0.20ab	2.50±0.10b	2.65±0.08bc	71.20±6.67a
		Source of variation		
Drought	0.95**	0.35	0.713*	-0.613
EIE	-0.014	0.231	0.323	0.466

Note. Different lowercase letters indicate significant differences between the treatments ($p < 0.05$); Analysis of variance results of the main effects and their interaction effect on the chlorophyll (Chl) and RWC are shown as * and ** as significantly as $p < 0.05$ and 0.01, respectively; CK = Well-watered; EIE1 = Drought with 1 g/L EIE; EIE3 = Drought with 3 g/L EIE; EIE5 = Drought with 5 g/L EIE; EIE = *Elusine indica* extract

Osmoprotectants, GSH, and MDA

Table 4 shows that drought and application of EIE had significant main effects on proline, sugar, protein, GSH, and MDA ($p < 0.05$ and 0.01). Drought significantly increased proline, protein, GSH, and MDA content by 50.6, 13.7, 308.3, and 1.78%, respectively, and decreased sugar by 31.4% compared to CK treatment ($p < 0.05$). In addition, application of EIE improved sugar by 15.7–20.7%, protein by 6.3–11.1%, GSH by 18.4–26.5%, and MDA by 18.5–14.4%, and decreased proline by 17.8–20.3% compared to drought treatment.

Correlation and Principal Component Analysis

The results presented in Table 5 indicated several noteworthy correlations among the examined characteristics. Notably, a significant positive correlation was observed between sugar content and fresh weight ($r = 0.742$), dry weight ($r = 0.783$), and number of leaves ($r = 0.754$) ($p < 0.01$).

Conversely, sugar content exhibited a strong negative correlation with proline (r = -0.754), MDA (r = -0.459), and GSH (r = -0.68). Furthermore, the data revealed a positive correlation between GSH and proline (r = 0.523) as well as MDA (r = 0.768). Additionally, proline exhibited a significant negative correlation with fresh weight (r = -0.726), dry weight (r = -0.751), and the number of leaves (r = -0.675) (p < 0.05 and 0.01).

Table 4
Effect of drought stress and foliar application on osmoprotectants, glutathione (GSH), and malondialdehyde (MDA) of maize

Treatment	Proline (µg/g)	Sugar (mg/g)	Protein (mg/g)	MDA (µmol/g)	GSH (mg/g)
CK	136.84±19.35c	2.04±0.09a	1.83±0.13b	13.19±0.60c	0.12±0.03c
Drought	206.12±13.95a	1.40±0.14c	2.08±0.11a	14.97±0.94b	0.49±0.07b
EIE1	164.24±15.98b	1.69±0.13b	2.21±0.16a	15.96±1.03ab	0.58±0.06ab
EIE3	169.40±17.11b	1.67±0.10b	2.26±0.14a	16.89±1.38b	0.59±0.13ab
EIE5	168.40±10.01b	1.62±0.09b	2.31±0.18a	17.12±1.51a	0.62±0.08a
Source of variation					
Drought	0.921**	-0.954**	0.848**	0.792*	0.969**
EIE	-0.797**	0.738**	0.268	0.535*	0.49*

Note. Different lowercase letters indicate significant differences between the treatments (p < 0.05); Analysis of variance results of the main effects and their interaction effect on the proline, sugar, MDA, and GSH are shown as * and ** as significantly as p < 0.05 and 0.01, respectively; CK = Well-watered; EIE1 = Drought with 1 g/L EIE; EIE3 = Drought with 3 g/L EIE; EIE5 = Drought with 5 g/L EIE; EIE = *Elusine indica* extract

Table 5
Correlation coefficients of total morphological, chlorophyll, and osmoprotectant characteristics

	PH	FW	DW	NL	Chl *a*	Chl *b*	Caro	RWC	Proline	Sugar	Protein	MDA	GSH
PH	1												
FW	0.31	1											
DW	0.159	0.971**	1										
NL	0.035	0.776**	0.814**	1									
Chl *a*	0.126	-0.54*	-0.657**	-0.613**	1								
Chl *b*	0.399	-0.038	-0.127	-0.235	0.446*	1							
Cart	0.262	-0.108	-0.226	-0.329	0.688**	0.865**	1						
RWC	0.25	0.234	0.229	0.02	0.097	-0.12	-0.105	1					
Pro	-0.18	-0.726*	-0.751**	-0.675**	0.446*	0.002	0.121	-0.305	1				
Sugar	0.133	0.742**	0.783**	0.754**	-0.689**	-0.032	-0.194	0.015	-0.754**	1			
Protein	0.256	-0.287	-0.425	-0.229	0.589**	0.096	0.266	-0.002	0.340	-0.445*	1		
MDA	-0.061	-0.011	-0.118	-0.212	0.566*	0.069	0.293	0.22	0.269	-0.459*	0.539*	1	
GSH	0.201	-0.294	-0.415	-0.491*	0.75**	0.182	0.409	0.131	0.523*	-0.68**	0.737**	0.768**	1

Note. PH = Plant height; FW = Fresh weight; DW = Dry weight; NL = Number of leaves; Chl *a* = Chlorophyll *a*; Chl *b* = Chlorophyll *b*; Caro = Carotenoid; RWC = Relative water content; MDA = Malondialdehyde; GSH = Glutathione; * and ** as significantly as p < 0.05 and 0.01, respectively

DISCUSSION

Water deficit reduces the plant's capacity to uptake carbon dioxide and decreases stomatal conduction, affecting photosynthesis, and ultimately limiting plant growth. In this study, foliar application of EIE can significantly mitigate negative effects caused by water deficit by regulating photosynthesis, osmotic adjustment, and antioxidant defence. Interestingly, drought stress decreased plant height, fresh and dry weight, and number of leaves of maize plants, whereas application of EIE exhibited positive effects on plant growth (Table 2). This result is consistent with Anjum et al. (2011), who reported that drought stress reduced the gas exchange, limiting maize growth and productivity. The improved effect in this study can be attributed to the fact that EIE is abundant in essential nutrients such as nitrogen (N), phosphorus (P), and potassium (K), as well as osmoprotectants like soluble sugars and proline, and antioxidant including phenolic compound and flavonoids (Table 1).

The presence of these crucial components in EIE gives it the potential to mitigate the adverse effects of drought stress. Moreover, Taha et al. (2020) reported that foliar spraying of pollen grain extract could enhance the growth characteristics of basil and pointed out that biostimulant alleviates drought stress by promoting cell division and elongation and repairing plant nutritional status. Additionally, our finding demonstrated that applying EIE improved the chlorophyll contents and leaf RWC of stressed maize plants, reflecting the enhanced photosynthetic capacity and water uptake efficiency. This finding further confirms the beneficial effects of EIE in enhancing drought tolerance.

Plants' exposure to drought will stimulate the regulation of their internal physiological and biochemical metabolic systems and reduce oxidative damage by enhancing drought tolerance (Rai et al., 2012). One protective mechanism is that accumulating osmoprotectants such as proline and soluble sugar helps plants regulate stomatal pressure and enhance photosynthesis (Gill & Tuteja, 2010). A previous study found that pumpkin seed protein hydrolysate could improve the tolerance of *Phaseolus vulgaris* to salt stress by increasing the content of essential nutrients, proline, and soluble sugar (Sitohy et al., 2020). Moreover, Taha et al. (2020) reported that pollen grains extract increased proline and soluble sugar content in *Ocimum basilicum*. In the present study, EIE treatments led to a significant increase in soluble sugar content while causing a decline in proline content under drought stress conditions (Table 4). This result is similar to those found by Pourghasemian et al. (2020), who thought that under drought stress, foliar application with biostimulants led to a substantial effect on inducing plants' drought tolerance by improving protein and antioxidant content as well as reducing proline levels, further enhancing gas exchange and chlorophyll content.

GSH is a major low molecular-weight antioxidant compound in plants, and it acts as a redox-active molecule and plays a

crucial role in regulating the ascorbic acid - glutathione (AsA-GSH) cycle (Galant et al., 2011). This study showed that applying EIE significantly increased GSH content compared to drought treatment (Table 4). This finding was consistent with Taha et al. (2020), who reported that GSH and AsA can alleviate the damage from excessive accumulation of ROS, allowing plants to establish an internal defence mechanism to resist drought-induced oxidative stress. MDA production can be used as an indicator to evaluate the oxidative stress-induced free radical damage of cell membranes (ElSayed et al., 2022). Wang et al. (2022) reported an increase in MDA concentration in tomato plants under drought stress, which was markedly reduced after pig blood-derived protein hydrolysate application. Similarly, Pourghasemian et al. (2020) reported that foliar application of liquorice extracts reduced MDA content in sesame and mitigated drought stress.

However, our study showed that the application of EIE increased the level of MDA and had a positive correlation with the increase in concentration. This may be due to improper use of phytostimulant concentrations or the presence of phytohormone-stimulated oxidation in EIE itself. In addition, the findings from Goñi et al. (2018) illustrated that after adding seaweed extract, the MDA concentration in plants exhibited an initial decrease over a specific period, followed by an increase towards the end of the experiment. This also indicated that the effect of biostimulants is time-sensitive, and the application frequency is critical for substantial effects. Overall, EIE as a biostimulant is more beneficial to plants than harmful. The rational utilisation of weed resources can effectively reduce the direct competition with crops for nutrients and provide a feasible formula for a biostimulant application strategy.

CONCLUSION

Foliar application of EIE significantly improves growth performance and photosynthesis capacity by increasing chlorophyll and leaf RWC and accumulating soluble sugar and GSH. This finding indicates that EIE has the potential as a mitigator to mitigate adverse effects caused by drought stress. Moreover, *E. indica* is easy to obtain. Thus, EIE can be used as an inexpensive biostimulant to enhance plant tolerance through foliar spraying. However, the effects of EIE application on maize yield and intrinsic gene regulation are unclear and will be verified in future field trials.

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Metabolic and Biochemical Performances of Abaca (*Musa textilis* Née) under Intermediate and Advanced Phase Agroforestry System

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ABSTRACT

Abaca, one of the potential fiber crops with high-quality fiber and promising economic value, is mainly established under the agroforestry system, for it is considered a non-primary crop. The study aims to observe the metabolic and biochemical performance as well as the fiber quality of abaca under the agroforestry system. The experimental design used in this study was nested with two types of agroforestry systems, i.e., intermediate phase (Fase Tengah, FT) and advanced phase (Fase Lanjutan, FL) and was conducted during the rainy season. Parameters observed in this study were divided into edaphic and climatic parameters, oxidative response parameters, foliage macro- and micronutrient, and fiber quality. Despite poor soil quality compared to FL, higher relative humidity (4.35%), lower temperature (2.73%), and lower shading intensity were observed in FT. Improved soil characteristics in FL, *viz.* soil water content (19.64%), organic carbon (72.89%), porosity (4.29%), cation exchange capacity (13.77%), and pH (35.13%), were unable to compensate plant stress induced by the high shading intensity at 83.99%. Consequently, it contributed to higher levels of malondialdehyde, superoxide anion, hydrogen peroxide, superoxide dismutase, peroxidase, and phenol by 0.07%, 1.86%, 32.66%, 0.08%, 14.63%, and 35.08%, respectively, due to shading stress. Nevertheless, ascorbic acid content in FL was lower (18.90%) compared to FT. Higher fiber diameter (23.53%) and tensile strength (18.77%) of abaca in FT were observed compared to

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FL. The improved microclimatic conditions under FT promoted the high adaptability of abaca to poor soil quality. Therefore, it contributed to enhanced growth and fiber quality compared to FL. Pruning is pivotal to managing shading intensity.

Keywords: Abaca, agroforestry, oxidative stress, shading stress

INTRODUCTION

Despite decreasing land productivity topping 1.66 million ha globally, increasing demand for productive agricultural land promotes land use competition between initial cash crops and other crops (Food and Agriculture Organization of the United Nations [FAO], 2022). At the same time, the rise of biomaterial utilization derived from natural fiber in the synthetic industry has been considered to promote a significant impact on people and the planet. However, due to high maintenance and complicated production processes, farmers show little interest in cultivating fiber crops in a monoculture system. Therefore, they only cultivate fiber crops as secondary or supplementary crops (Yokokura, 1992). Abaca is one of the potential fiber crops commonly utilized in textile, electronic, medical, and paper industries. Abaca fiber has better durability than other natural fibers (e.g., sisal). Hence, it is commonly used as currency paper (Franck, 2005). Historically, Indonesia cultivated abaca on a plantation scale under Dutch colonialization, primarily in North Sumatra, Lampung, and East Java (Sudjindro, 2008). In 2009, Indonesia exported 92 tons of abaca fiber, accounting for USD 13,000 (FAO, 2013). Despite its profitable quality, abaca is commonly cultivated in agroforestry systems instead of monoculture because it is not considered profitable as a cash crop.

The agroforestry system is suitable for abaca as it provides shade to avoid the detrimental effects of high light intensity (Siles et al., 2013). Besides, the species diversity contributes to high organic matter and improvement of soil quality (Asigbaase et al., 2020). Abaca belongs to C3 crops, indicating strong high-light sensitivity (Tian et al., 2017); therefore, shading is pivotal. However, growth inhibition induced by shading might lead to crop death, which is determined by shading intensity, shading degree, and shading duration (Y-.B. Wang et al., 2021). Suitable shading intensity for abaca growth ranges from 40-50%, using multi-purpose tree species (MPTS) as shade trees with appropriate planting distance (Petronilo et al., 2016). Light transmission in the abaca-MPTS system could reach 36 and 9% above and under the abaca canopy, respectively. The system showed a more effective growth environment than the abaca-coconut system because of better soil quality; however, shading intensity and species density should be managed through pruning and thinning (Bande et al., 2016). Abaca cultivated under 50% shade produced longer, larger, and heavier pseudostem with higher tensile strength than 0% shade (Bande et al., 2013).

Shading stress can adversely impact stomatal conductivity, which subsequently leads to the production of reactive oxygen species (ROS). Hydrogen peroxide (H_2O_2) and nitric oxide (NO) are signals promoting stomatal closure under darkness. The concentration of H_2O_2 increases due to the activation of respiratory burst oxidase homolog D/F (RBOHD/F), which subsequently induces signaling cascades to produce NO in guard cells, causing stomatal closure (Zhang et al., 2017). Oxidative stress triggers the production of enzymatic and non-enzymatic antioxidants to avoid cell damage. Soybean leaves under partial shading showed higher enzymatic antioxidants (e.g., superoxide dismutase [SOD], catalase [CAT], peroxidase [POD], and ascorbate peroxidase [APX]) at 25, 39, 24, and 18%, respectively, than without shading. Higher enzymatic antioxidant concentration was exhibited under full shading, indicating higher oxidative stress under respective conditions (Raza et al., 2020). To date, scientific study regarding abaca is limited to fiber quality under shading, microclimatic effect on biomass, or nutrient composition in vegetative and generative stages (Armecin, 2008; Armecin & Coseco, 2012; Armecin et al., 2011; Bande et al., 2012, 2016). Therefore, abaca's metabolic and biochemical performances will be investigated to comprehend the adaptability and fiber quality under the agroforestry system.

MATERIALS AND METHODS

Plant Materials and Growth Condition

Three-year-old abaca plants were established under two agroforestry systems in Watualang Village, Pitu Subdistrict, Ngawi, Indonesia in 2020 (Figure 1). Watualang village is located ±150 m above sea level. The experiment was conducted during December 2022–April 2023. An intermediate agroforestry system was established by combining abaca and MPTS (e.g., timber and fruit trees) and was planted in 2020 and 2019, respectively. Meanwhile, under an advanced agroforestry system, abaca was combined with a 20-year-old teak. The abaca plantation belongs to Getas Forest for Specific Purposes (Kawasan Hutan Dengan Tujuan Khusus [KHDTK]) management. It has flat terrain with a 0-8° slope.

Annual precipitation is approximately 2,000–2,400 mm. According to Schmidt-Fergusson, the climate is classified as climate zone D, with a Q value of approximately 60–100% (Chanan, 2019). The total rainfall during the experimental period was 337–751 mm, which is classified as very high according to the Meteorological Climatological and Geophysical Agency (2023). Meanwhile, the duration of daylight in the respective period was approximately 3–5 hr (Figure 2). Based on the International Union of Soil Sciences Working Group (2022) soil analysis, soil type in both agroforestry systems was classified as Pellic Vertisol (from Greek *pellos*: dusty) because the color and chroma of the upper 30 cm soil layer were 3 and 1, respectively, with soil texture classified as clay—silty clay.

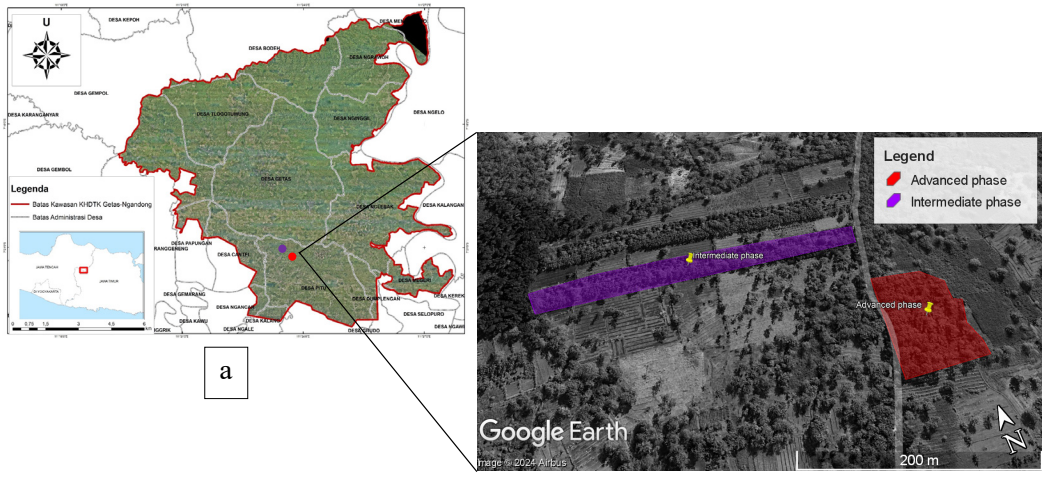

Figure 1. Geographical location of the study area: (a) Modified from Emma (2017); (b) Modified using Google Earth Pro

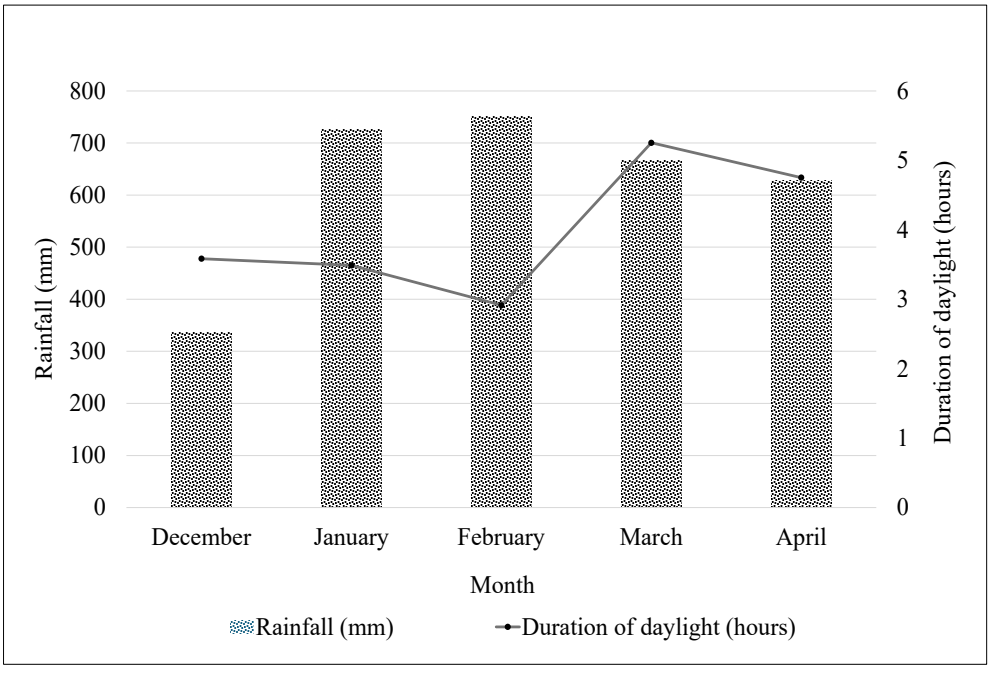

Figure 2. Rainfall intensity and duration of daylight during December 2022–April 2023 in Ngawi

Note. Rainfall range: <50 mm = Low; 50–150 mm = Moderate; 150–300 mm = High; >300 mm = Very high

Experimental Design and Sampling Method

The study was conducted using nested design with two treatments, viz. intermediate phase (Fase Tengah, FT) and advanced phase (Fase Lanjutan, FL) agroforestry, consisting of 5 blocks (12.5 m x 12.5 m) in each system. The planting distance of abaca is 3 m x 3 m, as well as for shading trees. The abaca agroforestry system was established by implementing an alternate cropping system. The agroforestry phase was determined based on Sasaki et al. (2020) and Suryanto et al. (2014), which classified the phase according to shading intensity. Intermediate phase agroforestry was categorized as abaca grown under 50–75% shading intensity; meanwhile, advanced phase was determined by >75% shading intensity. Shading intensity was determined based on canopy projection constructed using SeXI-FS software (ver. 2.1.1). The shading intensity range was adjusted based on the existing abaca cultivation at the location. Sampling locations in this research were determined based on three considerations related to uniformity in abaca age, cultivar, and plant management (*viz.* fertilization) to reduce any variability caused by non-treatment factors.

The growth dimension measured in abaca was diameter in different heights (10 cm, 30 cm, and 1.3 m), which was used in data analysis to estimate nutrient uptake based on an allometric equation for biomass estimation. Meanwhile, the growth dimensions of shading trees were height, diameter at breast height (dbh), branch-free height, outer crown height, and crown width in four directions. The shading intensity in both agroforestry systems was determined based on crown projection illustrated using SeXI-FS (ver. 2.1.1) as the preliminary observation. Canopy closure was determined by calculating intersection spots on the SeXI-FS (ver. 2.1.1) projection. The calculation was modified based on canopy closure measurement using spherical densiometer (Strickler, 1959) and calculated using the equation 1:

$$\text{Shading intensity} = \frac{\text{Shaded intersection spots}}{\text{Total intersection spots}} \text{ x } 100\% \quad [1]$$

Leaf (fully expanded) samples were taken from 3 plants chosen randomly, representing the first, middle, and last rows of the block in each agroforestry system (Figure 3). The part of leaves used as samples was the distal half, away from the middle rib on both surfaces and was chosen from healthy leaves (Ekanayake et al., 1994). The samples were used to measure oxidative stress parameters and macro- and micronutrients. Soil samples taken around abaca plants were conducted in the same manner as leaves and were used for physical and chemical analyses. Pseudostems, 250 in total, were harvested and processed using a decortication machine for fiber quality analysis (physical and mechanical characteristics). The physical and mechanical quality of abaca fiber was fiber diameter and tensile strength, respectively.

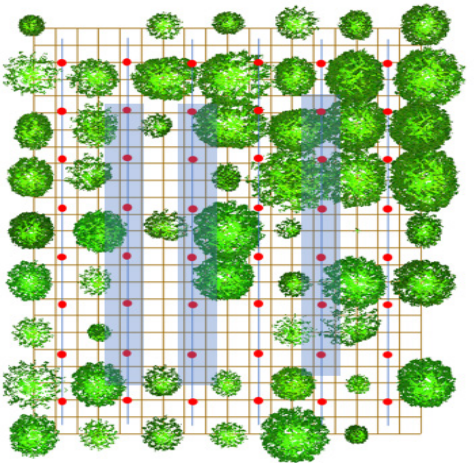

Figure 3. Sampling area of abaca leaves

Note. Red dot = Abaca; Blue blocks = Sampling area; The picture derived from block 1 of intermediate phase is an example of sampling area

ROS and Antioxidant Analyses

Oxidative stress parameters analyzed in the study were malondialdehyde (MDA), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), superoxide dismutase (SOD), peroxidase (POD), ascorbic acid, and phenol. MDA, $O_2^{\bullet-}$, H_2O_2, SOD, and POD analyses were conducted under low temperatures. The MDA was observed using the thiobarbiturate acid (TBA) method (Senthilkumar et al., 2021). The leaf sample (0.2 g) was homogenized using 3 ml of 0.1% (w/v) trichloroacetic acid (TCA, Merck, Germany) and centrifuged at 13,040 x *g* for 15 min. The supernatant (1 ml) was added into 2 ml of 0.5% TBA (Merck, Germany) in 20% TCA (Merck, Germany), then vortexed and incubated in a water bath at 90°C for 20 min. The mixture is later cooled in cold water for 10 min to stop the reaction. Absorbance was measured at 532 and 600 nm, respectively.

The $O_2^{\bullet-}$ and H_2O_2 were analyzed using the Griess method (Jahan et al., 2020) and the potassium iodide (KI) method (Zhou et al., 2006), respectively. Briefly, the $O_2^{\bullet-}$ analysis was conducted by homogenizing leaf sample (0.2 g) in 2 ml of 50 mM phosphate buffer (pH 7.8, Merck, Germany), then centrifuged at 13,040 x *g* and 4°C for 20 min. The supernatant (0.5 ml) was added into 0.1 ml of 10 mM hydroxylamine hydrochloride (Merck, Germany) and 0.5 ml of 50 mM phosphate buffer (pH 7.8, Merck, Germany). The mixture was incubated at 25°C for 30 min. Absorbance was measured at 540 nm.

The H_2O_2 was analyzed by homogenizing leaf sample (0.5 g) in 3 ml of 0.1% TCA and centrifuged at 13,040 x *g* and 4°C for 15 min. Active charcoal (0.15 g) was added into the supernatant in the new test tube and centrifuged at 5,796 x *g*, 4°C for 1 min. The reaction mixture was made by combining 0.5 ml of 100 mM potassium phosphate buffer (pH 7.4, Merck, Germany) and 2 ml of 1 M potassium iodide (KI, Merck, Germany) reagent, then 1 ml supernatant was added into the mixture and incubated in the dark for 1 hr. Absorbance was measured at 390 nm.

The SOD was analyzed according to pyrogallol autoxidation (Marklund & Marklund, 1974) with modification from Li (2012). The leaf sample (0.5 g) was homogenized using chilled 5 ml of 50 mM phosphate buffer (pH 6.5, Merck,

Germany) and centrifuged at 9,056 x *g*, 4°C for 15 min. The reaction mixture was made of 2.5 ml of 0.05 M Tris-HCl (pH 7.4, Merck, Germany), 100 µl of 1 mM disodium ethyleneiaminetetraacetate dihydrate (Na_2EDTA, Merck, Germany), 100 µl phosphate buffer (50 mM, pH 6.5), 100 µl sample extract, and 200 µl pyrogallol (Merck, Germany). Absorbance was measured kinetically at 325 nm for 3 min at 30-s intervals.

The POD was also analyzed based on pyrogallol autoxidation (Alexander, 1966). The leaf sample was homogenized using chilled 3 ml of 100 mM phosphate buffer (pH 7, Merck, Germany) and centrifuged at 11,866 x *g* and 4°C for 15 min. The reaction mixture was made of 2.1 ml ultrapure water, 0.32 ml of 100 mM phosphate buffer (pH 7, Merck, Germany), 0.16 ml of 0.15% H_2O_2 (Merck, Germany) and 0.32 ml of 5% pyrogallol (Merck, Germany). Absorbance was measured kinetically at 420 nm for 3 min at 30-s intervals.

Non-enzymatic antioxidants consisting of ascorbic acid and phenolic content were analyzed based on chromium reduction (Abera et al., 2020) and gallic acid method (Al-Saeedi & Hossain, 2015), respectively. The ascorbic acid assay was conducted by macerating 1 g leaf sample with 10 ml of 3% metaphosphoric acid (Merck, Germany) in 8% glacial acetic acid (Merck, Germany) and diluting until 100 ml with ultrapure water. The mixture was stirred for 45 min and protected from light. The reaction mixture was made of 2 ml of potassium dichromate ($K_2Cr_2O_7$, Merck, Germany) and manganese(II) chloride ($MnCl_2$, Merck, Germany) (0.335 mM: 0.185 mM) and 1 ml sample extract. Absorbance was measured kinetically at 350 nm for 5 min with 30-s intervals.

The phenolic content was assayed by macerating dried leaf powder (1 g) in 100 ml of 70% methanol (Merck, Germany). The mixture was incubated in the dark for 24 hr. The reaction mixture was made of 200 µl methanol extract (Merck, Germany) and 1.5 ml of 10% Folin-Ciocalteu (Merck, Germany). The mixture was incubated in the dark for 5 min, and later, 1.5 ml of 6% sodium carbonate (Na_2CO_3, Merck, Germany) was added, and it was incubated for 30 min in the dark. Absorbance was measured at 760 nm.

Leaves Nutrient Analysis and Nutrient Uptake based on Allometric Estimation

Macro- and micronutrient analyses were conducted to estimate nutrient uptake in abaca leaves. Dry matter of abaca leaves was used for N, P, K, Ca, Mg, S, Fe, Mn, Cu, Zn, Cl, and B analyses. N and K were assayed based on Kjeldahl and flame photometric analyses. P and B were assayed using a spectrophotometer. Cl was assayed based on the argentometric method. Meanwhile, others were assayed using an atomic absorption spectrophotometer (AAS). Subsequently, the result was used to estimate nutrient uptake based on the foliage biomass (*Y*) allometric equation. The equation was based on 3 different

pseudostem diameters *viz.* diameter at 10 cm (d_{10}), 30 cm (d_{30}), and 1.3 m (d_{13}) height (R^2 = 0.67) (Negash et al., 2013).

$$Y = 3.4 \times 10^{-3}\left(d_{10}^{1.982} d_{30}^{1.154} d_{1.3}^{0.863}\right) \quad [2]$$

Fiber Quality Analysis

The fiber quality analyzed in this study was fiber diameter and tensile strength. Fiber diameter was assayed by macerating abaca fiber in acetic acid (CH_3COOH, Merck, Germany) and H_2O_2 (Merck, Germany). After the fiber swelled, it was soaked and boiled in hot water for 4 hr. Fiber diameter was cut using microtome transversally and observed using a digital microscope Dino-Lite (Taiwan). Subsequently, it was calculated based on the cross-sectional area of the average of five diameters arranged at angle intervals of 36°. Tensile strength was measured based on ASTM D3379-75 (American Society for Testing and Materials [ASTM], 2003) by cutting 50 fiber samples to 90 mm length. The samples were measured using a universal testing machine (Instron 3360c, USA) at the speed of 1 mm/min.

Statistical Analysis

Analysis of variance (ANOVA) and independent *t*-test analysis were conducted using RStudio (ver. 2023.03.0). The relationship between soil, nutrient, and biochemical parameters was analyzed based on Pearson correlation analysis. Stepwise regression analysis forward mode was conducted at α = 0.20 using MINITAB (ver. 21.4) to develop a prediction model on fiber diameter and tensile strength. SeXI-FS software (ver. 2.1.1) was used to construct three-dimensional vegetation coverage.

RESULTS AND DISCUSSION

Climatic and Edaphic Characteristics

Different plant compositions and crown layers in the FT and FL systems promoted specific microclimatic conditions in each agroforestry. Analysis of crown projection showed that shading intensity in FL was 38% higher than in FT (Table 1), which could promote shading stress. Higher relative humidity in the FT system (4.35%; p<0.0001) and lower temperature (2.73%; p<0.0001) compared to FL indicated that the

Table 1
Climatic characteristics in abaca agroforestry

Variables	Agroforestry system		CV (%)
	FT	FL	
Relative humidity (%)***	65.980±0.40[a]	63.233±0.90[b]	0.27
Temperature (°C)***	32.835±0.17[b]	33.757±0.23[a]	0.25
Light interception (%)***	92.776±1.13[b]	95.691±2.10[a]	1.44
Shading intensity (%)*	59.82 (M)	83.99 (H)	-

Note. FT = Intermediate phase agroforestry; FL = Advanced phase agroforestry; *** = p<0.0001; M = Moderate; H = High; * = Based on a modified spherical densiometer calculation (Strickler, 1959); Values (mean ± standard deviation) indicated by the same letters showed no significant difference at α = 0.05

multilayer system in FT (Table 1) promoted a cooling effect, which in turn created specific microclimate conditions. Multilayer systems and various vegetation structures contributed to improving the cooling effect and humidity regulation in a stand (H. Wang et al., 2023). Besides, a multilayer system with various crown architecture in FT could potentially reduce turbulence (mixing air) compared to FL with high branch-free height teak trees. Decreasing turbulence kinetic energy occurred as increasing crown depth (Dupont & Brunet, 2008).

Higher plant density in the FT system (254 shading trees) compared to the FL system (81 teak trees and 66 teak saplings) promoted lower temperature and high relative humidity. The low plant density of cacao-rubber agroforestry contributed to higher temperatures by 3°C compared to higher plant density in the cacao-cabruca agroforestry system in Brazil (Heming et al., 2022). On the other hand, light interception in the FL system (3.14%; $p<0.0001$) was significantly higher than in the FT system (Table 1), indicating higher shading intensity as stated by Delagrange et al. (2006) regarding light interception in broad-leaved forest. Plants exposed to high shading intensity continuously could lead to retarded growth resulting from decreasing physiological activity and activation of certain oxidative stress mechanisms.

Abaca agroforestry systems showed various soil physical and chemical characteristics. FL system, comprised of 20-year-old teak, showed an improvement in soil characteristics compared to the FT system. Soil water content (SWC) value, organic carbon, porosity, CEC, and pH of the FL system were significantly ($p<0.05$) higher than FT by 19.6, 72.7, 4.30, 13.8, and 35.26%, respectively (Table 2). Higher organic carbon in FL contributed to lower bulk density (9.65%) compared to FT. Soil organic carbon negatively correlated (R^2 = -0.83) with bulk density, indicating its importance in determining soil impedance. Agroforestry can provide different organic carbon levels in different soil depths and alleviate soil bulk density (Cherubin et al., 2019). Nevertheless, soil permeability in both systems was classified as very slow (Table 2). High bulk density promotes waterlogging and decreases soil permeability, inhibiting root growth due to soil impedance (Marschner, 2012).

The SWC, organic carbon, CEC, and pH are pivotal factors determining the nutrient uptake ability of plants. Soil organic content contributed to higher CEC and pH, particularly in silty clay texture (Brady, 1984). Soil CEC determined the soil's ability to hold exchangeable cations and release them for plants. Therefore, it contributed significantly to soil classification (Mattila & Rajala, 2022). It was also confirmed by the strong correlation between organic carbon and CEC (R^2 = 0.92) as well as its correlation with pH (R^2 = 0.98) in this research. The high pH value of FL soil promoted high N and Mg availability (Table 3), indicated by the positive correlation between pH and available N (R^2 = 0.83) as well as Mg (R^2 = 0.97). In contrast, the low soil pH of FT contributed to the high

availability of P, Fe, Cu, and Zn, indicated by a negative correlation among them (R^2 P = -0.94; R^2 Fe and Cu = -0.98; R^2 Zn = -0.66). Soil available P, Fe, Zn, and Cu is very low, particularly in alkaline soil (Marschner, 2012). Increasing soil pH at 7 or 8 contributes to decreasing soil availability of Fe and Zn (Brady, 1984).

Table 2
Soil characteristics in abaca agroforestry

Variables	Unit	Agroforestry system		Rating		CV
		FT	FL	FT	FL	
SWC*	%	48.807±3.10[b]	58.393±1.60[a]	Very high	Very high	4.61
Organic C*	%	2.447±0.43[b]	4.230±0.06[a]	Moderate	High	9.21
BD*	g/cm^3	1.143±0.05[a]	1.033±0.03[b]	Low	Low	3.54
Porosity*	%	49.493±1.96[b]	51.620±1.19[a]	Very high	Very high	3.21
CEC*	me%	40.953±2.14[b]	46.593±1.31[a]	Very high	Very high	4.05
Permeability		0.037±0.01[a]	0.067±0.02[a]	Very slow	Very slow	35.34
pH*		5.703±0.24[b]	7.707±0.16[a]	Slightly acidic	Neutral	3.05

Note. FT = Intermediate phase agroforestry; FL = Advanced phase agroforestry; SWC = Soil water content; BD = Bulk density; CEC = Cation exchange capacity; me% = Milliequivalents per 100 g of soil; CV = Coefficient of variation; * = $p<0.05$; Values (mean ± standard deviation) indicated by the same letters showed no significant difference at α = 0.05; Rating was based on Sulaeman et al. (2005) and United States Department of Agriculture (USDA) (1999)

Table 3
Soil available nutrients in the abaca agroforestry system

Soil available nutrients	Unit	Agroforestry system		CV (%)
		FT	FL	
N*	ppm	184.893±20.49[b]	330.117±78.37[a]	22.24
P*	ppm	24.803±4.01[a]	3.270±1.48[b]	21.52
S	ppm	114.197±17.97[a]	104.887±39.65[a]	28.09
K*	me%	0.860±0.07[a]	0.530±0.03[b]	7.75
Mg*	me%	7.080±0.45[b]	10.693±0.50[a]	5.36
Ca*	me%	112.427±8.10[a]	63.407±0.27[b]	6.52
Fe*	ppm	73.503±13.56[a]	21.423±8.66[b]	23.96
Mn*	ppm	71.747±0.65[b]	73.357±0.29[a]	0.69
Cu*	ppm	6.840±0.08[a]	4.887±0.06[b]	1.22
Zn	ppm	1.963±1.07[a]	0.667±0.12[a]	58.22

Note. FT = Intermediate phase agroforestry; FL = Advanced phase agroforestry; CV = Coefficient of variation; me% = Milliequivalents per 100 g of soil; * = $p<0.05$; Values (mean ± standard deviation) indicated by the same letters showed no significant difference at α = 0.05

Foliage Nutrient Uptake

Overall analysis of leaves' macronutrients and micronutrients showed higher nutrient content in abaca leaves of the FL system compared to FT; however, only Ca, Fe, and Mn showed significant differences between both systems (Table 4). Soil CEC and water content of FL were higher than FT; therefore, plants in FL had higher uptake. Besides, higher organic carbon in FL than in FT might increase nutrient mineralization (J. R. Sarker et al., 2017). Ca and Fe content of abaca grown in FL was 81.25% ($p<0.05$) and 23.53% ($p<0.05$) higher than that of FT. Meanwhile, the Mn content of abaca leaves in FL was 78.50% ($p<0.05$) lower than that of FT. Higher Mn content in abaca leaves under FT compared to FL could be associated with lower soil pH in FT. Therefore, it was more suitable for Mn availability (Barrow & Hartemink, 2023) and contributed to higher Mn uptake by plants. High P availability in FT could induce Mn uptake by plants, indicated by the strong positive correlation between these factors ($R^2 = 0.966$). A synergistic relationship between Mn and P was also observed by Barben et al. (2011) in potatoes and by Berríos et al. (2019) in ryegrass. Meanwhile, higher Ca and Fe content in abaca leaves of FL is related to their interaction with other nutrients.

Table 4
Macronutrients and micronutrients of abaca leaves under the agroforestry system

Foliage nutrients	Unit	Agroforestry system		CV (%)
		FT	FL	
N	%	2.557±0.61[a]	2.270±0.14[a]	18.24
P	%	0.253±0.03[a]	0.183±0.05[a]	18.23
K	%	2.510±0.14[a]	2.670±0.07[a]	4.30
S	%	1.593±0.52[a]	1.837±0.31[a]	24.82
Mg	%	0.089±0.00[a]	0.091±0.00[a]	1.70
Ca*	%	0.163±0.01[b]	0.293±0.01[a]	7.37
Fe*	%	0.017±0.00[b]	0.021±0.00[a]	6.03
Mn*	%	0.107±0.01[a]	0.023±0.01[b]	8.88
Cl	%	0.610±0.06[a]	0.552±0.04[a]	8.03
Cu	ppm	12.780±1.70[a]	16.087±3.87[a]	20.71
B	ppm	314.333±43.36[a]	304.333±45.50[a]	14.37
Zn	ppm	22.953±0.40[a]	24.860±3.56[a]	10.60

Note. FT = Intermediate phase agroforestry; FL = Advanced phase agroforestry; CV = Coefficient of variation; * = $p<0.05$; Values (mean ± standard deviation) indicated by the same letters showed no significant difference at $\alpha = 0.05$

Significantly higher N availability in FL (78.55%; $p<0.05$) system compared to FT could induce higher Ca uptake in FL. It could be associated with the activation of transcription factor nitrate transporter 1 (NRT1) when soil nitrate was high. The activation of NRT1 could induce phospholipase C enzyme activity (PLC), subsequently increasing Ca uptake in the cytosol (K.-H. Liu et al., 2020). High Ca uptake could occur under high soil N availability to maintain N balance in leaves (Xing et al., 2022). Meanwhile, higher Fe leaf content in abaca grown in FL was associated with its antagonistic interaction with soil available P (Figure 4). High Fe and P availability in the FT system was associated with declining Fe and increasing P content in leaves. However, high Fe and low P availability in FL could induce declining P and increasing Fe leaf content. Zheng et al. (2009) also observed interaction between Fe and P in rice.

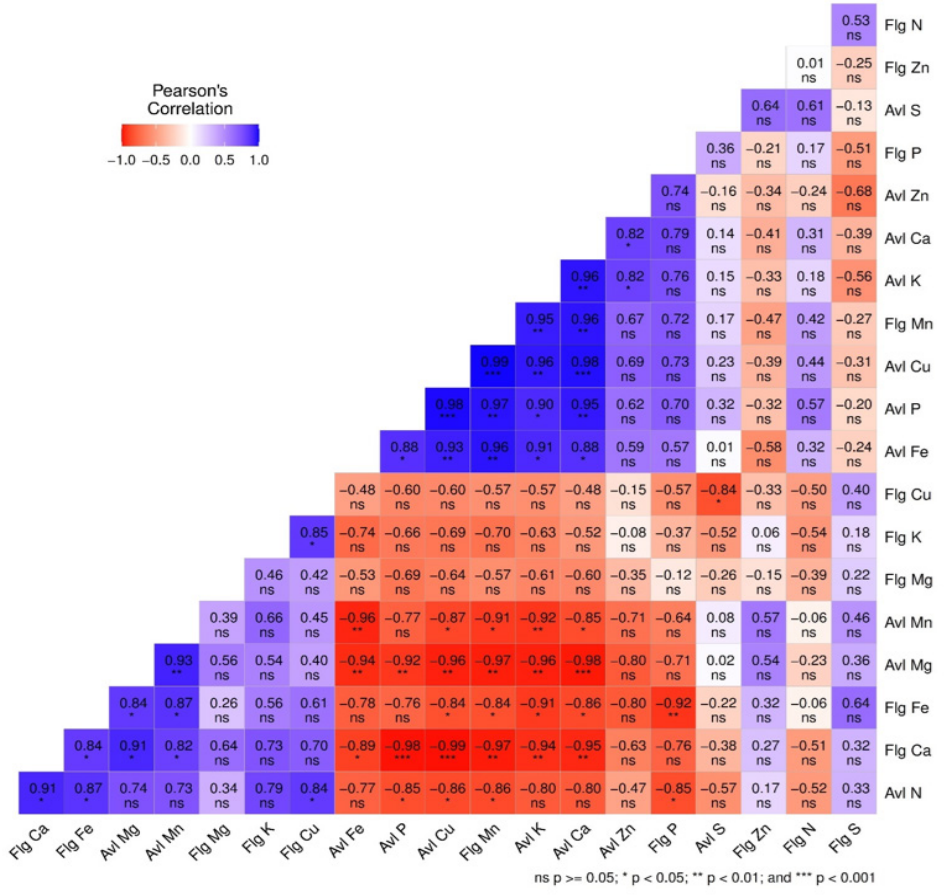

Figure 4. Correlation between soil available nutrients and foliage nutrients

Note. Avl = Available; Flg = Foliage

Nutrient uptake was analyzed based on allometry specifically used for foliage biomass estimation. Macronutrient uptake estimation by abaca in FT and FL, from the largest to the smallest, were N > K > S > P > Ca > Mg and K > N > S > Ca > P > Mg, respectively. Meanwhile, micronutrient uptake in FT and FL were Cl > Mn > B > Fe > Zn > Cu and Cl > B > Mn > Fe > Zn > Cu, respectively (Table 5). Lower nutrient uptake estimation in FL compared to FT was associated with lower biomass estimation of abaca in FL due to lower diameter at 10, 30, and 130 cm height. It indicated that improved soil characteristics in FL could not compensate for growth inhibition caused by shading stress. The high shading intensity in the FL system could hinder abaca growth. Therefore, pruning is essential to compensate for nutrient competition between abaca and shading trees (Raza et al., 2019).

Table 5
Nutrient uptake based on allometry estimation of abaca foliage

Nutrients	Nutrient uptake		CV (%)
	FT	FL	
N* (kg/ha)	107.490±28.07[a]	6.314±0.93[b]	34.90
P* (kg/ha)	10.597±2.11[a]	0.496±0.04[b]	26.94
K* (kg/ha)	104.768±14.37[a]	7.507±1.73[b]	18.22
Ca* (kg/ha)	6.795±0.72[a]	0.828±0.22[b]	14.00
Mg* (kg/ha)	3.691±0.31[a]	0.254±0.05[b]	11.39
S* (kg/ha)	66.528±23.00[a]	5.196±1.57[b]	45.46
Fe* (kg/ha)	0.736±0.08[a]	0.058±0.02[b]	14.99
Mn* (kg/ha)	4.429±0.25[a]	0.064±0.02[b]	7.74
Cl* (kg/ha)	25.512±4.26[a]	1.533±0.21[b]	22.31
Cu* (g/ha)	53.526±11.29[a]	4.622±1.91[b]	27.85
Zn* (g/ha)	95.627±9.58[a]	6.931±1.44[b]	13.35
B* (g/ha)	1317.270±286.43[a]	85.110±19.92[b]	28.95

Note. FT = Intermediate phase agroforestry; FL = Advanced phase agroforestry; CV = Coefficient of variation; * = $p<0.05$; Values (mean±standard deviation) indicated by the same letters showed no significant difference at $\alpha = 0.05$

ROS and Antioxidant Production

Abaca is considered a C3 plant, indicating high sensitivity to photorespiration; therefore, shading trees is required to avoid oxidative damage from high light intensity. However, through specific mechanisms, oxidative damage caused by ROS production is triggered by high light intensity and continuous low light intensity. The oxidative stress response observed in this study was significantly exhibited by abaca grown in the FL system, particularly H_2O_2 and phenolic content. H_2O_2 production in abaca leaves grown in FL was 32.66% ($p<0.001$) higher than that of FT.

Low light intensity could activate RBOHD/F, part of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase in the apoplast, which is involved in ROS production, particularly H_2O_2 (Zhang et al., 2017). Zhang et al. (2017) also observed that H_2O_2 production triggered by low light intensity could lead to NO production through signal cascading, subsequently causing stomatal closure. Low light intensity could trigger ion release from vacuoles in guard cells (Shimazaki et al., 2007), leading to lower carbon assimilation and higher ROS production (Shafiq et al., 2021).

Higher H_2O_2 led to higher phenolic content in abaca leaves of FL by 35.08% (p<0.0001) compared to that of FT (Tables 6 and 7). Phenol is one of the major H_2O_2 scavengers with an aromatic ring structure composed of -OH or -OCH, which plays an important role in trapping free radicals (Dumanović et al., 2021; Sadak et al., 2019; Sadak & Ramadan, 2021). On the other hand, ascorbic acid content was found to be higher (23.31%; p<0.0001) in the abaca of the FT system compared to that of FL (Table 7).

Table 6
Oxidative damage indicators of abaca leaves under agroforestry system

Agroforestry system	Oxidative damage indicators		
	MDA (nmol MDA/g FW)	H_2O_2** (µmol H_2O_2/g FW)	$O_2^{\bullet-}$ (µmol $O_2^{\bullet-}$/g FW)
FT	14.822±4.03[a]	125.000±29.14[b]	17.169±9.86[a]
FL	14.828±3.01[a]	165.830±64.74[a]	17.487±11.98[a]
CV (%)	22.21	18.44	46.86

Note. FT = Intermediate phase agroforestry; FL = Advanced phase agroforestry; MDA = Malondialdehyde; H_2O_2 = Hydrogen peroxide; $O_2^{\bullet-}$ = Superoxide anion; ** = p<0.001; Values (mean ± standard deviation) indicated by the same letters showed no significant difference at α = 0.05

Table 7
Non-enzymatic and enzymatic antioxidants of abaca leaves under agroforestry system

Agroforestry system	Non-enzymatic antioxidants	
	Phenol (mg GAE/g DW) ***	Ascorbic acid (mg AAE/100 g sample) ***
FT	3.045±0.92[b]	216.010±8.05[a]
FL	4.116±0.98[a]	175.180±45.18[b]
CV (%)	5.82	1.33
	Enzymatic antioxidants	
	SOD (U SOD/g FW)	POD (U POD/min g FW)
FT	114.830±16.87[a]	0.412±0.16[a]
FL	114.930±35.21[a]	0.467±0.07[a]
CV (%)	22.58	20.89

Note. FT = Intermediate phase agroforestry; FL = Advanced phase agroforestry; SOD = Superoxide dismutase; POD = Peroxidase; mg GAE/g DW = mg gallic acid equivalent per gram dry weight; mg AAE/100 g sample = mg ascorbic acid equivalent per 100 g sample; U/g FW= unit per gram fresh weight; **= p<0.001; Values (mean±standard deviation) indicated by the same letters showed no significant difference at α = 0.05

Higher ascorbic acid production is associated with activation of L-galLDH as electron transport for ascorbic acid biosynthesis in high light intensity conditions compared to low light intensity (Bartoli et al., 2009). It was also observed in satsuma mandarin oranges by Izumi et al. (1992) and in *Arabidopsis* by Heyneke et al. (2013). Additionally, ascorbic acid biosynthesis in plants mainly occurs through the L-galactose pathway (Smirnoff-wheeler pathway [SW pathway], L-gulose, D-galacturonic, and D-glucuronic, which relies on P content as a major enzyme component involved in ascorbic acid biosynthesis (Chaturvedi et al., 2022). A positive correlation between P leaves content and ascorbic acid ($R^2 = 0.766$) in abaca indicated a strong association between P leaves content and ascorbic acid.

In contrast, other oxidative stress responses, viz. MDA, $O_2^{\bullet-}$, SOD, and POD were observed to be not significantly different between abaca in FT and FL (Tables 6 and 7). However, the overall expression of the respective indicators was higher in FL than in FT. MDA and $O_2^{\bullet}$ of abaca growth in FL were higher by 6.75 and 1.86%, respectively, than in FT. Non-significant MDA and $O_2^{\bullet-}$ differences between the two agroforestry systems might be associated with chlorophyll degradation. Chlorophyll *a* and chlorophyll *b* content, total chlorophyll as well as chlorophyll *a*/*b* of abaca leaves grown in both agroforestry systems showed no significant difference (Table 8). Lipid peroxidation could promote chlorophyll degradation, resulting from oxidated polyunsaturated fatty acid (PUFA) by lipoxygenase (LOX) enzyme activity (Jakhar & Mukherjee, 2014). It can be assumed that no physiological plasticity, particularly chlorophyll degradation, was observed in the abaca of both agroforestry systems; therefore, MDA and $O_2^{\bullet-}$ contents were not significantly different. Other studies by Chaves et al. (2008) and Saleem et al. (2019) also observed similar MDA expression between high and low light intensity. Electron excitation between unshaded and shaded plants might show similar plasticity in transport electron or energy dissipation (Chaves et al., 2008).

The significant differences in SOD and POD between abaca leaves in FT and FL were also absent, despite the higher SOD (8.71%) and POD (14.63%) content

Table 8
Chlorophyll content of abaca leaves under agroforestry system

Parameters	Agroforestry system		CV (%)
	FT	FL	
Chlorophyll *a* (mg/g FW)	0.445±0.09 [a]	0.412±0.06 [a]	15.91
Chlorophyll *b* (mg/g FW)	0.806±0.16 [a]	0.745±0.11 [a]	15.92
Total chlorophyll (mg/g FW)	0.662±0.14 [a]	0.613±0.09 [a]	19.09
Chlorophyll *a*/*b*	0.553±0.00 [a]	0.553±0.00 [a]	0.01

Note. CV = Coefficient of variation; mg/g FW = mg per g fresh weight; Values (mean ± standard deviation) indicated by the same letters showed no significant difference at α = 0.05

of abaca leaves in FL compared to that of FT (Table 7). It might be associated with several nutrients related to the biosynthesis of the respective antioxidants, which were also observed to be not significant, except for POD. Cu and Zn were known to form Cu, Zn-SOD complex with Cu as an active cofactor to directly fix superoxide ions (Alscher et al., 2002). Cu and Zn content in abaca leaves of both agroforestry systems, which were also observed to be not significant (Table 4), might be associated with the absence of significant differences in SOD. A positive correlation between Cu and SOD (R^2 = 0.552) was also observed in this study. Approximately 90% of total SOD in eucaryotic organisms is composed of Cu, Zn-SOD complex rather than Mn-SOD or Fe-SOD (Pelmenschikov & Siegbahn, 2005). Meanwhile, POD might be associated with Fe leaf content, indicated by the positive correlation between POD and Fe (R^2 = 0.546). Despite significant differences between both systems for Fe (Table 4), POD content showed no significant difference. Fe is the major component of the heme complex in POD (Campa, 1990).

Fiber Quality

The fiber quality observed in this study was diameter and tensile strength. Abaca fiber is shaped like an ellipse rather than circular; therefore, a cross-sectional area was used to measure its diameter (K. Liu et al., 2013). Fiber diameter of abaca grown in FT was higher (23.53%; p<0.0001) compared to FL (Table 9). The outer and inner layers of abaca pseudostem grown in FT showed different fiber diameters, ranging from 0.112–0.318 and 0.161–0.287 mm, respectively. Meanwhile, the smallest and largest fiber diameter of the outer layer of abaca pseudostem grown in FL was 0.070–0.240 mm, while the inner layer ranged from 0.066–0.285 mm.

Table 9
Diameter and tensile strength of abaca bast fiber in agroforestry system

Agroforestry system	Fiber diameter (mm)***	Tensile strength (MPa)*	CV (%)
FT	0.208±0.01[a]	379.980±81.07[a]	7.90
FL	0.168±0.02[b]	319.940±107.99[b]	19.01

Note. FT = Intermediate phase agroforestry; FL = Advanced phase agroforestry; *** = p<0.0001; * = p<0.05; Values (mean±standard deviation) indicated by the same letters showed no significant difference at α = 0.05

Significant differences in fiber tensile strength in both systems were also observed. The higher tensile strength of abaca in FT (18.77%; p<0.05) was observed to be significantly different from that of FL (Table 9). The tensile strength of the outer layer of abaca pseudostem grown in FT and FL were 345.78 and 416.06 MPa, respectively. Meanwhile, the tensile strength of the inner layer of abaca pseudostem in FT and FL was 409.41 and 232.45 MPa, respectively. Higher fiber diameter could promote lower tensile strength.

Increasing fiber diameter could adversely affect tensile strength due to increasing defects in cross-sectional area. The fiber diameter and tensile strength of abaca in both agroforestry systems were exponentially distributed (Figure 5). Leaf fiber of abaca showed fiber diameter distribution at 55.6–197.6 µm, with a similar decreasing trend of tensile strength as increasing fiber diameter (Munawar et al., 2007). Decreasing tensile strength and increasing fiber diameter might be associated with alteration of lignocellulose content, microfibril angle, cell volume, and fiber defect density (Lewin, 2007). Despite the positive correlation between higher cellulose and increasing tensile strength in Enset, higher defects due to increasing fiber diameter could decrease tensile strength (Dessie et al., 2023).

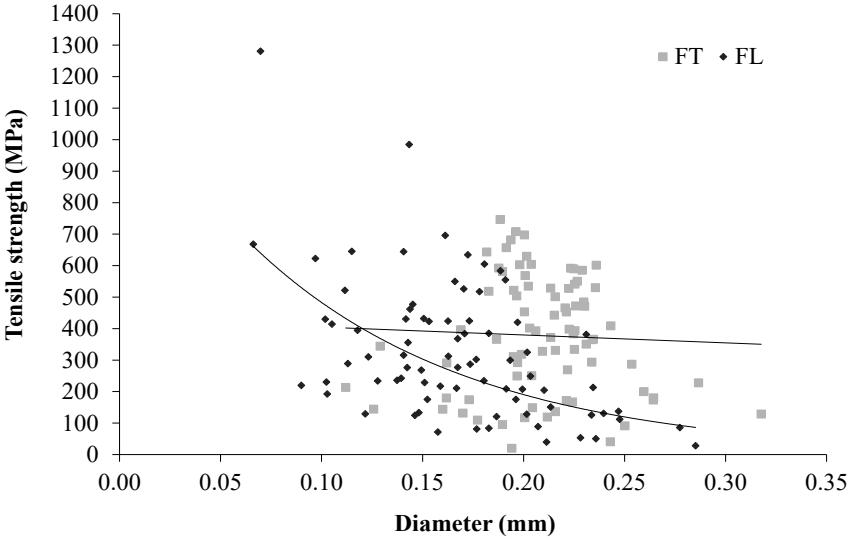

Figure 5. Relationship between diameter and tensile strength of abaca fiber

Note. FT = Intermediate phase agroforestry; FL = Advanced phase agroforestry

A forward stepwise regression analysis was conducted to determine factors that could explain the variable contributing to fiber diameter and tensile strength. Macro- and micronutrient content were used as independent variables in this analysis. The threshold *p* value used in both models was 0.20. The significance level of 0.20 was between the acceptable range (0.15–0.25) suggested by Derksen and Keselman (1992) to allow the authentic variables to be included in the model instead of noise variables. The results showed that Ca, Fe, and K were the variables that explained most of the variance in fiber diameter, indicated by their significant effect on the model (Table 10). These variables exhibited a positive effect on the model. The fourth variable that improved the model is N, which had a negative effect. The positive effect of Ca,

Fe, and K on fiber diameter was assumed to be associated with fiber uniformity. Smaller fiber diameter is attributed to tapered fiber tip morphology, which results in higher fiber uniformity (Graham & Haigler, 2021).

As one of the essential macronutrients, Ca plays an important role in pectin biosynthesis and strengthening cell walls through its bonding with carboxylate ions (de Bang et al., 2021). A positive correlation was observed between Ca content and fiber uniformity in cotton (Fortier et al., 2021). Increasing fiber uniformity of jute (S. R. Sarker et al., 2008) and cotton (Sawan et al., 2008) was attributed to increasing K content. Improved fiber uniformity of 47.5% was observed under 50 kg/ha iron sulphate ($FeSO_4$) fertilization in cotton (Sankaranarayanan et al., 2010). In contrast, higher N content promoted low fiber uniformity in cotton (Hassanzadehdelouei et al., 2022), while N fertilization on *Populus trichocarpa* exhibited no significant effect on fiber diameter (Pitre et al., 2010).

Table 10
Stepwise regression analysis on fiber diameter

		Independent variables					R^2 (%)	Adjusted R^2 (%)
		Constant	Ca	Fe	K	N		
Model 1	Factor	0.117	0.313				57.75	56.25
	SE	0.018	0.073					
	p-value		0.003					
Model 2	Factor	0.028	0.171	6.360			68.70	65.09
	SE	0.016	0.071	0.927				
	p-value		0.025	0.026				
Model 3	Factor	0.105	0.222	5.963	0.031		73.33	69.06
	SE	0.015	0.069	0.920	0.003			
	p-value		0.002	0.003	0.014			
Model 4	Factor	0.107	0.206	6.495	0.033	-0.002	78.06	72.33
	SE	0.014	0.052	0.907	0.001	0.001		
	p-value		0.016	0.017	0.025	0.153		

Note. SE = Standard error

Most variances in tensile strength were attributed to Mg and B content, which indicated a high-significance effect on the model (Table 11). The positive effect of Mg on tensile strength could be associated with its role in cellulose translocation, sucrose loading and unloading through phloem (Ahmed et al., 2020; Pandey, 2018). Increasing tensile strength of cotton by 2.3% was observed under Mg nano-fertilizer (Kanjana, 2020). Meanwhile, the positive effect of B on tensile strength could be associated with its role in cell wall strengthening. As an essential micronutrient, B plays an important role in cell wall structural function through

Table 11
Stepwise regression analysis on tensile strength

		Independent variables			R^2 (%)	Adjusted R^2 (%)
		Constant	Mg	B		
Model 1	Factor	534.1	51.4		81.47	74.41
	SE	45.75	14.20			
	p-value		0.180			
Model 2	Factor	318	53.7	1.103	87.01	78.35
	SE	9.68	13.78	0.119		
	p-value		0.002	0.003		

Note. SE = Standard error

the crosslinking of pectic polysaccharide rhamnogalacturonan II (RG II) (Miwa & Fujiwara, 2010) and resulted in increasing tensile strength in fiber plant (de Souza Júnior et al., 2022).

CONCLUSION

The advanced phase agroforestry system contributed to improved soil characteristics compared to the intermediate phase. However, high shading intensity under the advanced phase could promote abaca growth inhibition and oxidative stress responses. Consequently, it resulted in lower fiber quality compared to intermediate-phase agroforestry. Therefore, pruning management was substantial in improving the growth condition and fiber quality of abaca under agroforestry.

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Meta-analysis of the Use of Leaf Extract as Alternative Growth Promoter in Broiler Chickens

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ABSTRACT

Plants, especially on the leaves, have various bioactive compounds capable of becoming natural growth promoters. Plant leaf extracts have been widely studied for their ability as an antibiotic substitute for broiler chickens. This meta-analysis study was aimed to assess the effectiveness of supplementations with leaf extract on the growth performance of broiler chickens, using average daily feed intake (ADFI), average daily gain (ADG), final body weight (FBW), and feed conversion ratio (FCR) as responses observed criteria. The meta-analysis study was based on the articles published from 2006 to recent years as several countries started to ban in-feed antibiotics. Databases (PubMed, Scopus, Directory of Open Access Journals [DOAJ], and ScienceDirect) were searched for peer-reviewed randomised controlled trials (RCTs) published in English. The meta-analysis included 19 research papers that met the criteria. Overall results showed a significant increase ($P <$ 0.001) in ADFI by 0.56 g/day (95% confidence interval [CI] = 0.02 to 1.11), in ADG by 1.57 g/day (95% CI = 0.77 to 2.36), and in FBW by 2.28 (95% CI = 1.40 to 3.16). At the same time, the FCR was reduced ($P < 0.001$) by -1.25 (95% CI = -1.76 to -0.73) relative to controls taking cognisance of publication bias and heterogeneity. Results in the current meta-analysis study indicated that herbal feed additives were proven to be effective as growth promoters in broiler chickens.

Keywords: Broiler chickens, growth promoter, leaves extract, meta-analysis

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INTRODUCTION

Phytogenic feed additives (PFAs), which are also known as herbs or phytobiotics (Gheisar and Kim, 2018), are less toxic and less residue natural bioactive substances found in the organs of some plants (M. L. Wang et al., 2008). Research by Windisch et al. (2008) has shown that the potency of dietary leaf extract can change with the presence of in-feed antibiotics. Another study (Mohamed and Hassan, 2023) showed that dietary PFAs showed promising results in the growth performance of broiler chickens. Improved growth performance by including plant materials in the diet occurs due to the increased nutrient digestibility, digestive enzyme activities, and reduced intestinal pathogens, which allow increased availability of essential nutrients in the intestine for absorption and helps improve growth performance (Hasemi et al., 2008). The principal mode of action of phytobiotics arises from the beneficial influence of the gastrointestinal microbiota ecosystem through controlling potential pathogens (Meng et al., 2023; Qureshi et al., 2015), improving immunodeficiency and intestinal vegetation (Chen et al., 2020), as well as enhance intestinal tract performance associated with villus height and crypt depth improvement (Song et al., 2023).

Several studies recommended a potential part of phytogenic as safe growth promoters in poultry nutrition. Phytogenic possesses some substances that enhance dietary palatability, gut health, growth performance, and meat production (Mohamed and Hassan, 2023). In recent years, several researchers from various countries have explored herbal supplementations in poultry production. Research in tropical countries, such as Indonesia (Anggrain et al., 2014), Malaysia (Basit et al., 2020), India (Kumar et al., 2021), Nigeria (Alabi et al., 2017), and in subtropical countries, such as China (Zhao et al., 2019), Iran (Azimi et al., 2020), and Japan (Nakamura et al., 2022) have shown the beneficial effects of the in-feed herbal additives on growth performance of poultry.

The advantageous multifunctional effects of dietary supplementations with phytogenic feed additives were attributed to the presence of biologically active constituents in plants, such as terpenoids, phenolics, glycosides, and alkaloids (Huyghebaert et al., 2011). In recent years, research on natural plants that can be used as alternative growth performance in broiler chickens has continued to be carried out. Sun et al. (2020) showed that the dietary addition of *Achyranthes japonica* Nakai improved nutrient utilisation, growth performance, and meat quality of broiler chickens. S. J. Liu et al. (2021) found that supplementation of capsicum extract in feed improved nutrient digestibility, immune status, meat quality, and growth performance. Zhang et al. (2022) reported that the dietary addition of *Glycyrrhiza uralensis* Fisch improved immune response, intestinal microflora population, and growth performance. Amongst the organs of plants, leaves were the most used in plant part (38%), followed by fruit (22%), whole plant (9%), rhizome (8%), roots (7%), flowers and bark (4% each), sap (3%), stem and tuber (2% each),

and seeds (1%) (Elfrida et al., 2021). These findings prompted researchers to explore the beneficial effects of herbal feed additives as alternative growth promoters for broiler chickens, starting from leaves.

Several studies have shown that the efficacy of herbal feed additives varied due to several variables, such as geographical origin (Kokkini et al., 1994), genotype (Aligiannis et al., 2001), location (Lambert et al., 2001), processing methods (Delespaul et al., 2000), seasonal variations (McGimpsey et al., 1994), and climatic variations (Salgueiro et al., 1997). Therefore, a meta-analysis in this study was carried out from several published data in international journals to evaluate the effect of giving plant leaf extracts on the growth performance of broiler chickens.

MATERIALS AND METHODS

Literature Search and Study Selection

A comprehensive search for studies discussing the effect of leaf extract on broiler productivity was carried out in several scientific web databases, namely PubMed Central (www.ncbi.nlm.nih.gov/pmc/), ScienceDirect (www.sciencedirect.com), DOAJ (https://doaj/org/), and Scopus (www.scopus.com). An article search used the keywords 'leaf extract' and 'broiler performance'. The last search was carried out in February 2023. The criteria for articles to be included in the database were: (1) reporting the use of leaf extracts as a feed additive used to substitute antibiotics, the article must use levels as treatment and control (without additive supplementation), (2) *in vivo* studies on broiler chickens, which were peer-reviewed journals in English, (3) reports at least two performance variables: ADFI, FBW, ADG, and FCR, with the respective variance (standard deviation or standard error), (4) reports the number of replications and broiler strains. Studies using a combination of additive and challenging studies (infection of viruses/diseases, limited consumption, heat stress) were not included.

The selection process reported in Figure 1 resulted in a final paper that met the selection criteria to produce data used as a database (Page et al., 2021), information following the meta-analysis guidelines adopted based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). The database consisted of 19 studies with 153 treatments, as presented in Table 1. In tabulating data into the database, data relating to the parameters were converted into the same measurement units to facilitate further analyses.

Information includes: (1) name of authors and year of publication, (2) country of origin of research, (3) plant species, (4) solvent used, (5) bird strain, (6) number of birds, (7) study durations, and (8) level of treatments. Measures (standard deviation, standard error, or standard error of mean) of ADFI, ADG, FBW, and FCR were included in the database.

Meta-analysis Procedure

The effect size was calculated as the standardised mean difference (SMD) according to the method of Hedges (1981) for

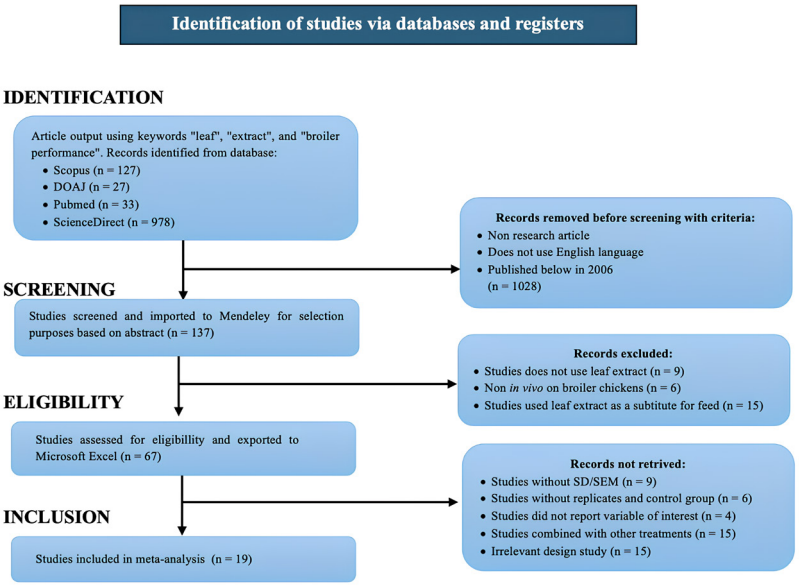

Figure 1. Flow chart of the article selection process utilised for the meta-analysis

Note. SD = Standard deviation; SEM = Standard error of the mean

Table 1
Studies included in the meta-analysis

Study	Reference	Country	Plant species	Solvent	Strain	Birds	Period	Level (ppm)
1	Al-khalaifah et al. (2020)	Egypt	*Allium ampeloprasum*	Ethanol	Hubbard	250	1–42	0–200
2	Al-Masari and Al-Himdany (2022)	Iraq	*Cynara scolymus* L.	Not available	Ross	135	1–42	0–1,000
3	Al Salman and Al-Gharawi (2019)	Iraq	*Eucalyptus viminalis*	Distilled water	Ross	240	1–35	0–8,000
4	Al-Musawi et al. (2019)	Iraq	*Petroselinum sativum*	Water	Ross	240	1–35	0–15,000
5	Alabi et al. (2017)	Nigeria	*Moringa oleifera*	Water	Hubbard	240	1–56	0–150,000
6	Alabi et al. (2020)	Nigeria	*Moringa oleifera*	Water	Hubbard	240	1–42	0–120,000

Table 1 *(Continue)*

Study	Reference	Country	Plant species	Solvent	Strain	Birds	Periods	Level (ppm)
7	Duskaev et al. (2020)	Rusia	*Eucalyptus viminalis*	Distilled water	Abror Acres	120	1–35	0–200
8	Erener et al. (2020)	Turkey	*Olea europeae* L.	35% ethanol	Ross	375	1–42	0–600
9	Fathi et al. (2022)	Iran	*Ocimum basilcum*	80% ethanol	Ross	450	1–42	0–600
10	Khan et al. (2022)	Saudi Arabia	*Moringa oleifera*	Distilled water	Hubbard	350	1–35	0–12,000
11	Onu (2012)	Nigeria	*Telfairia occidentalis*	Water	Marshal	200	8–28	0–16,000
12	Racanicci et al. (2011)	Brazil	*Ilex paraguariensis*	Water	Cobb	100	1–25	0–1,000
13	Rasouli et al. (2020)	Oman	*Salvia officinalis* L.	Water	Ross	300	1–42	0–400
14	Sahu et al. (2017)	India	*Cassia tora*	Methanol	Cobb	90	1–42	0–400
15	Sani et al. (2013)	Iran	*Artemisia annua*	Methanol	Cobb	240	1–42	0–4,000
16	Shen et al. (2019)	China	*Bambusoideae* sp.	Not available	Abror Acres	576	1–42	0–5,000
17	Teteh et al. (2013)	Togo	*Moringa oleiifera*	Ethanol	Ross	615	1–28	0–2,000
18	Xie et al. (2022)	China	*Olea europaea* L.	75% ethanol	Abror Acres	720	1–35	0–5,000
19	Yan et al. (2022)	China	*Eucommia ulmoides*	Water	Wen Tianlu	252	1–51	0–1,000

fixed effect models as well as DerSimonian and Laird (1986) for the random effect model. The classic meta-analysis approach was carried out according to the studies used, which had variables with the same unit of measurement. The calculation was done with the raw mean difference, which allows the interpretation of the effect size in the original unit of measurement (Ranga Niroshan Appuhamy et al., 2013).

Forest plots were made to show the effect of giving plant leaf extracts on the growth performance of broiler chickens, which were represented by points and confidence intervals. Forest reports plots of effect sizes and weighted contributions to the fixed-effect and random-effect study models. The approach to random effects meta-analysis was performed using simple moment-based estimation of the between-

study variance (heterogeneity) of the true effect (θ) (DerSimonian & Laird, 1986). Heterogeneity was reported with the I^2 statistic, which correlates with ($\tau 2$) as a percentage of the total variability. The statistical model used in this study was based on a *P* value with a significant effect indicated by $P < 0.05$. All statistical analysis was performed with OpenMEE software (Wallace et al., 2017).

RESULTS AND DISCUSSION

Study Characteristic

A total of 19 studies conducted in 12 countries worldwide were aggregated, mainly shown in Iraq (15.78%), Nigeria (15.78%), and China (15.78%). Several broiler chicken strains were used, with Ross being the most common (36.84%), followed by Hubbard (21.05%). The solvent used for the leaf extract was water (57.89%). The range used for the studies was 90 to 720 birds, with the highest period 1–42 (47.36%) (Table 1). The level of ≤1,000 ppm (42.11%), followed by 1,000 ≥ 10,000 ppm (31.57%) and 10,000 ≥ 100,000 ppm (26.31%) was included in the experiment. Based on the statistical description, the difference in the values of the average observed parameters was caused by environmental conditions, experimental settings, and housing management reported in meta-analysis studies.

Average Daily Feed Intake

Meta-analysis in the current study evaluated the effects of leaves extract supplementations as a feed additive in broiler chickens was performed using 16 articles that had a significant effect on increasing the average daily feed intake ($P < 0.001$) with SMD value of 0.56 (0.02 to 1.11) and heterogeneity among the studies ($I^2 = 81.95\%$) (Figure 2). Thus, treatment was aggregated to identify the effects of the covariates (strain and species of leaf extract) by using a subgroup meta-analysis. The strain showed an increasing effect on ADFI compared to the control in Ross ($P < 0.005$). The addition of plant extracts made from *Allium ampeloprasum* L., *Petroselinum sativum*, *Eucalyptus viminalis*, and *Bambusoideae* sp. showed increasing ADFI ($P < 0.001$). At the same time, *Artemisia annua* decreased ADFI ($P < 0.001$), as shown in Table 2. The meta-analysis results were in line with several studies showing that plant extract supplementations as a natural feed additive have a trend of increasing daily feed intake compared to controls (Ali et al., 2020; Wallace et al., 2017). Onu (2012) reported that broiler supplementation with *Telfairia occidentalis* resulted in a decrease in ADFI with increasing levels of supplementation.

A subgroup meta-analysis study on the Ross strain supplemented with leaf extract also showed increased ADFI. Supported by the research findings of Rasouli et al. (2020), adding 200 ppm of *Salvia officinalis* L. extract increased ADFI. However, the results differ from those of Alabi et al. (2020), i.e., an increase in supplementation leaf extract can affect lower daily consumption compared to controls. Overall, the phytochemical compound in the leaf extract caused an improved ADFI value.

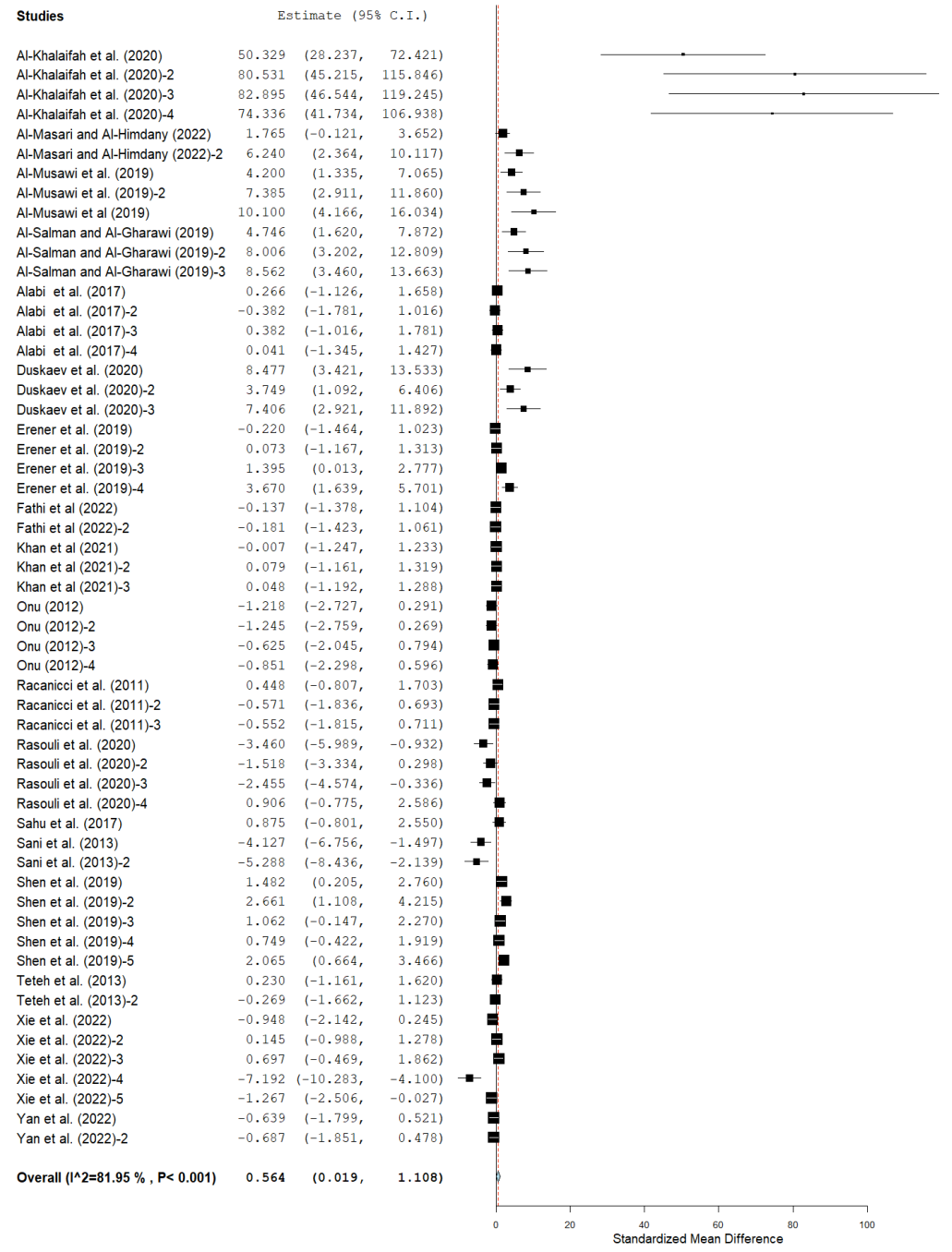

Figure 2. Forest plot of the effect of feeding leaves extract on ADFI of broiler chickens

Table 2
Subgroup analysis of the effect of leaf extract supplementation on average daily feed intake of broiler chickens

Covariates	N	SMD	95% CI		SE	*P*-value	Heterogeneity	
			Lower	Upper			I^2	*P*-value
Strain								
Hubbard	11	0.98	-0.79	2.75	0.90	0.29	87.55%	<0.001
Ross	20	1.48	0.48	2.49	0.51	<0.01	80.96%	<0.001
Arbor Acres	13	0.97	-0.13	2.07	0.56	0.08	84.45%	<0.001
Marshal	6	-0.97	-1.71	-0.24	0.36	0.01	0%	0.92
Cobb	4	-1.07	-2.46	0.32	0.71	0.13	76.18%	0.001
Wen Tialu Black	2	-0.66	-1.49	0.16	0.42	0.11	0%	0.96
Leaf extract								
Allium ampeloprasum	4	67.43	50.70	84.20	8.55	<0.001	18.13%	0.30
Cynara scolymus L.	3	3.70	-0.67	8.01	2.21	0.10	75.84%	0.04
Petroselinum sativum	3	6.49	3.16	9.82	1.70	<0.001	45.90%	0.16
Moringa oliefera	9	0.04	-0.40	0.49	0.23	0.85	0%	0.99
Eucalyptus viminalis	6	6.01	4.22	7.80	0.91	<0.001	19.73%	0.29
Olea europaea L.	9	-0.15	-1.24	0.95	0.56	0.79	82.32%	<0.001
Ocimum bassicum	2	-0.16	-1.04	0.72	0.45	0.72	0%	0.96
Telfaria ociidentalis	4	-0.97	-1.71	-0.24	0.38	0.01	0%	0.92
Ilex paraguariensis	3	-0.22	-0.95	0.51	0.37	0.55	0%	0.44
Salvia officinalis L.	4	-1.50	-3.38	0.38	0.96	0.12	71.61%	0.01
Cassia tora	1	0.88	-0.80	2.55	0.86	NA	NA	NA
Artemisia annua	2	-4.60	-6.62	-2.59	1.03	<0.001	0%	0.58
Bambusoideae sp.	5	1.49	0.85	2.13	0.33	<0.001	17.50%	0.30
*Eucommia ulmoide*s	2	-0.66	-1.49	0.16	0.42	0.11	0%	0.96

Note. N = Number of comparisons; SMD = Standardized mean differences between the leaves extract treatment and controls; CI = Confidence interval; *P*-value = Probability value; SE = Standard error; NA = Data non-available; SMD and I^2 were considered significant at $P<0.05$

Phytogenic compounds may influence the flavour of diets, which improves appetite and consumption; they might increase feed intake (Brenes & Roura, 2010). Several bioactive substances from plants that release scents stimulate the chicken's appetite, which could be the reason for the increased appetite. On the other hand, Masyita et al. (2022) reported that terpenes and terpenoids, the secondary metabolite substances found in plants such as eugenol, geraniol, carvone, thymol, and menthol, are responsible for the production of characteristic aromas. However, the detailed explanation regarding this matter is still very limited.

In contrast, research conducted by Aroche et al. (2018) showed that diet additions containing polyphenols, the

greatest of which are tannins, can limit feed intake due to astringent qualities, consequently generating protein links between saliva lubricants and hydrogen bonds. When broiler chickens are fed herbs containing bioactive components, their appetites might rise or fall, depending on the dosage supplementations. The amount administered must be carefully monitored to achieve the intended effects. Additionally, research has suggested using PFAs with nanotechnology (Baskara et al., 2021; Ibrahim et al., 2022; Xu et al., 2024).

Average Daily Gain

A total of 14 studies consisting of 32 comparisons were eligible to evaluate the leaf extract treatment effect on ADG, as summarised in Table 3. The pooled effect estimates from SMD revealed that it had a significant effect on increasing ADG (SMD 1.57 [0.77 to 2.36], shown in Figure 3). High heterogeneity within studies was 86.47% ($P < 0.001$). Ross showed an increasing result in ADG ($P < 0.001$) when studies using strains in Hubbard, Arbor Acres, Marshal, Cobb, and Wen Tialu Black showed no effect due to leaf extract supplementation. In this meta-analysis, increasing ADG was found on diet supplementations with leaf extract of *Allium ampeloprasum*, *Cynara scolymus* L., *Petroselinum sativum*, *Artemisia annua* ($P < 0.001$), and *Eucalyptus viminalis* ($P < 0.005$). At the same time, *Salvia officinalis* L. supplementation decreased the ADG of broiler chickens ($P < 0.001$).

Table 3
Subgroup analysis of the effect of leaf extract supplementation on average daily gain of broiler chickens

Covariates	N	SMD	95% CI		SE	*P*-value	Heterogeneity	
			Lower	Upper			I^2	*P*-value
Strain								
Hubbard	8	5.35	0.57	10.13	2.44	0.03	92.85%	<0.001
Ross	20	3.60	1.92	5.28	0.86	<0.001	88.33%	<0.001
Arbor Acres	8	0.59	-0.52	1.70	0.57	0.30	78.29%	<0.001
Marshal	4	0.23	-0.48	0.93	0.36	0.53	0%	0.82
Cobb	6	1.44	-0.49	3.38	0.99	0.14	71.35%	0.02
Wen Tialu Black	2	1.04	-0.05	2.12	0.55	0.06	35.52%	0.21
Leaves extract								
Allium ampeloprasum	4	51.94	25.86	78.01	13.30	<0.001	84.14%	<0.001
Cynara scolymus L.	2	8.54	4.93	12.16	1.84	<0.001	0%	0.68
Petroselinum sativum	3	9.80	5.42	14.18	2.23	<0.001	35.41%	0.21
Moringa oliefera	6	0.11	-2.60	2.83	1.39	0.94	89.35%	<0.001
Eucalyptus viminalis	6	5.16	1.93	8.39	1.65	<0.01	81.86%	<0.001

Table 3 *(Continue)*

Covariates	N	SMD	95% CI		SE	*P*-value	Heterogeneity	
			Lower	Upper			I^2	*P*-value
Olea europaea L.	9	1.19	-0.14	2.51	0.68	0.08	81.86%	<0.001
Ocimum basilicum	2	-0.18	-1.06	0.70	0.45	0.69	0%	0.99
Telfairia occidentalis	4	0.23	-0.48	0.93	0.36	0.53	0%	0.82
Ilex paraguariensis	4	-0.75	-1.50	0.00	0.38	0.05	0%	0.37
Salvia officinalis L.	4	-2.28	-3.34	-1.22	0.54	<0.001	0%	0.42
Cassia tora	1	2.64	-0.45	4.83	1.26	NA	NA	NA
Artemisia annua	2	4.68	2.76	6.60	0.98	<0.001	0%	0.38
Eucommia ulmoides	2	1.04	-0.05	2.12	0.55	0.06	35.52%	0.21

Note. N = Number of comparisons; SMD = Standardized mean differences between the leaves extract treatment and controls; CI = Confidence interval; *P*-value = Probability value; SE = Standard error; NA = Data non-available; SMD and I^2 were considered significant at $P<0.05$

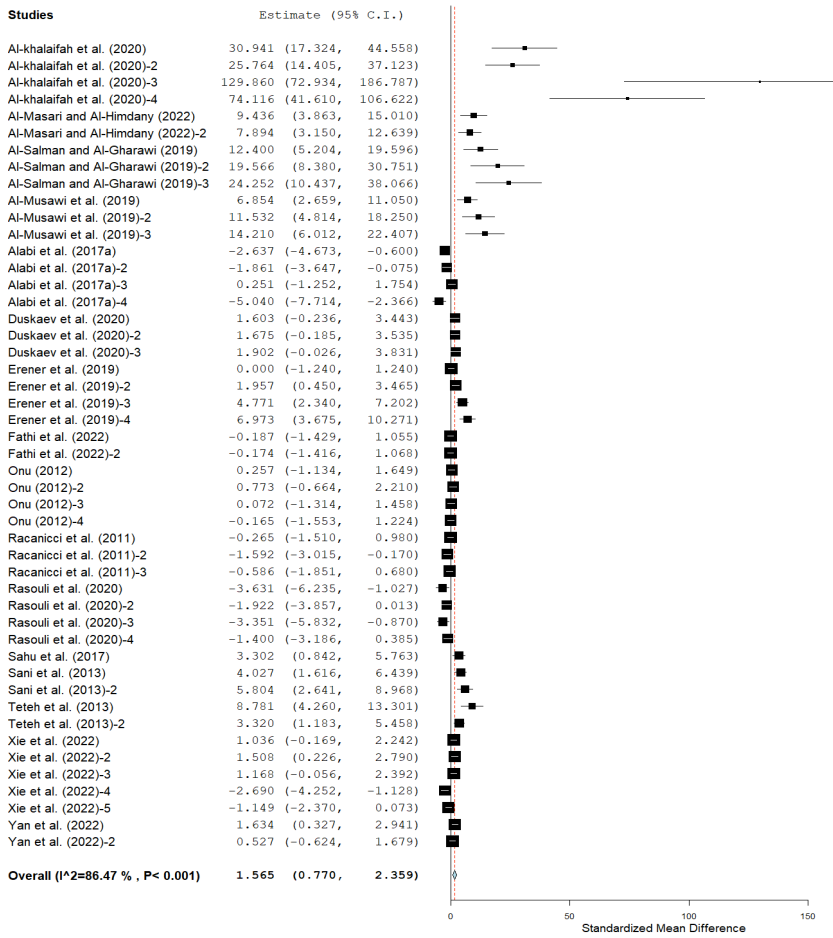

Figure 3. Forest plot of feeding leaves extract effect on broiler chickens' average daily gain

Based on this meta-analysis study, dietary leaf extract supplementations improved the ADG of broiler chickens compared to that of the control group ($P < 0.001$). In line with this result, several experiments showed that dietary supplementation of plant extracts increased the ADG of broiler chickens (Erener et al., 2020; Yan et al., 2022). The increased value of ADG showed a linear relationship with ADFI, which is one of the results of this meta-analysis study. Cheng et al. (2021)'s study showed that lotus leaf extract supplementation improved the growth and population of beneficial bacteria in the intestine and reduced the colonisation of pathogenic bacteria, resulting in the reduction of toxic metabolites produced by the pathogens. Reduction of toxic substances might reduce mucus secretion and increase micro-nutrient absorption, which in turn will optimise the conversion of nutrients into meat (Aljumaah et al., 2020).

Subsequently, the increase in daily body weight of birds might also be related to the antioxidant attributes in secondary plant metabolites. Platzer et al. (2022) showed that phenolic compounds are the most important natural antioxidants and can be classified into subgroups based on their structural properties. The terpene group has activity in counteracting free radicals and preventing oxidative stress (Luo et al., 2022). Apart from that, plants containing alkaloids can also reduce free radicals, disrupting the immune system, changing gene expression, and protecting against degenerative diseases, which can cause abnormal protein production (Albarrak, 2021).

Oxygen is essential for aerobic organisms and produces reactive oxygen species (ROS). ROS are molecules formed sequentially, adding electrons to oxygen, making them more reactive. However, when present in excessive amounts, it shows adverse effects on cells, including deoxyribonucleic acid (DNA) changes, lipid peroxidation, and protein degradation (Escorcia et al., 2020). Plant-derived supplementation can prevent various oxidative stress-induced diseases that lead to decreased productivity and quality in livestock. Supporting this statement, the study by M. Liu et al. (2023) showed that dietary supplementation of a polyherbal mixture containing various bioactive compounds, e.g., flavonoids, alkaloids, and essential oils, increased broiler chickens' daily body weight. This increase might be due to the presence of antioxidant properties of the bioactive compounds in herbs.

Research findings of Yang et al. (2015) also showed that a mixture of herbs containing capsicum, cinnamaldehyde, and carvacrol correlated with modifying the Nrf2 and NF-κB pathways to protect against oxidative stress with improved growth performance. In another study, the increased total antioxidant capacity in a broiler meat-fed diet containing herbal additives increased the activity of the superoxide dismutase (SOD) enzyme and a decrease in malondialdehyde (MDA) levels, which ultimately had the effect of reducing protein denaturation in the meat, resulting in increased meat quality (S. Wang et al., 2017).

Final Body Weight

As summarised in the random effect models (REM) analysis in Table 4. A total of 18 publications, which met the inclusion criteria were used in the meta-analysis to estimate the effect of supplementation leaves extract on the FBW of broiler chickens. The overall estimate of the SMD suggested that the increased levels of supplementation enhance FBW ($P < 0.001$) (SMD = 2.28; 95% CI = 1.40 to 3.16) and heterogeneity among the studies ($I^2 = 86.65\%$) (Figure 4). Subgroup analysis by the strain indicated that leaf extract supplementations increased on FBW ($P < 0.001$) in Ross and Arbor Acres (Table 3). Moreover, treatment using leaf extracts derived from *Allium ampeloprasum*, *Cynara scolymus* L., *Petroselinum sativum*, *Eucalyptus viminalis*, and *Bambusoideae* sp. increased FBW of broiler chickens ($P < 0.001$). The final body weight from the current meta-analysis study showed an increase in value at the end of the period compared to the control ($P < 0.001$). This finding was similar to the result of Al and Al-Gharawi (2019), who reported a gradual increase in body weight gain when the dose of *Eucalyptus viminalis* was 4,000 ppm or more.

Table 4
Subgroup analysis of the effect of leaves extract supplementation on final body weight of broiler chickens

Covariates	N	SMD	Ci 95%		SE	*P*-value	Heterogeneity	
			Lower	Upper			I^2	*P*-value
Strain								
Hubbard	15	0.48	-1.17	2.13	0.84	0.56	89.03%	<0.001
Ross	10	9.22	6.79	11.65	1.24	<0.001	48.56%	0.04
Arbor Acres	13	3.14	2.29	4.00	0.44	<0.001	34.32%	0.15
Marshal	8	0.56	-0.42	1.55	0.50	0.26	43.86%	0.15
Cobb	4	-0.08	-1.45	1.29	0.70	0.91	71.35%	0.02
Wen Tialu Black	2	1.01	-0.04	2.06	0.53	0.06	31.76%	0.23
Leaves extract								
Allium ampeloprasum	4	51.65	25.78	77.51	13.20	<0.001	83.98%	<0.001
Cynara scolymus L.	2	8.51	4.91	12.10	1.84	<0.001	0%	0.68
Petroselinum sativum	3	9.61	5.46	13.76	2.12	<0.001	31.17%	0.23
Moringa oliefera	13	0.12	-1.08	1.31	0.61	0.85	83.92%	<0.001
Eucalyptus viminalis	6	7.20	3.48	10.91	1.90	<0.001	77.89%	<0.001
Telfaria ociidentalis	4	0.56	-0.42	1.55	0.50	0.26	43.86%	0.15
Ilex paraguariensis	3	-0.67	-1.55	0.20	0.45	0.13	25.6%	0.26
Cassia tora	1	2.64	-0.45	4.83	1.12	NA	NA	NA
Bambusoideae sp.	5	3.11	1.99	4.23	0.57	<0.001	52.49%	0.08
Eucommia ulmoides	2	1.01	-0.04	2.06	0.53	0.11	31.76%	0.23

Note. N = Number of comparisons; SMD = Standardized mean differences between the leaves extract treatment and controls; CI = Confidence interval; *P*-value = Probability value; SE = Standard error; NA = Data non-available; SMD and I^2 were considered significant at $P < 0.05$

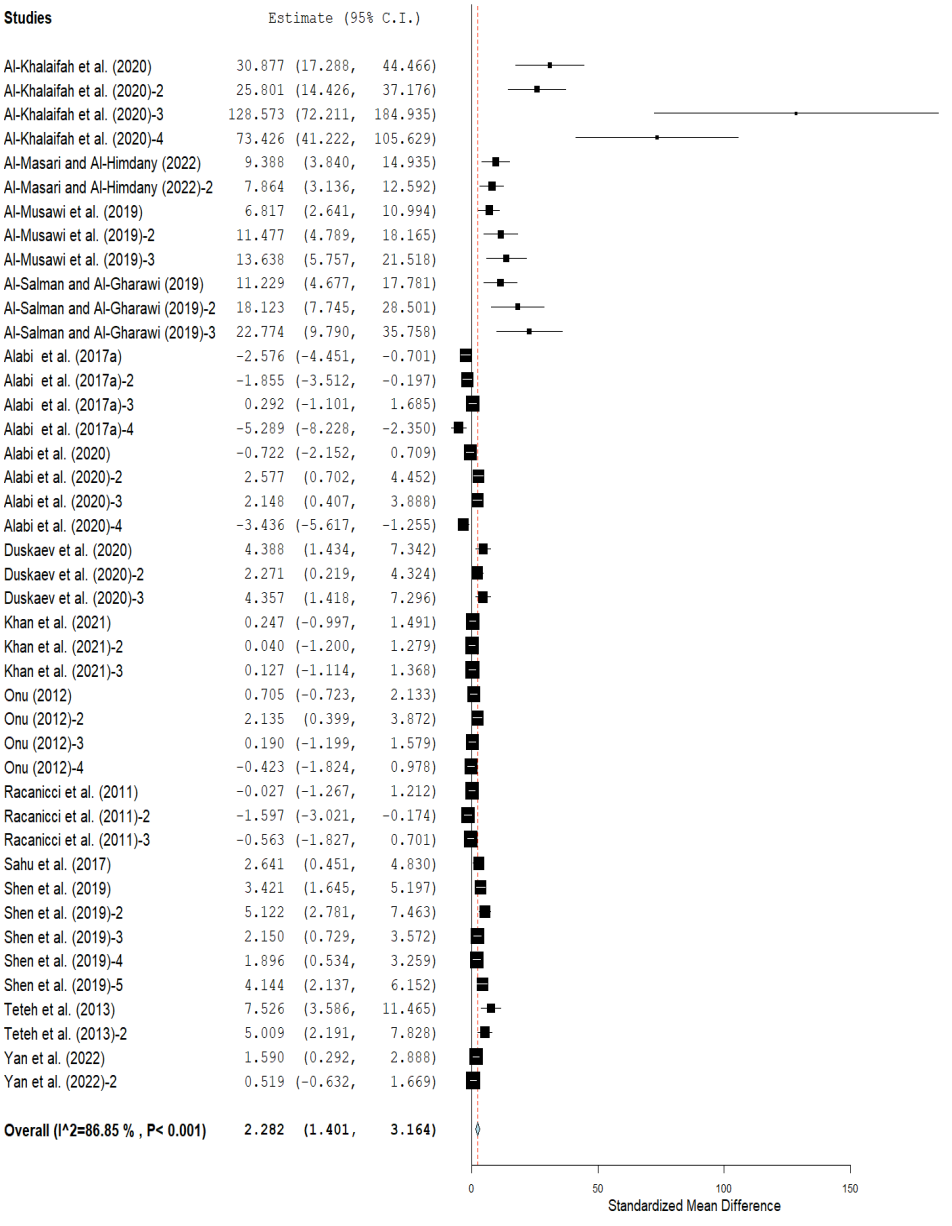

Figure 4. Forest plot on the effect of feeding leaves extract on final body weight of broiler chickens

Supplementations of 100 to 150 ppm *Ilex paraguariensis* reduced the BW of broiler chickens (Racanicci et al., 2011). The changes in the intestinal epithelium, resulting from poor digestion and absorption of nutrients due to changes in the intestinal epithelial lining and reduced bird performance (Islam et al., 2023). Thus, leaf extract supplementations carried out in various studies have an impact on

stimulating the intestinal epithelium to work optimally. Improvement in final body weight in broilers associated with the supplementation of leaf extract might be due to the increased appetite shown by feed intake.

Furthermore, the intestine of broiler chicken contains important bacteria, including *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* (Clavijo & Flórez, 2018). In the cecum, colonised microbiota also plays an important role in immune function, compromising the impact of nutrient absorption on the ultimate heaviness of the chicken body. The mode of action when using natural herbal additives could be responsible for the decreased number of hydrophobic pathogenic bacteria, thereby affecting the surface properties of microbial cells (Rashid et al., 2020). Pathogenic bacteria in the intestine are inhibited, but the growth of beneficial bacteria is promoted, which in turn will be important for nutrient absorption by the wall of the intestine (Han et al., 2016). Eventually, nutrient accumulation in the meat could increase as intestinal absorption increases.

Feed Conversion Ratio

Assessing the effect of leaf extract supplementation on the FCR of broiler chickens in 18 articles with 56 comparisons that met the eligibility rule for inclusion in the meta-analysis was used (Table 5). The addition of leaf extract as a feed additive for chickens had a significant effect on decreasing FCR ($P < 0.001$) with an SMD value of -1.25 (-1.76 to -0.73) and heterogeneity among the studies ($I^2 = 81.95\%$) (Figure 5). Arbor Acres and Wen Tialu Black showed a decreasing result in FCR ($P < 0.001$). The decrease in FCR was consistent in the supplementation leaf extract of *Petroselinum sativum*, *Olea europaea* L., *Artemisia annua*, *Bambusoideae* sp., and *Eucommia ulmoides* ($P < 0.001$). At the same time, the increase in FCR was found in *Allium ampeloprasum* L. ($P < 0.005$). The result of dietary supplementation of leaf extract shown a decrease in FCR ($P < 0.001$).

Various studies indicated that supplementations of plant extracts reduced FCR (Erener et al., 2020; Teteh et al., 2013; Yan et al., 2022). The lower FCR indicated the higher feed efficiency (Huang et al., 2022). Better growth performance, as shown in the lower FCR, could be attributed to the presence of various secondary metabolites in leaf extract, which increased the number of lactic acid bacteria, increased crypt depth and villus height, and hence improved nutrient absorption (M. Liu et al., 2023). A study performed by Parobali et al. (2024) showed that absorption of dietary nutrients into the body of birds through the bloodstream leads to increased feed utilisation efficiency, shown by increased villus height, crypt depth, and the ratio of villus height and crypt depth. A shorter villus may indicate inflammation, infection, nutritional deficiencies, and various diseases in the walls of the digestive tract. In addition, small villus can also be related to the small number of secretory and absorptive cells.

Overall, the decrease in the intestinal wall environment could have implications on micro-nutrient absorption and utilisation and, therefore, must be maintained properly (Prakatur et al., 2019). In addition, results in the current study suggest that the low value is closely related to the high values of ADFI and BW.

Table 5
Subgroup analysis of the effect of leaf extract supplementation on feed conversion ratio of broiler chickens

Covariates	N	SMD	95% CI		SE	*P*-value	Heterogeneity	
			Lower	Upper			I^2	*P*-value
Strain								
Hubbard	11	-0.82	-2.20	0.57	0.71	0.25	85.92%	<0.001
Ross	20	-1.65	-2.74	-0.57	0.56	<0.01	82.06%	<0.001
Arbor Acres	13	-1.37	-1.79	-0.96	0.21	<0.001	14.19%	0.30
Marshal	4	-0.84	-1.57	-0.10	0.38	0.03	0%	0.58
Cobb	6	-2.39	-5.34	0.56	1.51	0.11	89.35%	<0.001
Wen Tialu Black	2	-2.11	-3.12	-1.11	0.51	<0.001	0%	0.39
Leaves extract								
Allium ampeloprasum	4	6.81	-11.12	-2.51	2.20	0.01	89.16%	<0.001
Cynara scolymus L.	2	-0.06	-4.62	4.50	2.33	0.98	89.66%	<0.01
Petroselinum sativum	3	-5.28	-8.20	-2.37	1.49	<0.001	49.26%	0.12
Moringa oliefera	9	0.12	-0.86	1.11	0.50	0.81	73.68%	<0.001
Eucalyptus viminalis	6	-5.57	3.48	10.91	1.82	<0.01	83.38%	<0.001
Olea europaea L.	9	-1.51	-2.24	-0.78	0.37	<0.001	59.13%	0.01
Ocimum basilicum	2	0.24	-0.64	1.12	0.45	0.59	0%	0.86
Telfaria ociidentalis	4	-0.84	-1.57	1.10	0.38	0.03	0%	0.58
Ilex paraguariensis	3	-0.67	-1.55	0.20	0.41	0.04	10.82%	0.33
Salvia officinalis L.	4	0.82	-0.04	1.67	0.44	0.06	0%	0.51
Cassia tora	1	-5.55	-9.08	-2.03	1.80	NA	NA	NA
Artemisia annua	2	-30.71	-41.52	-19.90	5.51	<0.001	0%	0.40
Bambusoideae sp.	5	-1.50	-2.07	-0.92	0.30	<0.001	0%	0.60
Eucommia ulmoides	2	-2.11	-3.12	-1.11	0.51	<0.001	0%	0.40

Note. N = Number of comparisons; SMD = Standardized mean differences between the leaves extract treatment and controls; CI = Confidence interval; *P*-value = Probability value; SE = Standard error; NA = Data non-available; SMD and I^2 were considered significant at $P < 0.05$

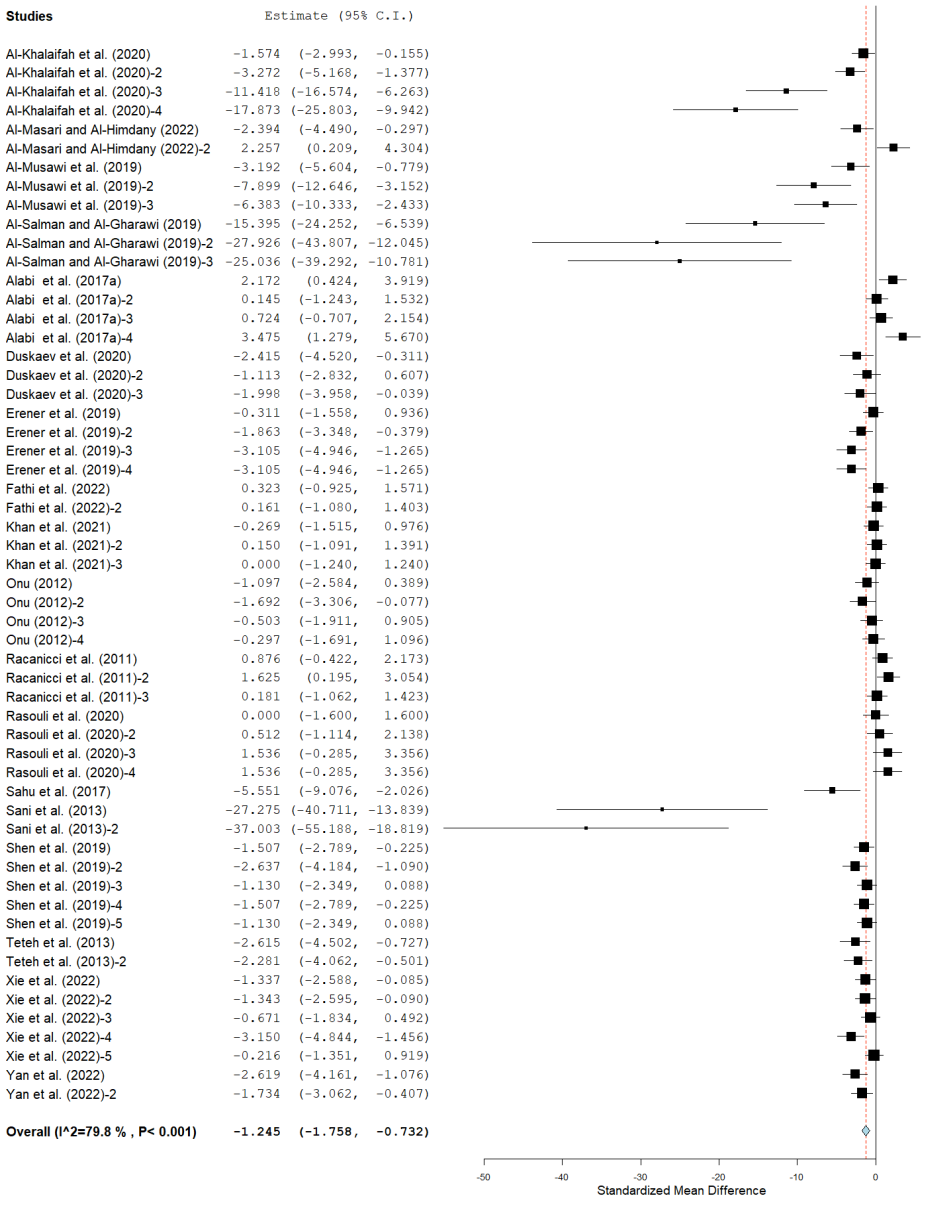

Figure 5. Forest plot on the effect of feeding leaves extract on broiler chicken's feed conversion ratio

CONCLUSION

The results of this meta-analysis study provided promising data on leaf extracts' potency as a natural growth promoter in broiler chickens. Future research should focus on the proper dosages of plant extract supplementation to change the presence of in-feed antibiotics.

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Initiation of Cassava Callus Culture and Its Prospect for Starch Production: A Systematic Mapping Study

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ABSTRACT

Mass propagation of cassava on several hectares of arable land due to increasing demand for its starch is not feasible due to land availability, pests and disease invasion, and long cultivation period. Plant cell culture technology is a promising solution despite the scarcity of cassava callus culture for starch production applications. Therefore, a systematic mapping study (SMS) was performed to identify the applications of cassava tissue culture and its prospects in starch production and investigate the important parameters for cassava callus culture initiation. The SMS began with formulating research questions (RQs), conducting searches on various databases, collecting and screening related articles, and extracting and mapping the selected articles. A total of 56 of 589 articles in the initial searching phase were chosen to be used as references to answer each RQ. The extracted data indicates that cassava tissue culture was mostly used for micropropagation, while starch production from its tissue culture is still limited. Basal medium and plant growth regulators influence cassava callus culture initiation most. The findings of the SMS offer a better understanding of cassava tissue culture and the prospects of producing cassava starch.

Keywords: Cassava, *Manihot esculenta* Crantz, plant tissue culture, starch, systematic mapping study

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INTRODUCTION

Manihot esculenta Crantz, known as cassava, tapioca, yuca, and mandioca, is a tuberous edible plant that possesses many desirable characteristics, such as able to withstand climate change, could be propagated on low fertility soils and tolerant against major diseases and pests (Tokunaga et al., 2020). It is the third most important source of calories in the tropics and is deemed the sixth most important crop after sugar cane, maize, rice, wheat, and potato (Parmar et al., 2017). Cassava is a woody plant with a rigid upright stem and spirally arranged in lobes whose tapered edible roots usually have brown, white, and reddish hues (Lim, 2016). The roots contain 80% dry weight of starch, which is usually utilized as raw materials in manufacturing industries for the production of bioethanol, bioplastics, dextrin, glucose, fructose, lactic acids, and baker's yeast (Parmar et al., 2017). Starch is a natural biopolymer comprising two types of polymer chains: amylose and amylopectin (Robyt, 2008). It is considered the most important polysaccharide, with beneficial characteristics such as being renewable, biodegradable, low-cost, and obtainable from abundant plant sources. It is also a good oxygen barrier, rendering it suitable for integration into raw materials for the plastic industry (Adewale et al., 2022). Starch has been widely used in various industries, such as in the food industries as a thickening, smoothening, and clarifying agent, and in the paper industries as a flocculant, retention, bonding, and binding agent (Vamadevan & Bertoft, 2015).

Although cassava starch is in high demand due to its various applications, there are limitations in conducting large-scale or mass-propagation of cassava, such as the availability of lands and long cultivating period, besides pest and disease invasion (Howeler et al., 2012). These challenges could be potentially solved using plant cell culture technology. The term refers to the aseptic culture of cells, tissues, organs, or whole plants under controlled nutritional and environmental conditions (Hussain et al., 2012). *In vitro* cultivation of cassava is advantageous to its mass propagation through a higher rate of multiplication, protection against infection during propagation, and the ability to produce disease-free cassava plants.

However, reports regarding the application of cassava tissue culture for starch production are limited. Therefore, this systematic mapping study (SMS) aims to identify the recent applications of cassava tissue culture and their prospects in starch production. In addition, this study also investigates the important parameters for the initiation of cassava callus culture. Data acquired from various databases (Academia, Academic Journals, African Journal Online, ResearchGate, SpringerLink, Taylor and Francis Online, and Wiley Online Library) were extracted to answer all constructed research questions (RQs) based on the study's objectives. This SMS can be a platform for further experimental studies on initiating cassava callus culture and open new possibilities to produce starch using plant cell culture technology.

METHODS

SMS is the research method used in this study to map and classify previous research studies based on the research area, patterns of evaluation, and research gap to recommend more areas of study (Petersen et al., 2008). All existing evidence on specific topics is screened and selected through a detailed search strategy to meet the eligibility criteria to answer the RQs and achieve the targeted objectives. The procedures in SMS involve the identification of RQs according to the study's objectives, followed by the development of a search strategy, execution of the screening stage, and data extraction and mapping. Figure 1 illustrates the steps in conducting SMS, where the first and second screening stages were done using Parsifal software (version 1.0).

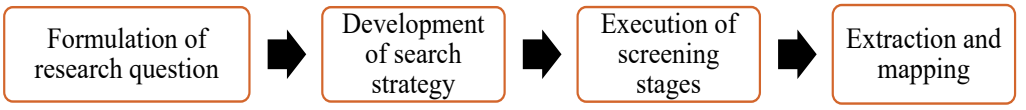

Figure 1. Flow chart of systematic mapping study

Formulation of RQs

One important step of SMS is formulating the RQs since they guide the search process. Clear and defined questions are necessary before executing all SMS procedures. Several research questions were constructed using the population, concept, and context (PCC) method to achieve this aim and purpose, as shown in Table 1 (Petersen et al., 2008).

Table 1
Research questions formulated using the population, concept, and context method

Research question (RQ) 1	
RQ 1	What applications of cassava cultures have been reported so far?
Sub-RQ 1	What are the types of cultures used for *in vitr*o propagation of cassava?
Sub-RQ 2	What are the varieties of cassava chosen in the articles?
Sub-RQ 3	What are the countries or the origin of the reported articles?
Research question 2	
RQ 2	What are the important parameters for initiating cassava callus culture?
Sub-RQ 1	What type of explants were used to study cassava callus culture?
Sub-RQ 2	What is the preparation method to surface-sterilize the explants to avoid contamination in the cassava callus culture?
Sub-RQ 3	What are the analyses used to quantify the growth of callus?
Sub- RQ 4	What statistical analysis is used to find the most significant parameters/ plant growth regulator (PGR) concentration reported in the articles?

Development of Search Strategy

The primary search process in this SMS involved various online databases, such as Academia, Academic Journals, African Journal Online, ResearchGate, ScienceDirect, SpringerLink, Taylor and Francis Online, and Wiley Online Library. A comprehensive search strategy ensures that the constructed search strings cover both objectives. Thus, the search strategy from Zein et al. (2016) was adopted to construct the search strings as follows:

- The search was conducted based on synonyms and other alternative words suitable for use as keywords.
- The use of Boolean OR to integrate the alternative keywords and suitable synonyms.
- The use of Boolean AND to connect the important terms.
- Double quotations are used for the composite words.
- Paratheses are used to separate keywords and synonyms.

Several trials were conducted, and the final search strings chosen are as follows: ("tissue culture" OR "*in vitro* propagation") and cassava.

Execution of Screening Stages

All articles went through three screening stages, which were iterative and incremental. In the first screening stage, advanced search features provided by the online databases were utilized by applying the search string. All articles were screened and selected according to the study's title and uploaded to Parsifal. Duplicated articles from the databases were removed with the help of Parsifal. The second screening stage was done by going through the abstract, where articles that did not mention the use of tissue culture for propagating cassava were removed. The selected articles eligible for selection criteria were passed to the third screening stage, i.e., reading the full-text articles, which includes the introduction, methodology, and conclusion. Toward the end of the SMS, all important information from the selected articles was extracted to unravel the RQs and achieve the targeted objectives. Table 2 lists the essential selection criteria for screening and selecting related articles.

Table 2
Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Studies must be published between 2011 and 2021	Studies published before the year 2011
Studies must be in English	Non-English articles
Studies must describe the use of plant tissue culture for *in vitro* propagation of cassava	All systematic review and meta-analysis articles
The study must mention using cassava or *M. esculenta* Crantz as an explant for tissue culture	All book chapters and Doctoral and Master's theses
Studies must be journal articles or conference papers only	Studies do not properly describe the use of tissue culture in propagating cassava

RESULTS AND DISCUSSION

Search Result

About 589 articles were initially retrieved from databases such as Academia, Academic Journals, African Journal Online, ResearchGate, ScienceDirect, SpringerLink, Taylor and Francis Online, and Wiley Online Library. Since there is the possibility of duplicate articles in all databases, it is crucial to filter them out first. A total of 32 duplicates were removed using Parsif.al. Table 3 summarizes the number of articles accepted in the first, second, and third screening stages of this SMS, while Figure 2 illustrates the results of the first screening stages in percentage.

Table 3
List of sources and results of the screening stages

Online databases	Stage 1	Stage 2	Stage 3
Academia	8	6	5
Academic Journal	14	13	12
African Journal Online	19	12	8
ResearchGate	47	25	20
ScienceDirect	12	5	2
SpringerLink	18	10	4
Taylor and Francis Online	8	5	4
Wiley Online Library	6	2	1
Total	132	78	36

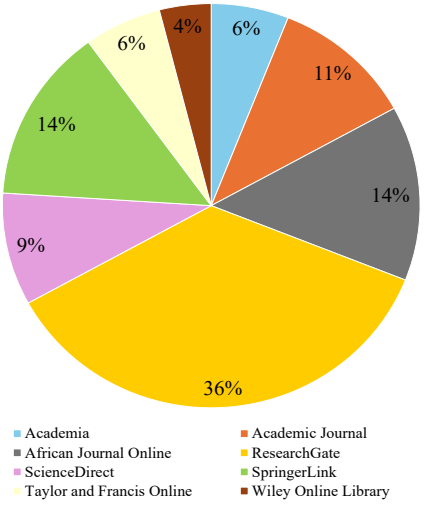

Figure 2. Stage 1 screening result distribution in percentage

From the recorded number of articles in Table 2, 557 were screened in the first screening stage after removing duplicates in the initial search result. A total of 132 articles were selected in the first screening phase, excluding articles with titles unrelated to the study. Figure 2 illustrates the overall result from the first screening stage, where ResearchGate contributed the highest percentage (36%), followed by African Journal Online and SpringerLink at 14%. From the second screening stage, 78 articles were accepted after going through their respective abstracts. At this stage, any articles that did not mention the use of plant tissue culture in propagating cassava were excluded from the SMS. Finally, after the third screening phase, only 56 articles were selected and chosen to answer the constructed RQs as they met all requirements listed in the inclusion criteria (Table 2).

The second objective involved further screening to choose the studies that

utilize callus culture for *in vitro* cassava propagation. Articles in the third screening stage must clearly describe the protocols and important parameters needed to initiate cassava callus culture. Only 11 articles were selected at the third screening stage, which exhibits all the information needed to complete the second objective of this SMS (Figure 3). Five articles were obtained from the Academic Journal online database, followed by the African Journal Online, with three. None of the articles from ScienceDirect, SpringerLink, Taylor and Francis Online, and Wiley Online

Library discussed the protocols and important parameters to initiate cassava callus culture. All selected articles in the SMS are listed in Table A1 in the Appendix.

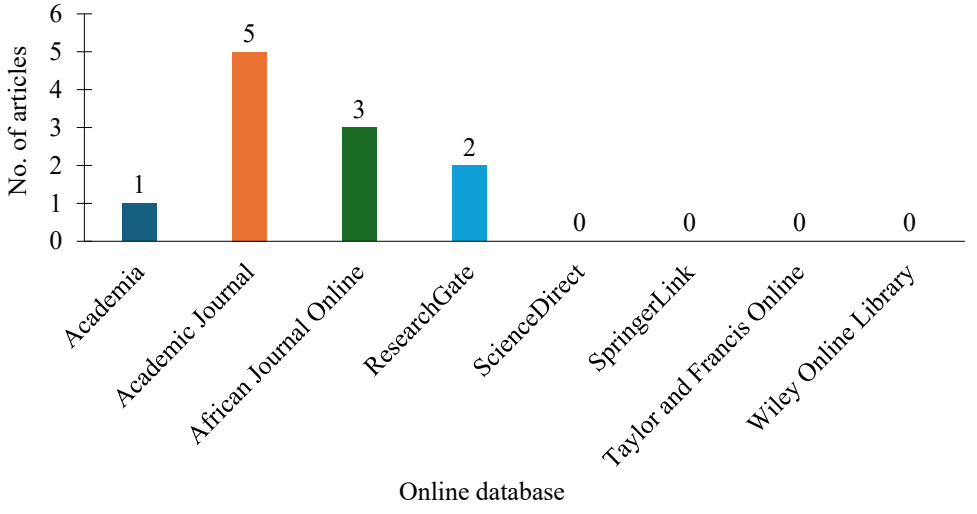

Figure 3. The number of articles selected to achieve Objective 2 after the third screening stage

Answering the RQs

All RQs constructed at the beginning of the SMS are answered according to the information extracted from the 56 chosen articles.

RQ 1: What are the Applications that Have Been Reported So Far?

The applications of cassava cultures are illustrated in Figure 4 from the final selection of 56 articles.

Figure 4 depicts that *in vitro* cultivation of cassava is mostly used for micropropagation purposes, as indicated by the 75% distribution in the pie chart. Micropropagation refers to the rapid production of plantlets, resulting in uniform genetic characteristics and free from disease and pests as they grow in a controlled environment (Mahdi & Edward, 2018). Most of the objectives in the studies involving micropropagation of cassava were to investigate the best media formulation and culture conditions for a high cassava yield.

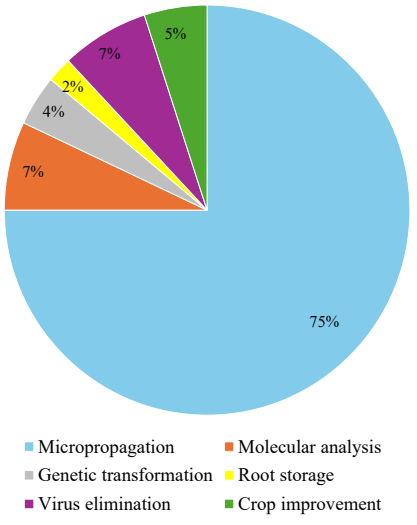

Figure 4. Applications of *in vitro* cultivation of cassava-based on 56 studied articles

Meanwhile, about 7% of the articles (Figure 4) refer to using plant tissue culture in cassava for molecular analysis and virus elimination, corresponding to 4 articles. Cassava is usually infected with viral diseases such as cassava mosaic disease (CMD), which can cause a yield loss from 20 up to 95% (Sessou et al., 2019). Pest problems in cassava plantations, especially whitefly species (*Bemisia tabaci*), can become a weed host on more than 600 crops, which can cause the spread of *Sri Lankan cassava mosaic virus* (SLCMV) and *Indian cassava mosaic virus* (ICMW; Duraisamy et al., 2013). Thus, various studies on cassava tissue cultures were geared to eliminate these viruses, and molecular analysis were conducted to help alleviate the issue.

Finally, the cassava root storage formation study using cassava tissue culture has the lowest distribution, i.e., 2% (Figure 4). According to the study by Yao et al. (2013), calcium significantly influences the *in vitro* growth of cassava root formation and its starch accumulation. Calcium is important in cell wall synthesis, membrane function, and cell signaling; thus, it needs to be supplemented in the culture medium to support *in vitro* growth of plant cultures (Smith, 2013). Results showed that although the addition of calcium positively affects the diameter of *in vitro* cassava storage roots, it is detrimental to the induction rate and starch content. The observation of *in vitro* cassava roots and field cultivation of cassava roots through the scanning electron microscope (SEM) showed that starch grains formed by these two different ways were similar in size and shape, indicating that the study of starch synthesis from *in vitro* induction of cassava roots is feasible (Yao et al., 2013).

SUB 1 RQ 1: What Types of Cultures are Used for *In Vitro* Propagation of Cassava? The SMS successfully highlights six types of plant tissue culture that have been used for *in vitro* cassava propagation, and the statistics are illustrated in Figure 5. The shoot and meristem cultures have the highest distribution among all cultures, i.e., 61%, with 36 articles. Shoot culture refers to *the in vitro* cultivation of shoot tips to produce shoot clumps from axillary or adventitious buds. It is also widely used for clonal propagation (Smith, 2013). The shoot culture contributed the highest in generating cassava *in vitro* because it has proven very effective in plant micropropagation (Gusain et al., 2021). Besides, the risk of getting bacterial or fungal contamination is very low in shoot culture, and the cultivation time is shorter compared to other types of culture.

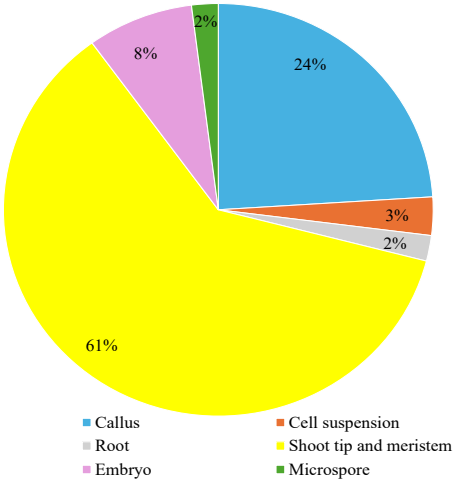

Figure 5. Types of cassava cultures for *in vitro* propagation of cassava

Callus culture refers to the growth of an unorganized, growing, and dividing mass of cells with the supply of auxin and cytokinin, where any part of the plant can be used as an explant (Smith, 2013). This type of cassava culture has the second highest distribution (24%), with 14 articles. Callus culture has been widely used as an experimental system to solve a wide range of basic research problems regarding plant cytology, morphology, physiology, anatomy, pathology, biochemistry, and genetics. It has also been used to solve research problems related to the propagation of horticultural and agronomic plants, especially in organogenesis and embryogenesis sections (Abdalla et al., 2013). Microspore culture and root culture are the least utilized, i.e., at 2%, with 1 article each. Microspore culture refers to cultivating cassava haploid tissue using pollen or anthers as an explant (Smith, 2013). More than 250 plant species have been propagated using microspore culture; however, only a handful of studies have reported developing *in vitro* double haploid protocols in cassava (Perera et al., 2014).

SUB 2 RQ 1: What are the Varieties of Cassava Chosen in the Articles?

Cassava has a broad range of different types of cultivars, which are distinguished or grouped by certain characteristics in terms of their morphology or physiology. These characteristics are important in horticulture, agriculture, or forestry applications. Researchers and farmers require plants with a particular characteristic or adaptation to their environment and cultivation practices to suit the purposes of cultivating the plant either *in vitro* or traditionally.

Detailed information on cassava varieties used in the 56 articles can be found in Table A1 (Appendix). All varieties used in the experimental studies were well established either directly from the greenhouse, farm fields, or experimental fields or obtained from research institutes. Generally, before the cassava plants were used as the explant for *in vitro* cultivation, they were well petted and grown under almost perfect conditions at 28°C under fluorescent lights with 70 $\mu mol.m^{-2}.s^{-1}$ and 12 hr light/12 hr dark of photoperiod regime (Tokunaga et al., 2020). Some researchers described the reasons for choosing specific cassava cultivars for their studies. The most common factor in cassava variety selection is due to the farmer's preference genotype that exhibits good performance, such as early maturity, high productivity,

palatability, and resistance to disease and pest (Fletcher et al., 2011; Maruthi et al., 2019; Sesay et al., 2018).

SUB 3 RQ 1: What are the Countries or the Origin of the Reported Articles? The origin or country of all 56 articles obtained in this SMS is depicted in Figure 6. Africa contributes the highest to the publication of studies on the *in vitro* cultivation of cassava, at 55%, with 31 articles. It reflects that cassava is the major staple food for millions in East and Central Africa, especially in rural areas. The tuberous roots of cassava, rich in carbohydrates, are suitable for human consumption after being processed and cooked (Osena et al., 2017). Besides, cassava can be utilized for bioenergy generation, biomaterial production, and animal feed because of its quality and quantity of starch content and high biomass productivity. Thus, any efforts to increase the production of cassava crops in Africa are supported by the government, and millions of dollars are allocated annually for cassava research and development to combat diseases and pests that affect crop yield (Fotso et al., 2014). Africa is actively involved in studies to produce cultivars that are resistant to the *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV), where these viruses are transmitted by whiteflies (*B. tabaci* Gennadius) to the planting materials and can cause 100% yield losses (Kidulile et al., 2018).

Meanwhile, China is the second highest contributor to the study of *in vitro*

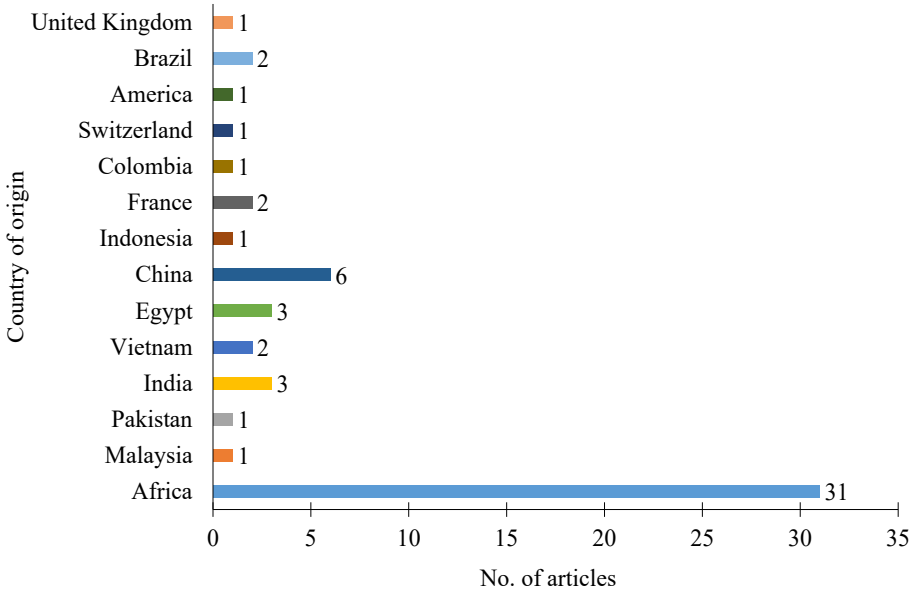

Figure 6. Origin of the published articles on *in vitro* propagation of cassava

propagation of cassava, at 11%, with six articles (Figure 6). Cassava research in China is mainly focused on the storage capacities of starchy cassava roots, and most of the articles found for this SMS were more focused on developing its tuber roots and storage capacity. For instance, Wu et al. (2014) concluded that sucrose plays a crucial role in cassava tuberous root formation and saccharide accumulation. Likewise, Yao et al. (2013) reported that calcium concentration in the medium influenced *in vitro* growth of cassava root formation and its starch accumulation (Yao et al., 2013). Figure 6 depicts almost similar distribution in the studies of cassava using plant tissue culture in other remaining countries, at about 3–5%, with a range of 1–3 published articles.

RQ 2: What are the Important Parameters to Initiate Cassava Callus Culture?

The important parameters to initiate cassava callus culture are divided into three categories: (1) the basal medium, (2) the PGRs, and (3) the growth condition and cultivation periods. Plant culture media used in the *in vitro* cultivation of cassava comprises essential elements or mineral ions (supplied as a complex mixture of salts), an organic supplement (supplied as vitamins and/or amino acids), and a carbon source (usually supplied as sucrose). Basal medium refers to the basic media without supplementation, which aims to support the growth of plant cell culture. From 11 articles extracted from SMS for the second objective, ten articles used the Murashige and Skoog (MS) basal medium, while another opted for the Gresshoff and Doy (GD) basal medium. Both MS and GD basal media comprise microelements, macroelements, iron sources, and organic supplements needed for the growth of plant cell culture, albeit with slight differences in the concentration of each element inside the basal medium. Moreover, some elements are only available in the MS medium but not in the GD medium and *vice versa*. MS medium is regarded as the most commonly used basal medium and is a basis for other media formulations (Smith, 2013).

PGRs are important in determining the pathway of plant cell development; they stimulate cell division and regulate the growth and differentiation of shoots and roots of cultured explants. For instance, the optimum type and PGR concentration for callus induction and growth for a particular plant do not necessarily have a similar impact on the development of shoots or roots (Gusain et al., 2021). Five main PGRs are used in plant culture systems: auxins, cytokinin, gibberellins, abscisic acid, and ethylene. The most common PGRs mentioned in this study's articles are auxins and cytokinin.

Auxins, which are 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), and picloram, are commonly used in studies of cassava callus culture initiation to promote cell division and growth. 2,4-D is the most used and effective auxin in the induction of plant cell culture (Smith, 2013). In a comparison between 2,4-D and picloram, the former exhibited a better response in the induction of callus culture, giving a

slightly higher frequency of callus formation (%). However, the highest frequency of callus formation for both PGRs was achieved at the same concentration, i.e., 8 mg/L (Ngugi et al., 2015). In a study to determine the best 2,4-D concentration that produces a high frequency of callus formation, a higher level of 2,4-D (12–16 mg/L) proved to be the best callus inducer (Elibariki et al., 2014). Meanwhile, Abdalla et al. (2013) demonstrated that the best 2,4-D concentration for inducing cassava callus culture is 15 mg/L, with 100% callus formation frequency. In addition, an experimental study on the effect of three types of cytokinin, i.e., thidiazuron (TDZ), 6-benzylaminopurine (BAP), and kinetin, showed that TDZ with a 1.5-µM concentration produced the highest frequency of callus formation (%; Sessou et al., 2019). It is not feasible to generalize the optimum PGRs for cassava callus culture due to the difficulty in predicting its effects on plant cell culture due to the differences in culture response between species, cultivars, and plants grown under different conditions. Further studies are required to confirm the optimum type and concentration of PGR for callus induction.

Table 4
Types of explants used to initiate cassava callus culture

Type of explant	No. of articles
Shoot	2
Leaf (immature leaf lobes, young leaf lobes, meristematic leaf lobes)	7
Stem internodes	4
Root	1
Axillary buds	1

Finally, the cultural environment is important to promote the growth of cassava callus culture. From the chosen articles in this SMS, the optimum temperature to cultivate the cassava callus culture is 22 to 28ºC, and most articles reported the incubation of callus culture in a dark room with a photoperiod of mainly 12 to 16 hr. The main benefit of using plant tissue culture in cultivating cassava is the ability to control the culture condition, which could prevent factors such as drought, rain, diseases, and pests from affecting the cultures.

SUB 1 RQ 2: What is the Type of Explants Used to Study Cassava Callus Culture?

Explants refer to small pieces of plant parts or tissues being cut aseptically and used to initiate a culture in a nutrient medium (Putri et al., 2019). Explants can be taken from different plant parts as long as they can be differentiated into totipotent cells. The types of explants used to initiate cassava callus culture extracted from the articles are listed in Table 4.

From Table 4, leaf explants, including immature leaf lobes (ILL), young leaf lobes, and meristematic leaf lobes, are widely used to initiate cassava callus culture. ILL and stem explants demonstrated different abilities to initiate callus culture in terms of the duration, characteristics, and frequency of callus formation (%), where ILL can generate callus on an average of 10 days while stem explant took up to 15 days (Syombua et al., 2019). The callus culture formed from the ILL explant is more translucent, gelatinous, and highly

embryonic than the stem explant, giving rise to mainly loose, friable, and non-embryonic callus. Syombua et al. (2019) further mentioned that the leaf explant gave higher frequencies of callus formation (%) than the stem explant. This notion is similar to the report by Abdalla et al. (2013).

Stem nodes are the second most frequently used type of explant to initiate callus culture, followed by shoot, root, and axillary bud explants (Table 4). The correct selection of explant material is important to successfully establish a tissue culture. The factor in choosing an explant depends on the type of culture to be initiated, the application of the proposed culture, and the plant species to be used.

SUB 2 RQ 2: What is the Preparation Method to Surface-sterilize the Explants to Avoid Contamination in the Cassava Callus Culture? Microbial contamination in plant tissue culture has become a constant problem compromising the *in vitro* culture development, especially when the explant is originally from field-grown plants (Putri et al., 2019). Surface sterilization is a pre-treatment method to prepare an explant before being cultured in the growth media. It refers to the process of immersing the explant in an appropriate concentration of chemical disinfectant(s) or sterilant(s) for a specific duration, resulting in the establishment of a contamination-free culture (Bello et al., 2018). This method is the most crucial step in developing plant tissue culture protocol, where inappropriate concentrations of chemical disinfectant or sterilization duration can have a lethal effect on the division of cells and restrict plant growth and development. Thus, it is necessary to take heed of the chemical sterilant's concentration, combination, and exposure duration to ensure a successful *in vitro* culture establishment.

Most of the studies required first cleaning the explants under running tap water and cleaning with soap to remove any physical dirt and surface debris attached to the explants (Elibariki et al., 2014; Faye et al., 2015; Fotso et al., 2014; Sessou et al., 2019). Next, explants are immersed in 70% ethanol, i.e., the concentration that effectively kills microbes, bacteria, and other microorganisms on the surface of the explant. The duration of explant immersion in 70% ethanol should not exceed 5 minutes as it can destroy its cells (Elibariki et al., 2014; Fotso et al., 2014; Sessou et al., 2019).

The immersion of explants in bleach is also crucial to further ensure the removal of contaminants from the explants, especially microorganisms. Sodium hypochlorite, commercially known as laundry bleach, is the most frequent choice, where it will be diluted to 25% for surface sterilization purposes. A balanced concentration and duration of immersion must be determined correctly to prevent phytotoxicity on the explant, which usually takes between 20 s and 20 min, depending on the concentration and type of explant (Elibariki et al., 2014; Fletcher et al., 2011; Sessou et al., 2019). The addition of Tween 20 in the bleach solution reduces the surface tension of the explant and allows better contact of the

surface with the surfactant(s). Calcium hypochlorite has also been reported (Faye et al., 2015). It is highlighted that this kind of bleach may cause less injury to plant tissues than sodium hypochlorite. Finally, the most important step in the surface sterilization method is to rinse the sterilized explant thoroughly with sterile distilled water, usually three to four times within each step.

SUB 3 RQ 2: What Analyses are Used for Quantifying Callus Growth? The growth of cassava callus, but Table 5 lists the most used procedures or analyses for observing, monitoring, and evaluating the success of initiating cassava callus culture.

The most common way to quantify the growth of callus culture is by determining the frequency of callus formation (%, Table 5). The frequency of callus formation is expressed as the percentage of the calluses produced by the explant per total number of explants being used in the experiment (Sessou et al., 2019), as presented in Eq. (1):

No additional procedures are required for this analysis compared to the fresh weight (FW) or dry weight (DW) analyses,

$$\text{Frequency of callus formation (\%)} = \frac{\text{Explant produced callus}}{\text{Total cultured explants}} \times 100\% \quad [1]$$

whereby specific and additional procedures must be conducted to determine the results. Besides, the callus formation method frequency is non-destructive and very convenient, considering that in the early stage of callus initiation, its amount is limited. Other quantifying methods, i.e., the size and weight of the callus, are the least preferred by researchers (Table 5). In the FW technique, the jar and media will be pre-weighed on the analytical weighing balance, and the measurement will be denoted as A before inoculating the explant. The data will be collected after four weeks of cultivation. The callus will be taken out from the glass jar and dabbed with filter paper to remove any moisture on the surface of the callus (Fotso et al., 2014). The callus, the jar and the medium will be weighed using the analytical weighing balance. The measurement will be recorded and denoted as B. Thus, the FW of the callus will be denoted as C, and the calculation will be made according to Eq. (2):

Table 5
Quantifying analysis for the growth of cassava callus culture

Quantifying analysis	No. of articles
Frequency of callus formation (%)	8
Size of callus	3
Fresh and dry weight	3

$$\text{Fresh weight of callus (g)}, C = B - A \quad [2]$$

For the DW, after determining the callus FW, the sample will be dried in the oven at 40°C for 24 hr in the Petri dish. The callus sample is left to cool to room temperature in a desiccator containing silica gels to prevent the cells from absorbing any moisture in the surroundings. The sample will be then weighed on the analytical weighing balance, and the measurement will be recorded.

Then, the callus sample will be returned to the drying oven for another 4 hr, and the step will be repeated until the sample reaches a constant weight (Abdalla et al., 2013). The constant weight is denoted as D, whereas the pre-weighed jar and medium are denoted as A. The FW of the callus culture is calculated based on Eq. (3):

$$\text{Dry wright of callus (g), } E = D - A \quad [3]$$

The FW and DW methods of callus growth quantification hinder the callus from being used for plant regeneration since the culture is exposed to high temperatures and contamination during the procedures. This disadvantage is especially evident when the amount of callus established is small, specifically when the research is at the beginning stage (initiation of callus culture).

SUB 4 RQ 2: What Statistical Analysis was Used to Find the Most Significant Parameters/PGR Concentration Reported in the Articles? Statistical analysis refers to collecting and evaluating data to identify patterns and trends. Table 6 summarizes the statistical analyses and tests conducted by the researchers to investigate the most optimum variables or parameters to induce the highest frequency of calluses (%).

Table 6 lists that all articles used ANOVA to determine any statistically significant differences between the means of two or more independent groups. ANOVA can be done through various programs, such as Minitab, SAS, SPSS, and PRISM software. Tukey analysis, also known as Tukey's range test, is used to find significantly different means. Meanwhile, Newman-Keuls, also known as Student-Newman-Keuls, can be run once ANOVA has given statistically significant results to see which specific pairs of means are different. *F*-test is the most often used to compare statistical models fitted to a data set to identify the model that best fits the population from which data were sampled. All the tests were done based on the number of variables manipulated in the experimental studies.

Table 6
Statistical analyses used in cassava callus culture

Test	Analysis	Software
Tukey analysis	ANOVA	Minitab
Student, Newman, and Keuls analysis	ANOVA	CoStat, PRISM
F-test	ANOVA	SAS
Least significant difference test	ANOVA	SAS
Duncan's multiplication range test	ANOVA	SPSS

CONCLUSION

This paper reviews cassava tissue cultures focusing on their applications and prospects for starch production, as well as the important parameters for initiating cassava callus culture using SMS. A total of 589 research articles published between 2011 and 2021 obtained from various databases were analyzed. After passing through three screening stages, which involved the implementation of the exclusion and

inclusion criteria, only 56 articles were used to extract important information to answer all RQs. About 42 articles discussed the micropropagation of cassava plants using tissue culture with various objectives, such as media optimization and protocol development. However, only one article highlighted *in vitro* cassava root formation and its starch accumulation. It demonstrates that studies of cassava tissue cultures on starch production are still scarce. Interestingly, using cassava tissue cultures, starch production is possible by supplementing specific nutrients to the growth medium. Therefore, the prospect of using cassava tissue cultures for starch production will grow based on the current research trend. The type of basal medium, PGRs, and culture environment play important roles in initiating cassava callus culture. Other than that, surface-sterilization methods and the choice of explant are crucial to ensure the success of cassava callus culture initiation. It is best to use 70% ethanol and 5% sodium hypochlorite in the surface-sterilization steps and the leaf as the explant source. All formulations, media, and techniques have been proven to produce the highest frequency of callus formation (%). To conclude, the information from this SMS can serve as a future reference for practical experimentation relating to the initiation of cassava callus culture and starch production.

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SUPPLEMENTARY DATA

Table A1

List of articles used in this systematic mapping study

No.	Title
1	Evaluation of regeneration potentials of farmer-preferred cassava (*Manihot esculenta* Crantz) landraces to unlock cassava transformation barriers
2	*In vitro* propagation of Malaysian cassava (*Manihot esculenta* Crantz) variety through low-cost tissue culture media
3	Rapid propagation of a biodiesel plant cassava (*Manihot esculenta* Crantz) through tissue culture
4	Optimization of *in vitro* propagation of cassava (*Manihot esculenta* Crantz) genotypes
5	Molecular studies on the transmission of *Sri Lankan cassava mosaic virus* (SLCMV) in cassava by *Bermisia tabaci* collected from cassava and cassava breed crops
6	Callus induction in three mosaic disease-resistant cassava cultivars in Benin and genetic stability of the induced calli using simple sequence repeat (SSR) and sequence-characterized amplified region (SCAR) markers
7	Cost-effective medium for *in vitro* propagation of Tanzanian cassava landraces
8	Development of *in vitro* propagation protocol for some recalcitrant cassava (*Manihot esculenta* Crantz) genotypes in Sierra Leone
9	Effects of different hormones on organogenesis *in vitro* of some varieties of cassava (*Manihot esculenta* Crantz) grown in Senegal
10	Explant type and hormone regime influence somatic embryogenesis and regeneration in cassava
11	Exploring the induction of doubled haploids in cassava through gynogenesis
12	Generating virus-free cassava plants by *in vitro* propagation with chemical and heat treatment
13	*In vitro* embryo rescue and plant regeneration following self-pollination with irradiated pollen in cassava (*Manihot esculenta* Crantz)
14	Response of four cassava cultivars (*Manihot esculenta* Crantz) plantlets free of cassava mosaic virus to micropropagation in different media
15	Shoot nodal culture and virus indexing of selected local and improved cassava genotypes (*Manihot esculenta*) from Sierra Leone
16	Somatic embryogenesis in two Nigerian cassava cultivars (Sandpaper and TMS 60444)
17	Use of multivariate analysis to evaluate the effect of sucrose on *in vitro* cassava conservation
18	An efficient protocol for *Agrobacterium*-mediated transformation of β-glucuronidase (*Gus/Gusplus*) gene into cassava plants (*Manihot esculenta* Crantz)
19	Callus induction, regeneration, and molecular characterization of cassava (*Manihot esculenta* Crantz)

Table A1 *(Continue)*

No.	Title
20	The effects of calcium in the *in vitro* cassava storage root formation
21	Somatic embryogenesis and organogenesis of biofuel plant cassava (*Manihot esculenta* Crantz) Chinese cultivars
22	Effect of 1-naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) on *cassava's in vitro* regeneration and hardening (*Manihot esculenta* Crantz)
23	Differing sucrose requirements for *in vitro* conservation of cassava genotypes
24	Effects of plant growth regulators on shoot multiplication and root induction of cassava varieties *in vitro* culture
25	Regeneration of Kenyan cassava (*Manihot esculenta* Crantz) genotypes
26	*In vitro* micropropagation of cassava through low-cost tissue culture
27	Effects of sucrose on tuberous root formation and saccharide accumulation in *Manihot esculenta* Crantz *in vitro*
28	Elimination of *Cassava brown streak virus* from infected cassava
29	Shoot induction from the axillary bud of β-carotene enriched *Manihot esculenta* Crantz and molecular stability of regenerants
30	Efficacy of chemotherapy and thermotherapy in eliminating cassava mosaic virus from Tanzanian cassava landrace
31	An efficient method of propagating cassava plants using aeroponic culture
32	Somatic embryogenesis and regeneration of five multipurpose cassava landraces extensively integrated into the African cropping system
33	Somatic embryogenesis and plant regeneration of cassava (*Manihot esculenta* Crantz) landraces from Cameroon
34	Effect of 2,4-D, explants type and cultivar on the callogenesis expression of cassava (*Manihot esculenta* Crantz) in Ghana
35	Responses of cassava (*Manihot esculenta* Crantz) varieties to *in vitro* mannitol-induced drought stress
36	Production of virus-free cassava through hot water therapy and two rounds of meristem tip culture
37	The comparatively proteomic analysis in response to cold stress in cassava plantlets
38	Evaluation of *Agrobacterium*-mediated transformation of two Nigerian cassava (Manihot *esculenta* Crantz) cultivars TME 419 and “Okwuoto"
39	A milestone in the doubled haploid pathway of cassava
40	Robust transformation procedure to produce transgenic farmer-preferred cassava landraces
41	Evaluation of cassava plants generated by somatic embryogenesis in different stages of development using molecular markers

Table A1 *(Continue)*

No.	Title
42	*In vitro* selection and characterization of salt tolerant cell lines in cassava plant (*Manihot esculenta* Crantz)
43	Genetic stability of cassava plants regenerated through organogenesis using microsatellite markers
44	*In vitro* clonal propagation of cassava plant
45	*In vitro* multiplication of cassava varieties
46	Inducing autotetraploids in cassava using oryzalin and colchicine and their *in vitro* morphophysiological affects
47	Micropropagation for rapidly multiplying planting material in cassava (*Manihot esculenta* Crantz)
48	A method for generating virus-free cassava plants to combat viral disease epidemics in Africa
49	Effect of types and concentrations of auxins on callus induction and primary somatic embryogenesis in low cyanide cassava cultivars (*Manihot esculenta* Crantz)
50	Cost-effective nutrient sources for tissue culture of cassava (*Manihot esculenta* Crantz)
51	Fruit, seed, and embryo development of different cassava (*Manihot esculenta* Crantz) genotypes and embryo rescue
52	Plant regeneration via protoplast electrofusion in cassava
53	Effects of plant growth regulators on *in vitro* cultured nodal explants of cassava (*Manihot esculenta* Crantz) clones
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Shoot Production and Metabolite Content of *Cosmos sulphureus* Cav. Leaves with Different Rates of Goat Manure

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ABSTRACT

Cosmos sulphureus Cav. is a plant species commonly cultivated for ornamental purposes, with its young leaves being consumed as vegetables. Therefore, this study aimed to investigate the effect of goat manure rates and different harvest criteria on shoot production as well as the metabolite of *C. sulphureus*. The experiment was conducted in Kuningan Regency, West Java, Indonesia, from October to November 2022, using a factorial randomized complete block design. Goat manure rates examined were 0, 100, 200, and 300 kg N/ha, equivalent to 0, 6, 12, and 18 tons of goat manure/ha. Shoot harvest criteria were two and four top nodes, with each treatment replicated three times. The result showed that applying goat manure significantly increased the shoot production of *C. sulphureus*. The harvest criteria affected the shoot weight per plant, where the four-node harvest was 98.3% higher than the two-node. The metabolite content, such as crude fiber, phenolic, and antioxidant activity, was not significantly different between the upper and lower leaves. However, the lower leaves had a total flavonoid and sugar content of 38.9 and 12.6%, higher than the upper leaves.

Keywords: Antioxidant, crude fiber, flavonoid, leaves age, phenol

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INTRODUCTION

Cosmos sulphureus Cav. is a plant species from Mexico (Vargas-Amado et al., 2020) and a member of the Asteraceae family. Furthermore, it is characterized by orange or yellow flowers and is often cultivated for ornamental purposes (Puttock, 2022). The various uses of *C. sulphureus* include as a bioherbicide (Respatie et al., 2019), natural dyes from its flower (Rahayuningsih et al., 2016), refugial flowering plants (Aldini et al., 2019), and the young shoots and leaves are eaten as vegetables (Lim, 2013).

The consumption of vegetables in Indonesia, currently at approximately 122 g, is still lower than the recommended daily intake of 300–400 g (Vermeulen et al., 2019). To address this issue and increase the consumption of various diversities, introducing underutilized vegetables to the community is crucial to overcoming malnutrition problems (Jena et al., 2018). Consequently, optimizing the use of underutilized vegetables is crucial, such as *C. sulphureus*, including *Cosmos caudatus* in Indonesia (Santosa et al., 2015).

Vegetables are essential for human diets, providing vitamins, minerals, fiber, and bioactive compounds (Ramya & Patel, 2019). Several types of vegetables have high bioactive content and antioxidant properties, such as polyphenols (Lima et al., 2014). A previous study showed that *C. sulphureus* has a high phenolic, flavonoid, and tannin content, serving as an antioxidant (Ortega-Medrano et al., 2023). The potential use of *C. sulphureus* as vegetables and some of the bioactive compounds in its leaves requires the proper cultivation techniques to maximize the yield of shoot production and metabolite content.

Fertilization is an essential aspect of plants, which is carried out by applying inorganic or organic fertilizers to provide nutrients to plants. The advantage of organic fertilizers also includes improving the physical and biological properties of the soil to prevent acidification and increase organic matter content (H. Wang et al., 2019). Furthermore, using organic fertilizers such as goat manure potentially produces high yields and quality leafy vegetables (Steiner et al., 2019). The addition of goat manure to the soil is capable of increasing pH, organic matter, total nitrogen, and available P (Uwah & Eyo, 2014), which are included in the metabolic process in plants (Tripathi et al., 2014).

Nitrogen is an essential nutrient plants need (Krapp, 2015) and is usually applied to enhance vegetative growth (Aminifard et al., 2012). Sufficient nitrogen supply must be considered to increase the growth and shoot production of *C. sulphureus*. Rates of nitrogen also affect metabolite content in leaves, while increasing organic fertilizer in plants decreases the antioxidant activity (Hassan et al., 2012). It shows that higher nutrient uptake in plants reduces the accumulation of phenolic compounds (Olarewaju et al., 2018). Therefore, it is necessary to study the appropriate nitrogen rates to optimize the growth and metabolite levels of *C. sulphureus* leaves.

The shoot production of *C. sulphureus* is also affected by harvest techniques,

requiring further investigation to obtain optimal yields because *C. sulphureus* shoot is obtained by cutting some of the top nodes, where their differences affect the leaves harvested. The younger leaves have higher nitrogen and lower fiber content (de los Santos et al., 2016), while the age of the leaves influences the metabolite content. Total phenolics and flavonoid content increased with *Moringa oleifera* leaves' maturity (Nobossé et al., 2018).

The number of harvested nodes also affects future shoot production. This phenomenon occurs because harvesting more nodes leads to increased lateral shoots, originating from leaves axils, reducing the number of shoots available for the next harvest. Therefore, this study aims to analyze the interaction effect of organic fertilizer rates and harvesting criteria on the repeated production and metabolite content of *C. sulphureus*.

MATERIALS AND METHODS

Study Area

The experiment was conducted from September to November 2022 at Mandirancan, Kuningan District, West Java, Indonesia, with coordinates 6°48'0.31" S, 108°28'17.5" E, and an altitude of 296 m above sea level. Laboratory analysis was carried out at the Postharvest Laboratory Department of Agronomy and Horticulture IPB University and Food and Biochemistry Laboratory, Agriculture Faculty, Sebelas Maret University, Indonesia.

Materials

The materials used in this experiment included seeds of *C. sulphureus* with orange flowers. The seeds were obtained from cultivated *C. sulphureus,* and the organic fertilizer was composted goat manure.

Procedures

The experiment was carried out using a factorial randomized complete block design. The first factor was rates of goat manure consisting of four levels, namely 0, 100, 200, and 300 kg N/ha. The proportion of C-organic, N total, phosphorus pentoxide (P_2O_5), and potassium oxide (K_2O) concentrations were 34.20, 2.45, 1.70, and 2.01%, respectively. The amount of goat manure applied to the soil was based on the concentration of nitrogen (2.45%) and water content at 33%, yielding rates of 0, 6, 12, and 18 ton/ha. The second factor was harvesting criteria comprising the harvesting of two and four top nodes. Each treatment was repeated thrice, resulting in 24 experimental units, represented by a plot of 2 m x 3.5 m, individually.

Cosmos sulphureus seeds were collected from the plants and soaked with water for two hours. Subsequently, the seeds were sown for 12 days using soil, manure, and rice-hull charcoal media. The seedlings were planted in a plot with a plant spacing of 30 cm x 50 cm, and goat manure was applied two weeks before planting.

Shoot harvesting was carried out twice in one week, with the first harvesting executed six weeks after planting (WAP). The yield observation included internode

length and shoot weight per plant in the first and second harvests, harvested shoot number, and shoot weight in the second harvest. The criteria for the second harvest were the same as those for the first harvest.

The metabolite content of *C. sulphureus* leaves was analyzed in the first harvest. The leaves were categorized based on their position in the first and second nodes (upper leaves) and the third and fourth nodes (lower leaves). The observations included leaf N, P, and K concentration, total sugar, crude fiber, phenol, flavonoid, and antioxidant activity.

Sample Preparation

The leaves dried using the drying oven at 50°C for 24 hr were ground with mortar, and the powder form obtained was stored in a refrigerator for analysis.

Determination of Nitrogen Concentration

Approximately 0.250 g of leaf powder was weighed and put into a digest tube, followed by adding 1 g selenium mixture (Merck, Germany) and 3 ml concentrated sulfuric acid (H_2SO_4, Merck, Germany). The solution was digested to 350°C for 3–4 hr, complete when white steam came out and obtained a clear extract. Subsequently, the extract was diluted with deionized water to 50 ml and shaken until homogenous, and the solution was left overnight for the particle to settle. A total of 10 ml of extract was piped into a vaporizer tube and added with 10 ml of 40% sodium hydroxide (NaOH, Merck, Germany). Erlenmeyer containing 10 ml of 1% boric acid (Merck, Germany) and three drops of Conway indicator (Merck, Germany) were prepared as a reservoir for the released ammonia (NH_3). Distillation was carried out until the container volume reached 50-75 ml. The distillate was titrated with 0.05 N H_2SO_4 (Merck, Germany) to achieve a pink color, and the titration volume was recorded. The formula for calculating N levels was as follows:

$$\text{N (\%)} = \frac{(V_{sample} - V_{blank}) \text{ x } 14.08 \text{ x Dilution factor x Correction factor x Normality}}{\text{Sample weight (mg)}} \text{ x } 100\%$$

where, N = nitrogen content (%), V = titration volume (ml).

Determination of Phosphorus and Potassium Concentration

The weight of 0.5 g of leaf powder was put into the digest tube, 5 ml of nitric acid (HNO3, Merck, Germany) and 0.5 ml of concentrated perchloric acid ($HClO_4$, Merck, Germany). The solution was left to stand for one night and heated at 100°C for 1.5 hr after incubation. Subsequently, the temperature was raised to 150°C for 2.5 hr until the yellow steam was exhausted and further increased to 165°C for 1 hr to achieve white steam formation. For P and K measurements, the extract was diluted with deionized water to a volume of 50 ml, shaken until homogenous, and left overnight. A total

of 1 ml of each sample extract was piped and standard series into a test tube for P measurement, added with 9 ml of deionized water and shaken. The 1 ml of diluted extract was pipetted, and the standard series was introduced into the test tube and added with 9 ml of color reagent of P. The solution obtained was incubated for 30 min, and the phosphorus concentration was measured using a spectrophotometer (Shimadzu UV 1800, Japan) at a wavelength of 889 nm. For K measurement, 1 ml of each sample extract was pipetted, and the standard series was introduced into a test tube. It was followed by adding 0.25% lanthanum chloride ($LaCl_3$, Merck, Germany) and shaking until homogenous, while K was measured using atomic absorption spectrophotometry (AAS, PG Instrument PG-990, China).

Determination of Leaf Sugar Content

Approximately 100 mg of leaf powder sample was placed into a centrifuge tube, and 10 ml of 80% ethanol (Merck, Germany) was added. A glass ball was placed on top of the tube and kept in a water bath at 80–85°C for 30 min. The solution was centrifuged, decanted into a 50 ml beaker glass, and repeated three times. Subsequently, the alcohol extract was evaporated in a water bath at 80–85°C to remove most alcohol. The distilled water was added to 25 ml, while 5 ml of extract was transferred to a 100 ml volumetric flask of distilled water. The 5 ml of diluted sugar extract (Merck, Germany) was put into a Pyrex test tube and an ice bath. To each tube, 10 ml of the anthrone reagent (HiMedia, India) was slowly added, allowing the reagent to run down the side of the test tube and stir with a glass rod. The tubes were put into a boiling water bath for 7.5 min and immediately cooled in ice, and the absorbance was measured at 630 nm.

Determination of Crude Fiber Content

The crude fiber was measured using the gravimetric method (Igile et al., 2013). During this process, filter paper was dried in an oven at 105°C and weighed. One gram of leaves powder was weighed, placed in Erlenmeyer glass, added with 200 ml of H_2SO_4 (Supelco, Germany), and boiled for 30 min. The solution was filtered with a Buchner funnel, and the residue was washed using hot distilled water until the washing water was no longer acidic. The residue obtained was transferred from filter paper to Erlenmeyer, and 200 ml of NaOH (Supelco, Germany) was added. The solution was boiled for 30 min, filtered with a Buchner funnel, and washed with hot distilled water to a non-alkaline state, followed by washing with 15 ml of 10% K_2SO_4 (Merck, Germany). The residue was washed with hot distilled water and added to 15 ml of 95% alcohol (PT. Brataco, Indonesia). Subsequently, the remnant was left on filter paper, dried in an oven at 105°C to achieve a constant weight equivalent to crude fiber, and cooled in a desiccator.

Determination of Total Phenol and Total Flavonoid Content

Total phenol was measured using Folin-Ciocalteau, while total flavonoid was

determined with aluminum chloride colorimetric (Vongsak et al., 2013). Initially, 0.01 g of leaf powder was added with 4 ml of ethanol and macerated for 72 hr at room temperature and in a dark room. The solution was added to 10 ml with 70% ethanol (Supelco, Germany), shaken, and centrifuged at 4,427 x *g* for 7 min. Total phenol levels were measured by pipetting 100 µl, added with 2.5 ml of distilled water and 100 µl of Folin-Ciocalteau reagent (Merck, Germany). The solution was vortexed and incubated at 45°C for 15 min, followed by absorbance measurement using a spectrophotometer at a wavelength of 765 nm with the gallic acid standard. Flavonoid levels were measured by pipetting 2 ml of supernatant, adding 2 ml of 2% aluminum chloride ($AlCl_3$, Merck, Germany), and homogenizing. The solution was incubated for 10 min at room temperature and in a dark room. After incubation, the absorbance was measured using a spectrophotometer (Shimadzu UV-Vis Type 1280, Japan) at a wavelength of 415 nm with quercetin standard.

Determination of Antioxidant Activity

Antioxidant activity was measured using the 2.2-diphenyl-1-picrylhydrazyl (DPPH) method (Cheng et al., 2016) and expressed by percentage of inhibition. The sample solution was made by dissolving the leaves powder with methanol in a ratio of 1:10. Subsequently, 0.1 ml of sample solution was pipetted to the test tube and added with 1 ml of 0.35 mM DPPH (Sigma Aldrich, Germany), and 4 ml ethanol (Supelco, Germany) was added, followed by vortexing, and incubation in a dark room as well as at room temperature for 30 min. The absence was measured using a spectrophotometer (Shimadzu 1240 UV-VIS, Japan) at a wavelength of 517 nm. The percentage of inhibition was calculated in the formula as follows.

$$\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \text{ x } 10$$

Data Analysis

The data were tested for normality using Shapiro-Wilk at $\alpha = 5\%$. Meanwhile, the normal distribution data were analyzed using analysis of variance (ANOVA) at $\alpha = 5\%$. When treatment had a significant effect, the analysis continued with Duncan's multiple range test at $\alpha = 5\%$. Shoot weight per plant at the first, second, and total harvest were analyzed using regression analysis.

RESULTS AND DISCUSSION

Soil Properties and Climate Conditions at Experimental Site

The soil properties showed a pH of 5.6 (slightly acid), with low C-organic and N-total at 1.77 and 0.18%, respectively. The content of total P_2O_5 was 203.51 mg/100 g (very high), total K_2O was 23.61 mg/100 g (medium), and the soil texture was silty clay loam. Consequently, *C. sulphureus* benefited from increased nitrogen input through fertilizer to enhance its vegetative growth.

The application of goat manure as organic fertilizer increased plant nutrient availability and the C-organic, including the N content of soil (Ekwealor et al., 2020). Furthermore, organic fertilizer increases soil pH, which correlates with plant nutrient availability and other biogeochemical processes such as soil enzyme activities, rhizosphere, and mineralization of organic matter (Neina, 2019).

In this study, an increase in soil pH was observed after planting. Nutrients absorbed by plant roots reduce the nutrient content in the soil. The addition of goat manure minimized the reduction of N, with P_2O_5 total increasing at 200 and 300 kg N/ha rates, while K_2O content increased at 200 and 300 kg N/ha, as shown in Table 1. Goat manure contains other macro and micronutrients (Uwah & Eyo, 2014). The P_2O_5 and K_2O content in goat manure was 1.7 and 2.01%, respectively. During the experiment, the average rainfall was estimated at 341.5 mm/month, with a temperature of 25.2°C and relative humidity of 68%.

Table 1
Soil properties before planting and after planting with the addition of different rates of goat manure

Parameters	Before planting	After planting with different rates of goat manure (kg N/ha)			
		0	100	200	300
pH H_2O	5.6 (SA)	5.9 (SA)	5.9 (SA)	6.3 (SA)	6.3 (SA)
C-organic (%)	1.77 (L)	1.32 (L)	1.39 (L)	1.66 (L)	1.61 (L)
N-total (%)	0.18 (L)	0.17 (L)	0.17 (L)	0.18 (L)	0.18 (L)
P_2O_5 (mg/100 g)	203.51 (VH)	195.6 (VH)	198.01 (VH)	217.99 (VH)	216.05 (VH)
K_2O (mg/100 g)	23.61 (M)	19.92 (L)	27.72 (M)	61.41 (VH)	41.67 (H)

Note. H_2O = Water; P_2O_5 = Phosphorus pentoxide; K_2O = Potassium oxide; SA = Slightly acid; L = Low; M = Medium; H = High; VH = Very high

Shoot Production of *C. sulphureus*

The yield component of *C. sulphureus* shoot production was evaluated based on weight and shoot number. The internode length of *C. sulphureus* was affected by rates of organic fertilizer in the first and second harvests. Based on the results presented in Table 2, an increase in the rates of goat manure significantly led to elevated internode length. It was attributed to the influence of nitrogen, enhancing stem development, including cell division and elongation (Souza & Tavares, 2021). An increase in shoot weight per plant in both the two and four-node harvesting criteria was also observed. The optimum rate of goat manure at the four-node was 266 kg N/ha, while the curve in the two-node harvesting

Table 2
Effect of goat manure rates and harvest criteria on internode length of shoot

Rates of goat manure (kg N/ha)	Internode length (cm)	
	First harvest	Second harvest
0	6.02±0.50b	6.45±0.21c
100	7.80±1.48ab	8.60±2.03b
200	9.64±2.95a	9.23±1.57b
300	9.91±1.01a	11.28±2.24a

Note. The number followed by the same letter in a column is not significantly different by the Duncan's multiple range test at the level of α = 5%. Numbers were followed by ± standard deviation

criteria was linear, as shown in Figure 1. The increasing rates of shoot weight in four nodes due to the addition of nitrogen rates were higher than in two-node harvesting.

The second harvest was carried out one week after the first harvest by cutting all the lateral branches according to harvesting criteria. An interaction was observed between the effect on shoot weight in the second harvest. As shown in Table 3, an increasing shoot weight of two-node harvesting was obtained from 300 kg N/ha compared to the control treatment, while the four-node shoot weight increased to 100 kg N/ha. Shoot numbers in the second harvest were affected by harvesting criteria and rates of goat manure, where the two nodes yielded higher results, as shown in Table 4. This result variation was caused by fewer shoots that met the four-node harvesting criteria. More shoots were carried out at the first four-node harvesting from the bud at the leaves axillar or the nodes.

The increasing availability of nutrients by adding organic fertilizer also enhanced branch growth in the second harvest. Table 4 shows that the application of goat manure plays a significant role in vegetative growth, increasing the harvested shoot number in the second harvest. This result is in line with

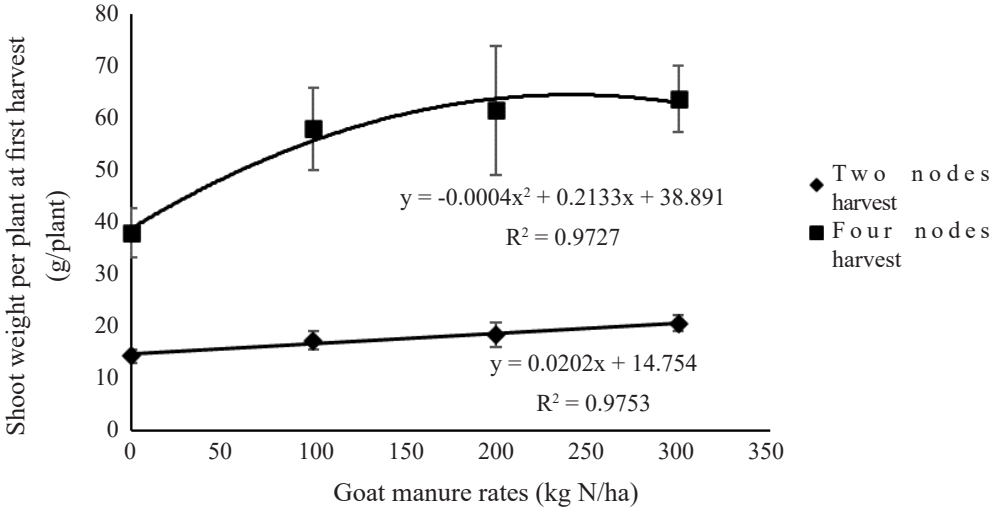

Figure 1. The increasing shoot weight per plant at several rates of goat manure at the first harvest

Table 3
Interaction effect of goat manure rates and harvest criteria on shoot weight (g) at second harvest

Treatment (kg N/ha)	Two-node	Four-node
0	4.81±1.71d	9.29±0.70c
100	5.42±1.85d	13.39±0.42b
200	5.37±1.51d	14.78±0.60b
300	6.16±0.92cd	17.91±0.46a

Notes. *Note*. The number followed by the same letter is not significantly different by the Duncan's multiple range test at the level of α = 5%. Numbers were followed by ± standard deviation.

Table 4
Effect of goat manure rates and harvest criteria on shoot number in the second harvest

Treatment	Shoot number
Harvest criteria	
Two-node	9.2±1.79a
Four-node	5.6±3.81b
Rates of goat manure (kg N/ha)	
0	6.2±1.79b
100	7.1±2.34ab
200	8.3±2.29a
300	8.1

Note. The number followed by the same letter in a column is not significantly different by the Duncan's multiple range test at the level of α = 5%. Numbers were followed by ± standard deviation

a previous study, where the application of organic fertilizer increased the significance of branches on chili (Khandaker et al., 2017). Similarly, there was an increase in shoot weight and shoot weight per plant in the second harvest at an optimum rate of 300 kg N/ha. This condition showed that goat manure was needed for *C. sulphureus* shoot production with repeated harvesting. The optimum rates of goat manure to obtain maximum shoot weight per plant in the second harvest for two and four were 509.5 and 608.5 kg N/ha, respectively, as presented in Figure 2. The difference in the optimum points during the first and second harvests showed that adding goat manure repeatedly or increasing the goat manure rates to the soil was still necessary to obtain the maximum yields in the second harvest.

The total harvest was the accumulated shoot weight of the first and second harvests. Based on the results, harvesting four nodes of *C. sulphureus* resulted in a significant 98.3% increase in the average total shoot weight per plant. The 324.0 and 739.5 kg N/ha were the optimum rates to obtain the maximum yield of *C. sulphureus* with four and two-node harvest criteria, respectively (Figure 3).

Goat manure contains macro and micronutrients essential for plant growth, contributing significantly to soil fertility. Apart from nitrogen addition into the soil, goat manure was also added at rates of 6-, 12-, and 18-ton/ha to 69.4, 138.8, and 208.2 kg of P_2O_5, as well as 82, 164.1, and 246.1 kg of K_2O, respectively. Furthermore, goat manure enhances soil organic content, improving proper soil structure conducive to root growth and increasing plants' uptake of water and nutrients. Although N is essential in plant vegetative growth, it is also affected by macronutrients such as P and K. Phosphorus availability to plants is beneficial to root growth (D. Liu, 2021), causing an increase in nutrient

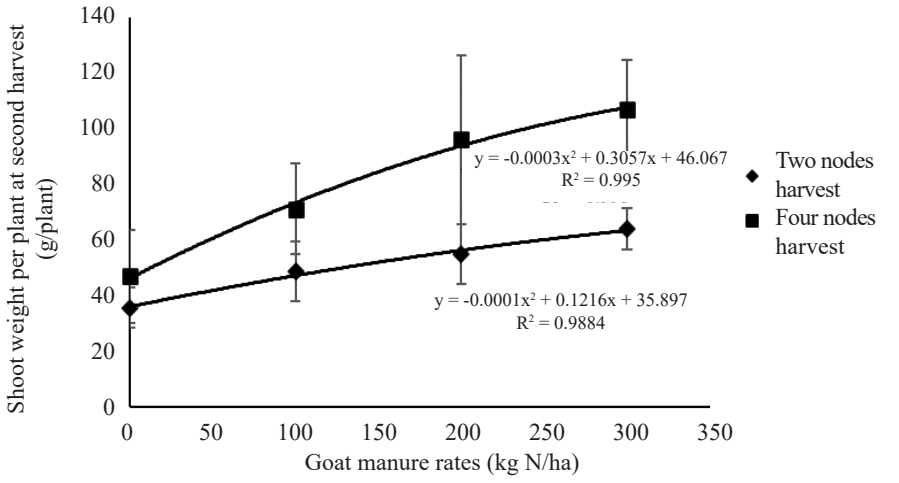

Figure 2. The increasing shoot weight per plant at several rates of goat manure at the second harvest

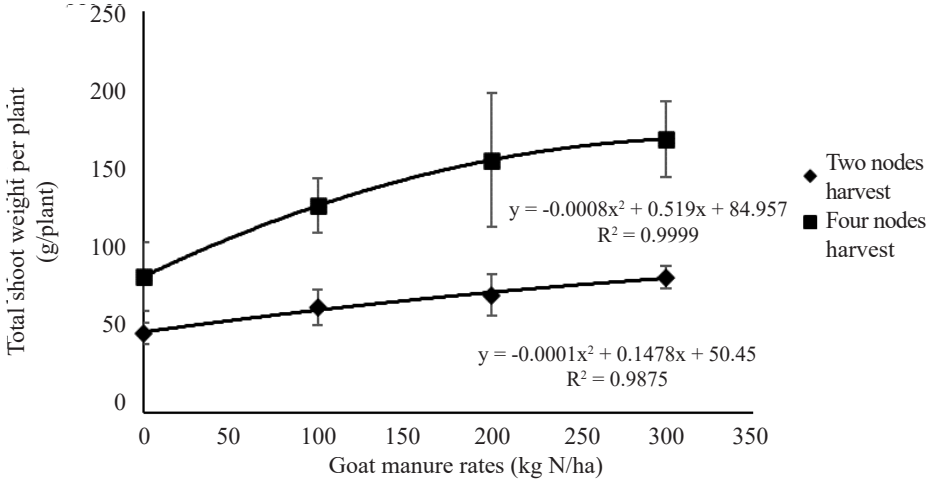

Figure 3. The increasing total shoot weight per plant at several rates of goat manure

uptake. Pearson correlation analysis showed that P concentration was positively correlated ($P<0.05$) with N ($r = 0.87$) and K concentration ($r = 0.46$) in leaves (Figure 4).

Phosphorus plays a significant role in adenosine triphosphate (ATP) formation and is included in some plant metabolism, such as photosynthesis, specifically in electron transport in light reactions (Carstensen et al., 2018). Potassium is also included in the essential macronutrients for plants, functioning in carbon dioxide (CO_2) assimilation in photosynthesis and photosynthate translocation from source to sink (Mengel, 2016). Photosynthesis is the primary plant metabolism that directly

affects plant growth and development. All the roles of the nutrient nutrients in organic fertilizer are included in photosynthesis, which affects plant growth.

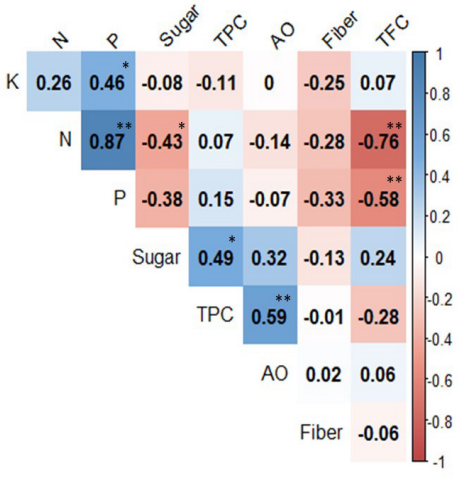

Figure 4. Pearson correlation analysis of metabolite content in leaves of *Cosmos sulphureus*

Note. TPC = Total phenolic content; TFC = Total flavonoid content; AO = Antioxidant activity; ** = Correlation significant at the 0.01 level; * = Correlation significant at the 0.05 level

Metabolite Content and Antioxidant Activity of Different Leaves Position

In this study, four-node harvest criteria showed a higher yield of *C. sulphureus*, showing the need to explore the physiological characteristics of leaves from different positions. Harvesting four included leaves in the third and fourth nodes below the first and second nodes. The results also showed that the lower leaves were older, showing an age difference between the two types. It suggested that the physiological characteristics of leaves, namely fiber and secondary metabolites, were also different (de los Santos et al., 2016; Nobossé et al., 2018). The nutrient content of leaves, including nitrogen, phosphorus, and potassium, was significantly different between the positions of leaves, as shown in Table 5. The upper leaves have higher N, P, and K content, which are the mobile nutrients in plant tissue. These elements were translocated from older leaves into young ones, resulting in decreased nitrogen content as the age increased (Onyango et al., 2012).

Despite goat manures supplying more plant nutrients, N, P, and K levels did not significantly increase in leaves. It was attributed to the characteristic slow release of nutrients from goat manure as organic fertilizer, resulting in an insignificant increase in nutrient levels. The nitrogen content in leaves also did not increase with manure application in amaranth, but a significant elevation was observed when mineral fertilizer was supplied (Onyango et al., 2012). However, nutrient uptake of *C. sulphureus* increased due to enhanced biomass (yield) and the addition of organic fertilizer. It is consistent with C.-W. Liu et al. (2014), who stated that applying organic fertilizer increased the dry weight of lettuce.

In contrast with N, P, and K concentrations, the sugar content was higher in the upper leaves (Table 6). As old leaves tend to meet senescence, nitrogen is translocated into young leaves, increasing the hexose/sucrose ratio at the start of senescence and accumulating soluble sugar in leaves (Agüera & De la Haba, 2018). However, the low content of minerals led

Table 5
Effect of goat manure rates and leaf position on N, P, and K concentration in leaves

Treatment	N (%)	P (%)	K (%)
Leaf position			
Upper leaves	5.01±0.17a	0.47±0.02a	2.35±0.63a
Lower leaves	4.25±0.20b	0.31±0.03b	1.91±0.54b
Rates of goat manure (kg N/ha)			
0	4.64±0.46a	0.38±0.10a	2.15±0.50a
100	4.55±0.50a	0.40±0.10a	2.20±0.80a
200	4.60±0.41a	0.39±0.08a	2.19±0.65a
300	4.73±0.44a	0.40±0.09a	1.98±0.62a

Note. The number followed by the same letter in a column is not significantly different by the Duncan's multiple range test at the level of α = 5%. Numbers were followed by ± standard deviation

Table 6
Effect of goat manure rates and leaf position on total sugar and crude fiber in leaves

Treatment	Total sugar (%)	Crude fiber (%)
Leaf position		
Upper leaves	2.30±0.39b	11.17±1.59a
Lower leaves	2.59±0.26a	12.31±2.13a
Rates of goat manure (kg N/ha)		
0	2.59±0.34a	11.01±1.40b
100	2.52±0.46a	11.13±1.18b
200	2.43±0.23a	11.41±1.22b
300	2.24±0.35a	13.42±2.77a

Note. The number followed by the same letter in a column is not significantly different by the Duncan's multiple range test at the level of α = 5%. Numbers were followed by ± standard deviation

to an increase in soluble carbohydrates (Sung et al., 2015), which occurred in the organic fertilizer rates treatments without affecting the sugar content of leaves. A negative correlation was also observed between nitrogen and leaf sugar content in *C. sulphureus* leaves ($P<0.05$, $r = -0.43$) (Figure 4). This condition occurred in stevia, where the increased nitrogen content was followed by a decrease in sugar levels (Sun et al., 2019).

The consumption of vegetables is essential for supplying dietary fiber, comprising residues from edible plant cells, including cellulose, hemicellulose, pectin, and noncarbohydrate residues such as lignin that resist hydrolysis by human digestive enzymes (Dai & Chau, 2017; DeVries et al., 1999). The fiber content in leafy vegetables must be considered due to its influence on consumer preference. Furthermore, texture and flavor are important characteristics of consumer acceptance of leafy vegetables

(Gil et al., 2012). This study observed no significant difference in crude fiber content between upper and lower leaves (Table 6). The fiber content of *C. sulphureus* leaves was affected by rates of organic fertilizer. The 300 kg N/ha rates significantly increased the crude fiber compared to lower nitrogen application. The fertilizer application of NPK and manure increased crude fiber content in amaranth compared to the control treatment (Oyedeji et al., 2014). In contrast with the previous study in radishes, high nitrogen input decreased crude fiber in leaves (Yousaf et al., 2021).

Phenols and flavonoids are secondary metabolites in plants, playing a significant role as antioxidants (Zeb, 2020). In this study, the total phenolic content (TPC) and total flavonoid content (TFC) of *C. sulphureus* leaves ranged from 19.05 to 20.66 mg GAE/g dry weight and 14.11 to 19.60 mg QE/g dry weight, respectively. These values were lower compared to a previous study, where the TPC of *C. sulphureus* leaves was 40.74 mg GAE/g dry extract (Ortega-Medrano et al., 2023). Although organic fertilizer rates and leaves position did not affect the phenol content, there was a difference in total flavonoid content between the upper and lower leaves. As presented in Table 7, the lower leaves had a 38.9% higher total flavonoid than the upper ones. In line with studies in *Moringa oleifera* leaves, TPC was increased with maturity (Nobossé et al., 2018), while total flavonoids decreased (B. Wang et al., 2018).

A negative correlation was found between nitrogen and total flavonoid content in leaves ($P<0.05$, r = - 0.76) (Figure 4). Meanwhile, nitrogen affected phenylalanine ammonia-lyase (PAL) enzyme activity that catalyzed flavonoid biosynthesis (Deng et al., 2019). The phenol and flavonoids were also synthesized through shikimate

Table 7
Effect of goat manure rates and leaf position on total phenolic contents, total flavonoid contents, and antioxidant activity in leaves

Treatment	TPC (mg GAE/g dw)	TFC (mg QE/g dw)	Antioxidant activity (% inhibition)
Leaf position			
Upper leaves	20.52±3.87a	14.11±2.77b	76.82±15.72a
Lower leaves	19.55±3.12a	19.60±3.63a	81.31±14.93a
Rates of goat manure (kg N/ha)			
0	20.66±3.01a	16.99±4.53a	74.07±15.91a
100	21.11±4.60a	18.86±3.86a	76.84±19.27a
200	19.05±3.06a	16.06±4.74a	76.94±16.78a
300	19.31±3.48a	15.50±4.05a	88.42±2.41a

Note. The number followed by the same letter in the column is not significantly different by the Duncan's multiple range test at the level of α = 5%. Numbers were followed by ± standard deviation; TPC = Total phenolic contents; TFC = Total flavonoid contents; GAE = Gallic acid equivalent; QE = Quercetin equivalent; dw = Dry weight

pathways, which catalyzed carbohydrates to produce aromatic amino acids. A positive correlation was observed between starch and C content with flavonoid content (Deng et al., 2019). Furthermore, total phenol content was positively correlated with sugar content ($P<0.05$, $r = 0.49$) (Figure 4). It showed that the high sugar content of *C. sulphureus* leaves also enhanced the phenol content.

The position of leaves and organic fertilizer rates did not affect antioxidant activity but were influenced by metabolite content. In this study, antioxidant activity was positively correlated with total phenol content ($P<0.05$, $r = 0.59$) (Figure 4). Some studies with different species also showed a correlation between TPC and antioxidant activities (Aryal et al., 2019). Furthermore, phenolic compounds have shown the ability to stabilize free radicals by donating H-atom to free radical substrate (Chen et al., 2020; Zeb, 2020).

CONCLUSION

This study showed that applying organic fertilizer increased the shoot production of *Cosmos sulphureus* Cav. The harvest criteria affected the shoot weight per plant, where the four-node harvest was 98.3% higher than the two-node. The characteristics of leaves and metabolite content consumers consider when consuming vegetables, such as crude fiber, phenolic content, and antioxidant activity, were not significantly different between upper and lower leaves. Furthermore, the lower leaves had a higher flavonoid and total sugar content at 38.9 and 12.6% than the upper leaves. Based on these results, a four-node harvest is recommended as the criteria for the shoot harvest of *C. sulphureus*.

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Novel Sustainable Bio-fertilizer Formulated with Mangrove-associated Bacteria Enhances Duckweed Growth and Protein Content

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ABSTRACT

Duckweed is a future food and a source of affordable protein that has the potential to replace animal protein. This study aims to formulate a bio-fertilizer consisting of mangrove-associated bacteria to boost the growth and protein of duckweeds as a sustainable approach to increase plant-based protein yields. The culture-depending technique was performed by using Aleksandrow agar, Pikovskaya's agar, and Jensen agar to screen potassium-solubilizing bacteria, phosphate-solubilizing bacteria and nitrogen-fixing bacteria, respectively, from mangrove soil sediments. Mangrove-associated bacteria that are close to *Acinetobacter radioresistens*, *Brachybacterium paraconglomeratum*, and *Enterobacter cloacae*, which are known as nitrogen-fixing bacteria, *Klebsiella quasipneumoniae*, *Bacillus tropicus*, and *Paenibacillus pasadenensis* known as potassium-solubilizing bacteria, and *Bacillus cereus* and *Bacillus thuringiensis* known as phosphate-solubilizing bacteria were identified through 16S rRNA gene sequencing. After that, three sets of bio-fertilizers were randomly formulated. Each set consisted of nitrogen-fixing bacteria, potassium- and phosphate-solubilizing bacteria, as well as commercial compost as a carrier. These formulated bio-fertilizers were evaluated for plant growth promotion and protein production on duckweed plants under temperatures between 26 and 30°C. The results showed that each set of our formulated bio-fertilizer can increase the

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nitrogen (N), phosphorus (P), and potassium (K), duckweed growth, and protein content when compared to the control group. It indicates that bio-fertilizers formulated with mangrove-associated bacteria and high NPK contents could enhance the growth of duckweed as well as its protein content, which could supply our future plant-based protein sustainably.

Keywords: Bio-fertilizer, duckweed, Lukut river Malaysia, mangrove-associated bacteria, nitrogen-fixing bacteria, phosphate-solubilizing bacteria, potassium-solubilizing bacteria

INTRODUCTION

Duckweed, the world's tiniest flowering plant, is gaining attention for its rich nutrient content and versatile applications in various industries, including animal feed, aquaculture, health supplements, bio-fertilizers, biofuels, and emerging human food products (de Beukelaar et al., 2019; Naseem et al., 2020). Its protein content, ranging from 20 to 30%, surpasses that of cereals, making it a sustainable and cost-effective source of protein (Appenroth et al., 2018; Yahaya et al., 2022). Duckweed's primary protein, ribulose-1, 5-bisphosphate carboxylase (RuBisCO), is an excellent source of essential amino acids and possesses favorable properties as a functional food (Chakrabarti et al., 2018). This aquatic green plant thrives in specific conditions, including an optimal temperature range of 17.5 to 30°C and ample sunlight, while also requiring supplemental nutrients like nitrogen (N), phosphorus (P), and potassium (K) for growth (Hasan & Chakrabarti, 2009). Additionally, duckweed is a phytoremediation plant capable of extracting pollutants, including metals and radionuclides, from wastewater and accumulating them in its tissues (Kamyab et al., 2017; Radulovic et al., 2018).

Therefore, to ensure the safety of duckweed as a food source for humans, it must be cultivated in a controlled environment, such as axenic culture, which can produce a substantial quantity of duckweed with minimal bacterial presence and fewer contaminants. However, setting up a laboratory for large-scale duckweed production is expensive. Hence, an open-air system that harnesses direct sunlight and minimizes nutrient usage becomes necessary to make duckweed a commercially viable future food option for low-income communities. In our previous study (Yahaya et al., 2022), we found that adding N:P:K fertilizer to the water containing duckweed served as the most effective growth medium, resulting in a substantial yield of duckweed. However, in the long-term condition, the commercial compost medium exhibited an even greater proliferation of duckweed. Furthermore, combining the commercial compost with the N:P:K fertilizer in water showed the potential to enhance the growth of duckweed and its protein content (Yahaya et al., 2022). However, excessive and long-term chemical application could cause negative effects on the environment and human health.

Bio-fertilizers are fertilizer preparations containing living cells or dormant cells of

effective strains of microorganisms that aid crop plant nutrient uptake through rhizosphere interactions (M. Kumar et al., 2022; Sarbani & Yahaya, 2022). The development of bio-fertilizers has become an important part of agriculture, which can help improve soil fertility and produce disease-resistant, stress-resistant plants with better nutrient uptake (Liu et al., 2023; Sahoo et al., 2018). Bio-fertilizers help maintain a nutrient-rich soil environment by facilitating processes such as N fixation, P and K solubilization, mineralization, synthesis of plant growth regulators, antibiotic production, and organic matter degradation in the soil (Mali & Attar, 2021; Shahwar et al., 2023). Bio-fertilizers containing *Azobacter* sp. and *Azospirillum* sp. bacteria exhibit growth-enhancing and essential oil production-increasing effects on basil (*Ocimum basilicum* L.) plants (Tahami et al., 2017), while bio-fertilizers containing *Rahnella aquatilis* and *Variovorax paradoxus* prove to be effective in promoting the growth of *Crocus sativus* (Saffron) plants and enhancing the production of secondary metabolites (Chamkhi et al., 2023) by facilitating plant-beneficial activities such as P solubilization, siderophore production, and auxin production.

Mangroves, found in the transitional zone between land and sea, are valuable sources of biotechnological resources like microbial cellulase, endophytes, and salinity-tolerant glucanase enzymes (Behera et al., 2017; Castro et al., 2018; dos Santos Goncalves et al., 2020). These ecosystems host 27 true mangrove species across 10 selected systems (Sreelekshmi et al., 2020), recognized for their carbon-rich nature (Adame et al., 2022; Gu et al., 2022; Morrissette et al., 2023). They harbor diverse microbial life, including rhizosphere bacteria with the potential to stimulate plant growth by producing phytohormones (Pham et al., 2022; Smaill et al., 2010; Talaat, 2019), mitigating environmental stressors (Chandra et al., 2021; David & Rostkowski, 2020; Muñoz-García et al., 2022; Ramakrishna et al., 2020), and preventing pathogen-induced diseases (Cheng et al., 2021; Gomez-Aparicio et al., 2022; Zhou et al., 2023).

There are limited studies regarding the effect of beneficial microbes on the growth and performance of duckweed cultivated in an open-air system. Hence, in this study, the integration of mangrove-associated microbes into our formulated bio-fertilizer was demonstrated, significantly boosting both duckweed growth and protein yield, underscoring the potential of duckweed as a viable future food option. In the past decades, many researchers have formulated various bio-fertilizers with extensive and advanced effects on crop growth to preserve the environment and ecosystem (Sarbani & Yahaya, 2022). Therefore, the bio-fertilizer formulation in this study aims to benefit plant and crop productivity.

MATERIALS AND METHODS

Sample Collection

Soil samples with a depth of 5 cm were collected at the freshwater riverine mangrove located at Lukut River, Negeri Sembilan,

Malaysia (coordinate 2° 35' 25.2342" N, 101° 48' 9.831" E) on 20th February 2020, during the low tide at three sampling points, with temperature ranging from 26.1 to 30°C. Soils 1 and 2 were collected in the 5 m x 5 m area populated by *Rhizophora mucronata* and *Avicennia officinalis* trees. Meanwhile, Soil 3 was collected from the riverbank near *the Nypa fruticans* tree and 50 m distance from Soil 1 and Soil 2. The samples were placed in a 50 ml tube and stored at -80°C until ready for analysis.

Screening of Nitrogen-fixing Bacteria, Phosphate-solubilizing Bacteria, and Potassium-solubilizing Bacteria

Soil samples were individually suspended in 0.85% of sodium chloride (NaCl, Merck, USA) (Yahaghi et al., 2018) for inoculation into Jensen agar (HiMedia Laboratories, India), Pikovskaya`s agar (HiMedia Laboratories, India), and Aleksandrow agar (HiMedia Laboratories, India). Jensen agar, Pikovskaya's agar, and Aleksandrow agar were used to screen and culture nitrogen-fixing bacteria, phosphate-solubilising bacteria, and potassium-solubilising bacteria, respectively. The Jensen medium was aerobically incubated at 27°C for 48 hr, while Aleksandrow agar and Pikovskaya agar were incubated at 35°C for 7 days after spreading the soil suspension.

DNA Extraction and 16S rRNA Gene Sequence Analysis

Bacterial cultures were prepared by proliferating a single colony from Jensen agar, Pikovskaya's agar, and Aleksandrov agar in a nutrient broth at 28°C with 200 rpm shaking and incubated for 48 hr to obtain the optical density at 600 nm. DNA from the bacterial cultures was extracted using the PROMEGA Wizard® Genomic DNA Purification Kit (USA). The purity, integrity, and quantity of extracted DNA were determined using agarose gel electrophoresis and a nanodrop (BioDrop, Thailand).

The PCR amplification of the 16S rRNA gene was performed by using a universal primer set of 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (GGT TAC CTT GTT ACG ACT T-3') (Zhou et al, 2009). The components for the PCR reaction were 12.5 µl of Go Taq® Green Master Mix 2X (Promega, USA), 0.5 µl of each forward and reverse primer (final concentration of each primer is 0.5 µM), 5 µl of DNA template (approximately 250 ng), and 6.5 µl of nuclease-free water. Amplification was performed on a Mastercycler nexus PCR cycler (Bio-Rad T100™ Thermal Cycler, USA), a program to perform an initial denaturation at 94°C for 5 min; 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C; and the final extension at 72°C for 10 min, followed by cooling to 4°C until the sample is recovered (Fatima et al., 2011). Amplicons were then visualized with a UV transilluminator after resolving 5 µl of the products in the 1% agarose gel and staining with GelRed (Biotium, USA) Lastly, purified PCR products were subjected to DNA sequencing at Next Gene Scientific Sdn. Bhd. (Malaysia).

The raw sequences of the 16S rRNA gene were analyzed and compared with sequences available in the National Centre for Biotechnology Information (NCBI) sequence database (MT367790, MN833494, MT214134, MT124566, KY908508, KY908503, MZ317924, ON222566, ON000559, KM100367, NR113987, OM510015, MW363212, KX216390, and OM351571). Then, MEGA 11 software performed the CLUSTALW alignment on the sequence. The maximum likelihood technique based on the Tamura-Nei model was applied to infer evolutionary history.

Development of Bio-fertilizers

At the beginning of the study, commercial compost (Growmate Eazy Mix, MR. DIY, Malaysia) was used as a carrier material for developing bio-fertilizer. The commercial compost was packed in autoclave polythene covers and sealed using an electric sealer. It was then sterilized at 121°C for 20 min to destroy contaminated microbes.

Nine bacterial isolates were cultured in nutrient broth (Oxoid, United Kingdom) at 28°C with shaking at 200 rpm for 48 hr until the optical density at 600 nm (OD) reached the value of 0.3 (10^7 CFU). The bacterial cultures were centrifuged at 11,057 × *g* at 4°C for 15 min and resuspended in sterilized nutrient broth. The process was repeated twice. Then, the pre-sterilized commercial compost was inoculated with bacteria at a ratio of bacteria pellet to pre-sterilized commercial compost 1:50, as recommended by Stella et al. (2019). Mangrove-associated microbes were randomly divided into three sets, each consisting of nitrogen-fixing, phosphate-solubilizing, and potassium-solubilizing bacteria. Set A bio-fertilizer consists of *A. radioresistens*, *K. quasipneumonia*, and *B. cereus*; Set B bio-fertilizer consists of *B. paraconglomeratum*, *B. cereus*, and *B. tropicus*; and Set C bio-fertilizer consists of *E. cloacae*, *P. pasadenensis*, and *B. thuringiensis*. The mixture was manually shaken by hand until the microbial inoculum was uniformly distributed in the commercial compost. Microbial inoculum and autoclaved commercial compost were packed into the polythene bag and immediately sealed. The package containing pre-sterilized commercial compost without bacterial inoculation was used as a control. All the packages were then incubated at 30°C for 7 days. After the 7th-day interval, formulated bio-fertilizers were tested for bacteria survivability and NPK content.

Bacterial Survivability Experiment

One gram of each sample was mixed with 9 ml of sterile distilled water in a ratio of 1:9 and allowed to mix thoroughly in a shaker for 1–2 hr. The suspension was serially diluted before being dispensed into the agar plate and incubated at 35°C for 24 hr. The number of bacterial growths on the plate was calculated using Equation 1.

$$\text{Population density (CFU/ml)} = \frac{\text{Number of colonies X Dilution factor}}{\text{Volume of culture plate}}$$

[Equation 1]

N, P, and K Determination in Mangrove Soils and Formulated Bio-fertilizer

After seven days of incubation, the formulated bio-fertilizers were analyzed for their N, P, and K contents. N content was measured using the Kjeldahl method. Meanwhile, P and K were analyzed using X-ray fluorescence (XRF).

Three samples of mangrove soil (Soils 1, 2, and 3) and three samples of formulated bio-fertilizers (Sets A, B, and C) and control were used for this analysis. The analysis was performed in triplicates. For the Kjeldahl method, a digestion tube mixed 1 g of soil samples or formulated bio-fertilizer with 10 ml of sulphuric acid (H_2SO_4, Merck, USA). A Kjeldahl tablet was then added as a catalyst to the sample solution. Then, the sample solution was digested using Gerhardt KJEDAHLTEM (Germany) for 60 to 120 min until the digestion solution turned clear green. After that, the digestion tubes were allowed to cool and placed in Gerhardt VAPODEST 500 (Germany) for titration and distillation processes. Finally, the N content of the samples was calculated based on a volumetric standard solution (Yahaya et al., 2022).

In XRF determination (S8 TIGER, Bruker, Germany), three soil samples and three sets of formulated bio-fertilizer and the control were dried, homogenized and sieved to get smaller particle sizes. Plastic cups lined with a 3.6 m thick Mylar® polymer were used to hold samples and placed inside the XRF analyzer. The X-ray tube operated at 15 W with a 50 kV generator in operation conditions. The spot size of the sample was typically 10 mm × 14 mm. The detector has a high resolution of 135 eV.

Duckweed (*Lemna minor*) Growth Experiment

A duckweed *L. minor* growth experiment was conducted to evaluate the efficiency of formulated bio-fertilizers. Ten fronds of *L. minor* plants were sterilized using 70% ethanol (R&M, Switzerland), bleach (CLOROX®, USA), and sterilized distilled water before being transferred into a container with a size of 122 mm x 173 mm x 62 mm that contains 200 ml of water and 25 g of control medium and three sets of formulated bio-fertilizer (Sets A, B, and C). In this experiment, duckweed plants were grown in a greenhouse with a temperature range between 26 to 30°C. The number of duckweed fronds was recorded every two days for 15 days (Figure 1).

Quantification of Protein Content in Duckweed Fronds

Fresh *L. minor* was dried in an oven at 65°C for 24 hr and ground into a fine powder. Dried duckweed was then soaked in sterilized distilled water at a ratio of 1:10 (1 g of dried duckweed in 10 ml of distilled water) overnight to allow cell expansion before applying physical enforcement to break the cells. Then, the soaked material and water were microwaved at 100 W for 15 min using a home-based microwave. The microwaved duckweed was filtered to separate the solids and the green juice. Protein content in the green juice was quantified using the Bradford reagent

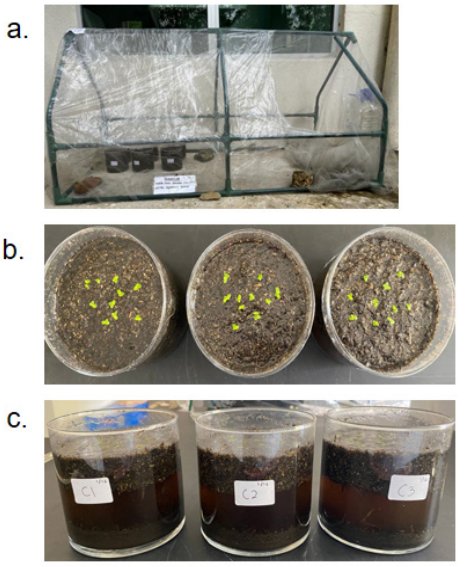

Figure 1. Experimental design for duckweed growth. (a) Greenhouse setup to grow duckweed, (b) top view, and (c) from the view of an experimental glass jar containing formulated bio-fertilizer

(Bio-Rad, USA) and measured using a UV spectrophotometer (Varian Cary 50 UV-Visible Spectrophotometer, Australia). The protein concentration was measured by using Equation 2.

$$y = mx + c \quad \text{[Equation 2]}$$

where, y = absorbance at 595 nm; x = protein concentration.

Statistical Analysis

All collected data on the elemental analysis, duckweed growth, and duckweed protein content were analyzed using MINITAB16 software (Minitab Ltd., Unted Kingdom) and analysis of variance (ANOVA) available in the software. Mean difference analysis was conducted using Tukey's method, with significant differences defined between the sample means ($P < 0.05$).

RESULTS

Morphology of the Bacteria Colonies Isolated from the Mangrove Soil

As the initial step in identifying the species of each bacterial isolate, the morphological characteristics of each were observed and recorded. The colonies of the selected isolates were characterized based on their shape, texture, and appearance. The nitrogen-fixing ability of isolated strains was determined on Jensen agar. Visible colony growth on the agar indicated positive nitrogen fixation, while bacteria unable to fix nitrogen did not grow on this medium. Specifically, J1, J2, and J3 were identified as nitrogen-fixing bacteria, exhibiting light yellow, yellow, and milky white colonies on Jensen agar.

On Pikovskaya's medium and Aleksandrow agar, the abilities of isolated strains to solubilize inorganic phosphorus and potassium were screened, respectively, using A1, A2, and A3 for potassium-solubilizing bacteria, and P1, P2, and P3 for phosphate-solubilizing bacteria. The isolates' potassium- and phosphate-solubilizing activities were qualitatively evaluated by the formation of halos (clear zones) around the colonies growing on Pikovskaya's medium. The bacterial colonies corresponding to Jensen agar, Aleksandrow agar, and Pikovskaya's agar are shown in Figure 2.

Microbial Identification Using 16S rRNA Gene Sequencing Analysis

The phylogenetic tree was constructed using Mega 11 software with the maximum-

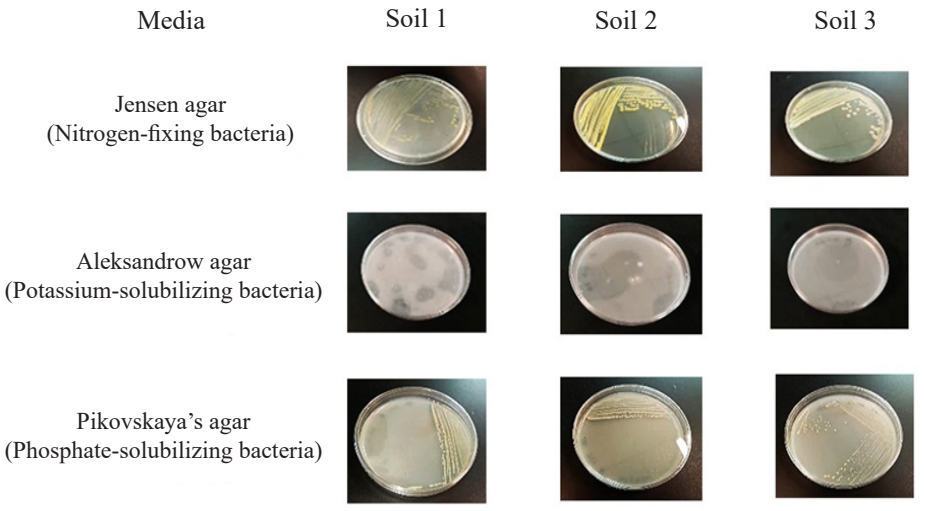

Figure 2. Morphologies of bacteria colonies isolated from soil 1, 2 and 3 on Jensen agar, Aleksandrow agar, and Pikovskaya's agar. Jensen agar, Pikovskaya`s agar, and Aleksandrow agar were used to screen and culture nitrogen-fixing bacteria, phosphate-solubilizing bacteria, and potassium-solubilizing bacteria, respectively

likelihood (Tamura-Nei model) analysis. The bootstrap values are presented as percentages of 1,000 replications at branch points. Subsequent Basic Local Alignment Search Tool (BLAST) analysis unveiled that all the sequences originated from bacteria commonly found in Earth's diverse habitats, including soils.

Taxonomic identification was carried out by analyzing the 16S rRNA gene sequence amplified from nine bacterial isolates. This analysis revealed nine distinct bacterial phylotypes exhibiting sequencing similarities ranging from 98 to 100% (Table 1). Interestingly, these phylotypes belonged to six genera: *Bacillus*, *Acinetobacter*, *Brachybacterium*, *Enterobacter*, *Klebsiella*, and *Paenibacillus*.

Of particular interest were three bacterial strains isolated from Jensen agar: (1) *Acinetobacter radioresistens* (J1), (2) *Brachybacterium paraconglomeratum* (J2), and (3) *Enterobacter cloacae* (J3). Notably, *A. radioresistens* strain OsEp Plm 15B15 (MT367790.1) demonstrated a remarkable 99.25% similarity with J1 isolate. Similarly, *B. paraconglomeratum* strain AS53 (MT214268.1) exhibited a high similarity value of 99.65%, closely matching J2. Likewise, *E. cloacae* strain SUK83 (KY908479.1) shared a significant similarity of 99.63% with J3 (Figure 3).

Meanwhile, the phylogenetic analysis of the 16S rRNA genes from three different strains isolated from Aleksandrow agar revealed their predicted identities as *Klebsiella quasipneumoniae* (A1), *Bacillus tropicus* (A2), and *Paenibacillus pasadenensis* (A3) with a sequence identity of 98–99%. The first bacterium isolated from Aleksandrow agar, *K. quasipneumoniae* strain cjy02 (MN177200.1), exhibited an impressive 99.70% similarity with A1. Additionally, *B. tropicus* strain WSB89

Table 1
A list of bacterial species obtained from soil samples 1, 2, and 3, and is provided through 16S rRNA sequencing

Samples	Identity	Query cover	Percent identity	Accession number
J1	*Acinetobacter radioresistens* strain OsEp Plm 15B15	100%	99.25%	MT_367790.1
J2	*Brachybacterium paraconglomeratum* strain AS53	99%	99.65%	MT_214268.1
J3	*Enterobacter cloacae* strain SUK83	99%	99.63%	KY_908479.1
A1	*Klebsiella quasipneumoniae* strain cjy02	100%	99.70%	MN_177200.1
A2	*Bacillus tropicus* strain WSB89	98%	98.67%	OP_630954.1
A3	*Paenibacillus pasadenensis* strain zp09	100%	99.33%	KM_100367.1
P1	*Bacillus cereus* strain R1	99%	99.34%	MN_213372.1
P2	*Bacillus cereus* strain E1	100%	100%	OP_597695.1
P3	*Bacillus thuringiensis* strain PDKV Bt I-3	100 %	99.74%	OP_209990.1

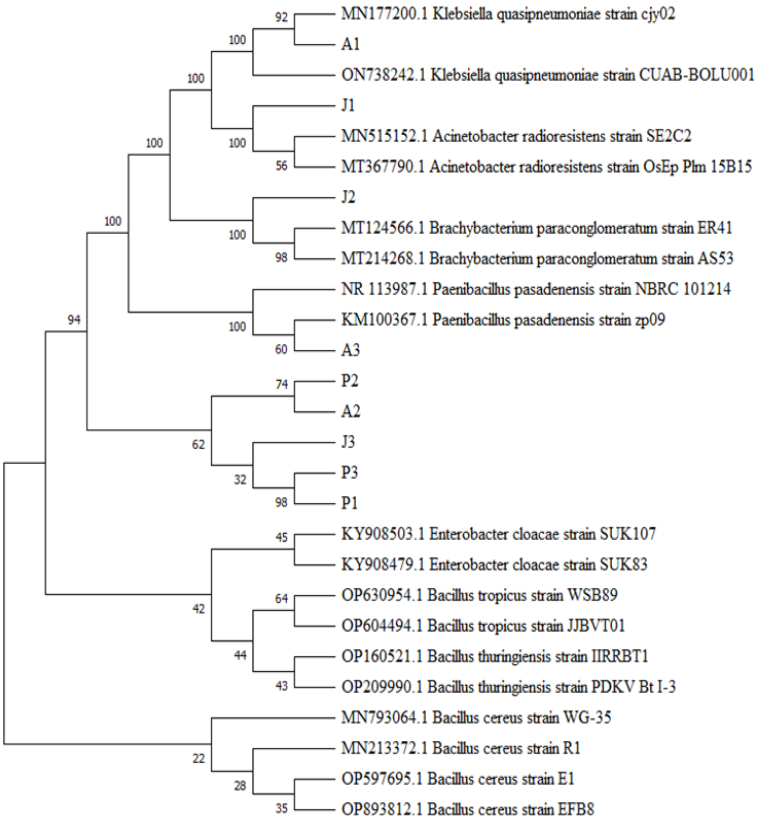

Figure 3. A phylogenetic bacterial species tree was isolated from three agar (Aleksandrow agar, Jensen agar, and Pikovskaya agar). The DNA of the bacteria was extracted, which then being sequenced with 16S rRNA sequencing and the sequences with National Center for Biotechnology Information

(OP630954.1) displayed a sequence similarity of 98.67% with A2, while *P. pasadenensis* strain zp09 (KM100367.1) showed a significant 99.33% similarity with A3.

Furthermore, the 16S rRNA gene sequence analysis identified two strains from Pikovskaya agar, similar to *B. cereus* (P2) and *B. thuringiensis* (P3). Intriguingly, one of the strains isolated from Pikovskaya agar demonstrated similarity with A2, which was earlier predicted to be *B. tropicus* (Figure 3). These findings contribute valuable insights into the phylogenetic relationships and taxonomic diversity of the identified bacterial strains, paving the way for further investigation into their ecological roles and potential applications in various scientific fields.

Bacterial Survivability in Formulated Bio-fertilizer

Total plate counting can be used to test the bio-inoculants in biofertilizers for their survivability. The microbial survivability results indicate that the bio-fertilizer sets A, B, and C had higher counts of viable microbes compared to the control group (Table 2).

Table 2
Total plate count in control and biofertilizer Sets A, B, and C

Samples	Mean $\log_{10}$ (cfu/g)
Control	6.20×10^6a
Set A	8.80×10^6b
Set B	8.00×10^6b
Set C	10^6b

Note. Means that do not share a letter between samples are significantly different ($P < 0.05$) based on Tukey's 95% simultaneous confidence intervals

N, P, and K Elements in Mangrove Soils and Formulated Bio-fertilizer

Next, the isolated bacteria were grouped into three sets of bio-inoculants, each exhibiting N fixation, P and K solubilization activities in three sets of formulated bio-fertilizers. Therefore, this study analyzed N, P, and K content in mangrove soils, as well as three sets of formulated bio- and compared them with the control (Figure 4). The analysis of chemical element content in mangrove soils revealed that the smallest trace element was P, followed by N. K element was the most abundant element in mangrove soil and showed a slight increase ($P = 0.003$) in Soil 2 compared to Soils 1 and 3.

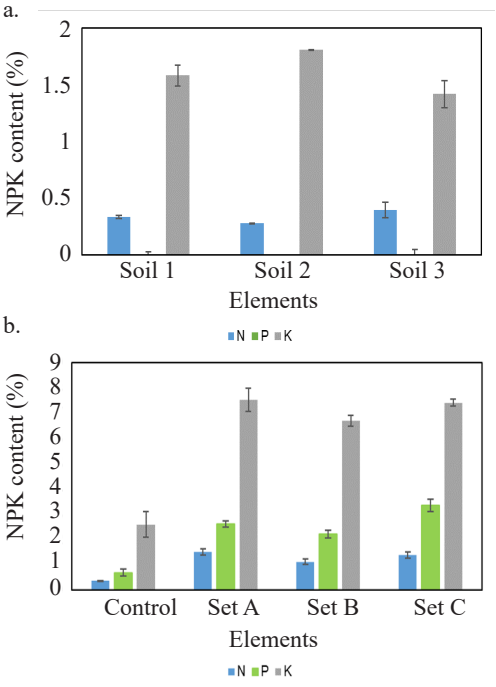

Figure 4. Nitrogen (N), phosphate (P), and potassium elements in (a) mangrove soils and (b) control and formulated bio-fertilizer Sets A, B, and C

Note. All data are mean±standard deviations (n = 3). Means that do not share a letter between samples are significantly different ($P < 0.05$) based on Tukey's 95% simultaneous confidence intervals

Meanwhile, from the analysis of chemical element content in our formulated bio-fertilizers, it was found that the K element was also the most abundant in all three sets of formulated bio-fertilizers and showed a significant increase (P = 0.000) compared to the control, which is similar to N and P elements. However, P content was slightly increased in formulated bio-fertilizer Set C compared to Sets A and B. At the same time, the percentage of N element was similar in Sets A and C. The presence of mangrove-associated bacteria in formulated bio-fertilizer is known to reflect the amount of N, P, and K content.

Effect of Formulated Bio-fertilizer on the Growth of Duckweed Plants

The effectiveness of formulated bio-fertilizer sets A, B, and C compared to the control on the growth of duckweed plants is presented in Figure 5. The duckweed growth was evaluated in terms of the number of duckweed fronds. Relative to the control, formulated bio-fertilizer sets A, B, and C exhibited a significant increase (P = 0.00) in duckweed growth from Day 3 until Day 15. The result of this analysis also showed that formulated bio-fertilizer Set C is the most effective medium to boost duckweed growth compared to Sets A and B. Figure 6 shows the impact of formulated bio-fertilizers sets A, B, and C on duckweed fronds on Day 15, contrasting the results with Day 0 and the control group. The results obtained from this experiment indicate that duckweed growth is correlated with the chemical element content in formulated bio-fertilizer Set C (*E. cloacae*, *P. pasadenensis*, and *B. thuringiensis*), which showed an increase of P content compared to Sets A and B.

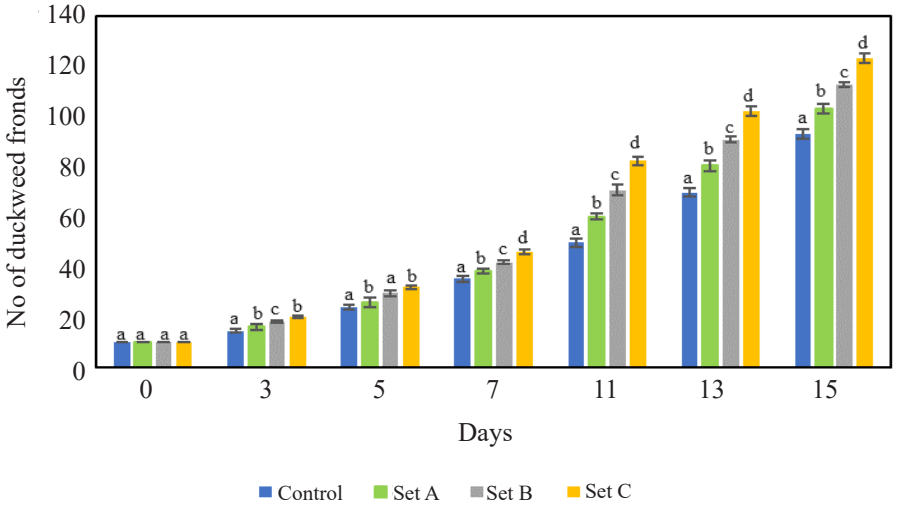

Figure 5. The growth of duckweed in control and formulated bio-fertilizer Sets A, B and C in 15 days

Note. All data are mean±standard deviations (n = 3). Means that those that share a letter between samples are significantly different (P < 0.05) based on Tukey's 95% simultaneous confidence intervals

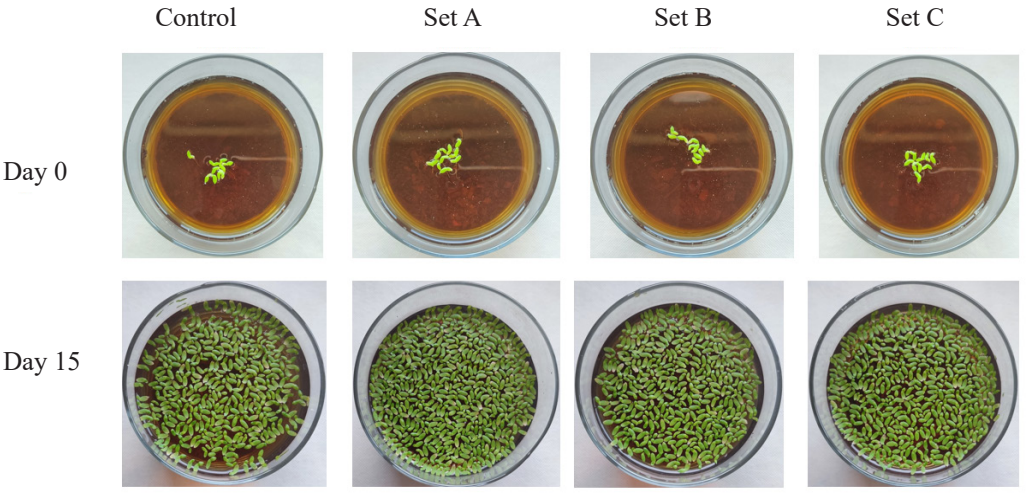

Figure 6. The growth of duckweed on 0 and 15 days in control and formulated bio-fertilizer Sets A, B, and C, respectively

Effect of Formulated Bio-fertilizer on the Duckweed Protein Content

The effectiveness of formulated bio-fertilizer sets A, B, and C compared to the control on the duckweed protein amount is presented in Figure 7. Overall, formulated bio-fertilizer sets A, B, and C displayed an increasing trend ($P = 0.00$) in the amount of protein in duckweed plants compared to the control. It indicates that the presence of mangrove-associated microbes in the formulated bio-fertilizer used as a duckweed growth medium is known to influence the amount of protein in this plant.

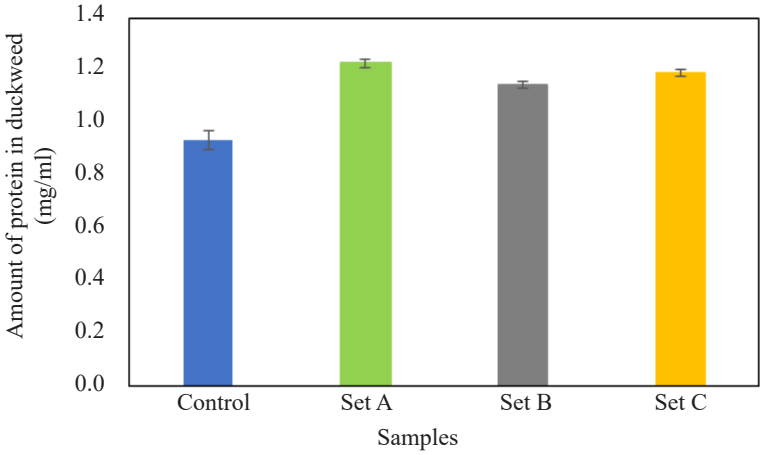

Figure 7. Amount of protein harvested from duckweed after 15 days grown in control and formulated bio-fertilizer Sets A, B, and C in 15 days

Note. All data are mean±standard deviations (n = 3). Means that those that share a letter between samples are significantly different (P <0.05) based on Tukey's 95% simultaneous confidence intervals

DISCUSSION

N, P, and K Content Analysis

Bio-fertilizer has been recommended as a more environmentally friendly substitute for conventional chemical fertilizers and pesticides. It has been established that rhizosphere soil has a rich source of plant growth promoting bacteria (PGPB) (Iniesta-Pallarés et al., 2023; Pii et al., 2015). In this study, bacteria were isolated from the mangrove soil. Nine bacterial strains were identified according to the 16S rRNA gene sequencing and grouped into three bio-fertilizer sets (Set A containing *A. radioresistens*, *K. quasipneumonia*, and *B. cereus*; Set B contains *B. paraconglomeratum*, *B. cereus*, and *B. tropicus*; and Set C containing *E. cloacae*, *P. pasadenensis*, and *B. thuringiensis*) with each having the ability to fix nitrogen, solubilize potassium and phosphorus, grown on the duckweed.

The results of bacterial screening on Jensen agar and microbial identification by using 16S rRNA analysis demonstrated that *A. radioresistens* (J1), *B. paraconglomeratum* (J2), and *E. cloacae* (J3) exhibit nitrogen-fixing capabilities. Some types of bacteria and cyanobacteria are essential to the nitrogen cycle as they can reduce or fix atmospheric nitrogen gas (N_2), rendering the element accessible to other organisms, including plants and animals (Saha et al., 2017). Soil microbes, influenced by soil nitrogen availability, impact the terrestrial carbon cycle through decomposition and the formation of soil organic matter (SOM) (Cotrufo et al., 2013). High-N substrates lead to rapid breakdown by bacteria, resulting in substantial microbial product accumulation and stable SOM creation. Cycles of extracellular enzyme production, primarily controlled by community composition, serve as markers for microbial nutrient demand, soil nutrient cycling, and soil respiration (Zechmeister-Boltenstern et al., 2015). Meanwhile, *B. cereus* (P2) and *B. thuringiensis* (P3) exhibit phosphate-solubilizing capabilities. Among the essential macronutrients, P plays a crucial role in plants' biological development and growth (Soetan et al., 2009). P solubilizers are crucial in solubilizing soil phosphorus by producing secondary metabolites, including enzymes such as acid phosphatases and phytases. Additionally, they generate phytohormones like indole-3-acetic acid (IAA) and siderophores, which further contribute to increased plant yield (A. Kumar et al., 2014; Kour et al., 2020).

Furthermore, *K. quasipneumoniae* (A1), *B. tropicus* (A2), and *P. pasadenensis* (A3) exhibit potassium-solubilizing capabilities. K is vital for plant development and growth as it involves numerous metabolic processes. It plays a crucial role in the plant's ability to withstand drought and diseases (Billore et al., 2009). Additionally, it contributes to starch production, controls root growth, regulates stomata movement within plant cells, activates enzymes, maintains cell turgor, and transports sugars as well as starches (Meena et al., 2014), ultimately influencing plant quality. Rhizospheric bacteria, known as potassium-solubilizing bacteria, have the ability to convert insoluble potassium into

soluble forms through acidolysis, chelation, exchange reactions, and complexation (Meena et al., 2015). Simultaneously, they decompose organic matter and crop residues to promote plant growth and increase yield (Etesami et al., 2017). Among soil microorganisms, potassium-solubilizing bacteria play the most significant role in plant potassium cycling (Sun et al., 2020). In the soil, K is found at a concentration of 1–2% as a soluble compound, with the other 90%+ present as insoluble rocks and silicate minerals (e.g., mica, muscovite, feldspar, microline, and orthoklas). In this way, the latter type is mostly inaccessible to plants (Parmar & Sindhu, 2013). Bacteria play a vital role in maintaining soil fertility by secreting organic acid during the degradation of silicate minerals, which release K, silicon, and aluminum.

Based on the results obtained from this study, Set C appears to be much better than Sets A and B as a bio-fertilizer option, as Set C showed an increase in the content of N, P, and K when compared to Sets A, B, and Control. Set C contains *E. cloacae*, *P. pasadenensis*, and *B. thuringiensis* in a formulated bio-fertilizer. The main advantage of Set C is its ability to enhance the P content in the soil. Several studies (Ali & Pati, 2023; Ansari et al., 2023; A. Kumar et al., 2014) have reported that *E. cloacae*, one of the bacteria in Set C, is a potent inorganic P solubilizer and can significantly increase P acquisition in plants. Moreover, *E. cloacae* exhibit a variety of growth-promoting actions, including P and K solubilization, as well as N fixation (Chin et al., 2017; Deepa et al., 2010; Ramesh et al., 2014). These actions improve plant health and soil fertility (Ghiglione et al., 2021). *Paenibacillus pasadenensis*, another component of Set C, has also been shown to be involved in the solubilization of soil phosphorus, the production of phytohormones and antimicrobial metabolites (Govindasamy et al., 2011), and is also known to be involved in the fixation of atmospheric nitrogen and the uptake of micronutrients, further benefiting plant growth (Grady et al., 2016). *Bacillus* sp. was also considered an effective nitrogen-fixing bacteria (Awasthi et al., 2011; Zhang et al., 2023). Multiple PGPB favorable characteristics were found in the genus *Bacillus*, including P solubilization and participation in the N cycle (Stegelmeier et al., 2022).

This study also shows that Set A bio-fertilizer, which contains *A. radioresistens*, *K. quasipneumonia*, and *B. cereus*, as well as Set B, which contains *B. paraconglomeratum*, *B. cereus*, and *B. tropicus*, exhibit increased levels of N, P, and K compared to control. Previous research demonstrated that *Acinetobacter* increased the amount of N that duckweed could extract from the pond water (Stegelmeier et al., 2022). *Acinetobacter* sp. is claimed to be a plant growth-promoting bacterium, as it has been found to enhance wheat growth (Egamberdieva et al., 2008). Furthermore, this study revealed that Set A is the second highest in increasing the P content in the soil, similar to Set B. This finding is supported by research conducted by Yamakawa et al. (2018),

in which *Acinetobacter calcoaceticus* P23 exhibited apparent P solubilizing activity. They claim that *Acinetobacter* can increase the amount of P in the cultivation of duckweed (Yamakawa et al., 2018). *Klebsiella quasipneumoniae*, a sister-like *of Klebsiella pneumonia*, is considered a human bacterial pathogen. However, it has been reported that a *Klebsiella* strain was present in the rhizosphere and exhibited PGPB traits (Tangapo et al., 2018). *Klebsiella pneumoniae* possesses various PGPB traits, namely the production of IAA, P solubilization, N–fixing ability, and several other traits (Ashfaq et al., 2022). Similar results have shown that K solubilizing bacteria have been successfully isolated from tobacco rhizosphere, such as *Klebsiella variicola* (Sun et al., 2020). There is extensive evidence that inoculation with *Klebsiella* sp. can increase the available K in the soil (Wang et al., 2020).

Set B is composed of *B. paraconglomeratum*, *B. tropicus*, and *B. cereus*. Results from this study show that Set B has the lowest increase in N, K, and P content compared to Sets A and C. *Brachybacterium paraconglomeratum*, which has demonstrated the ability to promote plant growth. This bacteria species can also produce the plant hormone IAA and siderophores. Furthermore, it can utilize 1-aminocyclopropane-1-carboxylic acid (ACC) as a sole source of N and exhibits ACC deaminase activity in plant growth promotion (Gontia et al., 2011).

The Effect of Bio-fertilizer in Improving Duckweed Growth and Its Protein Content

Microbes naturally found in plants are crucial in promoting plant growth, even in challenging conditions. Extensive research on bio-fertilizers has demonstrated their potential to supply vital nutrients to crops, enriching crop yields without harming the environment (Kour et al., 2020). However, not all microbes can interact with plants, making it essential to analyze the interactions of PGPB with their natural plant hosts (Zamioudis & Pieterse, 2012). Duckweed, a fast-growing aquatic plant, undergoes clonal duplication during its vegetative growth cycle, with a high number of fronds indicating healthy growth and reproduction (Tang et al., 2015). N fixation, P solubilization, and K solubilization have been identified as the mechanisms responsible for the symbiotic connections between bio-fertilizers and duckweed.

Studies have shown that bio-fertilizers from different sets can significantly enhance duckweed growth (Yoneda et al., 2021). Unlike in soil, plant-associated microorganisms in water must adhere to and colonize plant bodies to avoid being washed away by water currents. Aquatic PGPB is hypothesized to possess useful properties such as rapid adhesion and stable colonization. Based on the results obtained from this study, bio-fertilizers from Sets A, B, and C can enhance the growth of the duckweed, with Set C displayed as the best bio-fertilizer, corresponding to the highest number of duckweed fronts after 15 days.

The bio-fertilizer increased the number of duckweed fronds, which is consistent with the findings of Yoneda et al. (2021). Specifically, PGPB strains from the phyla Betaproteobacteria, Gammaproteobacteria, and Alphaproteobacteria have been observed to increase the number of duckweed fronds by more than twofold (Makino et al., 2022). Additionally, certain PGPB strains, such as *Pseudomonas* sp. Ps6 and *Ensifer* sp. strain SP4 have demonstrated the ability to accelerate duckweed growth (Toyama et al., 2017).

The potential for using this PGPB as a bioinoculant was demonstrated by exposing duckweed to all bacterial strains obtained in this study. The PGPB effects of all tested bacterial strains on duckweeds were comparable to those of the well-studied representative PGPB, *Acinetobacter calcoaceticus* strain (Makino et al., 2022; Toyama et al., 2017). The ability of *A. calcoaceticus* P23 to grow in both artificial media and environmental conditions makes it a potential bioinoculant for enhancing duckweed growth (Suzuki et al., 2014; Toyama et al., 2017; Yamaga et al., 2010). Several studies have shown that co-cultivation of duckweed with specific PGPB strains can lead to significant growth benefits. For instance, the rhizobacterium MH3 has been found to boost duckweed development, resulting in a 30% increase in frond number and a 50% increase in dry weight (Tang et al., 2015). Moreover, certain *Bacillus* strains present in different bio-fertilizer sets have successfully functioned as PGPB to stimulate rapid duckweed growth (Idris et al., 2007). The hypothesis of synergistic effects arising from the co-inoculation of these strains further supports establishing and maintaining a mutually beneficial plant-microbe relationship. Thus, the bio-fertilizer set can establish and maintain a mutually beneficial plant-microbe relationship. It is worth noting that the bio-fertilizer bacterial strain can potentially promote growth and rescue plants from growth inhibition synergistically.

In this investigation, bio-fertilizers denoted as Sets A, B, and C exhibited a notable augmentation in duckweed protein content in tandem with escalating concentrations of N, P, and K. These findings align with those reported by Li et al. (2016), observed a similar increase in protein content in *Spirodela polyhiza* duckweed as N and P concentrations were elevated (Li et al., 2016). The correlation between these studies suggests that higher nutrient levels are conducive to enhancing duckweed protein production. Notably, Set A displayed a higher protein content compared to Sets B and C, highlighting the influence of different bacterial species within the bio-fertilizer sets on protein content. Shuvro et al. (2023) also observed increased protein content in *L. minor* when cultured with *Azotobacter vinelandii* for 10 days, relative to the control. However, under stressful conditions, the protein production levels decreased (Shuvro et al., 2023). Furthermore, the growth factor and protein content of duckweed are impacted by light intensities (Petersen et al., 2022).

Our study highlights the potential of mangrove-associated bacteria identified as *A. radioresistens*, *B. paraconglomeratum*, and *E. cloacae*, which are known as nitrogen-fixing bacteria, *K. quasipneumoniae*, *B. tropicus*, and *P. pasadenensis* known as potassium-solubilizing bacteria, and *B. cereus* and *B. thuringiensis* known as phosphate-solubilizing bacteria when integrated into bio-fertilizers. These environmentally friendly alternatives to traditional chemical fertilizers and pesticides represent a novel approach. The microbial composition in each set of our formulated bio-fertilizer includes specific nitrogen-fixing species, potassium-solubilizing, and phosphate-solubilizing bacteria. This specificity distinguished our study distinct from previous research. The synergistic combination of these three types of bacteria in our bio-fertilizer formulation represents a promising strategy for developing bio-fertilizers to enhance plant growth. This study also contributes to the ongoing efforts in bio-fertilizer development by identifying specific microbial compositions that enhance plant growth. This knowledge is crucial for formulating effective bio-fertilizers that can be applied in various agricultural settings. In addition, highlighting the importance of mangrove-associated microbes in bio-fertilizers underscores the potential role of mangrove ecosystems in supporting agricultural practices. This information can contribute to conserving mangrove biodiversity for ecological and agricultural benefits.

CONCLUSION

Our study successfully achieved its aim by showcasing the impactful integration of mangrove-associated microbes into our formulated bio-fertilizer. The discernible outcome was a substantial enhancement in duckweed's growth and the subsequent protein yield. These findings contribute valuable insights into optimizing bio-fertilizer formulations and emphasize the significant potential of duckweed as a viable and promising future food option. As the challenges of sustainable food production are navigated, the demonstrated success of this integration underscores the importance of exploring innovative and environmentally friendly approaches to enhance agricultural productivity and advance the feasibility of alternative protein sources.

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Identification and Quantification of Cucurbitacins B and E in Different Parts of Bitter Gourd Plants Derived from Different Planting Methods

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ABSTRACT

Bitter gourd is a beneficial and easily accessible plant commonly utilised as a food source and medicinal herb. This plant produces numerous types of phytochemicals, especially when triggered by elicitors. It is also well known for its bitter taste, which is contributed by one of its phytochemical contents called cucurbitacin. This study determines the different levels of cucurbitacins B and E in the plants from two different planting methods, conventional and fertigation. Fruits, leaves, stems, and roots of bitter gourd plants from the two different planting methods were harvested for extraction using the sonication extraction method. The extraction solvents used were n-hexane, chloroform, and 80% ethanol. The extract's cucurbitacins B and E content were identified and quantified using high-performance liquid chromatography. A preliminary rapid test using the Salkowski's test to detect triterpenoids showed positive results for all sample runs. Results indicate significant variations in cucurbitacin levels across plant parts and cultivation methods. This study found that the content of cucurbitacin B in leaves of the fertigation planting method was the highest (208.0±0.4 ppm). Cucurbitacin B content in fruits was notably higher in both fertigation (200.0±1.3 ppm) and conventional (200.0±5.0 ppm) methods compared to other plant parts. However, leaves in the conventional method showed a significantly lower cucurbitacin B content (122.0±5.0 ppm). All plant parts were significantly different for cucurbitacin E, with the stem from the conventional planting method having the highest level

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of cucurbitacin E (31.0±1.0 ppm). Thus, it is concluded that plant parts and type of planting method can affect the cucurbitacin content in bitter gourd.

Keywords: Bitter gourd, cucurbitacin, fertigation, high-performance liquid chromatography, phytochemical

INTRODUCTION

Plants are a source of a large variety of organic compounds called secondary metabolites that have been utilised for a long time by human society. Secondary metabolites are complex compounds with unique carbon skeletons (Pagare et al., 2015). These compounds are present in all plant parts and involve various physiological processes as they can be synthesised in plant organs. They are not fixed to a single plant part but are defined by their biosynthesis and functions (Singh & Sharma, 2014). These processes are diverse and determined by cell type, environmental condition, and development stage (Kurepin et al., 2017; Li et al., 2020; Sanchita & Sharma, 2018).

Bitter gourd (*Momordica charantia*) is a food crop grown for food and medicinal purposes worldwide, especially in tropical regions. Bitter gourd is a family of Cucurbitaceae (Gayathry & John, 2022). According to studies by Jia et al. (2017) and Torre et al. (2020), bitter gourd produced a wide variety of secondary metabolites that give many beneficial properties, such as antioxidant, antimicrobial, anti-inflammatory, anticancer, and nutritional values. Aqueous, ethanolic, methanolic, and hexane extracts of bitter gourd contained alkaloids, glycosides, cholesterol, saponins, flavonoids, and terpenoids (Jia et al., 2017; Gayathry & John, 2022; Supraja et al. 2015). The primary constituents of this plant were cucurbitacins, sterols, saponin's cucurbitane-type triterpenoids (Chekka & Mantipelly, 2020), and vicine (Ahamad et al., 2017). Cucurbitacins are secondary metabolites found in many plant families but are highly common in the Cucurbitaceae plant family (Chanda et al., 2020; Haq et al., 2019). Cucurbitacin derivatives are differentiated by their structural skeleton and functional group arrangement (Haq et al., 2019). Cucurbitacins are known as the source of the cucurbitaceous plant's bitter taste (Kaushik et al., 2015). The cucurbitacin group currently consists of at least 19 members, which is differentiated by chemical structure variation between members named cucurbitacins A - T (Hunsakunachai et al., 2019). Similar to other cucurbitaceous plants, cucurbitacins are the main compound in bitter gourd (Maja et al., 2022).

Planting methods are one of the factors that affect the phytochemical accumulation and antioxidant activities in plants (Machado et al., 2018). The fertigation and raised bed (conventional planting) systems are two distinct growth conditions for plants, especially in media conditions. A fertigation system is a planting practice that allows precise and uniform amounts of water and nutrient supply to plants planted on non-soil or partial soil media through drips or sprinklers (Kant & Kafkafi., 2005). Raised bed planting is a planting technique on a bed above the existing soil level (Akbar et al., 2007). The raised bed is sometimes

covered with mulch to prevent water loss and suppress weeds. These two planting methods will give two distinct agronomic conditions affecting the plant's secondary metabolite (Neugart et al., 2018). Thus, different planting methods might stimulate the level of these factors that act as elicitors for secondary metabolite production.

From the understanding of the secondary metabolites synthesis mechanism and its beneficial effects on the *M. charantia* plant's defence system, the present project was planned to illustrate how the planting method impacted the production of the cucurbitacins in the bitter gourd plants.

MATERIALS AND METHODS

Plants Material

Seeds of bitter gourd were obtained from a local supplier. Seeds were soaked in water overnight to induce germination and sown in a germination tray filled with peat moss. After reaching the 3–4 leaf stages, the seedlings are transferred into a polybag prior to planting via conventional or fertigation systems. The planting beds were covered with silver shine and watered using a drip irrigation system for the conventional system. Plants in the conventional systems were fertilised manually with NPK fertiliser 15:15:15 one week after transplanting and NPK 12:12:17:2 on weeks 2, 5, and 8 at a rate of 30 g per plant (Anem, 2017). For the fertigation system, AB fertiliser was applied through a drip irrigation system (Kadir, 2020). There were 60 seedlings set up for each conventional and fertigation planting method. Both planting methods were conducted based on commercial practice described by Anem (2017) and Dhillon et al. (2017).

Preparation of Plant Extracts

The extraction method was conducted following the methods described by Attar and Ghane (2018), with slight modifications. Plant samples from both planting methods were harvested 60 days after sowing. Plant parts, including stems, roots, leaves, and fruits, were rinsed with distilled water, cut into small pieces and oven-dried at 50°C for 48 hr. The dried plant parts were then ground into powder. A total of 10 g of sample powder from each plant part was soaked into 100 ml of n-hexane (Merck, Germany). The mixture was placed in an ultrasonic bath (Elma Ultrasonic Cleaner 15 L Digital Ultra Sonic Tank Bath Cleaning Heater Timer PS-60, Germany) at 35°C for 40 min. The extract was filtered through a filter paper (Whatman™ 1001-125 Grade 1 Qualitative Filter Paper, diameter: 12.5 cm, pore size: 11 µm, USA). The filtrate was centrifuged (Hitachi, Japan) for 15 min at 905.58 g and filtered through the filter paper. The filtrate was re-extracted using chloroform (Thermo Fisher Scientific, USA) and 80% ethanol (Merck, Germany) following the n-hexane steps. The 80% ethanol (Merck, Germany) extraction filtrate was dried under vacuum at 40–60°C using a rotary evaporator (R-215, BUCHI, Switzerland).

Terpenoid Qualitative Rapid Screening (Salkowski's Test)

Qualitative phytochemical screening for steroids and triterpenoids in bitter gourd

plant parts was done using the Salkowski's test. A total of 1 g of plant extracts was exposed to a few drops of chloroform (Thermo Fisher Scientific, USA) and concentrated sulfuric acid (Grainger, USA) and let rest. After several minutes, a lower layer with a red-to-yellow colour indicated the presence of steroids and triterpenoids (Ramakrishna et al., 2019).

Standard Preparation

A total of 1 mg of each cucurbitacins B and E standard compound powder was dissolved in 1 ml acetonitrile (Merck, Germany) (stock solution). Further dilution was done by diluting the standard stock solution with acetonitrile to prepare a concentration range of 0.1–1.0 mg/ml for both standards. The standard solutions were filtered through a 0.45 µl syringe filter before injected into high-performance liquid chromatography (HPLC, Shimadzu/LC 20AT, Japan) and analysed to plot a standard curve. Both compounds from bitter gourd extracts were then identified and quantified by comparing them with the standards' standard curves and retention time.

HPLC Condition and Analysis

Compounds in the extract were identified and quantified using HPLC (Shimadzu/LC 20AT, Japan), following the method described by Zaini et al. (2018) with slight modifications. A total of 1 mg of each extract was dissolved in 1 ml of acetonitrile (Merck, Germany). The solution was filtered through a 0.22 µm syringe filter and pumped through the Syncronis™ C18 column (250 mm x 4.6 mm diameter, Thermo Fisher Scientific, USA). The flow rate and wavelength were optimised at 1 ml/min and 250-280 nm, respectively. A total of 2% acetic acid (Merck, Germany) in water (solvent A) and acetonitrile (Merck, Germany) (solvent B) were used as mobile phases with a gradient elution system. A 0.45 µm Millipore™ (Merck, Germany) membrane was used to filter the mobile phase, and the degas were filtered using sonication. The column temperature was maintained at 30°C, and the sample injection volume was set to 20 µl.

Data Analysis

The data obtained were analysed using analysis of variance (ANOVA). The means and standard deviation for the cucurbitacins B and E quantities were determined and compared using the Tukey test to find the significantly different mean within the same planting method, at the 5% significance level.

RESULTS AND DISCUSSION

Salkowski's Test

The Salkowski's test is a qualitative phytochemical assessment generally for terpenoids and their isomers, including tetracyclic triterpene cucurbitacins (Sasikala & Sundaraganapathy, 2018). This study used the Salkowski's test, a simple and supportive technique to detect triterpenoids. Crude extract of each bitter gourd plant part from both planting methods showed positive results, as presented in Figure 1. It indicated that triterpenoids and their isomers are present in the crude extracts

of all bitter gourd plant parts from both planting methods (Ramakrishna et al., 2019). It supports the assumption that cucurbitacins are present in the crude extract and demonstrates a promising result for HPLC analysis. All plant parts of bitter gourd contain terpenoids, including triterpenes's cucurbitacins (Ramakrishna et al., 2019). Thus, it is assumed that triterpenoids of cucurbitacins were among the detected terpenoids (Kaushik et al., 2015). The difference in intensity of the red-brownish colour that appeared might be affected by the concentration of the phytochemical compound (terpenoids). In the Salkowski's test, terpenoids are regarded as steroids due to their 5-5-carbon ring structure (Adedokun et al., 2023). The colour changes due to the reaction between sulphuric acid and the aromatic rings of steroids (Gupta, n.d.). It indicates that the reaction was straightforward and continues to occur if there are reagents. The chemical reaction might increase with increasing reagents (phytochemical and solvent concentration) (Key, 2014). However, despite the intensity of the colour formed, the criteria to accurately acknowledge the presence of the target compounds are only based on the empirical positive or negative result (Maharaj et al., 2017; Pochapski et al., 2011). Figure 1 shows that the stem extract from the fertigation method contains the highest terpenoid, including its derivative concentration, while the extract from the conventional method contains the lowest terpenoid concentration and its derivative.

Quantification of Cucurbitacins B and E

The standard curve R^2 values of 0.9986923 and 0.9961842 were obtained from the calibration curves of the cucurbitacins B

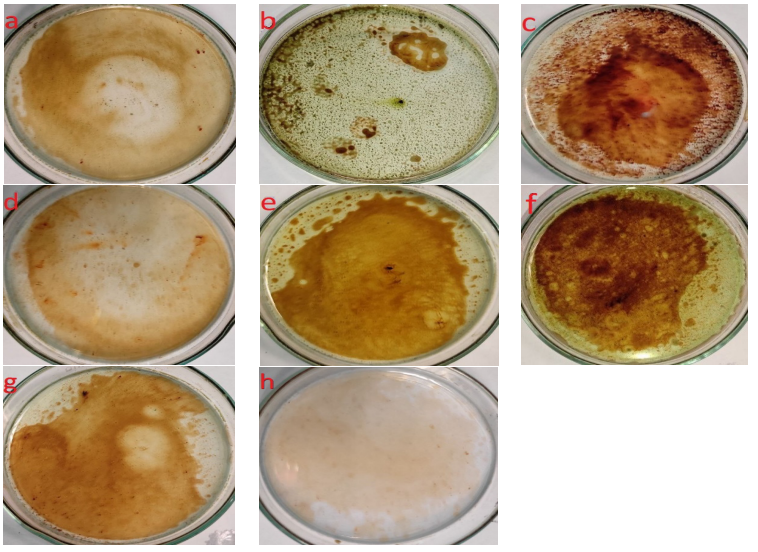

Figure 1. Phytochemical screening using the Salkowski's test for terpenoids of different plant parts of bitter gourd. The formation of a lower layer with a red to yellow colour indicates the presence of steroids and triterpenoid in (a) fruits from fertigation method, (b) leaves from fertigation method, (c) stems from fertigation method, (d) roots from fertigation method, (e) fruits from conventional method, (f) leaves from conventional method, (g) stems from conventional method, and (h) roots from conventional method, respectively

and E standard compounds, respectively. The levels of cucurbitacins B and E were compared between plant parts from the same propagation method and between the same plant parts from each of the different propagation methods.

For the fertigation planting method (Table 1), leaves contain the highest content of cucurbitacin E at 13.0±7.6 ppm and cucurbitacin B at 208.0±0.4 ppm. On the other hand, fruits have the lowest cucurbitacin E content at 2.0±0.6 ppm and the second highest cucurbitacin B content at 200.0±1.3 ppm. Cucurbitacins B and E content in stems is 185.0±0.4 ppm and 11.0 ±1.5 ppm, respectively. While cucurbitacins B and E in roots are 174.0±0.6 ppm and 5.0 ±0.0 ppm, respectively. It can be observed that the cucurbitacin B content in leaves of fertigation plants was significantly higher than that of stems and roots but not significant in fruits. For cucurbitacin E, leaves were only significantly higher than fruit, and there was no significant difference between fruits, stems, and roots.

For the conventional method, stems contain the highest content of cucurbitacin E at 31.0±1.7 ppm but the second lowest cucurbitacin B at 136.0±4.5 ppm. Fruits came with the highest content of cucurbitacin B at 200.0±5.0 ppm but also with the lowest content of cucurbitacin E at 5.0±0.1 ppm. Cucurbitacins B and E content in leaves was 122.0±10.5 ppm and 13.0±0.1 ppm, respectively. The root contains 9.0±1.2 ppm of cucurbitacin E and 166.0±2.5 ppm of cucurbitacin B. Analysis of variance showed that the content of cucurbitacin B in fruits was significantly higher than all other plant parts from the same planting method. All plant parts were significantly different for cucurbitacin E, with the stem having the highest level. The level of cucurbitacin B in all plant parts from both planting methods is higher than cucurbitacin E content. For comparison between planting methods, all plant parts from the fertigation method contain a higher level of cucurbitacin B compared to plant parts from the conventional method. However, only leaves and stems differ significantly.

Fruits and stems from the conventional planting method contain higher cucurbitacin E compared to fruits and stems from the fertigation method and are significantly different. Leaves from both planting methods are equal and not significantly different. At the same time, cucurbitacin E in roots from the conventional planting method is lower than in the fertigation method, and there is no significant difference either. Sikander et al. (2019) stated that cucurbitacins B and D are the most common cucurbitacins to be found in plants. Cucurbitacin B is also the highest contributor to the bitter taste in bitter gourd, while other cucurbitaceous plants with cucurbitacins combination (Luo et al., 2020; Maja et al., 2022).

Previous studies have also indicated that abiotic stress may increase the production of secondary metabolites while the crops are growing. *Cucumis prophetarum* callus cultured in media with sodium chloride as an imitation of salt stress contains a higher level of cucurbitacin compared to the absence of sodium chloride (Saker et al., 2010). The level of cucurbitacin E was also

recorded to be higher in fruits, leaves and roots of cucumber plants that were treated with potassium phosphate and chitosan as signalling compound elicitors compared to control with fruit has the highest content of cucurbitacin (Ramezani et al., 2017).

Overall, the content of cucurbitacin B in plant parts cultivated through fertigation and conventional methods exhibited variation, as statistically significant differences were observed. Furthermore, stems and roots from the conventional planting method demonstrated higher cucurbitacin E content compared to the fertigation method, whereas fruits and leaves showed similar levels. Notably, the highest content of cucurbitacin B was recorded in the leaves of the fertigation planting method. However, this difference was not deemed statistically significant compared to fruits from the fertigation and conventional planting methods. Similarly, the highest concentration of cucurbitacin E was observed in stems from the conventional planting method, with a significant difference noted compared to all other plant parts from both planting methods. No specific studies on the cucurbitacin B content of bitter gourd have been identified in previous research. However, Maja et al. (2022) reported cucurbitacin B content in the plant parts of various species. The leaves extract of *Citrullus lanantus* var. *lanantus* showed 0.3 ppm, *Citrullus colocynthis* exhibited 20.8 ppm (Kim et al., 2018), *Cucumis africanus* presented 0.082 ppm (Shadung & Mashela, 2016), and *Lagenaria siceraria* recorded 0.05 ppm (Mashilo et al., 2018). Furthermore, *Cucumis melo* displayed 65-75 ppm and 70-80 ppm in leaves and fruit extracts, respectively (Luo et al., 2020). Da Rocha Galucio et al. (2022) identified 79 ppm of cucurbitacin B in the ethanolic extract of *Luffa operculata* (L.) Cogn. fruits, considering it a substantial amount. It is noteworthy that the cucurbitacin B content reported in these previous studies was lower than the highest content observed in the present study. Contrastingly, for cucurbitacin E, previous studies present results opposite to the highest cucurbitacin E recorded in the present study. Chanda et al. (2019) extracted 356 and 663 ppm of cucurbitacin E from bitter gourd and *Cucurbita pepo* var. *pepo* fruits, respectively, higher than observed in the present study. The decrease in cucurbitacin E content in the present study is assumed to be caused by the modification of cucurbitacin E during the production of other cucurbitacin derivatives. Cucurbitacins B and E are the primary cucurbitacins that can be modified through the action of acetyl esterases into other cucurbitacins (Ahmed & Halaweish, 2014; Schabort et al., 1968).

The variation of cucurbitacins B and E level in different plant parts of the bitter gourd samples is consistent with the study by Kim et al (2018) and Luo et al (2020). Different concentrations of cucurbitacins were also recorded in other species of cucurbits (Maja et al., 2022). It is due to the differences in type and level of stress from the surrounding environment faced by each of the plant parts, even for the same individual plant, as the production of secondary metabolites is specifically based on organ, tissue, and even cell of the plants (Pagare et al., 2015; Ramezani et al.,

2017). Environmental changes can have a significant impact on the regulation of secondary metabolic processes (Sanchita & Sharma, 2018). Different plant sections have varied gene expression patterns associated with cucurbitacin production (Luo et al., 2020; Maja et al., 2022). Maja et al (2022) also suggested that different cucurbitacins have different cucurbitacins synthesis genes and gene expressions. It demonstrated that the level of cucurbitacins B and E recorded cannot solely be used to support the hypothesis that cucurbitacin content increases with stress. The production of secondary metabolites in plants is complex because other types of cucurbitacins may be produced during the stress, as the synthesise of cucurbitacins and other secondary metabolites is also genotype-dependent (Luo et al., 2020; Maja et al., 2022). Once elicitors, including abiotic stresses, are recognised by plants, the manufacture of genetic transcription factors that control plant secondary metabolism type and function will be initiated (Ramezani et al., 2017).

Table 1
Cucurbitacins quantity of bitter gourd plant parts

Phytoconstituent (Cucurbitacins)	Method	Quantity of cucurbitacins (ppm)			
		Fruits	Leaves	Stems	Roots
Cucurbitacin B	Fertigation	$^{ABa}200.0\pm1.3^{w}$	$^{Aa}208.0\pm0.4^{w}$	$^{BCa}185.0\pm0.4^{w}$	$^{Ca}174.0\pm0.6^{w}$
	conventional	$^{Aa}200.0\pm5.0^{w}$	$^{Bb}122.0\pm10.5^{x}$	$^{BCb}136.0\pm4.5^{x}$	$^{Ca}166.0\pm2.5^{w}$
Cucurbitacin E	Fertigation	$^{Aa}2.0\pm0.6^{x}$	$^{Ba}13.0\pm7.6^{y}$	$^{ABa}11.0\pm1.5^{y}$	$^{ABa}5.0\pm0.0^{x}$
	conventional	$^{Ab}5.0\pm0.1^{x}$	$^{Ba}13.0\pm0.1^{y}$	$^{Cb}31.0\pm1.7^{z}$	$^{Da}9.0\pm1.2^{x}$

Note. Different ABCD letters are significantly ($p < 0.05$) different data between plant parts; Different abcd letters are significantly ($p < 0.05$) different data between planting methods; Different wxyz letters are significantly ($p < 0.05$) different data between cucurbitacins on plant parts for both planting methods

CONCLUSION

This study reveals that plant parts and type of planting method can affect the cucurbitacin type and content in plants. This study also supported previous studies that cucurbitacin B is the common cucurbitacin in bitter gourd plants compared to cucurbitacin E. Type and imposition of elicitors and plant tissues are assumed to play an important role in defining the level and type of secondary metabolites, especially cucurbitacins in this study. This research sheds light on the interplay between planting methods, plant sections, environmental stresses, and secondary metabolite synthesis, allowing for a more nuanced understanding of cucurbitacin concentration differences in bitter gourd samples. Further analysis and experimentation would be needed to understand the underlying factors driving these differences in cucurbitacins B and E content between planting methods and plant parts.

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A Survey of Fruit Quality Properties, Growth, and Yield of Several Melon Varieties (*Cucumis melo* L.) Using Fertigation Approach

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ABSTRACT

Melons, *Cucumis melo* L., belongs to the family Cucurbitaceae. They are popular for their sweet, juicy fruit flesh with pleasant aroma. These melons are packed with numerous essential nutrients for the human body. In this study, the fruit quality properties, growth, and yield of nine different melon varieties were compared via a fertigation approach to deduce the best melon variety to be planted by melon farmers. Quantitative and qualitative traits of all nine melon varieties were collected and compared. As a result, it was discovered that the Japanese Rock Melon F1 Hybrid is an all-rounder best melon variety, with the highest seed germination percentage and fruiting percentage. On the other hand, the Sweet Green Melon F1 can also be considered the second-best choice, but the only downside is that the germination rate is significantly lower compared to others, at merely 37%. Hopefully, this study can serve as a foundation for developing a more comprehensive database for melon varieties to aid melon farmers in decision-making and further improve the industry's yield.

Keywords:*Cucumis melo* L., fertigation, fruit quality, germination rate, melon varieties

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INTRODUCTION

The melons (*Cucumis melo* L.) are family members of Cucurbitaceae. They are known vastly for their edible sweet, juicy and fleshy fruit enriched with a myriad of essential nutrients like magnesium, vitamin A, calcium, fibre, potassium, vitamin C, zinc, omega-3, iron, vitamin B6, and omega-6 (Mehra et al., 2015).

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Melons are cultivated especially in warm regions like India, Malaysia, and Iran. In Malaysia, melon production can yield as much as 5,845.81 metric tons from 313.4 ha plantation area in 2018 (18.65 metrics per ha), which was 4.63 times higher than that of paddy (4.031 metrics per ha) in the same year (Liyana & Pebrian, 2020; Statista Research Department, 2023). One of the most productive ways to cultivate melons is via the fertigation method. It is normally conducted in an open, closed or semi-closed fertigation system, requiring relatively less water consumption per unit area, space, and labour (Muhammad et al., 2017).

Interestingly, these melons come in a myriad of varieties, namely sweet green melon, sweet melon, green netted melon, rock melon, golden melon, and muskmelon (Silva et al., 2022). They share similar characteristics, such as sweet flesh, juicy texture, bright-coloured flesh, and skin with fragrance. Although they can easily be distinguished by their flesh colour, texture, skin colour, and pattern, selecting the most suitable melon variety for optimal growth, yield, and fruit quality remains a critical challenge for farmers. Existing literature does not comprehensively compare various melon varieties using fertigation, which combines irrigation and fertilization. Besides focusing on disease preventive studies on melon varieties, the focus should also be on the characterization of these melons in terms of their fruit quality, growth rate, and yield so that this documentation can facilitate melon farmers in the selection of the best melon variety to cultivate in the future (Jorge et al., 2022). In this study, nine melon varieties were documented based on their properties, and some of the best melon varieties were further deduced accordingly.

Culturing melons (*Cucumis melo* L.) is a vital aspect of the agricultural industry, given their popularity and nutritional significance. However, selecting the most suitable melon variety for optimal growth, yield, and fruit quality remains a critical challenge for farmers. Existing literature does not comprehensively compare various melon varieties using fertigation, which combines irrigation and fertilization. This research addresses this gap by thoroughly investigating the fruit quality properties, growth, and yield of nine different melon varieties, focusing on identifying the most promising variety for cultivation.

MATERIALS AND METHODS

Seedling Management

Nine melon seed types from different cultivar groups (Table 1) were bought from various seed companies. These seeds were germinated in the moistened peat moss-filled germination trays kept away from direct sunlight. The germination percentage was determined by calculating the germinated seedlings against the total planted seed. After these seedlings have expanded their true leaves (at least ten seedlings per melon variety), they are transferred to the greenhouse located at Semongok, and a complete randomized experimental design is adopted. On day 11 post-germination, these seedlings were transplanted into coco peat-filled polybags. They were maintained

carefully in a greenhouse equipped with an automatic fertigation system. The commercial premix AB fertilizers utilized in this study were bought from Lotus Farm Agritech Sdn. Bhd. (Malaysia) with fertilizer contents tabulated in Tables 2 and 3. Fertilizer was prepared by adding 100 L of water to 25 kg of fertilizer A and B separately. The 'TOP CLOUD' application was employed to manipulate the irrigation in an automated manner according to the schedule (Table 4). Foliar was performed every week (Table 5).

Table 1
List of melon varieties

Variety	Descriptive name	Cultivar groups
A	Golden Melon F1 Hybrid L	*cantalupensis*
B	Japanese Rock Melon F1 Hybrid	*reticulatus*
C	Hales Best Muskmelon	*reticulatus*
D	Rock Melon F1 Hybrid ML	*cantalupensis*
E	Sweet Melon F1 Hybrid SL	*cantalupensis*
F	Golden Melon F2 Hybrid L	*cantalupensis*
G	Golden Melon F1 Hybrid B	*cantalupensis*
H	Japanese Green Netted Melon F1 Hybrid	*reticulatus*
I	Sweet Green Melon F1	*reticulatus*

Table 2
The fertilizer contents

	Fertilizer content	Estimated amount (g)
Set A	Calcium nitrate ($CaNO_3$)	4863.2
	Ammonium nitrate (NH_4NO_3)	596.5
	Potassium nitrate (KNO_3)	1831.6
	Iron (Fe) EDTA	78.9
Set B	Monopotassium Phosphate (KH_2PO_4)	2045.5
	Magnesium sulphate ($MgSO_4$)	3578.1
	Manganese (Mn)	9.35
	Sodium borate ($Na_2B_4O_7$)	27.8
	Zinc (Zn) EDTA	8.8
	Copper (Cu) EDTA	2.8
	Ammonium molybdate ($H_8MoN_2O_4$)	0

Table 3
The estimated concentration of elements present in the fertilizers

Elements	Estimated concentration of element in fertilizer (ppm)
N	401.00
P	156.00
K	425.50
Ca	308.00
Mg	114.50
Fe	3.47
Mn	0.41
Zn	0.41
B	1.02
Cu	0.13
Mb	0

Table 4
The irrigation schedule

Day	Growth stage	Daily irrigation time
11	Seedling	8 a.m., 1 p.m., 6 p.m.
35	Flowering	8 a.m., 10 a.m., 1 p.m., 6 p.m.
45	Fruiting	7 a.m., 9 a.m., 11 a.m., 1 p.m., 3 p.m., 6 p.m.
50	Fruiting	7 a.m., 9 a.m., 11 a.m., 1 p.m., 3 p.m., 6 p.m.

Plant Management

The melon plants were given physical support as they were provided with a nylon string tied vertically to each polybag. Hands twined the newly sprouted melon stems onto the nylon strings. The axillary shoots were removed once the plant had grown more than five leaves to concentrate resources and nutrients for main stem development. For pollination purposes, the seventh to eleventh side shoots were untrimmed. Weekly chemical spraying was conducted to minimize fungal and pest manifestations (Table 6). The tallest height was recorded for each melon plant upon the emergence of the 24th leaf.

Pollination, Fruiting, and Harvesting

Manual pollination was performed on plants 35 days old and older, whereby the petals of the male flower were removed to reveal anthers before being introduced to the stigmas of the female flower (axillary shoot). Only a well-developed fruit (with the best morphological characteristics such as evenness, roundness, and plumpness) was selected for each plant, and nylon strings supported it, whereas other fruits were discarded.

Table 5
Weekly foliar application

Day	Foliar
10	Basfoliar® Aktiv SL (1 ml/500 ml)
18	Basfoliar®Avant Natur (20 ml/10 L)
25	Basfoliar® CaBMag (15 ml/10 L) + Basfoliar® Avant Natur (20 ml/10 L) + Fetrilon® Combi 2 (2.5 g/10 L)
32	Basfoliar® CaBMag (15 ml/10 L) + Basfoliar® Avant Natur (20 ml/10 L) + Fetrilon® Combi 2 (2.5 g/10 L)
39	Basfoliar® CaBMag (15 ml/10 L) + Basfoliar® Avant Natur (20 ml/10 L) + Fetrilon® Combi 2 (2.5 g/10 L)
46	Basfoliar® CaBMag (15 ml/10 L) + Basfoliar® Avant Natur (20 ml/10 L) + Fetrilon® Combi 2 (2.5 g/10 L)
53	Basfoliar® 19/19/19 (10 g/10 L)
60	Basfoliar® CaBMag (15 ml/20 L) + Fetrilon® Combi 2 (2.5 g/10 L)
67	Basfoliar® 19/19/19 (10 g/10 L)
74	Basfoliar® K (10 g/10 L)

Note. Brands of Basfoliar® Aktiv SL, Basfoliar®Avant Natur, Basfoliar® CaBMag, Fetrilon® Combi 2, Basfoliar® 19/19/19, and Basfoliar® K = COMPO EXPERT (Germany)

Table 6
Weekly chemical application

Day	Chemical
7	Previcur 840 (1 ml/500 ml) (before transplanting)
16	Fusilier (insecticide) 2.5 ml/10 L + Mancozeb MZ-45 (fungicide) 20 g/10 L
23	Fusilier (insecticide) 2.5 ml/10 L + Mancozeb MZ-45 (fungicide) 20 g/10 L
29	Nativo (2.5 g/10 L)
37	Nativo (2.5 g/10 L)
43	Nativo (2.5 g/10 L)
49	Deltamethrin 25% (2 g/10 L) + Fusilier (insecticide) 2.5 ml/10 L + Abamectin 1.8% w/w (8 ml/10 L)
51	Nativo (5.0 g/10 L)
58	Nativo (5.0 g/10 L)

Note. Previcur 840 (Bayer, Germany); Fusilier (insecticide) (Hextar, Malaysia); Mancozeb MZ-45 (fungicide) (Hextar, Malaysia); Nativo (Bayer, Germany); Deltamethrin 25% (Bayer, Germany), and Abamectin 1.8% w/w (Hextar, Malaysia)

The melon fruits were harvested by performing a T-shape cut (with at least 10 cm stem attached and one leaf attached) to ensure longer shelf life and reduce disease manifestation. Each melon fruit was documented with qualitative and quantitative data, such as weight, transverse circumference, and longitudinal circumference.

Sensory Test and Statistical Analysis

Seven randomly selected participants underwent a sensory test encompassing appearance, texture, juiciness, sweetness, and aromatics. One fruit from each melon variety was sliced and served. Each qualitative trait was graded on a scale of 5 to 1 (most preferred to least preferred).

The statistical tests done in this study include outlier detection, one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) post hoc tests with IBM® SPSS® Statistics Processor (version 28.0). These tests were performed on parameters such as weight and circumference.

RESULTS AND DISCUSSION

Germination and Fruiting Performance of Melon F1 Hybrid

The melon plant height after the emergence of the 24th leaf, germination, and fruiting percentage were tabulated in Table 7. The germination percentage for the melon variety I (Sweet Green Melon F1) was the lowest. In contrast, the melon variety C (Rock Melon F1 Hybrid ML) had the highest germination percentage, 93.3%. Other melon varieties had germination percentages ranging between 66.7% and 88.9%. As for the plant height, the shortest average height (214 cm) was seen in melon variety B (Japanese Rock Melon F1 Hybrid), while the greatest average height (244 cm) was observed in melon variety I (Sweet Green Melon F1). Looking at the fruiting percentage, melon variety C (Rock Melon F1 Hybrid ML) achieved the lowest fruiting percentage of 16.67%. In contrast, melon variety E (Sweet Melon F1 Hybrid SL) achieved the highest fruiting percentage of 83.33%, followed by melon variety B (Japanese Rock Melon F1 Hybrid) (80.77%). All melon varieties had achieved fruiting percentages greater than 50%, except for C and G (Rock Melon F1 Hybrid ML and Golden Melon F1 Hybrid B).

Table 7
The plant height, germination percentage, and fruiting percentage

Melon variety	Plant height (cm)	Germination percentage (%)	Percentage of melon plants with at least one fruit (%)
A	222	70.70	67.92
B	214	77.60	80.77
C	215	93.30	16.67
D	227	66.70	79.17
E	216	88.90	83.33
F	230	69.40	58.33
G	239	76.50	41.67
H	216	84.60	63.64
I	244	37.00	70.00

Note. A = Golden Melon F1 Hybrid L; B = Japanese Rock Melon F1 Hybrid; C = Hales Best Muskmelon; D = Rock Melon F1 Hybrid ML; E = Sweet Melon F1 Hybrid SL; F = Golden Melon F2 Hybrid L; G = Golden Melon F1 Hybrid B; H = Japanese Green Netted Melon F1 Hybrid; I = Sweet Green Melon F1

Differences in Fruit Weights Across Nine Melon Varieties

The morphology of nine melon varieties is shown in Figure 1. The fruit weight graph across all nine melon varieties is depicted in Figure 2. The melon variety H (Japanese Green Netted Melon F1 Hybrid) scored the highest mean fruit weight (1.637 kg). Upon the one-way ANOVA test, significant differences were found across all melon varieties. Together with the melon varieties B, C, and I (Japanese Rock Melon F1 Hybrid, Hales Best Muskmelon, and Sweet Green Melon F1), the melon variety H (Japanese Green Netted Melon F1 Hybrid) formed a significantly greater weights cluster in contrast with the other melon varieties. Melon variety G (Golden Melon F1 Hybrid B) scored the lowest mean fruit weight, i.e., 0.832 kg. On a side note, the two outliers from melon variety B and one outlier from melon varieties F and G were detected and removed from the analysis. Surprisingly, the mean melon transverse and longitudinal circumferences (Figures 3 and 4) did not show significant differences across all melon varieties.

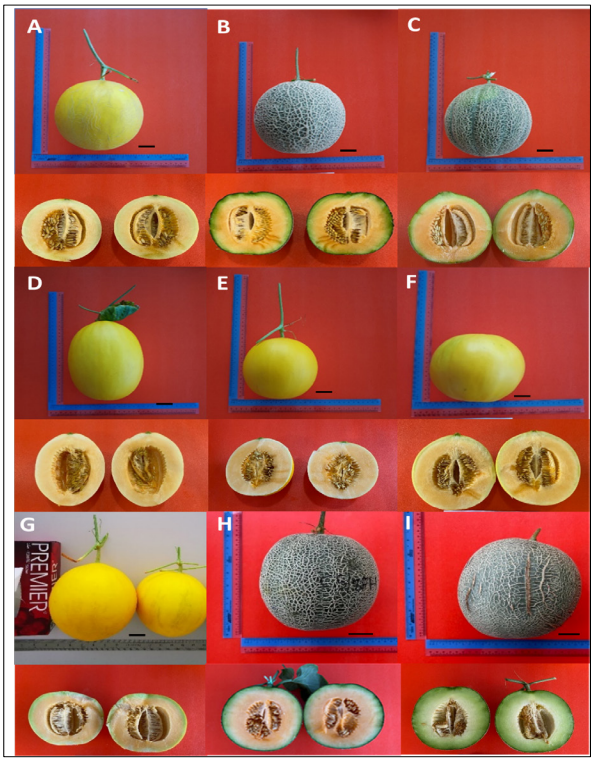

Figure 1. Morphology of nine melon varieties. The upper panel shows the whole fruit while the lower panel shows the transverse section (Scale bar = 2.5 cm measurement)

Note. A = Golden Melon F1 Hybrid L; B = Japanese Rock Melon F1 Hybrid; C = Hales Best Muskmelon; D = Rock Melon F1 Hybrid ML; E = Sweet Melon F1 Hybrid SL; F = Golden Melon F2 Hybrid L; G = Golden Melon F1 Hybrid B; H = Japanese Green Netted Melon F1 Hybrid; I = Sweet Green Melon F1

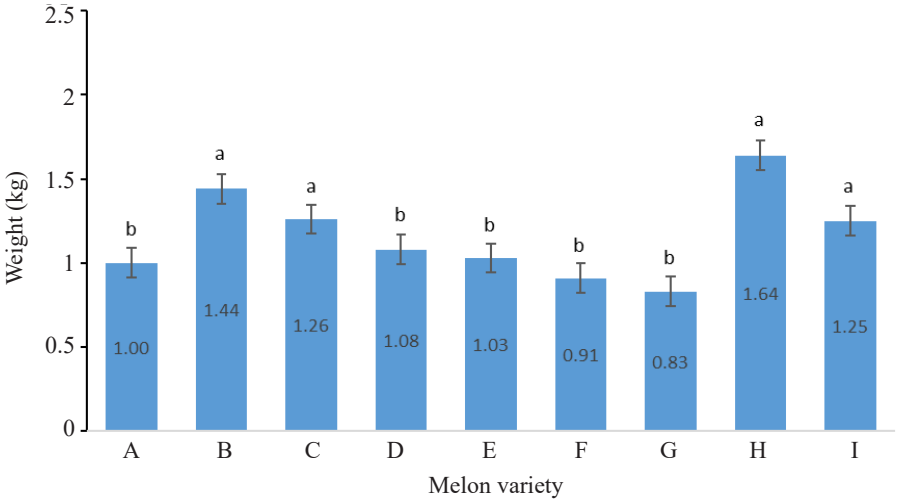

Figure 2. Mean melon fruit weight graph

Note. Significantly different groups were labelled with different lowercase alphabets after one-way analysis of variance and Tukey's honestly significant difference post hoc tests ($p \leq 0.05$); A = Golden Melon F1 Hybrid L; B = Japanese Rock Melon F1 Hybrid; C = Hales Best Muskmelon; D = Rock Melon F1 Hybrid ML; E = Sweet Melon F1 Hybrid SL; F = Golden Melon F2 Hybrid L; G = Golden Melon F1 Hybrid B; H = Japanese Green Netted Melon F1 Hybrid; I = Sweet Green Melon F1

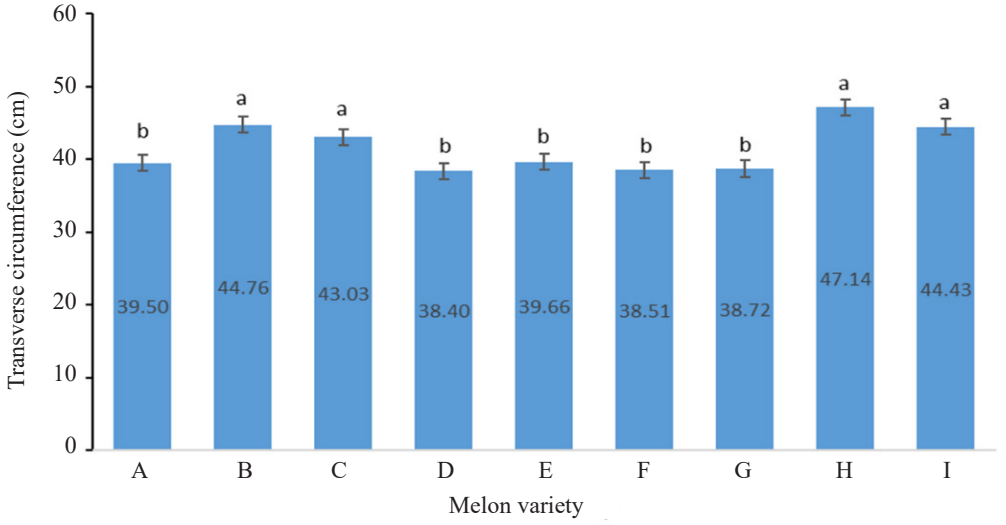

Figure 3. Mean melon transverse circumference graph

Note. Significantly different groups were labelled with different lowercase alphabets after one-way analysis of variance and Tukey's honestly significant difference post hoc tests ($p \leq 0.05$); A = Golden Melon F1 Hybrid L; B = Japanese Rock Melon F1 Hybrid; C = Hales Best Muskmelon; D = Rock Melon F1 Hybrid ML; E = Sweet Melon F1 Hybrid SL; F = Golden Melon F2 Hybrid L; G = Golden Melon F1 Hybrid B; H = Japanese Green Netted Melon F1 Hybrid; I = Sweet Green Melon F1

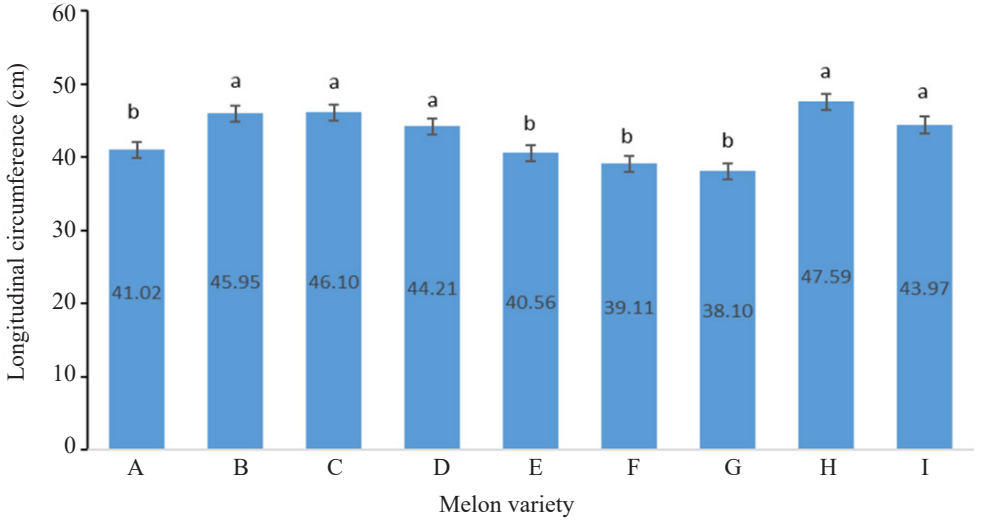

Figure 4. Mean melon longitudinal circumference graph

Note. Significantly different groups were labelled with different lowercase alphabets after one-way analysis of variance and Tukey's honestly significant difference post hoc tests ($p \leq 0.05$); A = Golden Melon F1 Hybrid L; B = Japanese Rock Melon F1 Hybrid; C = Hales Best Muskmelon; D = Rock Melon F1 Hybrid ML; E = Sweet Melon F1 Hybrid SL; F = Golden Melon F2 Hybrid L; G = Golden Melon F1 Hybrid B; H = Japanese Green Netted Melon F1 Hybrid; I = Sweet Green Melon F1

Sensory Tests on Five Qualitative Traits

The sensory tests were conducted with the participation of seven randomly selected individuals across five qualitative traits: fruit flesh texture, sweetness, appearance, juiciness, and aromatics (Figure 5). The melon variety D (Rock Melon F1 Hybrid ML) has the highest mean score of 4.143, whereas melon variety C (Hales Best Muskmelon) has the lowest mean score of 1 for fruit flesh texture. As for sweetness, the melon variety C scored the lowest (1.571), while the melon variety B (Japanese Rock Melon F1 Hybrid) scored the highest of all (4.143). The melon variety F (Golden Melon F2 Hybrid L) scored the lowest in aromatics compared to the melon variety C, which scored the highest (4). Interestingly, the melon variety B was given the highest score (4.714) with respect to appearance, while the melon variety C was voted to be the least attractive (2.571). The juiciest melon variety was voted to be the melon variety C (4.857), contrasting with the 2.4 score given to the melon variety F as the least juicy.

Best Melon Variety

When selecting the best melon variety, the goal to focus on is the consumers' preferences, as they are the endpoint determinant of the melon's price and sales based on its popularity (Bianchi et al., 2016). As such, in this case, the qualitative traits may appear to be much more essential to be taken into consideration when melon farmers are deciding on which melon variety to work on (Bianchi et al., 2016). With that in mind, the sensory tests conducted in

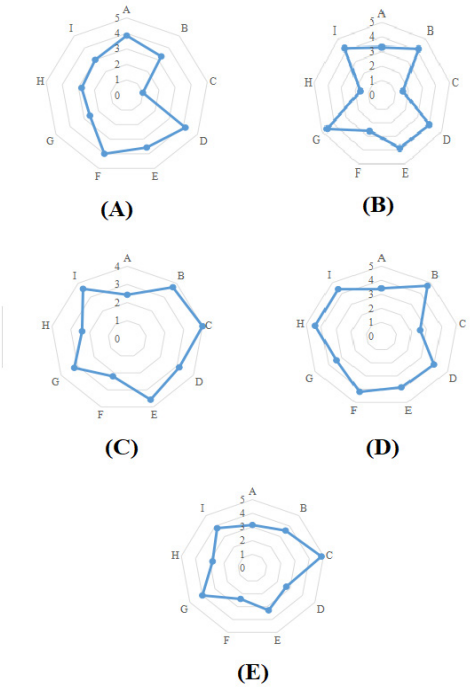

Figure 5. Mean scores for sensory tests qualitative traits: (A) flesh texture, (B) sweetness, (C) aromatics, (D) appearance, and (E) juiciness

this study unearthed two superior melon varieties, i.e., B and C, as both scored the highest in two of the five qualitative traits tested. Melon variety B excelled in sweetness and appearance, whereas melon variety C excelled in aromatics and juiciness.

In comparing the quantitative traits of melon varieties B and C, melon variety C recorded the highest germination rate (93.3%) among all the varieties. In contrast, the germination rate of melon variety B was not so bad either, with a percentage of 77.6%. Both mean plant heights of melon varieties B and C do not differ much (only 1 cm). However, the fruiting percentage of melon variety B (80.77%) far exceeded that of C (16.67%). The fruiting percentage is one of the most vital traits, directly affecting the yield. Since both varieties belong to the group *reticulatus* (i.e., Japanese Rock Melon F1 Hybrid and Hales Best Muskmelon) respectively, the fruit weights of melon varieties B and C are situated at the higher range together with the melon variety H and I, which also belongs to the *reticulatus* varieties (Sharma et al., 2014).

Undeniably, melon variety H scored quite well in most of the quantitative traits tested. However, qualitative traits scored quite low for four sensory traits: flesh texture, sweetness, aromatics and juiciness. I scored quite highly on the melon variety in appearance, juiciness, aromatics, and sweetness. However, the only downside is that the germination rate of melon I is significantly the lowest among all (37%), but the fruiting percentage is considerably good (70%). Utilization of genetic markers to assist in future breeding programs seems viable to allow better identification of the best melon with agronomic and qualitative traits (Flores-León et al., 2021; Luan et al., 2010).

The best all-rounder melon variety in this study would be the melon variety B (Japanese Rock Melon F1 Hybrid), as it possesses above-average quantitative traits and is generally popularly accepted by consumers at the same time. The melon variety I (Sweet Green Melon F1) could also be considered as it is quite positively voted by consumers, except that it has a very low germination rate in the agronomy aspect. Melon farmers could opt for melon variety

B for a better guarantee in terms of yield and quality. The melon variety can also be selected if the price of this melon can be marked up (due to popularity and market demand) to compensate for the downside of the predicaments faced by melon farmers in the low germination rate.

CONCLUSION

When selecting a melon variety to be planted to meet consumer market demand, qualitative traits should be considered ahead of quantitative traits. As such, melon variety B was deemed the all-rounder in almost all quantitative and qualitative traits tested in this study. However, a melon variety should also be considered if there is a markup in price due to popularity and market demand, as it only has one downside: low germination rate.

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Effect of Harvesting Time in Growth Performance and Energy Crops Productivity of Napier (*Pennisetum purpureum* cv. Taiwan) Exposed under CO_2 Elevated Conditions

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ABSTRACT

Napier grass is crucial in reducing greenhouse gas emissions by substituting non-renewable resources. When Napier grass is burned, the carbon dioxide (CO_2) released is roughly equal to the amount absorbed during its growth, making it a potentially carbon-neutral energy source. This study investigates the impact of ratooning (repeated harvesting) on various aspects of Napier grass, including growth, physiology, biomass production, nutrient content, and chemical analysis. It also explored the interaction between elevated CO_2 levels and ratooning. Two experiments were conducted over 12 months. Experiment 1 took place in an open field at the Faculty of Agriculture, Universiti Putra Malaysia (UPM), with two treatments: no ratooning and ratooning at three months after planting (MAP). Experiment 2 was conducted in an open field at UPM and a greenhouse at Tenaga National Berhad Research, Kajang, Selangor. Eight combination treatments were studied: (T1) 1-month elevated CO_2 ($MECO_2$) - no ratooned, (T2) 1 $MECO_2$-R at 3 MAP, (T3) 2 $MECO_2$-noR, (T4) 2 $MECO_2$-R at 3 MAP, (T5) 5 $MECO_2$-noR, (T6) 5 $MECO_2$-R at 3 MAP, (T7) 12 $MECO_2$-noR, and (T8) 12 $MECO_2$-R at 3 MAP. The results indicated that, in Experiment 1, no ratooning was more favourable for all parameters compared to ratooning. In Experiment 2, a 1-month exposure to elevated CO_2 showed better results compared to longer exposure periods. In conclusion, Napier grass performed

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better when not subjected to ratooning and exposed to short-term elevated CO_2 levels. This research highlights the potential of Napier grass as a sustainable and carbon-neutral energy source.

Keywords: Elevated CO_2, green energy, Napier grass, productivity, ratooning, renewable sources

INTRODUCTION

Pennisteum purpureum CV. Taiwan or Napier grass was originally developed and popularised in Taiwan and has great potential as a green energy source due to the conversion efficiency into various forms of renewable energy (Long et al., 2004). Before this, Napier grass was widely known as a forage crop for livestock feeding. However, it received attention as a bioenergy crop due to its high productivity, adaptability, and nutritional value (Ainsworth & Rogers, 2007). Besides that, this cultivar exhibits vigorous growth, and it is characterised by tall, robust stems that can reach heights up to 3–4 m, which can produce a substantial amount of production under favourable conditions (Osborne et al., 2008). *Pennisetum purpureum* cv. Taiwan also has relatively good nutritional content with appreciable crude protein, fibre, and other essential nutrients (Ansah et al., 2010). However, Napier grass quality depends on several factors, such as maturity and growing conditions, based on the purpose of use (Polle et al., 1997). This cultivar demonstrates excellent adaptability to a wide range of environmental conditions (Assuero & Tognetti, 2010). It thrives in warm and humid climates but tolerates moderate drought conditions (Namiki, 1990).

The high biomass yield of Napier grass makes it an excellent option for bioenergy production (Behnke et al., 2010). It can be harvested and processed to produce biofuels like biogas, bioethanol, and bio-oil (Rangnekar & Thorpe, 2001). Biogas can be generated through the anaerobic digestion of Napier grass, providing a renewable source of clean energy for electricity or heating generation (Bendary et al., 2013). Napier grass is rich in organic matter that is easily digestible and is an ideal material as a feedstock for biogas production (Boyer, 2015). Anaerobic digestion of the grass releases methane, a potent greenhouse gas, which can be trapped and fully utilised as a clean and sustainable energy source (Babbar et al., 2015). The dry matter of Napier grass is used as a combustion tool that can produce steam, which drives turbines to generate electricity (Gulfam et al., 2017). This process is considered carbon neutral as the carbon dioxide released during combustion is absorbed by growing Napier grass, making it a renewable energy source (Byers & Guerrero, 1995).

Known as a fast-growing grass with high carbon sequestration, it has enlightened the Napier grass to play a role in mitigating climate change (Caird et al., 2007). During the growing phase, it absorbs significant amounts of carbon dioxide from the atmosphere, helping to offset greenhouse gas emissions (Chan et al., 2008).

Practically, Napier grass is beneficial in developing regions as it can be an accessible and renewable energy resource for rural communities, which reduces dependence on non-renewable energy resources (Chaparro & Sollenberger, 1997). While Napier grass shows a promising outcome as a green source, the large-scale utilisation for energy production may need extra precautionary steps and management to avoid negative impacts on the environment, such as habitat loss and competition with food crops (Chen et al., 2004). Proper management, such as sustainable cultivation practices and integrated land-use planning, is essential to responsibly harnessing its potential as a renewable energy resource (Orodho, 2012). There are four objectives of the study: (1) to study the effect of ratooning on the growth, physiology, and biomass production of Napier grass, (2) to analyse the effect of ratooning on nutrient content, proximate, and ultimate analysis of Napier grass, (3) to identify the effect of elevated CO_2 ratooning on growth, physiology, biomass production, and chemical content of Napier grass, and (4) to analyse the interaction between elevated CO_2 and ratooning on growth, physiology, biomass production, and chemical content of Napier grass.

MATERIALS AND METHODS

Experimental Site

The experiment was conducted at two locations: Field 15, Universiti Putra Malaysia, Serdang, Selangor, Malaysia, with the GPS coordinate 2˚59'01.9" N, 101˚44'01.7"E (Figure 2), and a greenhouse at Tenaga National Berhad Research, Kajang, Selangor with the GPS coordinate 2.9683° N, 101.7326° E (Figure 3). The open field for experimentation was cleared using a bulldozer to remove all weeds and unwanted plants in the experiment area. The soil was ploughed 25 cm deep so the soil could turn over the uppermost soil. Ploughing soil needs to be done twice, two weeks after the first plough, to ensure the soil is uniformly ploughed. The soil was left for two weeks before the liming process, and after that, the ploughed soil needed to be tested for pH level. The plot size for the experiment was 23.0 m x 12.0 m. Each treatment has four replicates, which represent four plots. Each plot was raised by 2.0 m x 3.0 m and built with a separation block consisting of 6 crops that have been planted and prepared the cutting from a mature stem of Napier grass within 15 cm to 20 cm, which consisted of three nodes. The cuttings were prepared under two conditions: (1) planting in an open area and (2) exposure under different periods of elevated CO_2 conditions (800 µmol/mol) before being transplanted to an open field. The CO_2 greenhouse was constructed so that the plant can receive 12 hr photoperiod and average photosynthetic photon flux density of 800 µmol/mol. Day and night temperatures and relative humidity were recorded. Vapor pressure deficit ranged from 1.11 to 2.32 kPa. Pure CO_2 at 99.8% purity was supplied from a high-pressure CO_2 cylinder and injected through a pressure regulator into the fully sealed 5 m × 3.67 m growth houses (Figure 1). The

CO_2 concentrations were measured using SenseAir CO_2 Sensors (USA), designated to each chamber during CO_2 exposition (Figure 4a-4e). The CO_2 was elevated slowly to 800 µmol/mol. The Average rainfall documented for the experimented region was 117.06 mm with a minimum and maximum temperature of 21.9°C and 38.0°C, respectively.

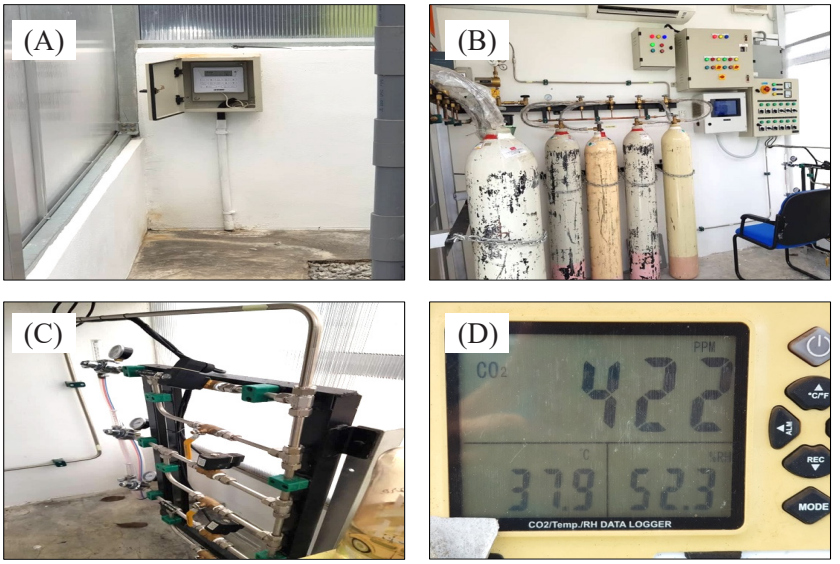

Figure 1. Monitoring of temperature, relative humidity (RH), and carbon dioxide (CO_2) level using a sensor (A); Source of pure CO_2 connected to the CO_2 greenhouse system (B and C); A data logger for CO_2, temperature, and RH was used in the greenhouse at Tenaga Nasional Berhad Research (D)

Figure 2. Open area experimental site

Figure 3. Greenhouse experimental site

Land Preparation and Fertilisation

The cutting propagated in polybag size, 61 cm x 61 cm arranged in the greenhouse and exposed at four different periods of elevated CO_2. At the same time, the ploughed land was applied with 300 kg/ha organic matter before crop transplanting. All cuttings were transplanted at the same time for open field and greenhouse. Sprinkler irrigation was the method of applying water in this experiment

at the greenhouse, which was applied at the necessary time. An overhead sprinkler was built in an open area surrounding the experiment area so that the flow rate reaches 60 to 70%. Surface broadcast is a fertiliser application method used in both experimental areas. The fertiliser urea (NPK 46-0-0, YaraTera™, Norway) was applied on the soil surface of an entire experiment plot either in granule or liquid form. The fertiliser was weighed with the amount of 10 kg/ha and applied after one week of planting and every month for up to 1 year to get the maximum yield of Napier grass as a bioenergy crop. Urea fertiliser (nitrogen) was applied as a single fertiliser to provide nutrients for Napier grass's shoot and root development.

Experimental Design and Treatments

Randomised complete block design (RCBD) was used for both experiments, with all assigned treatments randomly placed within each block. This design helps to control variability and ensures that each treatment has an equal chance of being influenced by different factors. Experiment 1 consisted of 2 treatments: T1: open field (OF)—no ratooned and T2: open field—ratooned at 3 MAP. In Experiment 2, the Napier grass was subjected to 2 factorials, i.e., ratooning and period of CO_2 elevation at 800 µmol/mol CO_2, for the ratooning plant was subjected to ratooned (R) and no ratooned (noR). The plant was exposed to a short period at 1-month elevated CO_2 ($MECO_2$) and 2 $MECO_2$ and a long period at 5 $MECO_2$ and 12 $MECO_2$, respectively. Eight combination treatments were studied: (T1) 1 $MECO_2$-no ratooned, (T2) 1 $MECO_2$-R at 3 MAP, (T3) 2 $MECO_2$-noR, (T4) 2 $MECO_2$-R at 3 MAP, (T5) 5 $MECO_2$-noR, (T6) 5 $MECO_2$-R at 3 MAP, (T7) 12 $MECO_2$-noR, and (T8) 12 $MECO_2$-R at 3 MAP. Both experiments were carried out for 12 months, which was a total of 10 treatments.

Data Collection

Plant growth was measured throughout the experimental period, while total biomass and proximate analysis were measured at 12 MAP.

Plant Growth

Field measurement and sampling were done every month for up to one year. At the time of field measurement, a 1 m x 1 m sample size from each plot was cut to measure all the growth parameters. The tiller number was counted in the sample size for each plot, including other growth parameters, plant height (PH), stem diameter (SD), and tiller bunch circumference (TBC).

PH and TBC were measured using measuring tape. The hook is a feature in measuring tape that helps measure one side of Napier grass. The measuring tape was stretched across the height or width of the Napier grass from the tip of the shoot to the root part or around the circumference of the Napier grass as straight as possible when a measurement was being done. The tape was locked, and the reading was written down.

SD was measured using a digital calliper. The device was re-zeroed before

use to minimise the instrumental error. If the device is not properly zeroing, the reading would be inaccurate. Technically, callipers have two jaws, i.e., large and small jaws. Large jaws are used to measure the outside of an object, while small jaws are used to measure the inside of an object. The large jaws were used to measure the stem diameter in this experiment. The calliper was unlocking the top lock screw before measurements. The large jaws were adjusted to widen the jaws by sliding the thumbscrew on the bottom to the right. The large jaws were placed around the stem. The slide should be moved to the left until the jaws are clamped around the Napier stem. The screw was locked to ensure the jaws were set to read.

Total Biomass

Biomass production is the net amount of plant-dried matter before and after the drying method at a certain harvesting period. Total biomass was measured on a plant or unit of land basis and closely related to the plant's carbon assimilation capacity. The plot size needed to harvest from each treatment was 3 m x 2 m to calculate the total biomass. All the fresh samples must be cleaned and weighed immediately after being harvested before the samples are brought back to the lab and dried. It is to minimise any errors that could happen during the total biomass calculation. The fresh weight of Napier grass was calculated by using the formula below:

$$\text{Crop yield (t/ha)} = \frac{\text{Fresh weight of tuber (g)}}{1{,}000} \text{ X } \frac{10{,}000\ \text{m}^2\ (1\ \text{ha})}{\text{Size of the quadrant (m}^2\text{)}}$$

Ultimate Analysis: Carbon (%), Hydrogen (%), Nitrogen (%), and Sulphur (%)

A sample was prepared by adding concentrated nitric acid to 1.0 g of dried sample and allowed to stand overnight before being digested for 2 hr at 115°C. A concentrated hydrochloric acid (LabChem, USA) was added to the sample mixture, and the digestion was continued until a clear solution was observed. The sample solution was then diluted into 100 ml with deionised water, filtered and left to dry before being burned and analysed in the ultimate analyser. An amount of 0.25 g from the prepared sample is put in a designated sample vial of refractory-grade clay. The designated vial was combusted at a temperature of 1,350°C. This sample was combusted in the ultimate analyser to determine the percentage of weight of carbon, hydrogen, nitrogen, and sulphur produced from the combustion process. All the parameters were determined simultaneously from the same sample in the analyser. Below are the total carbon, hydrogen, nitrogen, and sulphur calculations.

$$\text{Total carbon (\%)} = \frac{\text{Organic carbon + Inorganic carbon produced}}{\text{Total weight of sample}} \text{ x } 100\%$$

$$\text{Total hydrogen (\%)} = \text{Total hydrogen (ad) x } \left[\frac{100\% - \text{TM (\%)}}{100}\right] + [0.1119 \text{ x TM}]$$

$$\text{Total nitrogen (\%)} = \frac{\text{Weight of nitrogen produced}}{\text{Total weight of sample}} \text{ x } 100\%$$

$$\text{Total sulphur (\%)} = 100\% - (\text{C\%} + \text{H\%} + \text{N\%})$$

where,
ad = As determined
C% = Carbon in percentage
H% = Hydrogen in percentage
N% = Nitrogen in percentage
TM = Total moisture

Statistical Analysis

All data collected were analysed using a two-way analysis of variance (ANOVA) by Statistical Analysis System (SAS 9.4) for RCBD with factorial and replicated four times to determine the significant differences between treatment means. Difference between means separated using least significant difference (LSD) at $P<0.05$ level.

RESULTS AND DISCUSSION

Experiment 1

Plant Growth

Figures 4A and 4C prove that the open field-no ratooned (OF-noR) showed significantly higher than open field-ratooned (OF-R) at plant height and tiller bunch circumference reading by increasing 5.03% and 12.09%, respectively. At the same time, stem diameter showed significantly higher at OF-R compared to OF-noR, which increased by 18.92% (Figure 4B). Extending the planting period for both treatments can increase plant growth for OF-R, as the crops have more time to establish and grow new crops without relying on the regrowth of existing plants (Engineer et al., 2016). Previous studies showed that plant age significantly affected leaf area and height by 39% and 53%, respectively (Chun et al., 2003). At the same time, the previous study reported a significant effect on plant height and basal circumference as it increased by 41.02% and 23.84% after cutting interval (Durand & Kawashima, 1980).

Despite these findings, all OF-R treatments will surpass the OF-noR at a certain period as if it grows continuously (Côté et al., 2010). The OF-R start to cross the OF-noR line at 9 MAP for Figure 4A, while 4 MAP for Figure 4B. From Table 1, ratooning does not show a significant effect between OF-noR and OF-R, with only a 0.5% difference. The result was in line with previous reports; the regrowth method has reduced the maise grain yield by 15.9% compared to continued growth in 2019 (De Graaff et al., 2006). Although the yield has significantly reduced the yield, across the years, the grain yield increased by 2.7–10.8% in 2020, better than the continued growth (Durand & Kawashima, 1980).

Table 1
The effect of ratooning on plant height, stem diameter, tiller number, and tiller bunch circumference of Napier grass (Pennisetum purpureum *cv. Taiwan*)

Treatments	Tiller number
Ratooned (R)	
Open field-noR	13.68a
Open field-R	13.61a
LSD $P<0.05$	NS

Note. Means followed by the same letter within a column are not significantly different at $P>0.05$ by least significant difference (LSD) test with n = 32; NS = Not significant; noR = No ratooned

Ultimate Analysis: Carbon (%), Hydrogen (%), Nitrogen (%), and Sulphur (%)

The ratooning application significantly ($P<0.05$) decreased the nitrogen of Napier grass by 15.13% in Figure 5A. However, it showed a significantly increased sulphur of Napier grass by 18.75% (Figure 5B). Meanwhile, carbon and hydrogen showed no significant effect due to ratooning. The reason behind this result was that regrowth

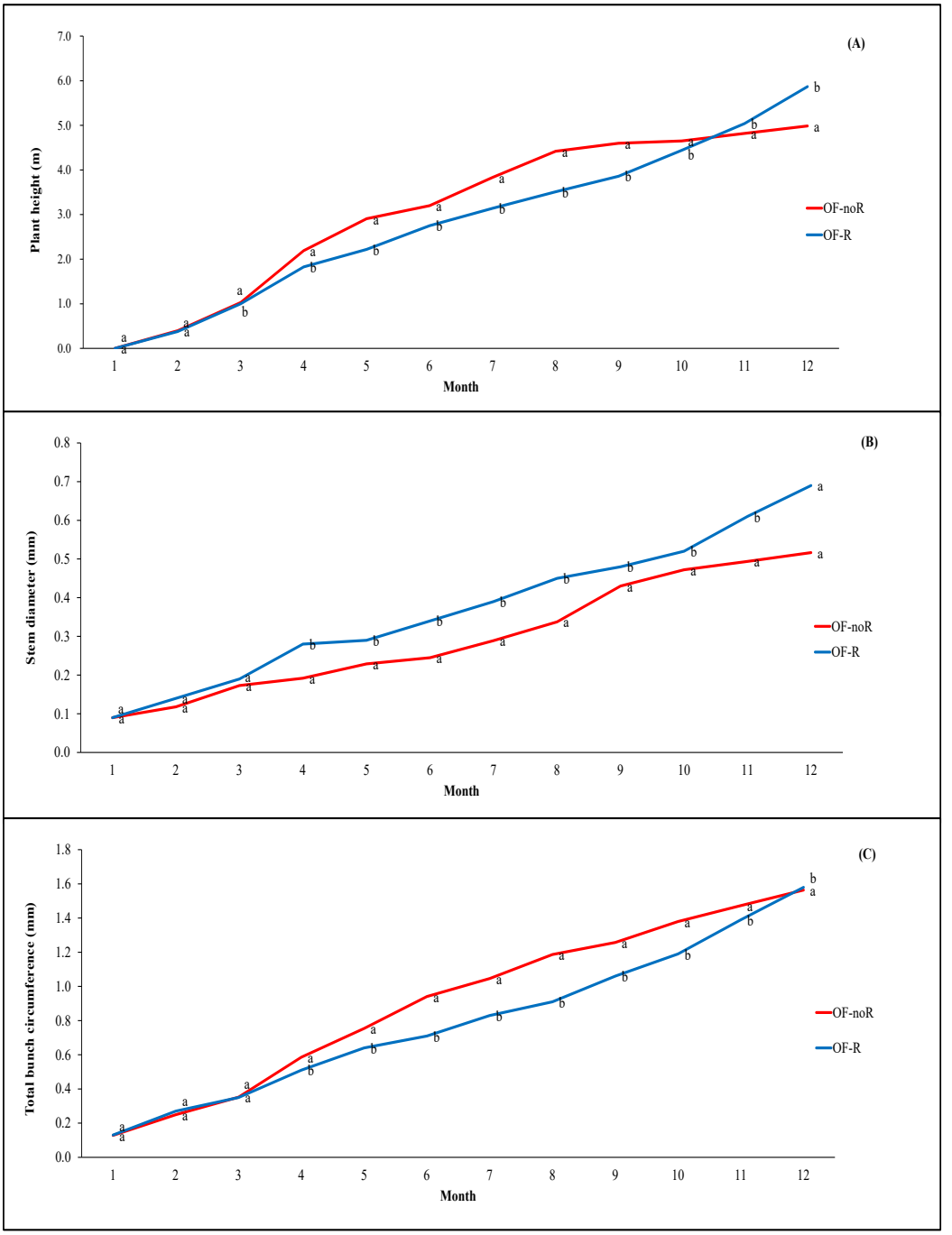

Figure 4. The effect of ratooning on (A) plant height, (B) stem diameter, and (C) tiller bunch circumference of Napier grass (*Pennisetum purpureum* cv. Taiwan). Mean values with the same letter are not significantly different at P>0.05 by the least significant difference (LSD).

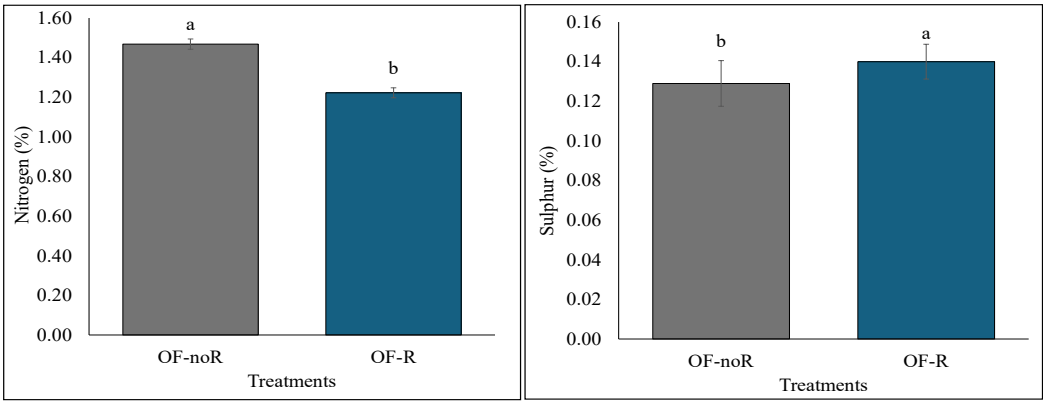

Figure 5. The effect of ratooning on (A) nitrogen and (B) sulphur contents of Napier grass (*Pennisetum purpureum* cv. Taiwan), respectively

Note. OF-noR = Open field-no ratooned; OF-R = Open field-ratooned; a and b indicate significant differences between means using the least significant difference at $p \leq 0.05$.

often consists of young, actively growing plant tissues that contain relatively higher levels of carbon and hydrogen compared to mature plant parts (Elehinafe et al., 2021). Carbon and hydrogen are key components of organic compounds in plant growth and development (Xu et al., 2021). During ratooning, plants need more nitrogen resources for protein synthesis. At the same time, their nitrogen-containing compound has already achieved continuous growth since day one transplant, resulting in mature plants containing high nitrogen (%) compared to young plants (Falster & Westoby, 2003). Ratooning can impact the cycling of sulphur in the soil (Imran et al., 2007). When the ratooning happens, the remaining sulphur content in the soil becomes a critical factor in determining the sulphur uptake by the regrown crop (Feng et al., 2008). Based on the results, the soil has sufficient sulphur levels to support the subsequent growth of Napier grass with adequate sulphur content. Thus, Napier grass must allocate nutrients differently during ratooning compared to the initial growth phase (Geuns, 2003). Nitrogen and sulphur are important for various physical and physiological reasons, including protein synthesis, enzyme activity, and plant growth (Grodzinski et al., 1996).

From the results, Table 2 shows no significant effects of ratooning on carbon (%) and hydrogen (%). These results were relatively due to their proportions in plant tissue, which remains stable (Gupta et al., 2016). Primarily, ratooning will affect only aboveground biomass, while the below-ground root system will remain intact and contribute to nutrient reserves (Haegele et al., 2017). Post-ratooning causes the stored nutrients in the roots to execute to support the regrowth process; thus, carbon and hydrogen content do not experience significant shifts (Hager et al., 2016). Unlike nitrogen and sulphur content, these elements were translocated from older to newer tissues after ratooning, but carbon

and hydrogen content do not undergo substantial redistribution within the plants, which reflects no fluctuate significant effect due to ratooning (Halim et al., 2013). Besides, the carbon and hydrogen turnover rate in plant tissues is also generally slower compared to nitrogen, which is involved in more dynamic processes (Pérez-López et al., 2010). Carbon and hydrogen take longer to manifest, making them less likely to show significant shifts within a short period, for example, between harvesting time and ratooning cycles (Hanna & Monson, 1988).

Table 2
The effect of ratooning on carbon and hydrogen of Napier grass (Pennisetum purpureum *cv. Taiwan*)

Treatments	Carbon (%)	Hydrogen (%)
Ratooned (R)		
Open field-noR	45.03a	5.50a
Open field-R	44.23a	5.59a
LSD $P<0.05$	NS	NS

Note. Means followed by the same letter within a column are not significantly different at $P>0.05$ by least significant difference (LSD) test with n = 32; NS = Not significant; noR = No ratooned

Total Biomass

Based on Figure 6, OF-noR showed significantly higher compared to OF-R on total biomass at every harvesting period, which increased by 63.60% (6 MAP), 64.28% (8 MAP), and 28.86% (12 MAP), respectively. This result corresponded with photosynthesis rate (PR), stomatal conductance (SC), transpiration rate (TR), shoot fresh weight (SFW), and shoot dry weight (SDW) (McDonald & Ho, 2002). When an aboveground portion of the crop is removed, the stubble and roots are left behind. As a result, the overall biomass of the plant is temporarily reduced (Pérez-López et al., 2010). The regrowth and biomass recovery rate will depend on crop type, growing conditions, and management practices (Harris, 1992). If ratooning is practised multiple times, the plant's biomass declines with each successive ratoon (Heijnen et al., 2001). This decline can be attributed to factors like nutrient depletion, exhaustion of energy reserves, and reduced plant vigour. As a result, the biomass of each subsequent ratoon is lower than the previous one (Jaafar et al., 2008). A previous study has shared the same result in their findings. The performance of Napier grass before ratooning was 63.16%, while the performance after ratooning was 42.11% and concluded the growing process was interrupted and led to a decrease in plant biomass (Wangchuk et al., 2015)

Normally, the first growth cycle benefits from all essential nutrients in the soil, establishing the root system and leading to robust and vigorous growth, resulting in relatively higher biomass (Polle et al., 1997). However, it is less likely to be available after ratooning (Imran et al., 2007). Due to ratooning practices, it can influence the number of nutrient reserves stored in the root systems, which affects the regrowth potential (Ibrahim et al., 2011). Adequate management with proper fertilisation, good irrigation, and close pest control monitoring can provide a conducive environment for

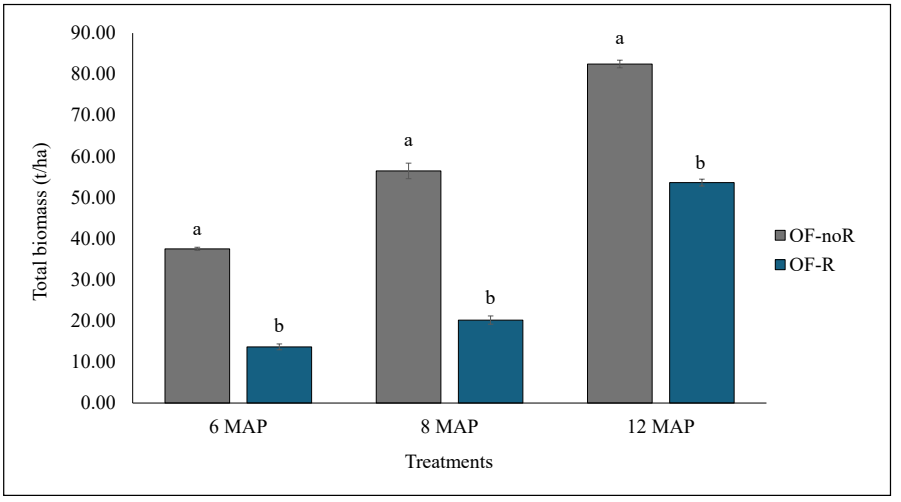

Figure 6. The effect of ratooning on total biomass of Napier grass (*Pennisetum purpureum* cv. Taiwan) at six months of planting (MAP), 8 MAP, and 12 MAP, respectively

Note. OF-noR = Open field-no ratooned; OF-R = Open field-ratooned; a and b indicate significant difference between means among MAP using least significant difference at $p \leq 0.05$

growth (Heijnen et al., 2001). A healthier soil environment positively impacts root growth and overall plant height (Ishii et al., 2015).

Experiment 2

Plant Height

Based on Figure 7A, the 5 $MECO_2$-noR showed a significantly higher plant height compared to another elevated CO_2, which increased by 22.15% (T1), 17.93% (T2), 30.57% (T3), 40.30% (T4), 22.57% (T6), 1.05% (T7), and 23.00% (T8), respectively. CO_2 is an important element during photosynthesis that produces carbohydrates and energy (Jaafar et al., 2008). The concentration of CO_2 stimulated photosynthesis, which affected sugar production and other compounds needed in plant growth (Chen et al., 2004). This potentially helps plants to grow taller in size as they have a sufficient amount of energy (Jørgensen et al., 2010). This result was in line with Jaafar et al. (2008), which proved the statement above based on his findings in the previous experiment. In Figure 7B, the 2 $MECO_2$–noR showed a significantly higher stem diameter compared to another elevated CO_2, which increased by 38.24% (T1), 42.16% (T2), 52.94% (T4), 56.86% (T5), 73.53% (T6), 59.80% (T7), and 72.55% (T8), respectively. Higher carbon dioxide levels can enhance the production of building blocks by growing and allocating more resources for cell division, expansion and elongation, which results in an increase in stem diameter (Jampeetong et al., 2014). These results were in line with the previous, which showed the effect of the tiller number on the C_4 crop by exposure to long CO_2 exposure (Zailan et al., 2016).

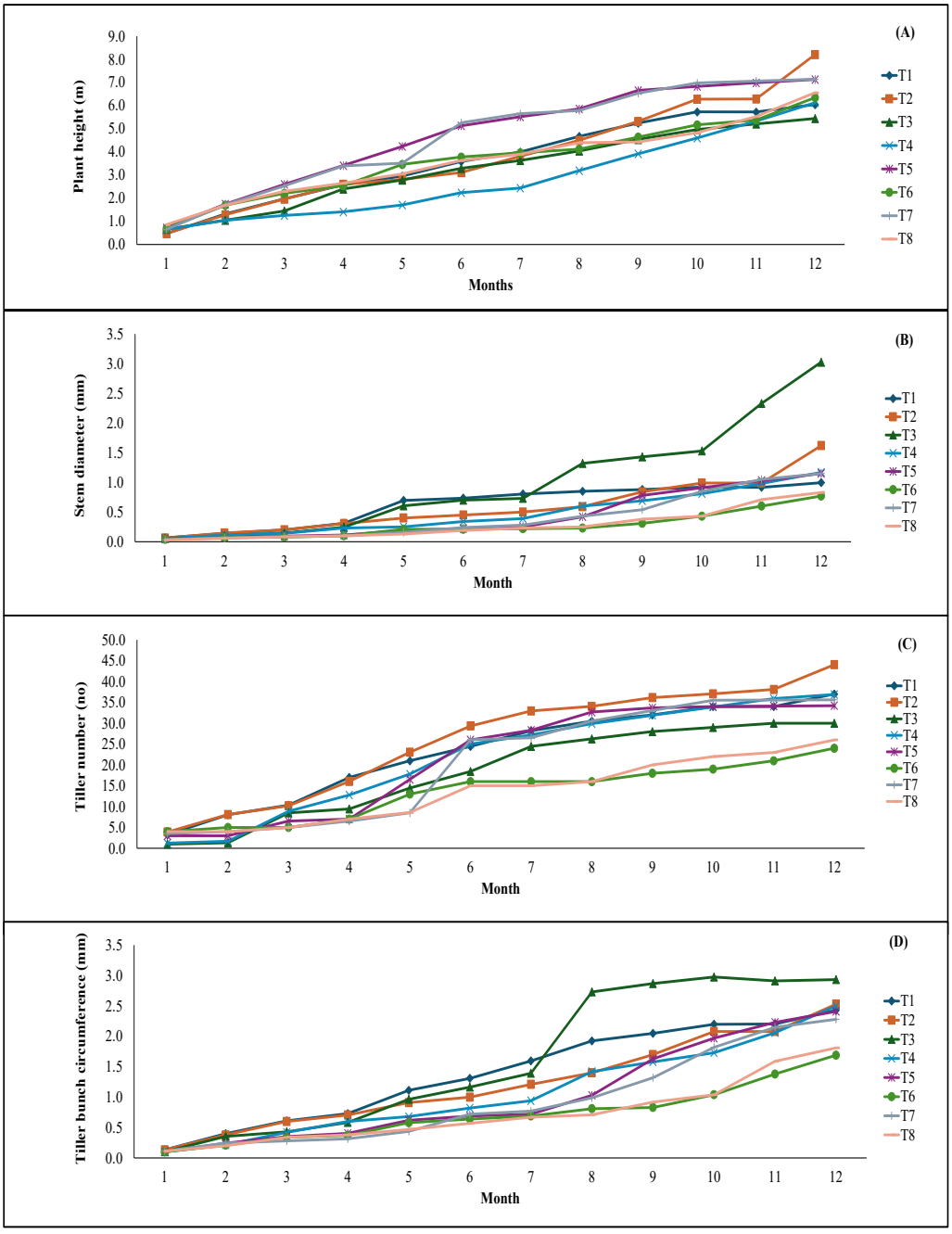

Figure 7. The interaction effect of ratooning and different periods of elevated carbon dioxide on (A) plant height, (B) stem diameter, (C) tiller number, and (D) tiller bunch circumference of Napier grass (*Pennisetum purpureum* cv. Taiwan)

Note. T1 = 1-month elevated CO_2 ($MECO_2$)-no ratooned (noR); T2 = 1 $MECO_2$-ratooned (R) at three months after planting (MAP); T3 = 2 $MECO_2$-noR; T4 = 2 $MECO_2$-R at 3 MAP; T5 = 5 $MECO_2$-noR; T6 = 5 $MECO_2$-R at 3 MAP; T7 = 12 $MECO_2$-noR; T8 = 12 $MECO_2$-R at 3 MAP

Whereas, in Figure 7C, the 1 $MECO_2$-R showed a significantly higher tiller number compared to another related CO_2, which increased by 10.58% (T1), 29.41% (T3), 15.91% (T4), 17.22% (T5), 47.58% (T6), 19.98% (T7), and 47.09% (T8), respectively. Elevated CO_2 and ratooning have been proven to increase aboveground biomass production in either shoot or tiller numbers (Long et al., 2004). The growth rate depends on the proportion of allocations; if the plant allocates essential nutrients and other elements to tiller parts, the tiller number increases biomass production (Poudel & Dunn, 2017). It can result in the initiation of more tiller buds and the subsequent growth of new tillers (Pritchard et al., 1999). On the other hand, the 2 $MECO_2$-noR showed a significantly higher tiller bunch circumference compared to another elevated CO_2, which increased by 14.01% (T1), 24.14% (T2), 32.70% (T4), 36.15% (T5), 55.29% (T6), 41.06% (T7), 54.62% (T8), respectively (Figure 7D). The longer Napier grass is exposed to elevated CO_2, the more significant changes in tiller bunch circumference (Rahman et al., 2019). However, if the impact has reached maximum production, any longer exposure will not help to increase the tiller bunch circumference (Kimball, 2016). The result corresponds with a previous study, which states that a high carbon supply under elevated CO_2 helps accelerate cell division and expansion in tissues and enhance early growth and development in the meristematic tissues of the plant. Kimball (2016) and Wangchuk et al. (2015) have proven that elevated CO_2 significantly affected plant height and tiller number with increasing plant height throughout the experiment.

Ultimate Analysis: Carbon (%), Hydrogen (%), Nitrogen (%), and Sulphur (%)

Table 3 shows a significant interaction between ratooning and different periods of elevated CO_2 in nitrogen and sulphur. While in Figure 8A, the 12 $MECO_2$-R at 3MAP shows the highest reading of total nitrogen (%) compared to other treatments, which are increased by 14.04% (T1), 11.70% (T2), 20.47% (T3), 17.54% (T4), 26.90% (T5), 9.37% (T6), 33.33% (T7), and 35.87% (T8) respectively. Elevated CO_2 levels will have different approaches to photosynthesis, transpiration, and stomatal conductance rates (Zakaria et al., 2019). High CO_2 concentration can theoretically lead to increased photosynthesis rate in plant growth, resource allocation, and tissue composition due to high carbon assimilation (Gulfam et al., 2017). Besides, elevated CO_2 plays an important role in microbial activities, which results in an increase in the decomposition rates of organic matter (Zhou et al., 2022). Thus, nitrogen fixation has increased due to the large amount of nitrogen compound released from organic matter (Long et al., 2006).

Elevated CO_2 concentrations can stimulate photosynthesis and enhance plant carbon uptake (Wangchuk et al., 2015). The increased carbon assimilation results in higher carbon content in organic materials (Lounglawan et al., 2014). Elevated CO_2 can improve plant nitrogen use efficiency,

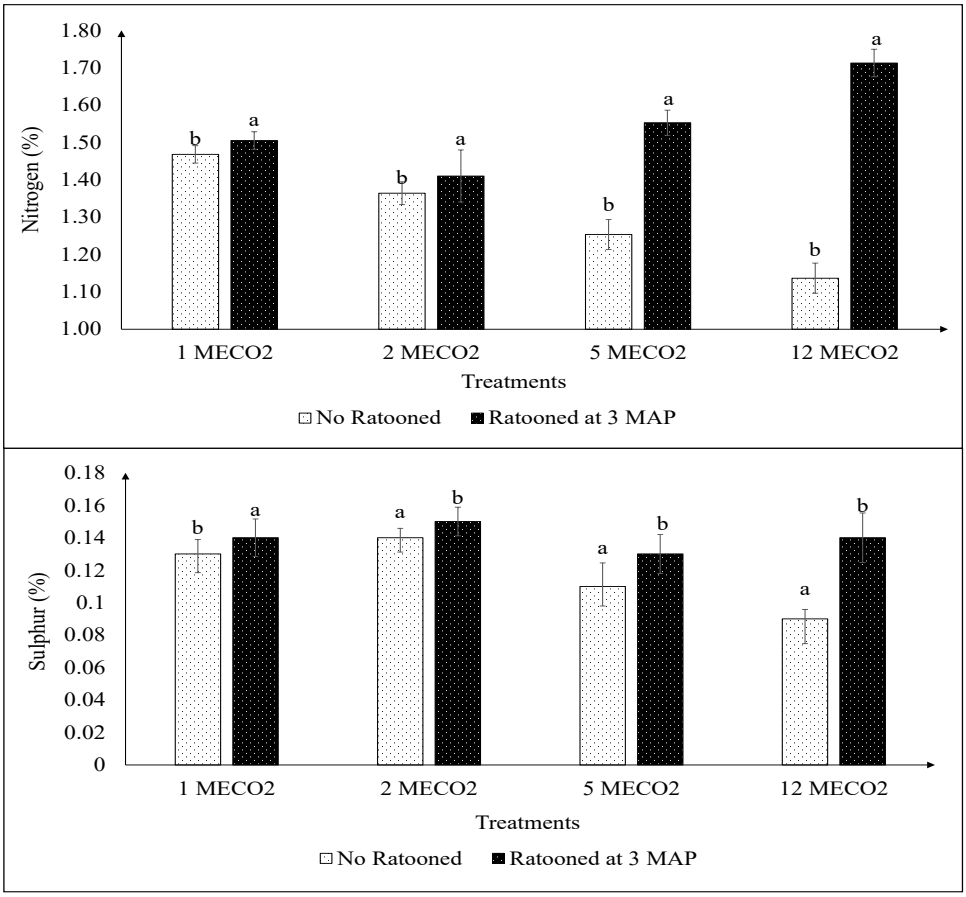

Figure 8. The interaction effect of ratooning and different periods of elevated carbon dioxide on (A) total nitrogen and (B) total sulphur of Napier grass (*Pennisetum purpureum* cv. Taiwan), respectively

Note. T1 = 1-month elevated CO_2 ($MECO_2$)-no ratooned (noR); T2 = 1 $MECO_2$-ratooned (R) at three months after planting (MAP); T3 = 2 $MECO_2$-noR; T4 = 2 $MECO_2$-R at 3 MAP; T5 = 5 $MECO_2$-noR; T6 = 5 $MECO_2$-R at 3 MAP; T7 = 12 $MECO_2$-noR; T8 = 12 $MECO_2$-R at 3 MAP; a and b indicate significant difference between treatment means using least significant difference at $p \leq 0.05$

potentially reducing nitrogen content (Zakaria et al., 2019). Additionally, elevated CO_2 can alter soil microbial communities' composition and abundance, affecting ecosystems' nitrogen availability and cycling (Leakey et al., 2009). These changes can indirectly influence the nitrogen content in organic materials (Mwendia et al., 2019). Elevated CO_2 can indirectly affect the sulphur content of organic materials by altering soil microbial activities and nutrient cycling (Norhaiza et al., 2009).

Based on Figure 8B, five treatments show the highest reading of total sulphur, which are 1 $MECO_2$-noR (T1), 1 $MECO_2$-R at 3 MAP (T2), 2 $MECO_2$-noR (T3), 2 $MECO_2$-R at 3 MAP (T4), and 12 $MECO_2$-R at 3 MAP (T8). Fertile soil contains enough

macronutrients to meet plant requirements, which include nitrogen, potassium, phosphorus, calcium, magnesium, and sulphur (Manyawu et al., 2003). Sulphur is categorised as a secondary element of macronutrients, which are required in smaller amounts than primary nutrients (Marafon et al., 2021). However, unlike carbon and nitrogen, sulphur is not a limiting nutrient in terrestrial ecosystems (Mason et al., 2008). Difference periods of elevated CO_2 can affect soil microbial communities and biological activities (Said et al., 2019). Microbes play a crucial role in the decomposition of organic matter and involve sulphur release through the sulphur cycle (Rengsirikul et al., 2013).

Total Biomass

In Figure 9, the highest total biomass of Napier grass was recorded at 1 $MECO_2$-noR (T1), which increased by 74.34% (T2), 10.08% (T3), 78.69% (T4), 91.86% (T5), 86.11% (T6), 23.59% (T7), and 88.59% (T8), respectively, for every period of harvest after planting followed by 2 $MECO_2$-noR (T2), 12 $MECO_2$-noR (T7), and other treatments. Elevated CO_2 levels typically stimulate photosynthesis and carbon assimilation in plants, leading to enhanced growth (Hampton et al., 2013). It can result in greater aboveground biomass, including stems, leaves, and fruits (Mukhtar, 2006). The increased availability of CO_2 allows plants to fix more carbon dioxide and produce higher amounts of carbohydrates, such as sugars and starches, which are the building blocks for biomass production (Zailan et al., 2016). However, Napier grass acclimates to elevated CO_2 levels, resulting in reduced growth response compared to initial exposure (Thompson et al., 2017). The reason behind this result was that Napier grass can adjust its physiological processes according to the new carbon dioxide environment (Sawasdee & Pisutpaisal, 2014). Ibrahim et al. (2011b) and Rambau et al. (2016) reported that crops exposed to elevated CO_2 positively increased the total biomass at week 12 by 6.08 t/ha. However, increasing the period of elevated CO_2 will

Table 3
The effect of elevated carbon dioxide (CO_2) period on carbon, hydrogen, nitrogen, and sulphur of Napier grass (Pennisetum purpureum *cv. Taiwan*)

Treatments	Carbon (%)	Hydrogen (%)
Ratooned (R)		
noR	44.34a	5.68a
R	43.42a	5.69a
LSD (P<0.05)	NS	NS
Elevated CO_2 (ECO_2)		
T1: 1-month ECO_2	44.95a	5.68ab
T2: 2-month ECO_2	44.67a	5.85a
T3: 5-month ECO_2	44.81a	5.76a
T4: 12-month ECO_2	41.10b	5.45b
LSD P<0.05	1.84	0.27
R x ECO_2	NS	NS

Note. Means followed by the same letter within a column are not significantly different at P>0.05 by least significant difference (LSD) test with n = 32; NS = Not significant; noR = No ratooned

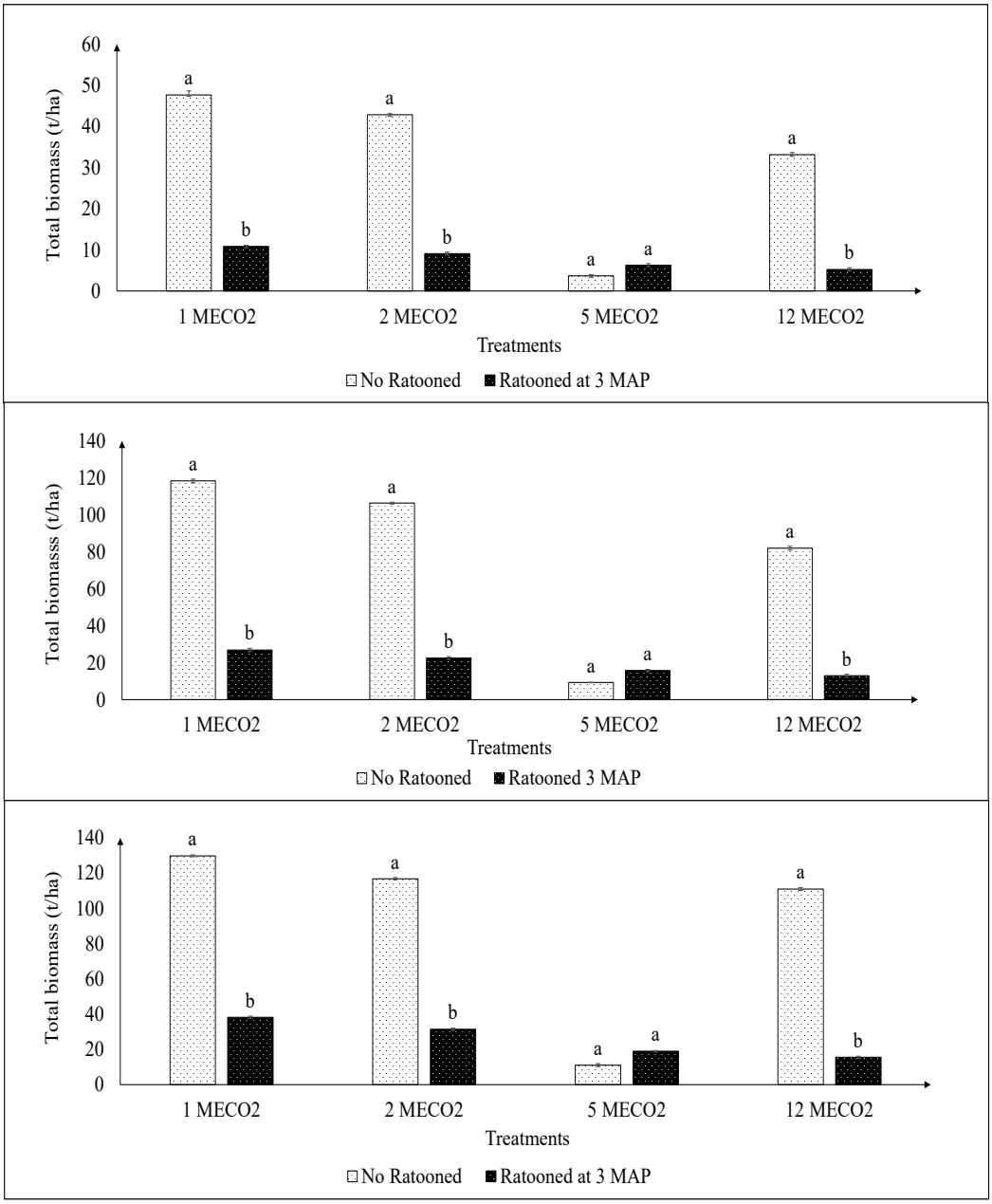

Figure 9. The interaction effect of ratooning and different periods of elevated carbon dioxide on total biomass of Napier grass (*Pennisetum purpureum* cv. Taiwan) at (A) 6 months after planting (MAP), (B) 8 MAP, and (C) 12 MAP, respectively; a and b indicate significant difference between treatment means using least significant difference at $p \leq 0.05$

Note. T1 = 1-month elevated CO_2 ($MECO_2$)-no ratooned (noR); T2 = 1 $MECO_2$-ratooned (R) at three months after planting (MAP); T3 = 2 $MECO_2$-noR; T4 = 2 $MECO_2$-R at 3 MAP; T5 = 5 $MECO_2$-noR; T6 = 5 $MECO_2$-R at 3 MAP; T7 = 12 $MECO_2$-noR; T8 = 12 $MECO_2$-R at 3 MAP

not eventually increase the total biomass at a certain time (Namiki, 1990). The plant initially responds positively to elevated CO_2 levels by exhibiting increased rates of photosynthesis (Akah & Onweluzo, 2014). It can lead to enhanced plant growth and increased biomass production, especially in the early stages of exposure (Niinemets & Valladares, 2006). This response is referred to as the "CO_2 fertilisation effect" (Collatz et al., 1992). However, the longer exposure of Napier grass to the high carbon dioxide percentage will not help increase the total biomass as it reaches maximum growth (Negawo et al., 2017).

CONCLUSION

Experiment 1 showed that Napier grass with no ratooned resulted in the highest readings in all parameters compared to ratooned in plant height, stem diameter, total bunch circumference, plant biomass, total nitrogen, and sulphur in the ultimate analysis. The ultimate analysis did not significantly affect tiller number, total carbon, or nitrogen.

Experiment 2 proved that Napier growth, such as plant height, stem diameter, tiller number, tiller bunch circumference, plant biomass, total nitrogen, and sulphur in the ultimate analysis, showed significant interactions between ratooning and elevated CO_2. The treatment that implied the highest result in all parameters was 1 $MECO_2$, which was able to promote similar results as the plant received treatments from 2, 5, and 12 $MECO_2$, which is better than control treatments. However, in Experiment 2, all treatments that had no ratooned showed the highest reading compared to ratooned treatments.

These results prove that crops were facing a slow recovery process after being ratooned, thus affecting crop productivity. In conclusion, Napier grass production will decrease with increasing crop age, either ratooned or no ratooned treatments. Long exposure under elevated CO_2 conditions did not significantly benefit plant growth in the study. Meanwhile, the results suggested that shorter exposure periods (1 and 2 $MECO_2$) led to more favourable outcomes in terms of growth performance, biomass quality, and biochemical accumulation. Napier grass has been considered for carbon dioxide sequestration due to its fast growth and high biomass production. The suggestion for utilising Napier grass in carbon dioxide sequestration areas is to build agroforestry systems that integrate or plant trees alongside Napier grass, creating a more diverse and resilient ecosystem and capturing carbon in the grass biomass and the trees. Besides, mixed cropping with other fast-growing plants or cover crops should be considered to enhance the overall carbon sequestration potential. Diverse plantings can also improve soil health and fertility. Next, Napier grass, such as biogas or bioethanol, can be utilised for bioenergy production. While burning biomass releases carbon dioxide, using it as a renewable energy source can potentially displace fossil fuels, contributing to a net reduction in atmospheric carbon.

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Addressing Nitrogen-rich Biomass Production Challenges in *Azolla microphylla* Cultivation from Varying Shading and Water Depth Dynamics

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ABSTRACT

Azolla microphylla, a rapidly growing aquatic fern with the unique ability to fix atmospheric nitrogen, presents significant potential for sustainable agriculture. Despite its nitrogen-fixing prowess, challenges persist in optimizing biomass production, prompting a detailed exploration of influential factors in this study. This paper addresses the persistent challenge of optimizing nitrogen-rich biomass production in *Azolla* cultivation. Employing a split-plot experimental design, the study investigates the influential factors of shading percentage (N) and water depth (G) in *Azolla* growth, systematically ranging from 0% (full sunlight/N1) to 75% (N3) shading percentages and 2.5 cm (G1), 5.0 cm (G2), and 7.5 cm (G3) water depths. In addition to assessing growth and production outcomes, this study explores the nitrogen content in *Azolla* under three different conditions: fresh, dried, and composted *Azolla*. Findings unveil the significant influence of shading percentage and water depth on *Azolla* growth, with the N1G2 treatment identified as the optimal condition for achieving maximum biomass production. Set against the backdrop of tropical agriculture, specifically within the high temperatures in Indonesia, our study underscores the resilience of *Azolla* to elevated temperatures, highlighting

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its potential as a nitrogen-fixing agent. Notably, fresh *Azolla* closely matches urea in nitrogen content, suggesting its potential as an organic fertilizer substitute for urea. This research sheds light on the critical challenges surrounding nitrogen-rich biomass production from fresh *Azolla*, emphasizing the necessity of temperature resilience and water depth optimization. The insights provided hold significance for tropical agriculture practices seeking to harness the potential of *Azolla* as a free-air nitrogen fixator.

Keywords: *Azolla microphylla*, nitrogen fixation, shading percentage, sustainable cultivation, tropical agriculture, water depth

INTRODUCTION

Azolla microphylla, commonly known as *Azolla*, represents a captivating aquatic fern within the Azollaceae family. Its distinct characteristics include a small stature, rapid proliferation, and a noteworthy symbiotic relationship with the nitrogen-fixing cyanobacteria *Anabaena azollae* (Abd El-Aal, 2022; Adhikari et al., 2020; Akhtar et al., 2021; G. Kumar & Chander, 2017). *Azolla*'s paramount attribute in agriculture lies in its proficiency as a biofertilizer. Its ability to fix atmospheric nitrogen provides a sustainable alternative to synthetic nitrogen fertilizers, thereby enhancing soil fertility and creating optimal conditions for crop cultivation (Adhikari et al., 2020; Ahmad & Tariq, 2021). This agricultural significance has positioned *Azolla* as a comprehensive subject for study and exploration. The distinctive nitrogen-fixing symbiosis of *Azolla*, where *Anabaena* resides within specialized dorsal hairs on the upper leaf surface, is a focal point for research into biological nitrogen fixation (Devaprakash et al., 2024). This symbiotic relationship is integral to the plant's role in promoting soil fertility and sustainable agricultural practices.

Beyond agriculture, *Azolla*, as a biomass source, has garnered attention for its potential in bioenergy production (Purnama et al., 2024). A study exemplified by Chupaza et al. (2021) has demonstrated the feasibility of utilizing *Azolla* biomass for bioethanol production. Its rapid growth rate and high biomass productivity make it an attractive candidate in the renewable energy sector. *Azolla*'s utility also extends to animal husbandry, where it serves as a valuable nutritional supplement in livestock diets. Rich in proteins, vitamins, and minerals, *Azolla* contributes to enhanced livestock health and productivity, as evidenced by the findings of G. Kumar and Chander (2017) as well as Nasir et al. (2022).

From an environmental sustainability perspective, *Azolla* cultivation seamlessly aligns with sustainable agricultural practices, alongside efforts to seek alternative pesticides that do not contaminate agricultural products (Purnama et al., 2023). Its ability to thrive in diverse water conditions and minimal environmental impact positions *Azolla* as an eco-friendly intervention in agricultural systems (Alam et al., 2023; Xu et al., 2017). Despite its numerous advantages, *Azolla* cultivation confronts

several challenges that hinder its widespread adoption. Environmental factors, such as extreme temperatures or fluctuations, can impede *Azolla* growth (Costarelli et al., 2021). High temperatures may induce thermal stress, affecting nitrogen fixation efficiency, while insufficient sunlight due to shading or low light conditions may restrict photosynthesis, hampering overall growth potential. Sunlight intensity plays a multifaceted role in the growth dynamics of *Azolla*, profoundly impacting photosynthetic activity, transpiration rates, and overall plant physiology (Kakaeian & Mohammadi, 2022; Pouil et al., 2020). Water depth also emerges as a crucial factor affecting *Azolla* cultivation, which exerts a substantial influence on the nutrient absorption rates and evapotranspiration of *Azolla* (Adzman et al., 2022; Kimani, Kanno, et al., 2020).

Numerous research endeavors have delved into mitigating environmental challenges, specifically addressing issues such as high light intensity and water depth in the cultivation of *Azolla*. The dynamic interplay between these factors and *Azolla*'s growth dynamics has been a focal point in the quest for sustainable and optimized cultivation practices. One significant facet of prior investigations lies in understanding the impact of high light intensity on *Azolla* growth. Studies exemplified by the work of Kakaeian and Mohammadi (2022) have underscored the need for nuanced light management strategies to harness optimal growth conditions. Similarly, research by Pouil et al. (2014) contributes valuable insights into the multifaceted effects of light intensity on *Azolla*, emphasizing its role as a pivotal factor influencing key physiological processes.

Another critical environmental parameter explored in past research is water depth. The depth of water habitats inherently influences *Azolla* cultivation, and studies such as those conducted by Adzman et al. (2022) have delved into the consequences of varying water depths on *Azolla* biomass production. These investigations have provided essential insights into the correlation between water depth and nutrient absorption rates, offering a foundation for developing cultivation strategies tailored to specific water depth conditions. However, studies combining shading and its variations have not been extensively explored, even though the integrative approach of these two factors provides a holistic understanding of the environmental parameters influencing *Azolla* growth. Given the intricacies of these interactions, there exists an urgent need to thoroughly investigate and establish the optimal combination of water depth and shading percentage that is environmentally conducive to the biomass growth of *Azolla*.

This study, which aims to identify optimal combinations of shading percentages and water depths conducive to promoting maximum nitrogen-rich biomass production, distinguishes itself by including an analysis of nitrogen content in *Azolla* biomass under three conditions: fresh, dried, and composted. This comprehensive approach involves a meticulous examination of synergies between shading and water depth

dynamics, highlighting the multifaceted nature of the study. The focus on addressing challenges related to nitrogen-rich biomass production in *Azolla* cultivation, along with including nitrogen content analysis from various biomass conditions, sets this research apart from previous studies. While prior research has provided valuable insights into individual factors, this study uniquely integrates them, considering their combined effects on biomass yield. The integrative approach contributes to a more holistic understanding of the cultivation dynamics and offers practical insights for optimizing *Azolla* cultivation practices in the context of nitrogen-rich biomass production.

MATERIALS AND METHODS

Experiment Design and Management

An eight-week field experiment aimed to assess *A. microphylla*'s response to various culture conditions, involving three water depth levels and three light level conditions. The split-plot experimental design included 27 ponds, oriented east-west, resulting in nine treatment conditions (N1G1, N1G2, N1G3, N2G1, N2G2, N2G3, N3G1, N3G2, N3G3) replicated thrice. The control condition exposed nine ponds to direct sunlight (0% shade net), while nine were covered by a 50% shade net and another nine by a 75% shade net, designated as N1, N2, and N3, respectively. Water depths (G) were 2.5 cm (G1), 5.0 cm (G2), and 7.5 cm (G3).

The experiment took place during the dry season in a designated artificial pond at Universitas Lancang Kuning's recycling center ("Waste Bank") in Pekanbaru, Indonesia. The pond (each consisting of 1 m^2 with a depth of 0.20 m), as shown in Figure 1, was positioned at coordinates 0°34'52.6"N 101°25'36.9"E and an altitude of 30 m. Continuous light intensity and air temperature monitoring were performed using a light/temperature meter (SMART SENSOR® AS803 Digital Lux Meter, China). Digital lux meters were strategically placed in three ponds per light condition at 5 cm above the water surface, capturing measurements during the morning (07:00-08:00 local time [UTC+0:700]), noon (12:00-13:00 local time [UTC+0:700]), and afternoon (16:00-17:00 local time [UTC+0:700]). The shading effect was achieved using locally available, cost-effective nylon materials, creating a shading gradient among the experimental conditions. The lux-recorded data for light intensity were transformed to μmol/m^2/s, following the methodology outlined by Pouil et al. (2022).

Plant Materials and Cultivation Procedures

Azolla specimens were sourced from the Faculty of Agriculture's small pond at Universitas Lancang Kuning, Pekanbaru, Indonesia (0°34'36"N 101°25'29"E, altitude 33 m). Several academic reasons underpin our decision to select *A. microphylla* as the focal species for this study over other *Azolla* species. Firstly, *A. microphylla* has been widely recognized for its suitability and adaptability to tropical environments. According to Sarah et al. (2023), *A.*

microphylla has demonstrated superior growth performance and environmental adaptability in tropical regions, making it a suitable candidate for cultivation in the Indonesian context. Additionally, *A. microphylla*'s ability to thrive in nutrient-rich aquatic habitats, as documented by Ting et al. (2022), renders it an ideal species for studying nitrogen-rich biomass production, a key focus of our investigation.

Azolla microphylla, inoculated at 100 g fresh weight (FW) per 1 m^2 pond (i.e., 100 g FW/m^2) followed previous research (Pouil et al., 2022), underwent cultivation. Each pond received 3 kg of cow manure (i.e., 30 tons/ha) one week before the experiment's initiation. Throughout the cultivation process, phosphorus fertilizer (superphosphate 36%) was applied three times at 20 g intervals (i.e., 200 kg/ha). The first application, 6 g per pond, occurred two weeks after the initial inoculation. The second application, 6 g per pond, followed the second harvest, with an additional 8 g per pond applied after the third harvest.

Figure 1. Experimental design overview with 1 m x 1 m artificial ponds

After each harvest, 100 g of *A. microphylla* per unit was redistributed, consistent with the initial inoculation treatment.

Total plant biomass assessments were conducted at the experiment's onset and three subsequent times after two weeks of phosphorus addition during the eight-week experiment by subtracting the initial seeding (total 300 g FW per pond). Samples were carefully rinsed and drained. Dry weight (DW) determination adhered to the procedures outlined in previous research (Kimani, Kanno, et al., 2020), wherein the samples were subjected to oven drying at 70°C for 48 hr and subsequently weighed (± 0.1 g). Productivity was calculated by dividing the total biomass of *Azolla* per square meter over the 60 days for each pond.

Nitrogen Analysis in *Azolla*

Nitrogen analysis in *A. microphylla* encompassed three distinct conditions: fresh, dried, and composted *Azolla*. Nine replicates for each condition were collected from experimental ponds, where fresh *Azolla* was directly harvested, dried *Azolla* was obtained after oven-dried, and composted *Azolla* was derived from the decomposition process based on previous research (Lestari et al., 2019). Two treatments were applied to composted *Azolla*: the first with cow manure and the second without. These samples were systematically taken from *Azolla*, which had been previously cultivated. Sample preparation involved using fresh *Azolla* without any pre-treatment, while dried and composted *Azolla* underwent grinding into a fine powder for homogeneity. The Kjeldahl

method was then employed for nitrogen extraction based on previous research with several modifications (Shamsudin et al., 2021), with 1 g of fresh *Azolla* and 0.5 g of dried or composted *Azolla* subjected to digestion.

Data Analysis

Collected observational data from the experimental treatments underwent statistical analysis using SPSS software (version 21). The analysis began with a significance assessment by comparing the calculated *F*-value with the critical *F*-table value at 5%. This step determined whether the observed differences were statistically significant or inconsequential. Subsequent analysis involved using Duncan's multiple range test (DMRT) at a 5% significance level to identify specific treatment groups that differed significantly. This detailed post-hoc analysis ensured a thorough examination and validation of any observed variations among treatment levels.

RESULTS AND DISCUSSION

Effects of Shading and Water Depth on *Azolla* Production

In the effort to unravel the intricacies of *A. microphylla* cultivation, investigating the relationship between shading percentage and water depth is imperative in this study. The experimental results unveil that the combination of N1G2, representing the treatment without shading or fully exposed to sunlight with a water depth of 5.0 cm, exhibits no significant difference in *Azolla* biomass compared to shaded conditions. Statistically, there is no significant difference compared to combinations with 2.5 cm and 7.5 cm water depths. Nevertheless, a numerical trend indicates an increase in biomass for the combination without shading and a water depth of 5.0 cm (Table 1).

The findings of previous research, as elucidated by Adzman et al. (2022) and Kimani, Kanno, et al. (2020), underscore a consistent pattern of *Azolla* thriving in environments characterized by deeper water depths. The fundamental mechanism driving this is *Azolla*'s ability to access sufficient dissolved oxygen (DO), a crucial resource for its growth. While *Azolla*, like other plants, relies on carbon dioxide (CO_2) in photosynthesis, it also undergoes respiration to generate the energy needed for growth, nutrient absorption through its roots, and the uptake of active ions. In this context, the submerged roots of *Azolla* require a continuous supply of oxygen to trigger these vital processes (Amit et al., 2016; Xu et al., 2017). As a result, lower water levels imply reduced availability of DO, while higher water levels enhance the DO levels, creating a supportive environment for *Azolla* growth.

These findings clarify the relationship between shading percentage and water depth influencing *Azolla* growth. Water depth is associated with optimal DO supply for *Azolla*. This understanding has practical implications for *Azolla* cultivation, as it indicates that the N1G2 combination condition, characterized by the absence of shading and a water depth of 5.0 cm, can play a crucial role in enhancing *Azolla* biomass, especially *A. microphylla*. By

Table 1
Total fresh biomass of Azolla microphylla *(g) across shading percentage and water depth (n = 9)*

Treatments	Total fresh biomass (g)
N1G1	4,059.78bc
N1G2	4,686.89c
N1G3	4,439.89c
N2G1	3,547.56ab
N2G2	3,309.11a
N2G3	3,740.67ab
N3G1	3,595.89ab
N3G2	3,615.00ab
N3G3	3,767.11ab

Note. Full sunlight or 0 % shade net (N1), 50 % shade net (N2), 75 % shade net (N3), water depth of 2.5 cm (G1), water depth of 5 cm (G2), and water depth of 7.5 cm (G3). The numbers followed by the same letter do not significantly differ based on the post hoc Duncan's multiple range test at a 5% significance level

leveraging these findings, farmers and agricultural practitioners can optimize *A. microphylla* cultivation techniques, contributing to sustainable farming practices by generating a natural nitrogen source and potentially reducing or replacing reliance on synthetic fertilizers (Jama et al., 2023). Furthermore, this research reinforces the confidence that *Azolla* can thrive and be applied to agricultural lands under high-temperature and sunlight-intensity conditions, particularly in flooded rice fields with varying water depths.

In the field, temperature and light intensity are also crucial determinants of *Azolla* growth rate, as evidenced by studies (Akhtar et al, 2020; G. Kumar & Chander, 2017; Pouil et al, 2020). Water depth significantly influences air temperature, with higher ponds maintaining cooler air temperatures. Lower temperatures result in denser air molecules, increasing DO levels (Ali et al., 2016). Conversely, higher air temperatures decrease oxygen solubility, ultimately determining DO availability for *Azolla*. Additionally, light intensity plays a vital role in plant physiology, significantly influencing the fundamental process of photosynthesis (Kakaeian & Mohammadi, 2022; Pouil et al., 2020). It is well-established that reduced light intensity will inevitably lead to decreased photosynthetic efficiency (Liu & Van-Iersel, 2021), a phenomenon of paramount importance in plant biology.

However, our research has revealed intriguing and unexpected findings that challenge conventional expectations. Contrary to the anticipated results, shading did not consistently yield a proportional increase in *A. microphylla* biomass, as presented in Table 1. To delve deeper into this interesting phenomenon, Table 2 and Figure 2 comprehensively illustrate the observed variations in temperature and light intensity at different time intervals. Concerning temperature, it is evident that the shade net used failed to significantly reduce temperature. For each treatment, the average morning, noon, and afternoon temperatures were 27–34°C, 29–41°C, and 27–36°C, respectively. *Azolla*'s ability to thrive over a wide temperature range, from 18 to 28°C (Korsa et al., 2024), 6 to 30°C (Sadeghi et al., 2012), with optimal growth observed at 25°C and rapid reproduction occurring between 18–26°C (Veerabahu, 2015), is one of the advantages

in *Azolla* cultivation. Temperature primarily influences photosynthesis when light is a limiting factor (Hussain et al., 2021; Moore et al., 2021). CO_2, coupled with limited light, can enhance photosynthesis. Furthermore, temperature has a significant impact on air absorption by plant roots, with increased absorption rates at higher temperatures (Cannavò et al., 2023). Changes in air viscosity, cell membrane permeability, and root cell activity cause this phenomenon. Temperature also affects nutrient absorption, with low temperatures inhibiting nutrient uptake due to reduced respiration activity or decreased cell membrane permeability (Bhattacharya, 2022).

Meanwhile, the sunlight intensity, particularly the treatment without shading (N1), consistently exhibited the highest light intensity throughout the observation period. Conversely, the treatment with 75% shade net consistently showed the lowest light intensity. This observation effectively underscores that shading restricts the quantity of incoming light (Timmermans et al., 2020). This phenomenon challenges our traditional understanding of the impact of shading percentages on plant growth, especially in the context of *A. microphylla* cultivation. It suggests that *A. microphylla* may have unique adaptations and responses to different light exposures compared to other plant species. Further investigation into the intricate interaction between *A. microphylla* and light intensity is required to unveil the fundamental mechanisms governing its growth dynamics and potentially optimize cultivation practices.

Effendi et al. (2019) reported that applying 30–50% shading during cultivation decreased biomass yield compared to the unshaded condition. Interestingly, shading percentages of 50–75% did not show a significant impact on the growth of *A. microphylla*, as supported by Pouil et al. (2020), reinforcing the findings in this study. Table 1 demonstrates an inverse relationship between shading percentage and *A. microphylla* biomass. Consistent with the research by Adzman et al. (2022), it is suggested that *A. microphylla* requires full sunlight exposure combined with a water depth of 20 cm, making field conditions such as fishponds ideal for *Azolla* cultivation (Pouil et al., 2020).

However, another critical factor influencing *Azolla* biomass production is the nutrient concentration within the pond. As commonly known, the water volume also expands with increasing water depth. Under such circumstances, the nutrient concentration will decrease if the same amount of fertilizer is applied to each treatment with varying water depths. Despite direct exposure to sunlight, *Azolla*'s growth and biomass production at a water depth of 7.5 cm is not as favorable as at a water depth of 5 cm. Therefore, future research should ensure uniform nutrient concentration by adjusting the amount of nutrients used according to the volume or depth of water treatment, as demonstrated by Adzman et al. (2022).

Table 2
Summary of the light intensity and temperature conditions measured throughout the experiment in the three shading conditions (n = 60)

Shading treatment	Light Intensity (µmol/m²/s)				Temperature (°C)			
	07:00–08:00 (UTC+0:700)	12:00–13:00 (UTC+0:700)	16:00–17:00 (UTC+0:700)	Day	07:00–08:00 (UTC+0:700)	12:00–13:00 (UTC+0:700)	16:00–17:00 (UTC+0:700)	Day
N1 (0% shade net)	74 ± 31	116 ± 29	66 ± 29	85 ± 37	29 ± 1.1	35 ± 2.8	32 ± 1.9	32 ± 3.0
N2 (50% shade net)	42 ± 13	42 ± 20	35 ± 24	51 ± 24	29 ± 1.3	36 ± 3.1	32 ± 1.9	32 ± 3.5
N3 (75% shade net)	34 ± 10	64 ± 22	42 ± 19	47 ± 22	29 ± 1.3	35 ± 2.8	32 ± 1.9	32 ± 3.2

Note. Data are means ± standard deviations

Figure 2. Biomass productivity due to the influence of shading and water depth

Note. N1 = 0% shade net; N2 = 50% shade net; N3 = 75% shade net

Nitrogen Content in *A. microphylla*

The exploration into the nitrogen content of *A. microphylla* has uncovered intricate dynamics, significantly contributing to our understanding of *Azolla*'s nutritional profile and its potential as an organic nitrogen source in agriculture. Fresh *A. microphylla* exhibited an exceptional nitrogen content of 40.3%, marking a pivotal discovery, as shown in Table 3. This high nitrogen concentration underscores the inherent capability of *Azolla* species in fixing atmospheric nitrogen, aligning with the seminal work of Yao et al. (2018), which emphasizes the nitrogen-fixing prowess of *Azolla*. Contrastingly, the dehydration process significantly influenced the nitrogen levels in dried *Azolla*, resulting in a substantial decrease of 3%. The impact of moisture loss on nitrogen concentration is well-documented, as reported by da Silva et al. (2022), further affirming the sensitivity of *Azolla*'s nitrogen content to processing methods.

Table 3
Total N from Azolla *in several conditions from this study (n = 9)*

No.	*Azolla* conditions	Total N (%)
1	Fresh	40.3 ± 1.76
2	Dried	3.00 ± 0.22
3	Composted without cow manure	2.76 ± 0.06
4	Composted with cow manure	1.94 ± 0.03

Note. Data are means ± standard deviations

Furthermore, the observed decrease in nitrogen content upon drying can be attributed to several factors. Firstly, water loss during dehydration leads to a concentration effect, where the nitrogen content becomes more concentrated in the remaining biomass, resulting in higher nitrogen content per unit weight. The dehydration process may also trigger biochemical reactions, such as enzymatic degradation or microbial activity, which could contribute to the breakdown or loss of nitrogen-containing compounds. Moreover, volatilization of ammonia, a common nitrogenous compound, may occur during drying, further diminishing the nitrogen content of the dried *Azolla* biomass (Bao et al., 2022). These mechanisms collectively contribute to the observed decline in nitrogen content following the dehydration process, highlighting the importance of considering processing methods when assessing the nitrogen content of *Azolla* biomass.

Composting dried *Azolla* introduced another dimension to the nitrogen dynamics, with composted *Azolla* displaying a nitrogen content of 2.76%. Incorporating cow manure as a composting agent further reduced the nitrogen content in the composite to 1.94%. The observed decrease in nitrogen content during composting can be attributed to several factors. On the one hand, microbial activity during composting leads to the breakdown of nitrogen-containing compounds present in *Azolla* biomass, resulting in the release of nitrogen in gaseous forms such as ammonia and nitrous oxide, as reported by Wong et al. (2017). On the other hand, incorporating cow manure, although rich in nitrogen, may promote microbial activity that accelerates the decomposition of organic matter, thereby leading to nitrogen loss through volatilization and leaching processes (Toledo et al., 2020). These processes cumulatively decrease nitrogen content observed during composting, underscoring the complex nitrogen transformation dynamics within *Azolla*-based composting systems.

The observed variations in nitrogen content across different *Azolla* conditions emphasize the critical role of processing methods and additives in shaping the nutritional composition of *Azolla* biomass. These nuanced findings are pivotal for optimizing the utilization of *Azolla* as an organic nitrogen source in agriculture, aligning seamlessly with the sustainable and eco-friendly farming paradigms advocated by Alam et al. (2023) and Xu et al. (2017). Moreover, the significance of the nitrogen content in fresh *Azolla* becomes even more pronounced as our investigation, based on an extensive literature review, reveals

a lack of previous studies comparing the nitrogen analysis of fresh, dried, and composted *Azolla*. Existing research, if any, predominantly focuses on the utilization of *Azolla* in cultivating various crops (Jama et al., 2023; G. P. Kumar et al., 2020; Marzouk et al., 2023; Seleiman et al., 2022), comparing the outcomes of using fresh, dried, and composted *Azolla*. Notably, our study aligns with the findings of Muñoz et al. (2008), who compared nitrogen content in fresh and composted poultry (*Gallus domesticus*) manure (57% : 14%).

This comprehensive exploration of nitrogen content in *A. microphylla* enriches our understanding of *Azolla*'s nutritional dynamics. It provides a robust foundation for leveraging its potential as a nitrogen-rich organic fertilizer. Considering its diverse applications within sustainable agriculture, these findings underscore the imperative for tailored approaches in *Azolla* utilization.

Azolla is an Eco-friendly Fertilizer

The substantial nitrogen content inherent in *A. microphylla* positions it as a promising and sustainable alternative to synthetic fertilizers, especially urea. This attribute has the potential to significantly reduce our dependence on inorganic fertilizers and mitigate the environmental impacts associated with their production and application. The growth and decomposition rates exhibited by *A. microphylla* make it a highly valuable resource in green manure and biofertilizers, particularly in paddy fields, as highlighted by Roy et al. (2016). Previous studies have consistently highlighted *Azolla*'s nitrogen-fixing potential (Devaprakash et al., 2024; Yao et al., 2018). This symbiotic relationship allows *Azolla* to convert atmospheric nitrogen into a form readily available to plants, contributing significantly to nitrogen enrichment in the soil (Akhtar et al., 2021; Ssenku et al., 2022).

Based on findings from previous research, *Azolla* can be seamlessly integrated into agricultural practices through two primary approaches. The first approach involves directly incorporating *Azolla* into paddy fields. Studies indicate that this method effectively enhances nitrogen availability in the soil, resulting in improved plant growth. Simultaneously, it offers additional benefits, such as weed control and enhanced water management in agricultural fields (Borkar et al., 2023; Kimani, Bimantara, et al., 2020). On the other hand, the second approach entails a controlled fermentation process applied to *Azolla* before its introduction to agricultural fields. This fermentation process provides flexibility in modifying the composition of *Azolla* based on specific agricultural needs. Research outcomes suggest that this approach provides greater control over the nutritional properties of *Azolla*, offering options to include or exclude additional organic fertilizers as per diverse agricultural requirements (Seleiman et al., 2022). Integrating *Azolla* into agricultural practices through these two approaches provides farmers with flexible and adaptable options tailored to local conditions and specific crop needs. It underscores *Azolla*'s position as

a reliable source of organic fertilizer that can be easily integrated into sustainable agricultural contexts.

Moreover, *A. microphylla* has demonstrated efficacy in improving crop yields. Studies by Ahmad et al. (2024) and G. P. Kumar et al. (2020) have shown positive effects on the growth and productivity of various crops when *Azolla* is integrated into agricultural practices. The nitrogen content in *A. microphylla*, especially in its fresh form, closely resembles that of urea, underscoring its potential as a sustainable nitrogen source (Jama et al., 2023). The use of *A. microphylla* as a fertilizer alternative aligns with sustainable agriculture principles. It addresses concerns related to the environmental consequences of conventional nitrogen-based fertilizers, such as groundwater contamination and greenhouse gas emissions (Adabembe et al., 2022; Akhtar et al., 2021). Furthermore, *Azolla* cultivation can be integrated into paddy fields, providing additional benefits such as weed suppression and enhanced water management (Ahmad et al., 2024; Hermawan et al., 2021).

Incorporating *A. microphylla* into agricultural systems as an environmentally friendly fertilizer alternative holds significant promise. The cumulative findings from various studies underscore its potential to contribute to sustainable agriculture, emphasizing the need for further research and practical applications to maximize its benefits.

CONCLUSION

This research contributes valuable insights to optimize *Azolla* cultivation, specifically *A. microphylla*, by revealing the intricate interplay between shading percentage and water depth. These factors influence temperature, light intensity, DO, and nutrient concentration, subsequently affecting *Azolla* growth. The findings not only advance our comprehension of *Azolla* cultivation but also foster ongoing research and the development of sustainable agricultural practices. Harnessing the nitrogen potential of *Azolla* presents an opportunity to contribute to an environmentally conscious and economically sustainable agricultural ecosystem, mitigating the environmental repercussions associated with synthetic fertilizers and reinforcing food security.

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Optimization of Medium for Lipid Production from *Lipomyces maratuensis* InaCC Y720 Using Statistical Experiment Design

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ABSTRACT

Lipomyces maratuensis InaCC Y720 is a potential novel oleaginous yeast. Media-based production optimization has never been carried out using this strain. This study aims to define an optimized medium from 12 medium component factors, where the Taguchi method is used for screening significant factors of medium and the response surface methodology (RSM) is used to optimize the concentration of significant factors. According to Taguchi, glucose, yeast extract, and magnesium sulfate ($MgSO_4$) have a significant influence on lipid accumulation, with their concentrations maintained at optimal levels through RSM optimization. Conversely, potassium dihydrogen phosphate, sodium hydrogen phosphate, and calcium chloride inhibit lipid accumulation, and copper(II) sulfate has the least influence, categorizing them as eliminated factors. The RSM-optimized medium increased lipid content by 3.6-fold compared to the initial medium. Glucose and yeast extract showed a positive correlation with lipid accumulation, suggesting potential for further optimization, while the optimum concentration for $MgSO_4$ was 0.15 g/L. This study is intended to serve as a reference for increasing lipid accumulation by *L. maratuensis* InaCC Y720.

Keywords: Initial media, nutrients, oleaginous yeast, optimized media, response surface methodology, Taguchi method

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INTRODUCTION

The expansion of microorganisms as renewable energy continues to be developed. One of them is the development of lipid accumulation from microbes. Microbial lipids, also known as single-cell oil (SCO), show the potential to substitute palm oil in biodiesel production due to their rapid

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production, minimal labor needs, resilience to seasonal changes, and ease of industrial-scale processing (El Kantar et al., 2021; Mhlongo et al., 2021). Microbes that can accumulate lipids of more than 20% from their dry biomass are referred to as oleaginous microorganisms (Anandan et al., 2016).

In the lipid accumulation by microbes, the composition of the media (macronutrients, micronutrients) and physical factors (temperature, pH, production time, and agitation) are crucial factors that can influence lipid accumulation (Duman-Özdamar et al., 2022; Sarkar et al., 2023). Lipid accumulation in oleaginous microorganisms occurs when nitrogen nutrients for cells are limited, and carbon is abundant (Qin et al., 2017). Regarding media composition, various macronutrients (C, N, Mg, P, Ca, Na, K) and micronutrients (Fe, Zn, Co, Cu, Mn) are combined to determine the effects of enhancement or inhibition on lipid production. Therefore, there is potential for higher lipid production under optimal cultivation media conditions.

In several studies optimizing cultivation media for lipid production by oleaginous microorganisms, statistical approaches such as the Taguchi method and response surface methodology (RSM) are utilized. The Taguchi method enhances the quality and performance of products and processes by systematically identifying and optimizing factors influencing variation. Meanwhile, RSM aims to study the relationship between input variables (factors) between input variables (factors) and the output response in complex systems. Based on the Taguchi method, it is known that glycine as an N source gives higher productivity (1.5 times), followed by urea and cellulose as a carbon source, significantly increasing biomass (2.4 times) and lipid (2.3 times) productivity in *Desmodesmus subspicatus* (Sarkar et al., 2023). Applying RSM, Duman-Özdamar et al. (2022) determined a carbon-to-nitrogen (C/N) ratio of 175 g/g for maximal oil production by *Cutaneotrichosporon oleaginosus* and a C/N ratio of 140 g/g for *Yarrowiaa lipolytica*.

Lipomyces maratuensis InaCC Y720 is a novel oleaginous yeast. It can produce up to 3.7 g/L of fatty acids when grown in a medium containing glucose and malt extract (Yamazaki et al., 2017). Based on its ability to accumulate high lipids, *L. maratuensis* InaCC Y720 has a potential for improvement through optimization of its growth medium, which has not been explored. For that reason, this study aims to define an optimized medium and determine the influence of component media on lipid production by *L. maratuensis* InaCC Y720 using the Taguchi method and RSM approach.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

Lipomyces maratuensis InaCC Y720 is an Indonesian culture collection (InaCC) isolated from Maratua Island, East Kalimantan, Indonesia (Yamazaki et al., 2017). The strain was grown on potato dextrose agar (PDA, Oxoid, United

Kingdom) and kept in a refrigerator. Seed culture was prepared by subculturing the yeast in potato dextrose broth (PDB, Oxoid, United Kingdom) at 28ºC with 200 rpm agitation for 48 hours.

Screening of Significant Medium Component using Taguchi

Medium components are screened using the Taguchi method with the "bigger is better" for the signal-to-noise ratio (SNR) parameter. The design of the experiment Taguchi consists of three levels and 11 factors (Table 1) that result in 27 medium variations. Level 2 medium compositions were based on the initial medium, according to Holdsworth and Ratledge (1988). All chemicals used in the medium were manufactured by Merck (Germany).

The experiment was carried out by inoculating 1 ml seed culture (OD_{600} = 0.8) into 50 ml varied medium in a 250 ml Erlenmeyer flask and then incubating at 28ºC with 200 rpm agitation for 72 hr. According to Bligh and Dyer (1959), yeast biomass and lipids were determined after 72 hr of incubation. All experiments were done in triplicates.

Optimization of Significant Factors Using RSM Method

Based on the Taguchi results, three significant factors (a, b, and c) were selected as independent variables and lipid production (mg/L) as dependent variables (y). The three significant factors are (a) glucose, (b) yeast extract, and (c) $MgSO_4$. Their concentrations were optimized using Box-Behnken design (BBD) and RSM. Each variable was studied in three levels (−1, 0, +1), and the experimental design included 15 runs with three replicates (Table 1). The mathematical relationship between the response variable (lipid production) and variables (a, b, and c) was projected according to the polynomial equation:

$$y = x + xa + xb + xc + xa^2 + xb^2 + xc^2 + xab + xac + xbc \quad \text{[Eq. 1]}$$

where, y = Dependent variables; x = Dependent variables; a = Glucose; b = Yeast extract; c = $MgSO_4$.

The precision of the above polynomial model was evaluated by the coefficient of determination (parity plot) and normal probability, and the *F*-test determined the statistical significance. The experiments

Table 1
Coded values of response surface methodology using three levels Box-Behnken design

Independent variable (g/L)	Symbol	Level		
		Low (-1)	Middle (0)	High (+1)
Glucose	a	30	50	70
Yeast extract	b	1.5	3.0	4.5
Magnesium sulfate	c	1.5	3.0	4.5

were carried out by adding 1 ml seed culture (OD_{600} = 0.8) to 50 ml medium in a 250 ml Erlenmeyer flask and then incubating at 28ºC with 200 rpm agitation for 72 hr. Biomass and lipids were collected after 72 hr and determined according to Bligh and Dyer (1959).

Growth Profile

The growth profile was observed by growing the yeast in 50 ml optimized medium (1 ml inoculum with OD_{600} = 0.8) at 28ºC and 200 rpm agitation for 72 hr. Biomass, lipid, glucose, and total nitrogen were measured every 12 hr. All experiments were done in triplicate.

Lipid Extraction and Analysis

Biomass was harvested by centrifugation (Hermle-Z326K, Germany) at 1,746 x *g* for 10 min, washed twice with 35 ml of distilled water, and dried in the oven at 70°C. Biomass is measured gravimetrically by comparing wet weight to dry weight. Lipid extraction was carried out according to Bligh and Dyer (1959). The cells were disrupted by adding 10 ml of 4 M hydrogen chloride (HCl, Merck, Germany) for 2 hr, then a mixture of chloroform (Merck, Germany) and methanol (Merck, Germany) (2:1 v/v ratio) was added before incubated for another 2 hr. The mixture was then centrifuged at 1,746 x *g* for 10 min, and the lower layer was transferred to a new glass vial and dried in the oven at 70°C. Lipid measured gravimetrically. The content of lipids was determined by comparing lipid weight to dry biomass weight, and lipid yield was determined by a gram of lipid per gram consumed glucose.

Analysis of Glucose Content

Reducing sugar is determined by the 3,5-dinitrosalicylic acid (DNS) method. DNS solution was carried out by dissolving 1 g DNS powder (Merck, Germany), 20 ml 2 M sodium hydroxide (NaOH, Merck, Germany), and 30 g Rochelle salt (Merck, Germany), then adding distilled water until the volume reached 100 ml. Reducing sugar concentration was measured by adding 1 ml sample, 1 ml DNS reagent (Merck, Germany), and 2 ml distilled water in a test tube heated for 1 min at 100°C in a water bath and then moving it into cold water. The absorbance of the sample was measured by a spectrophotometer at 540 nm (Wood et al., 2012).

Analysis of Total Nitrogen

The total nitrogen (%) in the sample was determined using the Kjeldahl method. Add 1 ml sample, 0.7 g catalyst (i.e., a combination of 250 g sodium sulfate [Na_2SO_4, Merck, Germany] + 5 g copper(II) sulfate [$CuSO_4$, Merck, Germany] + 0.7 g selenium [Merck, Germany]), and 4 ml concentrated sulfuric acid (H_2SO_4, Merck, Germany) into Kjeldahl flask, then destructed at 100°C in a fume hood until the color became light green. The sample cooled down, and 10 ml of distilled water was then distilled by adding 20 ml of sodium hydroxide-titanium (II) oxide (NaOH-TiO,

i.e., sodium hydroxide [NaOH 40%, Merck, Germany] + sodium thiosulfate [NaS_2O_3 5%, Merck, Germany]). The distillation used boric acid (H_3BO_3) 4%, which was given methyl red-bromo cresol green indicator (Mr-BCG, Smart Lab, Indonesia). This process ran until 60 ml distillate was obtained. 0.02 N hydrochloric acid (HCl, Merck, Germany) titrated the distillate. The residual nitrogen was measured as follows:

$$\text{Total nitrogen (\%)} = \frac{(\text{Titration volume} \times 0.02 \times 14)}{\text{Sample weight}} \times 100\% \quad \text{[Eq. 2]}$$

Data Analysis

The data was analyzed using Taguchi, RSM, and analysis of variance (ANOVA) on Minitab software (ver. 18) (Hamzaçebi, 2021; Roy, 1990).

RESULTS AND DISCUSSION

Screening of Significant Medium Component for Lipid Production by *L. maratuensis* InaCC Y720

The Taguchi method was utilized to screen significant factors and their concentration in the growth medium that affect lipid accumulation by *L. maratuensis* InaCC Y720. Subsequently, RSM was applied to optimize the concentrations of the identified significant factors as determined by the Taguchi method. *L. maratuensis* InaCC Y720 was cultured under 12 factors with three levels (Table 2) at 28℃ and 200 rpm for 72 hr. Biomass and lipids were collected for evaluation. Screening by Taguchi is presented in the SNR analysis of variance (Table 3) and the main effect plot for SNR (Figure 1).

SNR analysis of variance (Table 3) showed the significance and contribution of each factor to lipid accumulation. Three significant factors and their contributions were shown: yeast extract (16.88%), $MgSO_4$ (16.83%), and glucose (14.20%). The contribution of other factors is as follows: calcium chloride ($CaCl_2$, 10.69%), manganese sulfate ($MnSO_4$, 9.6%), zinc sulfate ($ZnSO_4$, 8.35%), sodium hydrogen phosphate (Na_2HPO_4, 7.93%), cobalt(II) nitrate ($Co(NO_3)_2$, 4.64%), potassium dihydrogen phosphate (KH_2PO_4, 3.61%), ammonium chloride (NH_4Cl, 3.36%), iron(III) chloride ($FeCl_3$, 1.98%), and copper(II) sulfate ($CuSO_4$, 1.36%).

Certain metal ions serve as micronutrients, supporting fungal growth and cellular metabolic activity. Zinc (Zn) deficiency impacts spore germination and fungal cell proliferation. Magnesium (Mg) functions as an intracellular divalent cation crucial for DNA and adenosine triphosphate (ATP) synthesis, as well as stimulating fatty acid synthesis. Mg deficiency in yeast can lead to distorted cell division, abnormal cell shape, and reduced viability, resulting in delays or alterations in the cell cycle. Mangan (Mn) serves various roles, such as acting as an intracellular regulator for enzymes, stimulating protein synthesis, and participating in thiamine biosynthesis. Iron (Fe) and copper (Cu) are recognized as cofactors. Phosphorus is a vital element in cells, commonly found in nucleic acids, phospholipids, and coenzymes, and can be stored as polymetaphosphate in cells.

Table 2
Factors and levels of experimental design Taguchi

No.	Factors	Level 1	Level 2	Level 3
1	D-glucose (g/L)	10	30	50
2	NH_4Cl (g/L)	0	0.5	1.0
3	Yeast extract (g/L)	0	1.5	3.0
4	KH_2PO_4 (g/L)	0	7.0	14.0
5	Na_2HPO_4 (g/L)	0	2.0	4.0
6	$MgSO_4.7H_2O$ (g/L)	0	1.5	3.0
7	$CaCl_2.2H_2O$ (g/L)	0	0.1	0.2
8	$FeCl_3.6H_2O$ (g/L)	0	0.008	0.016
9	$ZnSO_4.7H_2O$ (g/L)	0	0.001	0.002
10	$CuSO_4.5H_2O$ (g/L)	0	0.0001	0.0002
11	$Co(NO_3)_2.6H_2O$ (g/L)	0	0.0001	0.0002
12	$MnSO_4.5H_2O$ (g/L)	0	0.0001	0.0002

Note. NH_4Cl = Ammonium chloride; KH_2PO_4 = Potassium dihydrogen phosphate; Na_2HPO_4 = Disodium phosphate; $MgSO_4 \cdot 7H_2O$ = Magnesium sulfate heptahydrate; $CaCl_2 \cdot 2H_2O$ = Calcium chloride dihydrate; $FeCl_3 \cdot 6H_2O$ = Ferric chloride hexahydrate; $ZnSO_4 \cdot 7H_2O$ = Zinc sulfate heptahydrate; $CuSO_4 \cdot 5H_2O$ = Copper(II) sulfate pentahydrate; $Co(NO_3)_2 \cdot 6H_2O$ = Cobalt(II) nitrate hexahydrate; $MnSO_4 \cdot 5H_2O$ = Manganese(II) sulfate pentahydrate

Table 3
Analysis of variance of the mean plot for signal-to-noise ratio

Factors	df	SS	Contribution	MS	F	P
Glucose	2	194.025	14.200	97.012	24.630	0.039*
NH_4Cl	2	45.915	3.360	22.958	5.830	0.146
Yeast extract	2	230.573	16.880	115.287	29.270	0.033*
KH_2PO_4	2	49.313	3.610	24.657	6.260	0.138
Na_2HPO_4	2	108.388	7.930	54.194	13.760	0.068
$MgSO_4$	2	229.999	16.830	114.999	29.200	0.033*
$CaCl_2$	2	146.086	10.690	73.043	18.540	0.051
$FeCl_3$	2	27.118	1.980	57.056	3.440	0.225
$ZnSO_4$	2	114.113	8.350	9.315	14.490	0.065
$CuSO_4$	2	18.629	1.360	31.687	2.360	0.297
$Co(NO_3)_2$	2	63.374	4.640	65.465	8.050	0.111
$MnSO_4$	2	130.929	9.600	3.939		
Error	2	7.877	0.580			
Total	26	1,366.34	100			

Note. Df = Degrees of freedom; SS = Sum of squares; MS = Mean squares; * = Significant at P-value <0.05; NH_4Cl = Ammonium chloride; KH_2PO_4 = Potassium dihydrogen phosphate; Na_2HPO_4 = Disodium phosphate; $MgSO_4$ = Magnesium sulfate; $CaCl_2$ = Calcium chloride; $FeCl_3$ = Ferric chloride; $ZnSO_4$ = Zinc sulfate; $CuSO_4$ = Copper(II) sulfate; $Co(NO_3)_2$ = Cobalt(II) nitrate; $MnSO_4$ = Manganese(II) sulfate

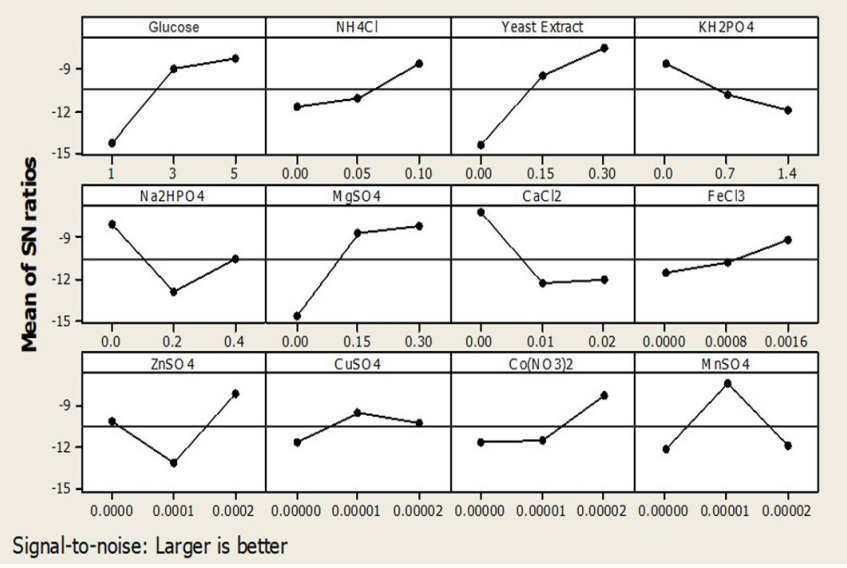

Figure 1. Main effect plot for signal-to-noise ratios (SNR) of lipid production using Taguchi method

Note. x-axis = Concentration of the factors (g/L) ; y-axis = The ratio of the mean (signal) to the standard deviation (noise) ; NH_4Cl = Ammonium chloride; KH_2PO_4 = Potassium dihydrogen phosphate; Na_2HPO_4 = Disodium phosphate; $MgSO_4$ = Magnesium sulfate; $CaCl_2$ = Calcium chloride; $FeCl_3$ = Ferric chloride; $ZnSO_4$ = Zinc sulfate; $CuSO_4$ = Copper(II) sulfate; $Co(NO_3)_2$ = Cobalt(II) nitrate; $MnSO_4$ = Manganese(II) sulfate

However, an excess concentration of metal ions can function as an inhibitor. Calcium (Ca) plays a role in metabolic responses, cell membrane stabilization, budding, and protein synthesis in cell walls. Two hypotheses explain the lipid-triggering effect of calcium deficiency. The first involves antilipolytic pathways mediated by the calcium-sensing receptor (CaSR), and the other centers on calcium ions' role in the basal sensitivity of the sterol-sensing mechanism in the sterol regulatory element binding protein (SREBP) pathway. Reduced calcium in the endoplasmic reticulum alters sterol distribution, enhancing SREBP activation and initiating neutral lipid synthesis (Arigony et al., 2013; Dzurendova et al., 2021; Ouedraogo et al., 2017; Wang et al., 2017). Studies regarding the effect of manganese on lipid accumulation in yeast are still few. Some studies suggest a positive impact on lipogenesis in *Mucor plumbeus* (Yoo et al., 1982), *Mortierella* sp. (Šajbidor et al., 1992), and *M. circinelloides* (under phosphorus-limiting conditions) (Dzurendova et al., 2020). However, in *Cunninghamella bainieri* 2A1, Mn appears to have no significant effect (Manikan et al., 2014).

Several studies highlight the influence of media components on lipid accumulation. A study by Shuib et al. (2014) showed that media containing ammonium tartrate, glucose, and metal ions (Mg^{2+}, Mn^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+}, Co^{2+}, and Zn^{2+}) increased lipid content by *C. bainieri*

2A1. In addition, it was observed that the cessation of lipid accumulation was caused by reduced enzyme activity as well as depletion of metal ion concentrations in the medium. A study by Zhao et al. (2016) showed that cobalt significantly inhibited the growth of *Y. lipolytica* but led to a slight increase in lipid content. Consistent findings from past to present studies suggest that phosphate limitation consistently increases lipid content. It was reported in *Rhodotorula glutinis* (Granger et al., 1993), *Rhodosporidium toruloides* Y4 (Wu et al., 2010), and a recent study by Morales-Palomo et al. (2023) revealed that *Y. lipolytica* ACA DC 50109 under phosphate limitation (0 g/L), the lipid content and lipid yield increased to 44.4% w/w lipid per dry weight. Phosphate ions (PO_4^{3-}) are vital for cofactors, phosphorylated proteins, and RNA/DNA synthesis. Limitation in PO_4^{3-} significantly impact cellular physiology and metabolism, hindering biomass production while promoting lipid accumulation (Wang et al., 2017). Dzurendova et al. (2020) studied the effects of metal in phosphorus-limiting media on lipid accumulation by *M. circinelloides*. Key points include Zn^{2+} enhancing biomass, Mg^{2+} optimizing both biomass and lipid production, Fe^{3+} deficiency causing inhibited growth, and reported lipid increase with Ca and Cu deficiency.

This study applied the "larger is better" approach in Taguchi for the main effect plot, where higher points on the horizontal axis signify increased lipid accumulation. The main effects observe each level concentration and offer valuable insights for factor and level assessments (Minitab, n.d.a). This plot draws trends and shows how each level influences *L. maratuensis* InaCC Y720 lipid accumulation. It makes this a consideration for this research in evaluating and determining the factors and their levels that will be optimized in RSM.

This study divides the factors into three categories: (1) optimized materials, (2) maintained materials, and (3) eliminated factors. Based on Figure 1, the higher the lipids accumulated, the more significant factors showed higher concentrations. Therefore, they are optimized with RSM and increased by one level (concentration) to find the optimum level. $MnSO_4$, $ZnSO_4$, $Co(NO_3)_2$, NH_4Cl, and $FeCl_3$ were categorized as maintained factors. They showed a contribution and a trend of increasing levels directly proportional to the increase in lipid accumulation. Therefore, Level 3 of them was applied in RSM even for NH_4Cl as the main N source, except $MnSO_4$ at Level 2. For the factors eliminated are KH_2PO_4, Na_2HPO_4, $CaCl_2$, and $CuSO_4$. Adding KH_2PO_4, Na_2HPO_4, and $CaCl_2$ in the media showed the highest lipid accumulation at the lowest level. It means that a higher concentration can inhibit lipid accumulation. However, at certain concentrations, it may increase lipid accumulation. While $CuSO_4$ showed the lowest percentage of contribution to increasing lipid accumulation.

Optimization of Conditions to Increase Lipid Production in *L. maratuensis* InaCC Y720

Based on our consideration and explanation above, the new formula medium for optimization in RSM contained (g/L) 0.1 NH_4Cl, 0.0016 $FeCl_3$, 0.00002 $ZnSO_4$, 0.00002 $Co(NO_3)_2$, and 0.00001 $MnSO_4$. This study applied the BBD in RSM, resulting in 15 variations. The BBD involves fitting a second-order polynomial equation to experimental data, enabling us to identify optimal conditions for the desired outcome. The experimental responses are presented in Table 4.

In the BBD experimental design, the ninth design was predicted to yield the highest lipids (1.68 g/L), but both the second and ninth designs in the experiments produced 1.66 g/L. With small residual values and no significant deviation from the prediction model, the second design was selected as the optimized media composition, utilizing less glucose (50 g/L compared to 70 g/L in the ninth design). Papanikolaou et al. (2002) explained that each microorganism has a different tolerance level to glucose concentrations in the environment. High initial glucose concentrations allow inactivating enzymes involved in lipid synthesis in some microorganisms. Studies by Mondala et al. (2012), 40 and 60 g/L glucose showed no difference in lipid production. In Braunwald et al. (2013), after 48 hr of incubation, no significant differences were noted among C/N ratios (glucose and NH_4^+) of 20, 70, and 120 by *R. glutinis*. However, higher lipid accumulation was apparent after 120 hr of incubation.

Table 4
Optimizing Lipomyces maratuensis InaCC Y720 *lipid content and comparing data with model predictions*

Run	Glucose (g/L)	Yeast extract (g/L)	$MgSO_4$ (g/L)	Lipid content (g/L)		
				Experiment	Predicted	Residues
1	50	4.5	4.5	0.40	0.49	-0.09
2	50	4.5	**1.5**	**1.66**	**1.61**	**0.05**
3	70	3.0	4.5	0.56	0.45	0.11
4	30	3.0	4.5	0.76	0.73	0.03
5	70	1.5	3.0	0.48	0.54	-0.06
6	50	3.0	3.0	1.03	0.87	0.16
7	50	1.5	1.5	0.45	0.36	0.09
8	30	4.5	3.0	0.99	0.92	0.07
9	**70**	**3.0**	**1.5**	**1.66**	**1.68**	**-0.02**
10	30	3.0	1.5	0.59	0.70	-0.11
11	50	3.0	3.0	0.99	0.87	0.12
12	70	4.5	3.0	1.31	1.33	-0.02
13	30	1.5	3.0	0.27	0.25	0.02
14	50	1.5	4.5	0.24	0.28	-0.04
15	50	3.0	3.0	0.59	0.87	-0.28

Note. $MgSO_4$ = Magnesium sulfate; The highlighted figures = Highest results in the experiments

The RSM can estimate the equation model of the medium concentration to obtain the optimum response of lipid accumulation. The experiment of the BBD was fitted with polynomial regression (Table 5) as follows:

$$y = -2.6187 + 0.2537a + 9.6074b + 7.5336c + 0.0122a^2 - 6.9568b^2 - 1.2901c^2 + 0.952ab - 1.0560ac - 11.5873bc \quad \text{[Eq. 3]}$$

where, y = lipid yield (g/L), a = glucose concentration (g/L), b = yeast extract concentration (g/L), c = $MgSO_4$ concentration (g/L).

Based on the ANOVA of the polynomial equation model (Table 5), yeast extract and the interaction of glucose*$MgSO_4$ and yeast extract*$MgSO_4$ exhibit p-values of less than 0.05, signifying their significant impact on lipid accumulation. The lack-of-fit value is crucial for assessing the reliability of the regression model derived from the experimental design (Minitab, n.d.c). In this case, the model's lack-of-fit *p*-value is greater than 0.05, indicating no significant difference. It implies that the response data aligns well with the model and that no statistically significant difference exists in responses.

In the contour plot (Figure 2), the correlation between glucose and yeast extract

Table 5
Analysis of variance for the polynomial model for optimization of lipid production (P<0.05)

Factors	df	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	9	2.811	2.811	0.312	9.000	0.013*
Linear	3	2.029	0.404	0.135	3.880	0.089
Glucose	1	0.024	0.029	0.029	0.830	0.403
Yeast extract	1	1.066	0.296	0.296	8.520	0.033*
$MgSO_4$	1	0.717	0.182	0.182	5.240	0.071
Square	3	0.106	0.106	0.035	1.020	0.459
Glucose*Glucose	1	0.015	0.009	0.008	0.250	0.636
Yeast extract*Yeast extract	1	0.088	0.090	0.090	2.610	0.167
$MgSO_4$*$MgSO_4$	1	0.003	0.003	0.003	0.090	0.777
Interaction	3	0.676	0.676	0.225	6.500	0.035*
Glucose*Yeast extract	1	0.003	0.003	0.003	0.090	0.771
Glucose*$MgSO_4$	1	0.401	0.401	0.401	11.570	0.019*
Yeast extract*$MgSO_4$	1	0.272	0.272	0.272	7.830	0.038*
Residual error	5	0.173	0.173	0.034		
Lack-of-fit	3	0.055	0.055	0.018	0.310	0.821
Pure error	2	0.118	0.118	0.059		
Total	14	2.985				

Note. R^2 = 0.9419; df = Degrees of freedom; Seq SS = Sequential sum of squares; Adj SS = Adjusted sum of squares; Adj MS = Adjusted mean square; * = Significant at *P*-value <0.05; $MgSO_4$ = Magnesium sulfate

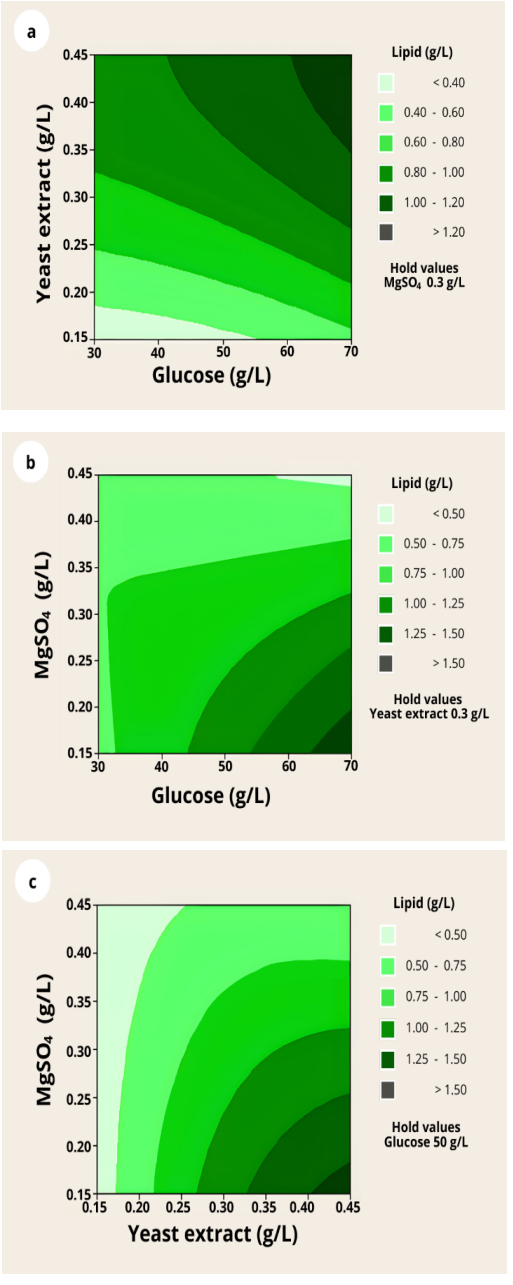

Figure 2. Contour plot of the glucose, yeast extract, and magnesium sulfate ($MgSO_4$) interaction in lipid accumulation. (a) Glucose*Yeast extract, (b) Glucose*$MgSO_4$, and (c) Yeast extract*$MgSO_4$

indicated that elevating the concentration increases lipid accumulation. While $MgSO_4$ showed the opposite interaction. Based on Taguchi and RSM, 0.15 g/L is the optimum concentration for $MgSO_4$. Glucose and yeast extract have not reached their peak point, meaning they can be increased to determine the optimal concentration. However, as explained above, longer incubation may be necessary at higher glucose concentrations. Glucose is a carbon source for forming lipid chains, and a high C/N ratio has been proven to stimulate increased lipid production (Lopes et al., 2020). Yeast extract contains amino acids and trace elements essential for biomass, growth, and lipid metabolism. These include glycine, proline, histidine, alanine, tyrosine, cysteine, arginine, asparagine, glutathione, dextran, mannan, trehalose, vitamin B, Fe^{3+}, Mg^{2+}, Mn^{4+}, K^{+}, Se^{2-}, Na^{+}, Zn^{2+}, Ca^{2+}, and Cu^{2+} (Ardiyanti & Guntoro, 2019; Tomé, 2021). In lipid metabolism, B vitamins play a role. Pyridoxine (B6) functions as a coenzyme, and inositol (B8) is a precursor of phosphatidylinositol, the main constituent of phospholipid membranes (Perli et al., 2020).

The model's statistical analysis included an assessment of accuracy and the normality of the residuals. The R^2 value indicates the polynomial equation's suitability to the experimental data. The parity plot (Figure 3) shows an R^2 value of 94.19%, meaning that the polynomial model cannot explain only around 5.81% of the total variation.

In addition, the residual data were analyzed using the Kolmogorov-Smirnov. It evaluates whether the residuals' distribution

significantly deviates from a normal distribution (Minitab, n.d.b). Figure 4, the probability plot of lipids showed a *p*-value of 0.085 (> 0.05). It suggests that the prediction model is not statistically significant for the experiment, indicating that the distribution of the residuals does not significantly differ from a normal distribution.

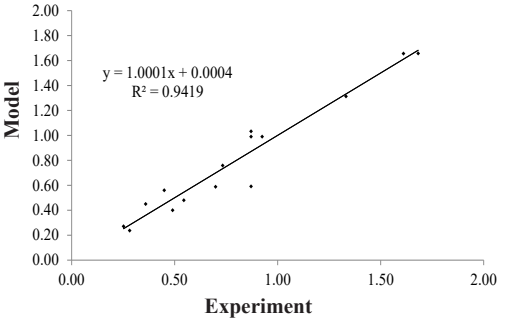

Figure 3. Parity plot of the model to the experiment (R^2 = 0.9419)

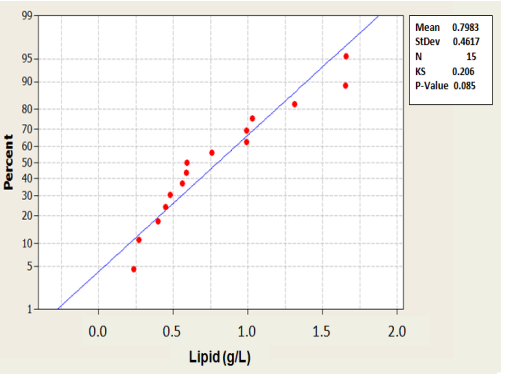

Figure 4. Normal probability plot for lipid production

Note. StDev = Standard deviation; N = Sample sizes or number of observations; KS = Kolmogorov-Smirnov

Profile of Lipid, Biomass, and Consumption of Carbon and Nitrogen

This study compares the profile of lipid, biomass, consumption of carbon and nitrogen of *L. maratuensis* InaCC Y720 in both media. The optimized medium by RSM, determined as the highest lipid production, comprises the following concentrations in g/L: 5.0 glucose, 0.45 yeast extract, 0.15 $MgSO_4$, 0.1 NH_4Cl, 0.0016 $FeCl_3$, 0.00002 $ZnSO_4$, 0.00002 $Co(NO_3)_2$, and 0.00001 $MnSO_4$. The results of this comparison are presented in Figure 5.

The optimized medium obtained 5.26 g/L biomass, 1.57 g/L (31.7%) lipid, with a yield of 0.0157, while the initial medium obtained 4.96 g/L biomass, 0.43 g/L (8.7%) lipids, with a yield of 0.0043. The optimized medium exhibited a significant 3.6-fold increase in lipid content. This improvement is attributed not only to the optimization of glucose, yeast extract, and $MgSO_4$ concentrations by RSM but also to the exclusion of KH_2PO_4, Na_2HPO_4, $CaCl_2$, and $CuSO_4$ based on Taguchi selection.

In this study, a decrease in lipid content percentage was observed (despite an increase in biomass) in the media after 60 hr, both in the initial and optimized media (Figure 5). It might be caused by the exhaustion of carbon in media that was detected to be depleted before 48 hr (Figure 6). Similar findings were reported by X. Zhang et al. (2017); there was a decrease in lipid content after 48 hr on glucose and 60 hr on glycerol. At the same time, the carbon source was detected as exhausting after 36 hr for the glucose medium and 48 hr for the glycerol medium, respectively.

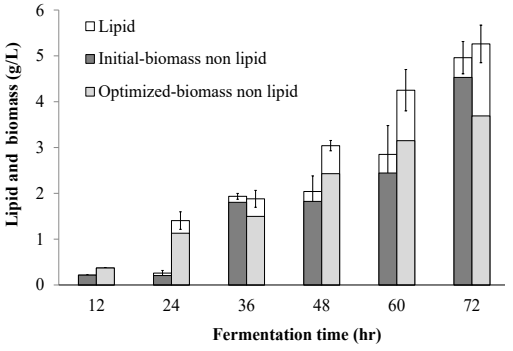

Figure 5. Lipid and biomass on initial and optimized medium

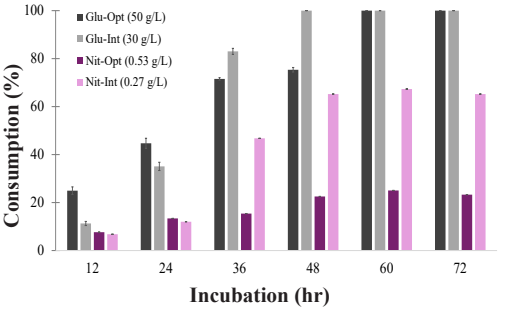

Figure 6. Glucose and nitrogen consumption (%) in initial and optimized media

Note. Glu = Glucose; Nit = Nitrogen; Opt = Optimized media; Int = Initial media

This study measured nitrogen consumption rates to identify when nitrogen limitation triggers accumulation (Figure 6). After 72 hr of incubation, nitrogen consumption reached 65.21% in the initial medium and 23.30% in the optimized medium. It assumed that the Kjeldahl Method is not suitable for this. It only measures nitrogen bound to organic components (proteins, amino acids, nucleic acids) and ammonium in samples (Muñoz-Huerta et al., 2013). In media, NH_4Cl is the main source of inorganic nitrogen, and yeast extract is the source of organic nitrogen and vitamins. It assumed that NH_4Cl was depleted in the media and that nitrogen was detected from extracellular enzymes and yeast extract. It is supported by the optimized media, which has a higher yeast extract concentration, thus explaining why lipid accumulation occurred when nitrogen was still present in this study.

L. Zhang et al. (2022) explain some effective strategies for enhancing lipid yields, such as optimizing parameters, employing two-stage systems, metabolic engineering, selective mutagenesis, and co-culture systems. Parameter optimization including physical parameters (agitation speed, temperature, inoculum age and size, optical density (OD), operation modes, feeding strategies, and fermentation period) and chemical parameters (concentration and source of carbon and nitrogen, C/N ratio, pH, micronutrients, and inhibitor concentration). In our study, some chemical parameters are optimized.

Yamazaki et al. (2017) reported that *L. maratuensis* InaCC Y720 accumulated 3.70 g/L total fatty acids after ten days in the 5G5M medium (50 g/L glucose, 50 g/L malt extract). This study only achieved 43% of that amount (1.66 g/L) within three days. It has shorter incubation and uses fewer carbon sources. Nonetheless, this research successfully optimized the Bligh Dyer medium for lipid accumulation by *L. maratuensis* InaCC Y720. It has optimized the concentration of media components and eliminated components considered inhibitors of lipid accumulation by *L. maratuensis* InaCC Y720.

Lipomyces, especially *L. starkeyi*, is a frequently utilized species in lipid production research using oleaginous yeast. *L. starkeyi* InaCC Y604 (isolated from Indonesia) grown in nitrogen-limited mineral medium (-NMM) ($MgSO_4$ 1.5 g/L, KH_2PO_4 7 g/L, Na_2HPO_4 5 g/L, $FeSO_4$ 0.08 g/L, $ZnSO_4$ 0.01 g/L, $CaCl_2$ 0.1 g/L, $MnSO_4$ 0.1 g/L, $CuSO_4$ 0.002 g/L, and $CoCl_2$ 0.002 g/L) containing glucose (50 g/L) and xylose (50 g/L) achieved lipid content and biomass 17% (w/w) and 25.02 g/L (60 hr), respectively (Agustriana et al., 2020). In a subsequent study, Juanssilfero et al. (2021) introduced variations in other carbon sources in the design (fructose, galactose, mannose, and cellobiose). It showed mixed glucose and xylose still the highest even compared with a single carbon source (lipid content and biomass of 64.19% (w/w) and 34.49±0.38 g/L [60 hr]).

In contrast, in this study, the attained lipid content and biomass were only 31% (w/w) and 5.25 g/L (60 hr). Apart from genetic differences, variations also exist in the C/N ratio, carbon concentration, sources, and extraction methods. Nevertheless, the potential and lipid production capabilities of *L. maratuensis* InaCC Y720 remain unexplored and open to optimization.

CONCLUSION

This study enhanced lipid production by *L. maratuensis* InaCC Y720 by applying Taguchi and RSM designs. The optimized medium resulted in a 3.6-fold increase in lipid production compared to the initial medium. Glucose, yeast extract, and $MgSO_4$ emerged as significant factors influencing lipid accumulation. $MnSO_4$, $ZnSO_4$, $Co(NO_3)_2$, NH_4Cl, and $FeCl_3$ showed a positive influence. KH_2PO_4, Na_2HPO_4, and $CaCl_2$ inhibit lipid accumulation, and $CuSO_4$ has the lowest contribution. According to RSM results, the optimized medium comprises the following concentrations in g/L: 5.0 glucose, 0.45 yeast extract, 0.15 $MgSO_4$, 0.1 NH_4Cl, 0.0016 $FeCl_3$, 0.00002 $ZnSO_4$, 0.00002 $Co(NO_3)_2$, and 0.00001 $MnSO_4$.

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Studies on Pre-harvest Spray of Alpha-NAA on Potato Crop in Relation to Enhance Potato (*Solanum tuberosum* L.) Tubers Storage

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ABSTRACT

The present investigation on a pre-harvest spray of alpha-1-naphthalene acetic acid (alpha-NAA) on potato crops in relation to improving the storage ability was undertaken in the Botany Department of Kurukshetra University, Kurukshetra, India on *Solanum tuberosum* cv. 'Kufri Chandermukhi'. Sprout initiation was observed in tubers on the 20th day, with 4.0 x 10^{-4} M application of alpha-NAA during storage. In control, it was prominently noticed on the first observation made on the 10th day (0.8 mm), whereas in the treated one, it was very small. These treatments were able to check the percentage of sprouting. Rottage was observed after the 40th day of storage. The decline in starch content was less in the treatment group than in the control group up to the 20 days, but a reverse trend was witnessed after that compared with initial values. The starch contents were significantly higher in treated tubers than untreated in most stages. After 40 days, per cent cumulative physiological weight loss values were 9.20, 9.62, and 10.33% in 4.0 x 10^{-4} M alpha-NAA, 5.5 x 10^{-4} M alpha-NAA, and control, respectively.

Keywords: Alpha-NAA, pre-harvest spray, potato, sprouting, storage

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INTRODUCTION

Potato (*Solanum tuberosum* L.) is a stem tuber crop belonging to the family Solanaceae. It is a unique crop that can supplement mankind's food needs substantially (Scott et al., 2019). In world history, potatoes have been a well-known food commodity, and whenever there has been an insufficiency of food grains for people, the potato has come to rescue life (Neeraj et al., 2019). It produces well-balanced protein content and additional calories/unit area and is produced in less per unit time than other cereal crops. Therefore, it is the most suitable non-traditional crop to ward off hunger. Potato tubers are important for human food and nutrition, employment, and income in Africa, Asia, and Latin America, which is self-apparent from the steady expansion in the area and production of tubers in developing countries (Devaux et al., 2020).

Out of the total dry matter accumulation in potatoes, about 80% is starch, which mainly contributes to the calorific value of the potato. Sugars occur in varying quantities, being very low at harvest, and could increase to a very high level at the end of the storage period (Neeraj et al., 2019). Potato proteins are better than cereal proteins because of their balanced amino acid composition, equivalent to some proteins of animal origin (Kapoor et al., 1975). Among the various vitamins in potatoes, vitamin C is the largest.

Asian farmers produce potatoes during the *rabi* season and store them for hot summers. The coming sowing season depends upon storage conditions to guarantee a sufficient and balanced supply of this fragile product (seed and table potatoes) (Lu et al., 2012). The potato tubers' storage is not only important from the marketing point of view, but it is also required for their utilization as seed potatoes for crop cultivation. In India, refrigerated storage facilities are expensive, insufficient, and unevenly distributed; therefore, during surplus availability of potato tubers in the vegetable market, growers get big financial losses as well as waste of commodity.

According to Kaguongo et al. (2014), the post-harvest tuber losses at farmer's fields, vegetable markets, and processing facilities are estimated at 12.8, 24.4, and 25.0%, respectively. Sprouting is mainly responsible for weight loss as well as degradation in the marketable quality of potato tubers and seed tubers (Suttle, 2003). Problems related to storage have been identified as the major constraints in achieving a further increase in the production of potatoes in India (Neeraj et al., 2019). The potato seed industries rely mainly on stored tubers. Losses in stored potatoes are due to sprouting, shrinkage, and rotting. Therefore, adequate storage infrastructure is necessary to prevent potato rot, sprouting, and physiological weight loss. The time of onset of sprouting is determined by the length of the dormancy period of the tubers. Shrinkage occurs due to the loss of water from tubers to air in stores along a water vapour pressure gradient and the consumption of respiratory substances. The main purpose of potato storage is to maintain tubers in their most edible and saleable condition.

Not all farmers have access to a refrigerated cold storage facility in nearby areas. Due to this lack of facility, tubers sprout during long-duration storage (Foukaraki et al., 2016), and this problem may be solved with the help of sprout inhibitors and growth regulators. Therefore, the present study was carried out on a pre-harvest spray of alpha-NAA on a potato crop to improve its storage ability.

MATERIALS AND METHODS

Experimental Crop and Application of Alpha-NAA

'Kufri Chandermukhi' was selected as a plant material for the present investigation, undertaken in the Botany Department of Kurukshetra University, Kurukshetra, *Solanum tuberosum* cv. seed tubers were obtained from Central Potato Research Institute, Substation Modipuram (Utter Pradesh). The experimental crop was grown in nine beds in the University Botanical Garden, Kurukshetra University, Kurukshetra (N 29° 57' 26.3124", E 76° 48' 59.742"). The area of each experimental bed was 1 x 3 m^2.

Three beds were selected for applying each alpha-NAA concentration (4.0 x 10^{-4} and 5.5 x 10^{-4} M, ChemSupply, Australia) as pre-harvest treatments six weeks before harvesting. Three beds were sprayed with distilled water and maintained as controls. No rainfall was received after the application of treatments until the harvesting of tubers, although arrangements were made for rain in this area.

Tubers Storage and Observations

The crops were lifted when haulms died down naturally. Harvesting operations were done in dry weather with hand hoes. After harvesting, potatoes were spread on the floor at room temperature for five days.

Potato tubers from treated and control plants were collected separately, kept in cold storage (1–3°C with 90%RH) for 120 days, and then brought to the laboratory for physiological and biochemical analysis at room temperature 30±2°C.

Physiological Analysis

After 120 days of cold storage, periodic observations up to 40 days at room temperature were made for percentage crude protein weight loss (CPWL), sprouting behaviour and percentage rottage after a regular interval of 10 days using 70–90 g weight category of potato tubers.

Biochemical Analysis

Biochemical analysis was carried out on starch, sugars (reducing, non-reducing, and total), ascorbic acid, and protein. The specific enzymatic activity of alpha-amylase and peroxidase was also determined (Association of Official Analytical Chemist [AOAC], 1990).

RESULTS AND DISCUSSION

Sprouting and Spoilage

Sprouting behaviour results presented in Table 1 reveals that pre-harvest alpha-NAA treatment had slightly delayed the initiation of sprouts. It also greatly reduced

the number of sprouts per tuber. The number of sprouts was brought down from 3.70 to 1.41, 4.76 to 2.11, and 7.21 to 5.01 per tuber by the lower concentration of this growth regulator (4.0×10^{-4} M) on the 20th, 30th, and 40th days, respectively. Sprout initiation was observed on the 20th day in tubers, which had 4.0×10^{-4} M application of alpha-NAA. In control, it was prominently noticed on the very first observation made on the 10th day (0.8 mm), whereas in the treated one, it was very small. These treatments were able to check the percentage of sprouting. Rottage was observed after the 40th day of storage.

Per cent cumulative physiological weight loss (%CPWL) data reveals that alpha-NAA treatment significantly influenced the %CPWL of tubers during room-temperature storage. After 40 days, values were 9.20, 9.62, and 10.33% in 4.0×10^{-4} M alpha-NAA, 5.5×10^{-4} M alpha-NAA, and control, respectively.

The significant results and interpretation of the present experimentation concurrs with those of previous research workers (Birbal et al., 2009). They found a decline in the total and physiological weight reduction in the tubers using growth regulators, i.e., gibberellic acid and auxins (NAA and indole-3-butanoic acid [IBA]), applied to the foliage. Additionally, the reduction in total weight loss resulting from applying gibberellins may be attributed to decreased activity of enzymes responsible for the hydrolysis of cell walls, as Miceli et al. (2019) observed.

These findings strongly agree with the previous research by Birbal et al. (2009), who also reported a reduction in tuber rottage with gibberellic acid application compared to the control. The reduction in sprouting with pre-application of gibberellic acid may develop the thicker cuticle (thick tuber skin) with more dry matter accumulation in the potato tubers. These structural modifications are apparent in reducing post-harvest losses coupled with the sprouting feature (Miceli et al., 2019). However, Nyankanga et al. (2018) observed that applying pre-ethrel could augment the sprouting percentage in potato tubers during storage.

According to Wang et al. (2009), the increase in the potato tubers dry matter content is accompanied with the application of growth regulation treatments. Likewise, Alexopoulos et al. (2006) also studied the effect of gibberellic acid on potato crop growth and development, and they reported that the time of spray of gibberellic acid is also important; it imparts the high accumulation of dry matter in haulms, leaves, and especially in below ground part (potato tubers) and more deposition of dry matter related to higher carbohydrate accumulation and sugars.

Changes in Biochemical Parameters

The protein content of tubers also shows a decreasing trend with an increase in the storage period (Table 2). Higher values were recorded in the cortex than in the pith. The protein value was lower in treated tubers than in control tubers. A maximum decrease in protein content was recorded during 30-40 days in untreated and treated tubers.

Ascorbic acid results based on both concentrations were able to reduce the loss of ascorbic acid, thus reflecting a gradual decrease during storage in all the tubers. The increment in ascorbic acid in the cortex of the tubers treated with 4.0 x 10^{-4} M alpha-NAA was significant compared with the control on the 20th, 30th, and 40th days. The amount was much higher in the pith than in the cortex.

Starch values initially were 57.48-58.41, 65.49-68.23, 68.13-68.83 mg/100 mg on a dry weight basis in control, 5.5 x 10^{-4} and 4.0 x 10^{-4} M alpha-NAA treated tubers, respectively, in pith-cortex regions. Significant decline was noticed in room temperature storage in treated and control tubers. The decline was less in the treatment group than in the control group up to the 20 days, but a reverse trend was witnessed after

Table 1
Effect of pre-harvesting alpha-naphthalene acetic acid treatment on sprouting, rotting, and water losses of potatoes at room temperature storage (30±2°C)

Storage days	Treatments	Per cent sprouting	Number of sprout/ tuber	Length of longest sprout (mm)	Range of sprout length (mm)	Per cent rotting	Per cent cumulative physiological weight loss
10	Control	13.50	1.21	0.80	J - 0.80	-	1.50
	4.0 x 10^{-4} M NAA	-	-	-	-	-	1.14
	5.5 x 10^{-4} M NAA	-	-	-	J - 0.20	-	1.19
20	Control	28.30	3.70	5.00	J - 3.00	-	3.48
	4.0 x 10^{-4} M NAA	15.00	1.41	2.00	J - 1.43	-	3.08
	5.5 x 10^{-4} M NAA	18.20	1.81	2.90	J - 2.10	-	3.36
30	Control	37.30	4.76	8.00	J - 8.00	-	7.07
	4.0 x 10^{-4} M NAA	30.10	2.11	5.42	J - 5.42	-	6.02
	5.5 x 10^{-4} M NAA	33.20	2.45	7.20	J - 7.20	-	6.44
40	Control	52.10	7.21	13.20	J - 13.20	50	10.33
	4.0 x 10^{-4} M NAA	48.80	5.01	10.00	J - 10.00	10	9.20
	5.5 x 10^{-4} M NAA	49.20	5.21	11.40	J - 11.40	15	9.62

Note. J = Just initiation; NAA = Naphthalene acetic acid

that when compared with the initial values. The starch contents were significantly higher in treated tubers than untreated in most stages.

Sugar content data showed increased total sugars with prolonged storage at room temperature. The pith region exhibited a slightly higher sugar content than the cortex. As the storage period advanced, the increase in total sugars was greater in treated tubers than in the control when the differences were calculated on the 40th day in cortex-pith from initial values.

The increase in total sugars was mainly due to reduced and non-reducing sugars in treated and untreated. The results showed an increase in reducing sugars with an increase in storage days. From the start, from zero-day onwards, there was no reduction in sugars; however, there was a decline with the increase in storage period. The pith region has a lower value than the cortex. The increase was significant from control to treated tubers on the 20th and 30th days. Potato tubers' total soluble solids (TSS) content is chiefly total sugars dominated and a small fraction of soluble protein, amino acids, and several other biological compounds (Bexiga et al., 2017).

The study implies that the spray of growth regulators on potato crops did not impart any considerable effect on the potato tubers' TSS content during the storage period. It may be because TSS content mainly depends on the inherent potential of variety, agronomic interventions, fertilizer application, and metabolic or functional status; hence, it was not inclined by growth regulators application. These results align with the earlier results of Sarkaria and Chinna (2021); they also observed that the TSS content in tubers was not significantly enhanced by using growth regulators application.

Changes in Specific Enzyme Activities

In the present study, the specific activity of alpha-amylase was significantly increased during storage in treated and control tubers for up to 30 days (Table 2). However, activity was lower in treated tubers than control ones; after 30 days, it registered a further decline in the case of 4.0 x 10^{-4} M alpha-NAA-treated ones. Pith samples exhibited higher activity than the cortex.

The specific activity of peroxidase was comparatively lower in treated than untreated tubers. The specific activity of peroxidase enzyme increased up to 20 days in untreated tubers, after which a gradual decline was noticed. Treated tubers registered a sharp fall at ten days, followed by further decline up to 40 days. The difference between the two concentrations of alpha-NAA was less marked. Peroxidase activity was comparatively lower in treated than untreated tubers.

The increase in total phenol content in potato tubers with applying gibberellic acid may be accredited to a resultant increase in the antioxidant activity associated with phenolic compounds, as Gilani et al. (2021) reported. The potato seed tubers, which are recently harvested, contain about 70% water and are more vulnerable to rot, blemish diseases, and galls while being stored under

Table 2
Effect of pre-harvesting alpha-naphthalene acetic acid treatment on biochemical changes of potato tubers at room temperature storage (30±2°C)

Treatment	Tuber part	0 day	10th day	20th day	30th day	40th day
Protein changes (mg/100 mg dry weight)						
Control	Pith	6.48±0.28	5.18±0.02	4.55±0.08	4.25±0.18	2.97+0.19
	Cortex	6.85±0.30	5.89±0.20	5.16±0.02	4.89±0.18	3.82±0.10
4.0 x 10^{-4} M NAA	Pith	5.87±0.26	4.81±0.04	4.10±0.18	3.57±0.04	2.51±0.02
	Cortex	6.16±0.27	5.38±0.01	4.51±0.01	3.92±0.01	3.32±0.09
5.5 x 10^{-4} M NAA	Pith	6.19±0.04	4.93±0.10	4.33±0.02	3.92±0.02	2.73±0.19
	Cortex	6.56±0.04	5.63±0.20	4.94±0.17	4.55±0.09	3.73±0.10
Ascorbic acid changes (mg/100 mg dry weight)						
Control	Pith	54.45±0.03	52.71±0.02	48.45±0.03	45.85±0.04	39.79±0.03
	Cortex	51.45±0.02	49.67±0.02	45.18±0.01	42.95±0.02	37.54±0.03
4.0 x 10^{-4} M NAA	Pith	57.14±0.02	53.19±0.02	49.54±0.02	47.35±0.03	41.85±0.03
	Cortex	53.65±0.04	50.16±0.03	47.81±0.04	45.25±0.02	40.39±0.04
5.5 x 10^{-4} M NAA	Pith	55.67±0.03	54.81±0.01	49.52±0.05	47.38±0.03	42.39±0.03
	Cortex	52.56±0.04	51.76±0.02	47.81±0.01	44.52±0.01	40.18±0.05
Starch changes (mg/100 mg dry weight)						
Control	Pith	57.48±0.33	43.83±0.56	43.61±0.31	39.33±0.91	37.52±0.63
	Cortex	58.41±0.85	50.47±0.83	43.47±0.51	42.83±0.13	41.61±0.66
4.0 x 10^{-4} M NAA	Pith	68.13±0.29	62.61±0.25	52.40±0.24	42.66±0.31	37.83±0.13
	Cortex	68.82±0.09	65.61±0.50	53.48±0.20	45.55±0.23	38.92±0.22
5.5 x 10^{-4} M NAA	Pith	65.49±0.01	57.16±0.59	53.40±0.48	42.65±0.41	35.88±0.39
	Cortex	68.23±0.11	57.40±0.50	56.03±0.26	46.55±0.80	37.22±0.22
Total sugars (mg/100 mg dry weight)						
Control	Pith	6.20±0.01	6.37±0.01	7.10±0.02	7.45±0.02	8.20±0.01
	Cortex	6.03±0.02	6.23±0.01	7.06±0.03	7.25±0.01	8.05±0.01
4.0 x 10^{-4} M NAA	Pith	6.49±0.01	7.14±0.01	8.16±0.01	9.15±0.28	10.27±0.01
	Cortex	6.37±0.02	7.04±0.02	8.04±0.00	9.24±0.03	10.20±0.01
5.5 x 10^{-4} M NAA	Pith	6.48±0.01	6.85±0.03	7.37±0.01	8.22±0.01	9.32±0.01
	Cortex	6.27±0.01	6.73±0.01	7.44±0.02	8.11±0.01	9.27±0.01
Reducing sugars (mg/100 mg dry weight)						
Control	Pith	3.07±0.03	3.42±0.06	4.99±0.19	5.39±0.13	6.32±0.09
	Cortex	2.74±0.01	3.16±0.07	4.49±0.11	4.73±0.13	6.04±0.13
4.0 x 10^{-4} M NAA	Pith	3.37±0.08	4.08±0.06	5.27±0.07	6.16±0.02	8.14±0.04
	Cortex	3.19±0.03	3.46±0.29	5.02±0.10	5.85±0.12	7.59±0.12
5.5 x 10^{-4} M NAA	Pith	3.17±0.05	3.24±0.01	5.11±0.09	5.70±0.13	7.24±0.13
	Cortex	2.89±0.04	3.62±0.08	4.72±0.12	5.55±0.11	7.09±0.13
Non-reducing sugars (mg/100 mg dry weight)						
Control	Pith	3.13±0.06	2.95±0.05	2.11±0.01	2.06±0.02	1.88±0.04
	Cortex	3.29±0.08	3.07±0.10	2.57±0.03	2.52±0.02	2.01±0.01
4.0 x 10^{-4} M NAA	Pith	3.12±0.10	3.06±0.07	2.89±0.12	2.99±0.04	2.13±0.20
	Cortex	3.18±0.03	3.58±0.03	3.02±0.08	3.39±0.06	2.61±0.01
5.5 x 10^{-4} M NAA	Pith	3.31±0.05	3.61±0.04	2.26±0.05	2.52±0.01	2.08±0.03
	Cortex	3.38±0.07	3.11±0.08	2.72±0.05	2.56±0.01	2.18±0.04

Table 2 *(Continue)*

Treatment	Tuber part	0 day	10th day	20th day	30th day	40th day
The specific activity of alpha-amylase (on a per mg protein basis)						
Control	Pith	0.43±0.01	0.49±0.01	0.59±0.01	0.61±0.01	0.64±0.03
	Cortex	0.42±0.01	0.48±0.01	0.54±0.01	0.60±0.01	0.65±0.01
4.0 x 10^{-4} M NAA	Pith	0.36±0.01	0.40±0.01	0.48±0.01	0.49±0.01	0.45±0.01
	Cortex	0.33±0.01	0.40±0.01	0.41±0.01	0.44±0.01	0.44±0.01
5.5 x 10^{-4} M NAA	Pith	0.37±0.01	0.39±0.01	0.48±0.01	0.41±0.01	0.45±0.01
	Cortex	0.34±0.01	0.39±0.01	0.42±0.01	0.45±0.01	0.44±0.01
Specific activity of peroxidase (on per mg protein basis)						
Control	Pith	0.31±0.01	0.29±0.00	0.32±0.00	0.15±0.01	0.11±0.01
	Cortex	0.29±0.01	0.28±0.01	0.38±0.01	0.12±0.01	0.08±0.01
4.0 x 10^{-4} M NAA	Pith	0.21±0.01	0.18±0.01	0.10±0.01	0.13±0.01	0.08±0.01
	Cortex	0.25±0.01	0.09±0.01	0.08±0.01	0.08±0.01	0.05±0.01
5.5x10^{-4}M NAA	Pith	0.28±0.01	0.12±0.01	0.10±0.01	0.13±0.01	0.09±0.01
	Cortex	0.27±0.01	0.08±0.01	0.09±0.01	0.10±0.01	0.05±0.00

Note. NAA = Naphthalene acetic acid

ambient conditions. Gilani et al. (2021) also stated that gibberellic acid stimulates pathogen defence-related enzymes, e.g., polyphenol oxidase and peroxidase, and elevates the phenolic substance, which imparts systemic resistance against pathogens.

In the present study, foliar spray of alpha-NAA six weeks before harvest was found effective and reduced per cent sprouting at concentrations of 4.0 x 10^{-4} and 5.5 x 10^{-4} M alpha-NAA when potatoes were maintained at room temperature after 120 days of initial cold storage. Besides causing a delay in sprout initiation, this growth substance also reduced sprout growth and the number of sprouts per tuber. Birbal et al. (2009) reported that NAA at a concentration of 60–100 ppm had delayed the tubers sprouting and suppressed the growth of sprouts in the variety Dargheeling Red Round. NAA applied at 120 ppm had only a small influence in delaying the sprouting of tubers.

More recently, Elsherbiny et al. (2023) stated the negative association between phenolic compounds and potato dry rot diseases. The earlier findings of Sourati et al. (2022) revealed that the IBAs performed better than NAAs among auxins to improve the potato tubers' quality. Likewise, Chandra and Mondy (1981) also observed that IBA and NAA affected the phenol and dry matter biochemical composition and quality of potato tubers as plant hormones alter growth and development. Kondhare et al. (2021) observed that the pre-harvest application of auxins significantly improved the quality attributes of potatoes. Busse and Bethke (2020) also advocated the foliar application of auxins as they enhanced the antioxidants and qualitative attributes. Clarke et al. (2020) also observed that low-dose auxin foliar application

reduced potato diseases, and they further stated that the auxin foliar application enhanced potato tubers' weight significantly compared to the control (no spray).

CONCLUSION

The sprouting of potato tubers may be reduced by the foliar spray of 4.0 x 10^{-4} M alpha-NAA on potato crops six weeks before harvest. Moreover, alpha-NAA foliar application significantly enhanced the weight of potato tubers and reduced peroxidase activity.

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Formulation, Characterization, and *In Vitro* Simulated Gastrointestinal Fluid Analysis of Chewable Yogurt Tablet Incorporated with Corncob Fiber

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ABSTRACT

This study formulates and evaluates a novel functional food, corncob fiber-infused chewable yogurt tablets, to enhance nutritional value. The tablets have the potential to alleviate gastrointestinal symptoms in the elderly and combat malnutrition in selective eaters, potentially replacing multiple supplement tablets. Four batches of tablets underwent rigorous evaluation, considering physicochemical properties, shelf life, and probiotic viability in simulated gastrointestinal conditions. All tablets exhibited robust stability against simulated fluids (85–90% survival rate) and met desired physicochemical benchmarks. Notably, F1 had the lowest hardness (9.50 kp/cm²), while tensile strength showed no significant variance (0.93–1.18 N/mm²) between tablets. However, F3 and F4 displayed significantly longer disintegration times (41.11–52.82 min). After three months, the average bacterial viability was 7 log no. CFU/g, highlighting the tablets' potential to deliver intact probiotics for immediate beneficial effects upon consumption. Thus, these

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chewable yogurt tablets offer a promising means to deliver probiotics effectively while addressing specific dietary challenges.

Keywords: Chewable yogurt tablet, corncob fiber, physicochemical properties, shelf-life study, simulated gastrointestinal fluid analysis

INTRODUCTION

Yogurt is normally obtained via milk fermentation by lactic acid bacteria such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Cajigas, 1990). It is a better-digestible dairy product with several known health benefits due to its high probiotics, calcium, and protein content (Kumar & Mishra, 2004; Mckinley, 2005). Past research has demonstrated that the lactic acid bacteria in yogurt act as probiotics, improving the properties of the indigenous microflora in the human gastrointestinal tract and reducing the risk of developing irritable bowel syndrome (EFSA Panel on Dietetic Products, Nutrition and Allergies [NDA], 2010; Guarino et al., 2013; Xie et al., 2023).

Due to people's awareness of its alimental values, yogurt consumption has increased tremendously in the past decade, making it the second-largest segment of the Asia-Pacific dairy market (26.28%) in 2023 (Mordor Intelligence, n.d.). As one of the functional food components potentially offering additional health benefits to customers, yogurt is undoubtedly worth studying (Granato et al., 2020). It has enormous potential to become the next big thing in the functional food industry, with a projected market value of USD 63.96 billion by 2029 (Mordor Intelligence, n.d.). Yogurt's high digestibility is one of the main factors contributing to the rising yogurt consumption in the Asia-Pacific region (Y. L. Chan et al., 2019). Asians avoid dairy products in their diets and shopping decisions since most (up to 95%) are lactose intolerant (Goh et al., 2018). Asians, often lactose intolerant, can still enjoy the benefits of dairy and probiotics by choosing yogurt owing to its low lactose content (3.5–6%) (Y. L. Chan et al., 2019). Hence, innovation in product development of yogurt's diversity to meet specific nutritional needs is crucial in contributing to and broadening the dairy market share and income.

Many new yogurt products have been developed over the past decades, such as plant-based yogurt, Greek yogurt, drinkable yogurt, yogurt pie, prebiotic enriched yogurt, yogurt ice cream, yogurt gummies, and yogurt puff (Malaysian-German Chamber of Commerce and Industry & Brandt, 2015). Yogurt products, however, are primarily categorized into two groups: (1) set yogurt, where probiotics are directly inoculated without fermentation, and (2) stirred yogurt, which is fermented by lactic acid bacteria (i.e., probiotics) (Hersh, 2021). The drawbacks of products from the group that does not undergo fermentation include lower adherence to existing food standards regarding labeling (e.g., active bacterial count) and the need to include additional ingredients (e.g., fillers, preservatives, flavorings, food coloring, sweeteners, acid

regulators, and food texture modifiers) to achieve the desired organoleptic properties of the product (Faccia, 2020; Y. L. Chan et al., 2019). Although direct incorporation of freeze-dried probiotic strains can guarantee the number of bacteria in pharmaceutical products, it fails to offer the advantages of fermentation, such as the production of natural aromatic compounds (like acetaldehyde), beneficial metabolites (like folic acid), and the conversion of lactose to galactose (Baglio, 2014; Li et al., 2023).

Moreover, researchers have successfully enhanced daily fiber intake by integrating fiber into yogurt, developing a novel functional food with a wide range of advantageous effects. Several studies reported that fiber enhances intestinal microflora growth and gastrointestinal immunity (Dabija et al., 2018; Daud et al., 2018; Gilliland, 1990; Shah, 2007). A person's health may also be improved by consuming more fiber as it lowers the risk of developing constipation, obesity, diabetes, cancer, hypercholesterolemia, gastrointestinal issues, ulcerative colitis, hyperlipidemia, hypertension, and heart disease (Hoppert et al., 2013; Ramirez-Santiago et al., 2010; R. K. Robinson, 1992; Tomic et al., 2017).

Fiber is an indigestible plant carbohydrate that travels through the stomach and intestines unchanged. There are two types of fiber: soluble (i.e., pectins, gums, and mucilage) and insoluble (i.e., cellulose, hemicelluloses, and lignin). Insoluble fibers are usually used as food fortification ingredients due to their beneficial function in relieving fiber-lacking-associated illnesses such as constipation (Bertolino et al., 2015; Dabija et al., 2018; Sah et al., 2016). Poor fiber intake is also one of the major contributors to the development of illnesses associated with malnutrition (Daud et al., 2018; R. K. Robinson, 1992). According to research by Reynolds et al. (2019) dietary fiber intake of at least 25 to 29 g per day was associated with a significant risk reduction for a range of critical outcomes (Reynolds et al., 2019). Despite this, many people fail to achieve the lower end of the suggested range.

Corn is the third main cereal crop in the world, after rice and wheat. The increase in corn production worldwide has led to an increase in corncob waste. These corncobs contain a significant amount of fiber (38.52%) and offer a desirable natural corn flavor (Lee et al., 2019). Moreover, it is a good bulking agent as it can promote satiety and play an essential role in weight management (Lee et al., 2018). Thus, the fiber powder from corncob can be a suitable insoluble fiber source that could be used as a food fortification ingredient.

Consumers increasingly seek convenient and quick nutritious foods to support a healthy diet. The development of these chewable yogurt tablets, enriched with corncob fiber, aims to address swallowing difficulties in the elderly and children while also offering a potential alternative to improve the nutrient intake among selective eaters, potentially reducing the need for multiple supplement tablets (Rana et al., 2011).

Thus, this study aims to formulate and assess chewable yogurt tablets enriched with corncob fiber. It investigated the impact of different formulation factors on the tablets' physical and chemical attributes (titratable acidity, hardness, tensile strength, pH, friability, disintegration, color profile), as well as the survival and stability of lactic acid bacteria (probiotics) within the tablets when subjected to simulated gastrointestinal conditions. Additionally, the study conducted a three-month shelf-life analysis of the optimal chewable yogurt tablet formulation under refrigerated conditions (4°C) to compare its lactic acid bacteria (probiotics) viability against a control group.

MATERIALS AND METHODS

Materials

Skim milk powder, whey protein isolate, fish gelatin powder (Halal), and zip-lock plastic bag were purchased from Edible Food Sdn. Bhd. in Kuala Lumpur, Malaysia. De Man-Rogosa-Sharpe (MRS) agar and peptone water were obtained from Oxoid, United Kingdom. Magnesium stearate, silicon dioxide, lactose, mannitol, talcum powder, starch, and sodium starch glycolate were procured from R&M Chemicals, Malaysia. Phenolphthalein, sodium hydroxide, hydrochloric acid, sodium acetate, acetic acid, ethanol and sterile Petri dish were obtained from Fisher Scientific (Malaysia). Corncob was purchased from NSK Trade City Sdn. Bhd. in Kuala Lumpur, Malaysia.

Methods

Preparation of Corncob Fiber (CF) Powder

Corncobs were cleaned with distilled water and cut into small pieces. After that, corncob pieces were dried in an oven (Memmert, Germany) at 60°C for 36 hr. Dried corncob pieces were ground into finer powder using a heavy-duty blender (Waring Commercial, USA). After that, they were sieved at 100 amplitudes using a vibratory sieve shaker AS 200 basic (Retsch, Germany) with a 40-mesh sieve. The fine powder obtained was sealed in a zip-lock plastic bag and stored at -18°C prior to any analysis (Lee et al., 2019).

Preparation of Fermented Yogurt Powder

A spray-dried fermented yogurt powder, possessing an active bacterial count of not less than 9 log no. CFU/g, was procured from Universiti Putra Malaysia. The manufacturing process involved spray drying a 10% skim milk solution, subjected to anaerobic fermentation at 38±2°C, with a 2% bacterial strain inoculation. The bacterial strain consisted of a mix-strain culture of *Lactobacillus delbrueckii* subsp. *bulgaricus BSL1* (ATCC® 11842™) and *Streptococcus salivarius* subsp. *thermophilus* (ATCC® 19258™) in a 1:1 ratio. The spray drying process parameters were maintained at an inlet temperature of 110±5°C, outlet temperature of 60±5°C, and a feed rate of 20 ml/min. The oral administration of this product is constrained in accordance with the guidelines set forth by the American Type Culture Collection (ATCC) (n.d.a, n.d.b).

Preparation of Chewable Yogurt Tablet

A chewable yogurt tablet was prepared via direct compression based on the formulation Khokra et al. (2012) suggested with some modifications. All the ingredients were weighed individually according to the formulation (F1, F2, F3, and F4) shown in Table 1 and sieved at 100 amplitudes by using a vibratory sieve shaker AS 200 basic (RETSCH, Germany) with a 40-mesh sieve. After that, all the ingredients were blended in a zip-lock plastic bag for 10 min. The above blend was then lubricated with glidants (silicon dioxide, talc, and magnesium stearate) for 2 min. The final blend was compressed into 18 mm diameter right circular cylinder tablets of 1,000 mg weight each using a manual tablet compression machine at 100 MPa.

Physicochemical Properties Analysis on Chewable Yogurt Tablet

Color Analysis. A CR-410 Chroma Meter (Konica Minolta, Inc., Japan) was used to analyze the color of the sample for the values of L* (lightness), a* (redness), and b* (yellowness). A white calibration plate was used to calibrate the system before sample analysis. Samples were filled and sealed into a transparent zip-lock plastic pouch (7.5 cm x 5 cm). The measuring head of the CR-410 Chroma Meter was then placed on top of the samples securely to obtain the color values in triplicate (Lee et al., 2019).

Table 1
Composition of chewable yogurt tablet

Ingredients	Formulation			
	F1	F2	F3	F4
Intragranular				
Fermented yogurt powder	47.50	47.50	47.50	47.50
Skim milk powder	2.50	2.50	-	-
Gelatin powder	-	-	2.50	2.50
Extragranular				
Whey protein isolate	17.50	7.50	17.50	7.50
Corncob fiber powder	-	10	-	10
Mannitol	12.50	12.50	12.50	12.50
Maize starch	10	10	10	10
Silicon dioxide	1.50	1.50	1.50	1.50
Magnesium stearate	0.75	0.75	0.75	0.75
Sodium starch glycolate	3.50	3.50	3.50	3.50
Talc	0.75	0.75	0.75	0.75
Lactose	3.50	3.50	3.50	3.50
Total (%)	100	100	100	100

Note. F1 = Chewable yogurt tablet (control); F2 = Chewable yogurt tablet fortified with 10% corncob fiber; F3 = Chewable yogurt tablet added with 2.5% gelatin; F4 = Chewable yogurt tablet added with 2.5% gelatin and fortified with 10% corncob fiber

Titratable Acidity. A 6 g sample was crushed into fine powder using a mortar and pestle. After that, the sample was well-mixed with 50 ml of distilled water. The analyte was then added with a few drops of phenolphthalein indicator and titrated with 0.1 M sodium hydroxide (NaOH) solution in triplicate. The titration endpoint was determined by the color change of the analyte from colorless to pink or the pH of the analyte reached pH 8.2 for the dark color sample. The endpoint of titration was recorded in ml of titrant used. The pH of the analyte was measured with a calibrated FIVEGO™ F2 Portable pH Meter (METTLER TOLEDO, USA). The percentage of titratable acidity can be calculated below using the milliequivalent factor of lactic acid = 0.09 (Sadler & Murphy, 2010).

$$\% \text{ Acid} = [(\text{ml of NaOH used}) \times (0.1 \text{ N NaOH}) \times (0.09)] / (\text{Grams of sample}) \times 100\% \quad [1]$$

pH Analysis. Sample preparation for pH analysis was standardized to the method of titratable acidity as mentioned previously. The sample (6 g) was blended with 50 ml of distilled water. The sample was crushed into a fine powder using a mortar and pestle in a prior experiment. FIVEGO™ F2 Portable pH Meter (METTLER TOLEDO, USA) was calibrated with pH buffer solutions (pH 4.0, 7.0, and 10.0) prior to analysis. The pH of the sample was measured and recorded using the calibrated pH Meter at room temperature in triplicate (Saint-Eve et al., 2008).

Tablet Weight Variation Measurement. Twenty tablets were weighed individually, and the average weight was calculated using Equation 2. Based on the United States Pharmacopeia method, the maximum acceptable percentage difference was set at 5% for tablets weighing more than 324 mg (United States Pharmacopeia [USP], 2012).

$$\mu = (\Sigma x_i) / n \quad [2]$$

where, μ = average weight (mg); Σx_i = total tablets weight (mg); n = number of tablets.

Friability. According to the United States Pharmacopeia method (United States Pharmacopeial Convention [USP], 2015), 10 tablets were de-dusted prior to weighing and the initial weight was recorded. Friability was tested using a friabilator (PI-FTV-01 Pharmag Instruments, India) with a rotation speed of 25±1 rpm. After 100 rotations, the tablets were collected and de-dusted, and the final weight of the tablet was recorded. Friability was calculated as below:

$$\text{Friability (\%)} = [(W_i - W_f)/W_i] \times 100\% \quad [3]$$

where, W_i = initial weight; W_f = final weight.

Disintegration. Based on the United States Pharmacopeia method with some modifications, 6 tablets per sample were tested using tab disintegrator (DT-1000, Pharmag Instruments, India) with basket-rack assembly (USP, 2015). A 1 L beaker was filled with 800 ml of distilled water to cover the basket fully and maintained at

37±2°C. One tablet was added to each glass tube. The basket was moved at a constant frequency rate between 30±1 cycles per min. The tablet's complete disintegration time was recorded in minutes.

Hardness and Tensile Strength. Five tablets' thickness, diameter and hardness were measured using a tablet hardness tester (ezTab400, Pharmag Instruments, India) with a constant loading speed of 20 N/s. The tablet was placed between the platen to allow diametrical compression to be applied up to fracture. The force required to cause fracture on the tablet was recorded as hardness (kilopond, kp). The thickness and diameter of the tablet were reported in centimeters (cm). According to the United States Pharmacopeia method (USP, 2015), tensile strength was calculated as equation below:

$$\sigma = 2F/\pi DH \quad [4]$$

where, σ = tensile strength (kp/cm_2); F = breaking force (kp); D = diameter (cm); H = thickness (cm).

Hygroscopicity. The hygroscopicity of the sample was determined using the method reported by European Pharmacopoeia (Barret, 2018). Approximately 0.2 g of sample and blank plastic Petri dish (without lid) were weighed accurately up to 3 decimal places on a calibrated Classic Plus analytical and top-loading digital balances (METTLER TOLEDO, USA). A surplus of saturated ammonium chloride solution (with excess crystal) was placed in the pit of the desiccator to provide an 80±2% relative humidity (%RH) environment. Samples were placed inside the tightly sealed desiccator and stored for a day in an IF450 convection force air incubator (Memmert, Germany) at a controlled temperature of 25±2°C. After 24 hr of storage, samples were removed from the desiccator and weighed on a calibrated analytical balance. The hygroscopic nature of samples was classified using Table 2. The hygroscopicity of the sample was calculated by the percentage increase in mass as follows:

$$\text{Percentage increase in mass (\%)} = [(M_2 - M_1) \times 100\%] / M_1 - M_0 \quad [5]$$

where M_0 (g) = weight of blank Petri plate; M_1 (g) = initial weight consisting of Petri plate and sample; M_2 (g) = weight consisting of Petri plate and sample after 24 hr.

Equilibrium Moisture Content (EMC). The sample's EMC was determined using the method reported by Callahan et al. (1982). Approximately 0.2 g of sample and bare plastic Petri dish (without lid) were weighed accurately up to 3 decimal places on a calibrated Classic Plus analytical and top-loading digital balances (METTLER TOLEDO, USA). A surplus of saturated salt solution (with undissolved crystal) was placed in the pit of the desiccator to provide a distinct moisture environment. Lithium chloride, potassium carbonate, sodium chloride, potassium bromide, and potassium nitrate created an environment of 11, 43, 75, 83 and 93%RH, respectively. Samples were

Table 2
Hygroscopicity classification as per European Pharmacopoeia (Barret, 2018)

Classification	Criteria
Non-hygroscopic	Increase in mass 0-0.012% w/w
Slightly hygroscopic	Increase in mass 0.2-<2% w/w
Moderately hygroscopic	Increase in mass 2-<15% w/w
Very hygroscopic	Increase in mass ≥15% w/w

placed inside the desiccator with different relative humidity percentages and tightly sealed. All desiccators were placed into the IF450 convection force air incubator (Memmert, Germany) and stored for a week at a controlled temperature of 25±2°C. After 7 days, samples were detached from the desiccators and the changes in moisture were calculated for each sample by obtaining the final equilibrium weight with a calibrated analytical balance. The initial moisture content of the samples was determined by using an XM120 moisture analyzer (Precisa Gravimetrics AG, Switzerland). The weight change at equilibrium (B) and initial moisture content (A) of the sample was required to determine the percentage moisture dry basis of the sample (P), and EMC values of the sample were calculated from P with the aid of Equations 6 and 7.

$$P = \{[(W \times A/100) - B] \times 100\%\} / [W - (W \times A/100)] \quad [6]$$

where, P (%) = percentage moisture dry basis; W(g) = initial weight of sample; A(%) = initial moisture content; B(g) = weight difference at equilibrium.

$$EMC = P / (P + 100) \quad [7]$$

where, EMC = equilibrium moisture content; P (%) = percentage moisture dry basis.

Proximate Composition Analysis. Samples were analyzed for their proximate composition based on the official AOAC methods (Latimer, 2023); the parameters that were determined were moisture, ash, fat, crude fiber, protein, and carbohydrate. The calorie content of food was determined from the three main components obtained in proximate composition analysis: carbohydrates, protein, and fat. Four (4) kcal was each provided by 1 g of protein and carbohydrate. However, 1 g of fat in food will provide 9 kcal. The calorie content of the sample was determined with the following formula:

$$\text{Calorie content (kcal/ 100 g)} = (\%\ C \times 4\ \text{kcal}) + (\%\ P \times 4\ \text{kcal}) + (\%\ F \times 9\ \text{kcal}) \quad [8]$$

where, % C = percentage of carbohydrate; % P = percentage of protein; % F = percentage of fat.

Enumeration of Bacterial Count

The number of lactic acid bacteria (probiotics) in the sample was analyzed using the pour plate method (Millette et al., 2013). Peptone water and MRS agar were prepared according to the directions recommended by Oxoid (United Kingdom), and autoclaved at 121°C for 15 min at 15 psi

in HV-50 Autoclave (Amerex Instruments, Inc., USA) prior to analysis. One g of the sample (tablet) was crushed into fine powder using a pestle and mortar. After that, the sample was transferred aseptically to a test tube filled with 9 ml of sterile 0.15% (w/v) peptone water and well-mixed using a vortex. Multiple tenfold dilutions in the ratio of 1:10 were required to obtain the targeted concentration, where 1 represents the amount of sample, and 10 represents the total size of the final sample. After the serial dilution, the sample was inoculated in MRS agar and incubated anaerobically at 38±2°C for 48 hr. The anaerobic incubation was carried out using a combustion-modified candle-jar system (Saha et al., 2016). Enumeration of bacteria was carried out in triplicate and reported in log no. CFU/g.

Stability Analysis of Lactic Acid Bacteria (Probiotics) in Chewable Yogurt Tablet under Simulated Gastrointestinal (GI) Fluid

Preparation of Simulated GI Fluid. To study the survival rate of lactic acid bacteria (probiotics) in chewable yogurt tablet samples, simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) was prepared according to the modified method developed by Millette et al. (2013). SGF was prepared by diluting 7 ml of 10 M hydrochloric acid (HCl) and 2 g of sodium chloride (NaCl) with distilled water up to 1,000 ml, and the pH was adjusted to pH 2.0 by using 0.2 M HCl or 0.2 M NaOH. SIF was prepared by diluting 0.9 g NaOH, 6 g bile salt, and 6.8 g monopotassium phosphate (KH_2PO_4) with distilled water up to 1,000 ml and the pH was adjusted to pH 6.8 by using 0.2M HCl or 0.2 M NaOH. Freshly prepared sterile SGF and SIF were stored in an incubator at 37±2°C for 60 min before the experiment to simulate body temperature (Nagashima et al., 2013).

Simulated GI Analysis. Two tablets of the same sample were crushed into chunks to simulate the actual swallowing size of the tablet after the chewing process. The tablet chunks were weighed at 1 g and mixed with 9 ml of sterile SGF to form suspension #1. Then, the suspension #1 was incubated at 37±2°C for 120 min. Meanwhile, the remaining part of the tablet (1 g) was crushed into powder and followed with the enumeration of bacterial count to verify the initial count of lactic acid bacteria (probiotics) in the chewable yogurt tablet sample. After incubation, 1 ml of the vortexed aliquot from suspension #1 was added to 9 ml of sterile SIF to form suspension #2 for further incubation (150 min) at 37±2°C. Another 1 ml of the vortexed aliquot was concurrently mixed with 9 ml 0.15% (w/v) sterile peptone water and proceeded with the enumeration of bacterial count to investigate the viability of probiotics in the chewable tablet after two hours of immersion in SGF. After 150 min of incubation, serial dilution was conducted with 1 ml of suspension #2 transferred into a dilution tube with 9 ml of 0.15% (w/v) sterile peptone water to check the final lactic acid bacterial (probiotics) count and evaluate the survival rate of probiotics after a total of 4.5 hr under simulated GI fluid condition (Nagashima et al., 2013).

Shelf-life Analysis

Bacterial count, pH, and titratable acidity are the required specifications in most food standards worldwide for yogurt products (Y. L. Chan et al., 2019). Thus, the survival rate of probiotics in the sample during storage in a securely capped amber glass bottle (Schott, Germany) at refrigerated temperature (4±2°C) was evaluated based on the method of Chaikham with some modifications (Chaikham, 2015). The samples with 0, 0.5, 1, 2, and 3 months of storage were extracted aseptically for microbiological enumeration analysis. The titratable acidity and pH of the sample were also analyzed to observe the changes in the sample during storage.

Data Analysis

All data were obtained in triplicate (n = 3) and subjected to analysis of variance (ANOVA) by using the MINITAB Release 14.12.0 Statistical Software (USA). Ryan Joiner's test was carried out to determine the normality of data distribution. Levene's and Barlett's tests were used to determine the equality of variances. Fisher's least significant difference (LSD) test was carried out to determine the significance of mean differences among samples (Granato et al., 2014). Results were expressed as mean ± standard deviation, and the significant level was determined at $p<0.05$.

RESULTS AND DISCUSSION

Physicochemical Properties of Chewable Yogurt Tablet

The physicochemical attributes of the chewable yogurt tablets are presented in Table 3. Ensuring compliance with the United States Pharmacopeia criteria, the tablet size was confined to 22 mm, thus fixing tablet weight and diameter at 1,000 mg and 18 mm, respectively (United States Pharmacopeial Convention, 2015). Minor weight fluctuations emerged due to ingredient loss during sieving and compression, resulting in non-significant differences ($p>0.05$) among tablets. The weight variation percentage ranged between 0.2 and 3.3%, abiding by the 5% limit prescribed by the United States Pharmacopeia for tablets exceeding 325 mg (United States Pharmacopeial Convention, 2015). The pH and titratable acidity of the samples ranged from 4.30 to 4.47 and 4.94 to 5.25, respectively, aligning with yogurt's pH range suggested by Baglio, from 3.9 to 4.6 (Baglio, 2014). Tensile strength, an indicator of chewability, showed no significant difference ($p>0.05$) among the samples (0.93–1.18 N/mm²). Notably, F1 exhibited the lowest hardness at 9.50 kp/cm², and other samples also maintained hardness levels below 12 kp/cm², in accordance with the United States Food and Drug Administration (US FDA) and Robinson et al. recommendations regarding chewable tablet hardness (Augsburger & Hoag, 2016; R. L. Robinson et al., 2001).

The chewable yogurt tablet was developed to enhance the availability of lactic acid bacteria (probiotics) by bypassing the digestive phase in the digestive system (Renu et al., 2015). For this purpose, a slower disintegration rate in distilled water at 37°C is ideal. As evident from Table 3 data, chewable yogurt tablet variants F3 and F4 exhibited significantly longer

Table 3
Physicochemical properties of chewable yogurt tablet

	F1	F2	F3	F4
Weight (mg)	998.30 ± 22.30	973.30 ± 23.40	985.00 ± 15.20	966.70 ± 15.10
Diameter (mm)	18.03 ± 0.02	18.01 ± 0.01	18.02 ± 0.01	17.97 ± 0.02
Tensile strength (N/mm^2)	0.93 ± 0.23^{a}	1.09 ± 0.23^{a}	1.16 ± 0.07^{a}	1.18 ± 0.08^{a}
Hardness (kp/cm^2)	9.50 ± 2.34^{b}	11.09 ± 2.35^{a}	11.86 ± 0.65^{a}	12.03 ± 0.86^{a}
pH	4.47 ± 0.01^{a}	4.45 ± 0.01^{a}	4.33 ± 0.01^{b}	4.30 ± 0.01^{b}
Titratable acidity (%)	4.94 ± 0.03^{b}	5.01 ± 0.11^{b}	5.19 ± 0.08^{a}	5.25 ± 0.07^{a}
Friability (%)	0.98 ± 0.03^{b}	0.15 ± 0.01^{a}	0.95 ± 0.03^{b}	0.27 ± 0.01^{a}
Disintegration (minutes)	21.81 ± 0.87^{a}	19.80 ± 0.84^{a}	52.82 ± 4.03^{b}	41.11 ± 2.73^{b}
Hygroscopicity (%)	1.62 ± 0.45^{b}	2.43 ± 0.50^{ab}	1.94 ± 0.17^{b}	4.20 ± 0.94^{a}
Color profile	L* = 88.05 ± 0.14^{b} a* = 0.13 ± 0.03^{a} b* = 7.94 ± 0.21^{a}	L* = 88.99 ± 0.24^{c} a* = 0.27 ± 0.04^{b} b* = 19.75 ± 0.25^{b}	L* = 88.42 ± 0.14^{a} a* = 0.14 ± 0.04^{a} b* = 7.69 ± 0.14^{a}	L* = 88.69 ± 0.37^{c} a* = 0.27± 0.02^{b} b* = 19.61 ± 0.25^{b}
Proximate composition (g/100 g of dry matter)				
Moisture	9.70 ± 0.03^{ab}	11.42 ± 1.19^{a}	9.03 ± 0.09^{b}	9.36 ± 0.16^{ab}
Ash	6.41 ± 0.06^{ab}	6.54 ± 0.20^{a}	5.92 ± 0.08^{b}	6.06 ± 0.24^{ab}
Crude fat	0.67 ± 0.01^{a}	0.58 ± 0.06^{a}	0.68 ± 0.02^{a}	0.55 ± 0.06^{a}
Crude fiber	0.04 ± 0.01^{b}	2.41 ± 0.16^{a}	0.03 ± 0.01^{b}	2.61 ± 0.11^{a}
Crude protein	29.36 ± 0.23^{b}	22.95 ± 0.81^{d}	31.19 ± 0.45^{a}	25.02 ± 0.18^{c}
Carbohydrate	53.86 ± 0.33^{b}	58.51 ± 2.25^{a}	53.20 ± 0.42^{b}	59.01 ± 0.74^{a}
Calorie (kcal/100 g of dry matter)	339.00 ± 1.41^{a}	331.50 ± 6.36^{a}	342.00 ± 1.41^{a}	341.00 ± 1.41^{a}

Note. F1 = Chewable yogurt tablet (control); F2 = Chewable yogurt tablet fortified with 10% corncob fiber; F3 = Chewable yogurt tablet added with 2.5% gelatin; F4 = Chewable yogurt tablet added with 2.5% gelatin and fortified with 10% corncob fiber. The values represented by different letters in the same row differ significantly ($p<0.05$); L* = Lightness; a* = Redness; b* = Yellowness

disintegration times (41.11–52.82 min) compared to F1 and F2. This outcome indicated that the addition of gelatin powder influenced the disintegration time. Including gelatin decreased tablet porosity due to cross-linking during gelatin, extending the disintegration process (Jones et al., 2011). Consequently, longer disintegration times result in reduced exposure of lactic acid bacteria (probiotics) to stomach acid and bile, aligning with the observations of Augsburger and Hoag (2016). Therefore, chewable yogurt tablets F3 and F4 hold the potential as effective carriers for delivering a higher number of probiotics (lactic acid bacteria) to the host's gut over an extended disintegration period.

Distinguishing trends emerged in color and friability within the tablet samples. Notably, the incorporation of corncob fiber powder significantly influenced the color profile and friability of F2 and F4 ($p<0.05$). These variants exhibited a more pronounced yellow hue (b* value: 19.61–19.75) attributed to carotenes and xanthophylls from corn (Floyd et al., 1995). Additionally, F2 and F4 demonstrated favorable friability (0.15–0.27%), which is essential for chewable yogurt tablets acting as probiotic carriers. Friability gauges the mechanical durability of tablets, shielding internal cells from gastric interactions. As per United States Pharmacopeia (United States Pharmacopeial Convention, 2015), minimal weight loss (≤1.0%) post-friability test implies sound structural integrity. It reflects the robust binding capability of corncob fiber powder.

Furthermore, corncob fiber-incorporated samples (F2 and F4) displayed moderate hygroscopicity (2.43 and 4.20%) compared to slightly hygroscopic F1 and F3 (1.62 and 1.94%) as per European Pharmacopoeia (Barret, 2018). Corncob fiber's addition likely contributed to hygroscopicity. Despite similar equilibrium moisture content (EMC) among all samples (1.64–14.97%) in relative humidity between 11 to 83% at 25°C, the EMC spiked to 31.23 and 39.25% (F2 and F4, respectively) at 93%RH, reflecting the moderately hygroscopic nature of corncob fiber (Figure 1) (Igathinathane et al., 2005). It aligns with the known moisture sensitivity of these materials.

By analyzing the proximate composition (Table 3), negligible distinctions ($p>0.05$) were observed in crude fat (0.55–0.68 g/100 g dry matter) and calorie content (331.5–342.0 kcal/100 g dry matter) among all chewable yogurt tablet formulations. However, F2 and F4 exhibited a significant decrease ($p<0.05$) in crude protein content (22.95 and 25.02 g, respectively) due to partial substitution of whey protein isolate with corncob fiber powder. On the other hand, the addition of corncob fiber powder remarkably elevated ($p<0.05$) crude fiber content in chewable yogurt tablets (F2 and F4) to 2.41 and 2.61 g, respectively, aligning with its high content (38 g/100 g dry matter) as reported by Lee et al. (2019). Rich in dietary fiber, these tablets have the potential to be a functional food through improved fiber and probiotic intake.

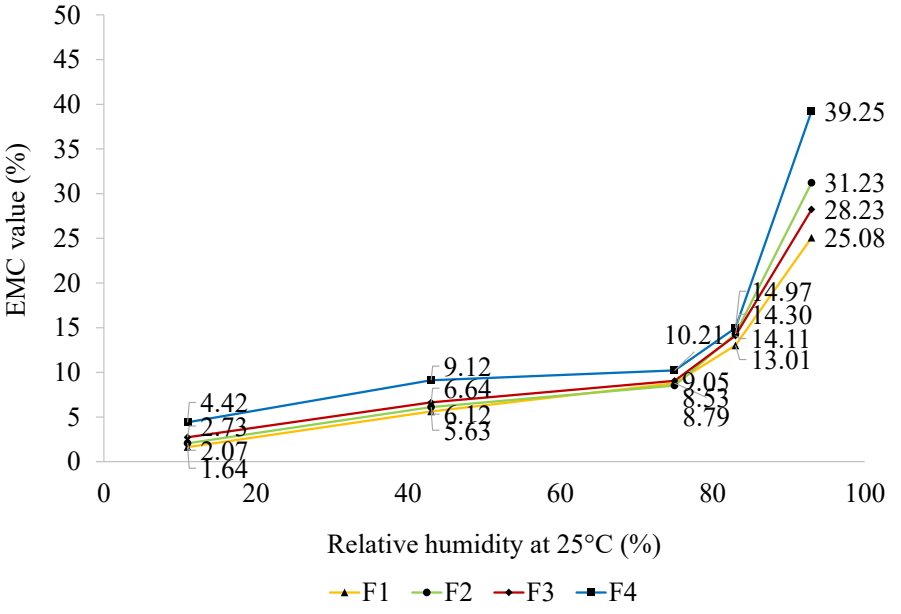

Figure 1. Equilibrium moisture content (EMC) of chewable yogurt tablets with different formulations (F1, F2, F3, and F4) and different relative humidity (11, 43, 75, 83, and 93%) at 25°C

Note. F1 = Chewable yogurt tablet (control); F2 = Chewable yogurt tablet fortified with 10% corncob fiber; F3 = Chewable yogurt tablet added with 2.5% gelatin; F4 = Chewable yogurt tablet added with 2.5% gelatin and fortified with 10% corncob fiber

Bacterial Count in Chewable Yogurt Tablet

As depicted in Figure 2, the initial bacterial count across the tablet formulations ranged from 9.742 to 9.806 log no. CFU/g. Following the tablet-making process, a reduction of approximately 1 log cycle was observed in the bacterial count, resulting in ranges of 8.489–8.809 log no. CFU/g. Notably, no significant differences (p>0.05) were discerned among the four chewable yogurt tablet variants, indicating that tablet formulation did not significantly influence bacterial count reduction during compression. This reduction is likely attributed to the mechanical stresses encountered during compression, which damage bacterial cell walls and membranes (e Silva et al., 2013; Klayraung et al., 2009). These findings align with E. S. Chan and Zhang's (2002) study, revealing that probiotics endure compression up to 30 MPa without noteworthy viability loss. However, viability gradually diminished to around 85% at 90 MPa, and above 90 MPa, bacterial survival declined linearly, with only 33% retention at 180 MPa.

Viability of Probiotics after Simulated Gastrointestinal Analysis

The exposure of bile in SIF poses a significant threat to the lactic acid bacteria (*L. bulgaricus* and *S. thermophilus*) within chewable tablets, as bile disrupts the integrity and permeability of the cell membrane in

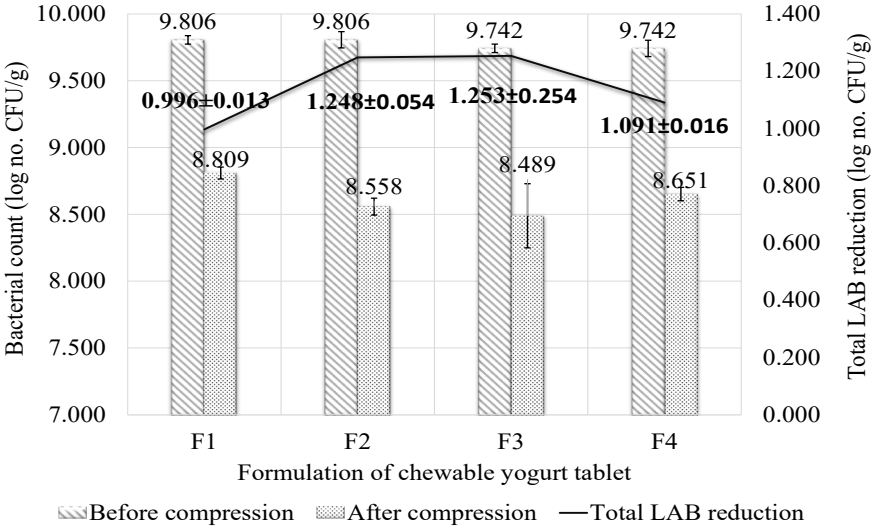

Figure 2. Bacterial count in chewable yogurt tablet before and after compression

Note. F1 = Chewable yogurt tablet (control); F2 = Chewable yogurt tablet fortified with 10% corncob fiber; F3 = Chewable yogurt tablet added with 2.5% gelatin; F4 = Chewable yogurt tablet added with 2.5% gelatin and fortified with 10% corncob fiber; LAB = Lactic acid bacteria

Gram-positive bacteria, ultimately causing membrane lysis and degradation (Begley et al., 2005). Survival outcomes of probiotics (lactic acid bacteria) within chewable yogurt tablet samples under simulated GI conditions are depicted in Figure 3. Notably, F3 and F4, benefiting from a resilient proteinaceous matrix formed by gelatin, exhibited relatively stronger resistance to simulated GI fluids compared to F1 and F2. This intricate protein matrix, created by entrapped gelatin, acted as a protective barrier against SIF (Maldonado-Valderrama et al., 2011). Consequently, F3 and F4 displayed a reduction of <1 log no. CFU/g, while F1 and F2 encountered a decrease of approximately 1.3 log no. CFU/g over 4.5 hours in simulated GI fluids. This outcome correlates with the gradual disintegration properties of F3 and F4, facilitated by gelatin. All chewable yogurt tablets performed admirably under GI conditions, yielding remarkable survival rates (85–90%). This success can be attributed, in part, to tablet friability (<1%), as outlined in the 'Physicochemical Properties of Chewable Yogurt Tablet' section. Adequate tablet friability effectively protects encapsulated cells against the challenging GI environment (Govender et al., 2014). A parallel study by Klayraung et al. similarly demonstrated that optimal tablet friability (<1%) sustained probiotic survival rates of up to 89.3% in simulated GI media (Klayraung et al., 2009). Notably, the results identify F4 (incorporating gelatin) as an optimal vehicle for probiotic delivery, enhanced by adding corncob fiber, significantly boosting viable cell delivery to the human intestine.

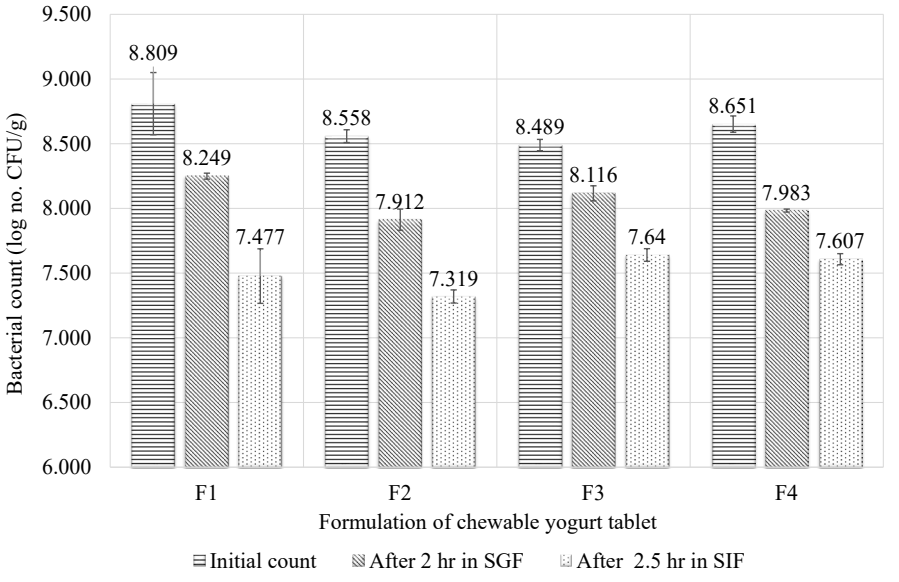

Figure 3. Viability of probiotics in chewable yogurt tablet after simulated gastrointestinal analysis

Note. F1 = Chewable yogurt tablet (control); F2 = Chewable yogurt tablet fortified with 10% corncob fiber; F3 = Chewable yogurt tablet added with 2.5% gelatin; F4 = Chewable yogurt tablet added with 2.5% gelatin and fortified with 10% corncob fiber; SGF = Simulated gastric fluid; SIF = Simulated intestinal fluid

Shelf-life Study

In the three-month shelf-life assessment (Figure 4), the tablets exhibited consistent pH and titratable acidity levels, indicating stability. However, the viability of probiotics gradually diminished across all tablets during storage at 4±2°C. While all four chewable tablet samples commenced with a similar initial bacterial count (around 8.6 log no. CFU/g), F2 and F4, incorporating corncob fiber powder, experienced a more substantial 2 log no. CFU/g reduction compared to F1 and F3 (1.3 log no. CFU/g) after three months. This decrease could be linked to corncob fiber powder's slightly higher equilibrium moisture content, impacting bacterial count during storage. Equilibrium moisture content holds significance for storage, handling, and processing due to its role in chemical and enzymatic reactions (Peng et al., 2007). Water presence affects shelf life and quality by triggering chemical and enzymatic reactions (Zhang & Mittal, 2013). Igathinathane et al. (2005) estimated corncob fiber powder's average equilibrium moisture content at 13.9 ±11.1% d.b. across temperatures (10–40°C). Atmospheric moisture continues to permeate the corncob fiber powder until equilibrium is reached. Elevated moisture content could lead to higher dissolved oxygen levels, potentially causing lactic acid bacteria to perish due to oxygen toxicity (Zayed & Roos, 2004). Notably, all tablets' average final bacterial viability remained at 7 log no. CFU/g aligns with worldwide food standards, requiring a minimum probiotic count of 10^7 (Y. L. Chan et al., 2019). Solutions to address

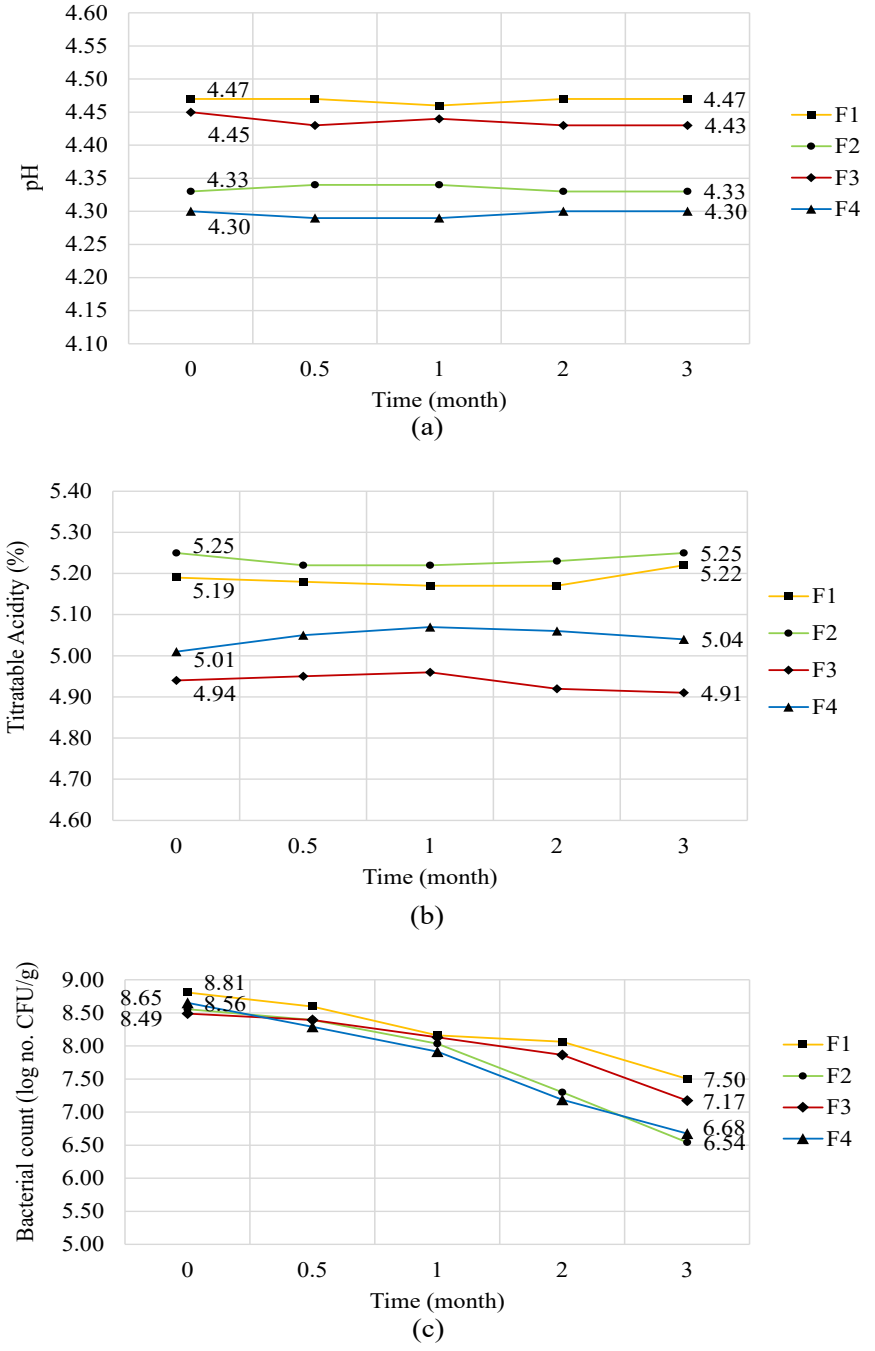

Figure 4. Changes in (a) pH value, (b) titratable acidity, and (c) viability of probiotics during refrigerated storage (4±2°C) for 0, 0.5, 1, 2, and 3 months

Note. F1 = Chewable yogurt tablet (control); F2 = Chewable yogurt tablet fortified with 10% corncob fiber; F3 = Chewable yogurt tablet added with 2.5% gelatin; F4 = Chewable yogurt tablet added with 2.5% gelatin and fortified with 10% corncob fiber

this challenge include incorporating food additives, silica gel desiccants, and vacuum packaging (Amarakoon & Navaratne, 2017).

CONCLUSION AND RECOMMENDATIONS

In this study, all tablets possessed good stability against the simulated GI fluids with an 85–90% survival rate and desired physicochemical properties (weight variation, hardness, tensile strength, pH, and friability). Furthermore, the F4 tablets added with gelatin and incorporated with corncob fiber had a good sustain-released property (longest disintegration time) that enhanced the delivery of probiotics and fiber to human guts. Adding corncob fiber powder in the formulation also imparted a pleasant maize flavor and natural yellowish color (higher b* value ≈ 19) to the chewable yogurt tablet with excellent friability (0.27%). The three-month stability study showed that all tablets encountered a marginally slow decline in terms of the viability of probiotics and had a final bacterial count ranging between 6.5 and 7.5 log no. CFU/g at the end of storage from the initial bacterial count of approximately 8.6 log no. CFU/g. However, there are no significant changes in physical parameters (titratable acidity and pH) during the three months of storage. Chewable yogurt tablet is considered to have good potential as a new dairy product or alternative supplement, as it is a functional, palatable, attractive, and innovative product that meets modern needs. F4 had decent physicochemical properties and sturdy resistance against the simulated GI fluid. Hence, it could be an ideal functional product to deliver probiotics and fiber to the guts in the most intact and viable form, which allows them to exert their beneficial effect immediately upon consumption.

For future research, it is suggested that the preservation of lactic acid bacteria in chewable yogurt tablets by using different packaging such as vacuum-packed, aluminum strip-packed, plastic blister-packed, amber bottle-packed, and packed with desiccants could be investigated to maintain the beneficial properties of probiotics in it. Moreover, an in-depth examination of the sensory attributes of this novel yogurt product may be conducted through methodologies such as volatile profiling, traditional acceptance testing, or text highlighting techniques. This exploration aims to enhance our comprehension of consumer preferences, a pivotal factor in achieving effective market positioning. Finally, an *in vivo* or clinical study on the treatment of gastrointestinal diseases by using chewable yogurt tablets incorporated with corncob fiber powder could be further researched.

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Flowering Phenology and Evaluation of Pollination Techniques to Achieve Acceptable Fruit Quality of Red-fleshed Pitaya (*Hylocereus polyrhizus*) in Sabah, East Malaysia

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ABSTRACT

As red-fleshed pitaya (*Hylocereus polyrhizus*) is not native to the tropical environment of the Malaysian state of Sabah, Borneo, little is known about its flowering phenology, pollination requirements, and potential pollinators, which has discouraged many farmers from growing this crop. Therefore, this study aimed to examine better pollination techniques to achieve acceptable fruit quality for red-fleshed pitaya production under local climatic conditions. For this purpose, stingless bees (*Tetragonula laeviceps*), self-pollination, natural pollination, and hand pollination were used. Pitayas were planted in the field from January 2018 to February 2022, and 40 flowers were observed to obtain data on flowering phenology and fruit quality. This study observed that anthesis of red-fleshed pitaya took about 24 hours in all treatments, depending on the local climate, starting at 6.30 p.m. and ending at 6.30 p.m. the next day. Besides self-pollination, the pitaya flowers were also successfully pollinated by natural, hand, and stingless bees. However, the fruits pollinated by stingless bees were the heaviest, longest, and thickest, indicating that the integration of pitaya cultivation and stingless bees is likely to improve the yield and quality of the fruits on the farm.

Keywords: Flowering phenology, fruit quality, *Hylocereus polyrhizus*, pollination technique

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INTRODUCTION

The pitaya is a cactus native to Mexico, south and central America, and is one of the fruits suitable for worldwide commercial cultivation. In Malaysia, the white-fleshed pitaya (*Hylocereus undatus*), the red-fleshed pitaya (*Hylocereus polyrhizus*), and the yellow-skinned pitaya (*Hylocereus*

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megalenthus) are considered the most suitable varieties for nationwide cultivation (Then, 2017). However, white-fleshed pitaya and red-fleshed pitaya are grown commercially mainly for their unique fruit shape, attractive red colour and relatively high antioxidant and nutritional value compared to other pitaya varieties (Renfiyeni et al., 2018; Then, 2017). Pitaya cultivation in Malaysia is promising as it benefits local farmers economically and can be exploited commercially. However, little is known about this crop's flowering phenology, pollination requirements, and potential pollinators under hot and humid tropical conditions. From 2000 until recently, hand pollination has been used by pitaya growers on commercial farms as the main technique for obtaining high-quality and large fruits (de Menezes et al., 2015; Li et al., 2022; Moreira et al., 2022; Renfiyeni et al., 2018). Natural pollination is also carried out, but fruiting success is reported to be poor, and the fruit produced is often of poor quality (Renfiyeni et al., 2018; Weiss et al., 1994). In self-pollination and natural pollination, the length between the stigma and the anther of the pitaya flower is considered one of the main obstacles to the success of fruit formation (Renfiyeni et al., 2018). Therefore, to achieve successful pollination of pitaya, it is important that pollination is done either manually or by a pollinator (Joanna & Ding, 2021). As the area under pitaya cultivation has increased exponentially in recent years, a systematic study is needed to understand how to achieve high-quality yields through efficient pollination techniques.

The red-fleshed pitaya is widely grown in Sabah, East Malaysia, because of its sweet taste and more expensive fruit compared to other pitaya varieties (Joanna & Ding, 2021, 2022; Phebe et al., 2009). It has also been observed that some farmers in commercial pitaya farms in Sabah use artificial light to attract nocturnal pollinators such as moths and bats. However, the success rate of pollination is uncertain due to a lack of consistent information. The short duration of the pitaya flower opening, which starts in the evening and closes completely the next morning, also reduces the time for pollination by diurnal pollinators such as bees and ants (Joanna & Ding, 2021; Renfiyeni et al., 2018). Therefore, hand pollination of pitaya has to be done at night as the natural pollinators of the flowers normally come during the day (Moreira et al., 2022). In Peninsular Malaysia, hand pollination on commercial farms is usually done at night from 8:00 p.m. to 2:00 a.m. but is very labour intensive (Ahmad Hafiz et al., 2019; Then, 2017; Then et al., 2020). In Mexico, the country of origin of pitaya, bats have been reported to efficiently pollinate flowers at night (Moreira et al., 2022; Valiente-Banuet et al., 2007), while some studies have found that bees can effectively pollinate flowers during the day and improve yield quality, although they have much less time to visit the flowers (de Oliveira Muniz et al., 2020; Indriyani & Hardiyanto, 2018; Valiente-Banuet et al., 2007). So far, the potential nocturnal and diurnal pollinators for the natural pollination of pitaya flowers in Sabah have not been documented, which is important for studies.

In contrast to common honeybees, studies on stingless bees as main pollinators of pitaya flowers are relatively rare worldwide, probably because they are restricted to subtropical and tropical regions (Jalil, 2017; Michener, 2007; Rahman et al., 2015; Rasmussen, 2008, 2013; Roubik, 1989; Sakagami, 1982; Sakagami et al., 1990). Like honeybees, stingless bees are effective pollinators because they have special structures for collecting pollen called corbicula that facilitate the transfer of pollen to other flowers. Previous studies have shown that stingless bees are used as primary pollinators in several tropical crops such as chilli (Putra et al., 2016), mustard (Atmowidi et al., 2007), *Brassica oleraceae* (Wulandari et al., 2017), *Foeniculum vulgare* (Layek et al., 2022), and rock melon (Azmi et al., 2019). In addition, pollination by stingless bees contributes significantly to crop production by increasing the number of pods, seeds per pod, seed weight per plant, and seed germination of mustard (Atmowidi et al., 2007) and kale (Wulandari et al., 2017) as well as improving the production quality of melon (Bahlis et al., 2021) and the quality and quantity of okra pods (Djakaria et al., 2022). Pollination by stingless bees has also been reported to increase fruit set, fruit size, and weight of strawberries (Alpionita et al., 2021; Roselino et al., 2009; Widhiono et al., 2012), so they may be suitable pollinators for pitaya flowers. This study selected *Tetragonula laeviceps*, one of Malaysia's most common species of stingless bees (Atmowidi et al., 2007), as the model organism for pitaya pollination. Previous studies have shown that *T. laeviceps* is suitable for integration into crops as it can cope better with hot and humid tropical weather conditions and can easily build its nests in new habitats (Agus et al., 2019; Atmowidi et al., 2007; Azmi et al., 2019; Bahlis et al., 2021; Djakaria et al., 2022; Layek et al., 2022; Roselino et al., 2009; Wulandari et al., 2017).

The lack of information on potential pollination and pollinator requirements for pitaya has limited understanding of the agronomic requirements for growing this non-native crop in Sabah, East Malaysia. As farmers learn more about this crop, its flowering phenology, pollination requirements, and potential pollinators, yields will likely increase, but information is currently limited. This study assessed the flowering phenology and quality of red-fleshed pitaya fruit obtained through stingless bee pollination, self-pollination, natural pollination, and hand pollination.

MATERIALS AND METHODS

Location of Study and Preparation of Materials

This study was conducted at the Insectarium, Faculty of Sustainable Agriculture, Universiti Malaysia Sabah (UMS), from January 2018 to February 2022. Sixteen polybags were planted with red-fleshed pitaya (*H. polyrhizus*), and all fertilisations were applied at the same rate and time. In this study, the red-fleshed pitaya plants took about two years to mature and produce flowers. Colonies of stingless bees, *T. laeviceps*, were kept in the field for

about three months under natural climatic conditions (22 to 31°C, 83% humidity) and acclimatised. Environmental parameters such as temperature (°C) and relative humidity (%) were recorded with the HOBO® Pro V2 data logger (USA).

Treatments and Experimental Design

The experiment was conducted with a complete randomised design (CRD), and the treatments for the plant in this study were (1) self-pollination (4 polybags), (2) natural pollination (4 polybags), (3) hand pollination (4 polybags), and (4) pollination by stingless bees (*T. laeviceps*) (4 polybags). Figure 1 shows the treatments for pollination of pitaya flowers. For self-pollination, flower buds were bagged in fine muslin bags (1 mm × 1 mm) 24 hr before anthesis to avoid visits by pollinators. In hand pollination, pollen was manually transferred from the stamens or the male part of the flower, to the pistil or the female part. The flower was bagged in the evening (4.00 p.m.) and hand-pollinated the next day at 6.00 a.m. in the morning. After manual pollination, the flower was bagged until the anthesis closed at 6.30 p.m. to avoid other pollinators. The flower was not bagged in natural pollination, so all pollinators could pollinate it. The types of potential pollinators visiting the flower during the anthesis period were visually observed twice within 24 hr (from 4.30 p.m. to 12:00 a.m. and from 4:00 a.m. to 6:30 a.m.) using the method described by de Oliveira Muniz et al. (2019). For pollination by stingless bees, three hives with strong colonies of *T. laeviceps* were placed next to the pitaya at least seven days before flowering to allow the stingless bees to acclimatise. The plants and hives were covered with mesh netting the evening before flowering and left covered for 24 hr. The foraging activity of the bees was observed at the three hives in front of the hive entrance tube for ten

(a) (b) (c) (d)

Figure 1. Treatment for pitaya pollination: (a) self-pollination; (b) natural pollination; (c) hand pollination; and (d) *T. laeviceps* pollination

minutes every hour from 6.30 a.m. to 4.30 p.m. on rain-free days, using the method described by de Oliveira Muniz et al. (2019).

Collection of Data

Flower Phenology Observation

For pitaya, each flower phenology was assessed on five consecutive days during several flowering phases from March 2020 to March 2021. For the study of flowering phenology, 40 pitaya flowers were randomly selected for data collection from all buds that had successfully flowered. All flower buds were observed throughout their development, and the length of the bud, the diameter of the open flower, the length of the stigma lobe and the length of the stamens were measured.

Fruit Quality Parameters Evaluation

Data were measured from 40 fruits (10 fruits × 4 treatments) for the fruit quality study. Pollination efficiency of pitaya was measured after fruit harvest, which usually occurred 28 to 33 days after the successful pollination of flowers. Fruit quality parameters were assessed, including weight, length, diameter, harvest age, and set formation. The value of fruit set formation was determined using the following formula:

$$\text{Fruit set rate (\%)} = \frac{\text{Total no. of fruitlets}}{\text{Total no. of flowers}} \times 100\%$$

(Renfiyeni et al., 2018)

Data Analysis

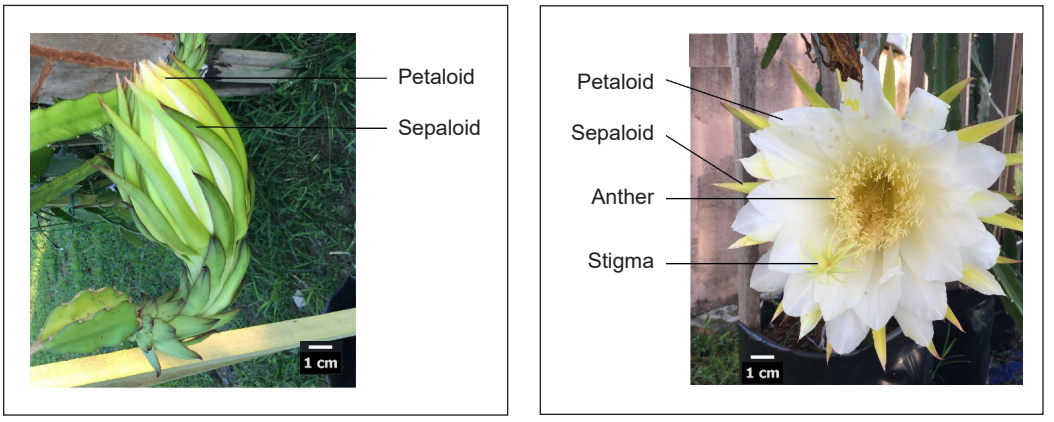
Statistical analyses were carried out using SPSS (version 26). One-way analysis of variance (ANOVA) was used to compare the morphological characteristics of the assigned flowers for each pollination type, the fruit characteristics and pollination types, and the fruit weight, length, and diameter.

RESULTS AND DISCUSSION

Flower Phenology of Red-fleshed Pitaya

In this study, pitaya was found to flower between one and a maximum of three clusters per month about two years after planting, which is also confirmed by Then et al. (2020). However, depending on climatic conditions, the flowering pattern and yield are not uniform. This study observed that a moderate temperature of about 32°C during the dry season is the most important factor influencing the flowering of red pitaya, compared to the rainy season with high and low temperatures.

In Figure 2a, the pitaya flowers appear at the edges of the stems and develop into flower buds in about 10 to 13 days following the emergence of spherical buttons from the stem margins. The whitish-green and cylindrical flower buds reach a length of about 30.55 cm, 3 to 4 weeks after the flower buds start to appear when anthesis takes place. The pitaya flowers started to open at 6.30 p.m. and were fully open by 8.00 p.m. on the first day. The next day, the pitaya flowers closed slightly around the afternoon (1.30 p.m.) and were fully closed by 6.30



(a) (b)

Figure 2. Floral morphology and anatomical structure of *Hylocereus polyrhizus* at the time of anthesis, different types of sepaloid and petaloid tepals: (a) Both male and female organs coexist in a flower; and (b) lateral view of a flower, stigma, and anther
Note. Scale bar represents 1 cm

p.m. (Figure 2b). The antithesis of pitaya flowers begins at 6.30 in the late afternoon in Sabah, one to two hours earlier than in Peninsular Malaysia (Then et al., 2020). It is probably related to the situation in the state of Sabah, where it gets dark at 6.30 p.m., whereas in Peninsular Malaysia, it usually starts at 7.30 p.m.

In this study, about four or five spherical buttons emerged from the areoles of the mature stems, but only two to three developed into flower buds, which took 3 to 4 weeks to grow and develop into mature flowers, which is also consistent with the observations of Then et al. (2020) in a pitaya farm in Peninsular Malaysia. The flowers of *H. polyrhizus* are white and sessile (Figure 2b), monoecious, with male and female organs and a long flower tube (Figure 2a), averaging 30.55 cm in length. Characteristics of *H. polyrhizus* are the typical white-coloured, sepaloid tepals, which may serve to increase the visibility of the flower to nocturnal and diurnal pollinators by maximising light reflection (Joanna & Ding, 2021). The perianth of the flower consists of two types of tepals: sepals, which cover the flower tube, and petaloid tepals, which sit on the edge of the flower cup. The flowers are zygomorphic as the pistil and stamens are in the ventral part of the flower. The average length of the filament is 19.75 cm. The flowers have a long stigma lobe with an average length of 25.90 cm (Figure 2b; Table 1). The length of the petals corresponds to the length of

Table 1
Pitaya flowers' morphological characteristics

Flower characteristics	**Morphological measurements**
Total flowers (N)	40
Mean of bud length (cm)	30.55±0.04
Mean of the diameter of opened flowers (cm)	27.40±0.06
Mean of the length of the stigma lobe (cm)	25.90±0.18
Mean of length of filament (cm)	19.75±0.13

the flower tube. The pistil connects the stigma on the upper side with the ovary on the lower side.

Fruit Quality Parameters Evaluation

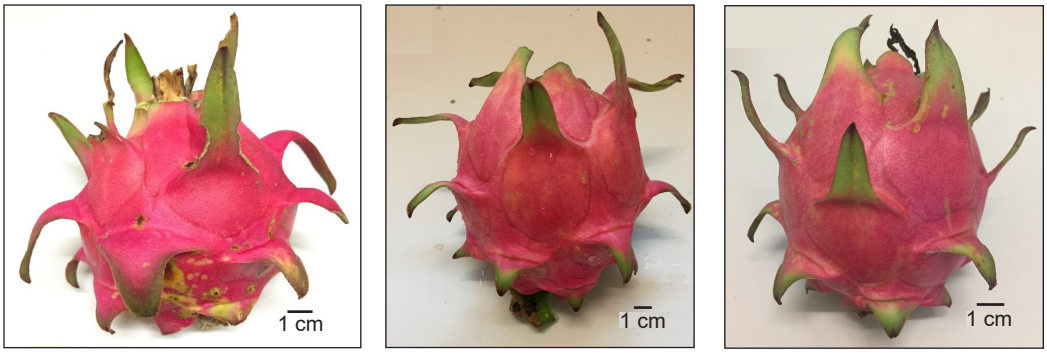
The data for self-pollination were excluded from the one-way ANOVA analysis because pollination was unsuccessful (Table 2). Apart from unsuccessful fruit pollination by self-pollination, no abnormal fruits were observed in other pollination techniques in this study (Table 2). This result is thus consistent with earlier studies by de Menezes et al. (2015), which reported that self-incompatible pitaya species only achieve the highest fruit quality through cross-pollination. The stigma of the red-fleshed pitaya flowers is higher than the anthers, which may have hindered self-pollination (Hadiati & Umjunidang, 2019; Joanna & Ding, 2021; Renfiyeni et al., 2018). This circumstance results in the pollen's inability to reach and adhere to the stigma. It is called self-incompatibility and is also due to genetic factors (Renfiyeni et al., 2018; Ruwaida, 2007). For this reason, pollination of this crop should be assisted by insects as pollinators or by humans through artificial pollination to maximise fruit set and quality (Merten, 2003; Nadila, 2014; Renfiyeni et al., 2018). Pollination and fertilisation of flowers are two interrelated processes whose success depends on the compatibility of pollen and stigma (Renfiyeni et al., 2018).

The fruit for each pollination treatment is shown in Figure 3. The different types of pollination had a significant effect on the trait's fruit weight ($F_{2,27} = 16.57$, $p<0.001$), fruit length ($F_{2,27} = 66.91$, $p<0.001$), and fruit diameter ($F_{2,27} = 59.46$, $p<0.001$), but showed no effect on harvesting age ($F_{2,27} = 0.052$, $p>0.05$) (Table 2 and Figure 3). As self-pollination of red pitaya flowers has been reported to be ineffective, pollination by natural or artificial pollination is strongly recommended to improve the efficiency of the pollination process (de Oliveira Muniz et al., 2020). De Oliveira Muniz et al. (2020) reported that pitaya appears to

Table 2
Fruit weight, fruit length and diameter, fruit set rate, and harvesting age of pitaya

Characteristics	Pollination types			
	Self-pollination	Natural pollination	Hand pollination	*Tetragonula laeviceps* pollination
Total flowers (N)	10	10	10	10
Mean of fruit weight (g)***	n.d.	670±55.31^{b}	548.50±34.98^{a}	676.50±51.89^{b}
Mean of fruit length (cm)***	n.d.	13.60±0.37^{b}	11.45±0.64^{a}	14.87±0.63^{c}
Mean of fruit diameter (cm)***	n.d.	9.45±0.37^{b}	7.83±0.63^{a}	11.22±0.83^{c}
Mean of fruit set rate (%)^{NS}	n.d.	100	100	100
Mean of harvesting age (days after anthesis) ^{NS}	n.d.	30.10±0.60	30.10±0.71	30.20±0.64

Note. *** = Significantly different at $p<0.001$; Means followed by another letter are significantly different at $p<0.05$ as measured by the post-hoc Tukey test according to analysis of variance; NS = Not significant; n.d. = No data (unsuccessful pollination)



(a) (b) (c)

Figure 3. Fruit of pitaya from three treatments: (a) hand pollination; (b) natural pollination; and (c) *Tetragonula laeviceps* pollination

be wholly or partially self-incompatible, and hand pollination is required to achieve commercial yields in cultivation. Pollination is successful when pollen adheres to the stigma and germinates (de Oliveira Muniz et al., 2020). This germinated pollen spreads downwards and penetrates the stigma lobe before reaching the ovary and attaching to the ovule (de Oliveira Muniz et al., 2020). The mature ovary becomes the fruit, and the mature ovule becomes the seed (Darjanto & Satifah, 1990).

In this study, the quality of pitaya fruit produced was also determined by the type of pollination technique ($p<0.001$). However, average fruit weight, length and diameter were highest when flowers were pollinated by stingless bees (*T. laeviceps*), followed by natural and hand pollination (Table 2). According to Zainudin and Hafiz (2015), pitaya fruits are classified as 'AA' for 500–800 g, 'A' for 350–450 g, 'B' for 250–350 g, and 'C' for less than 250 g. In this study, it was found that hand pollination and natural pollination produced pitaya fruits of the 'AA' class weighing 548 and 670 g, with a length of 11.45 and 13.6 cm, as well as a diameter of 7.83 and 9.45 cm, respectively (Table 2). Hand pollination is a necessary auxiliary method for pitaya production to achieve a high yield (Li et al., 2022). The slightly lower fruit weight, length, and diameter in hand pollination in this study could be due to the late timing of flower pollination, which took place in the morning, 12 to 13 hours after the flowers opened at 6.00 to 7.30 p.m. the previous day. In Guangzhou, China, Li et al. (2022) reported that choosing the right time for hand pollination is an important method for pitaya production in commercial farms to achieve high yields. Li et al. (2022) point out that the best pollination activity of pitaya flowers occurs at night immediately after flowering, between 8.00 p.m. and 2.00 a.m. and that late pollination activity after 4.00 a.m. can reduce fruit size by 10.5%. Therefore, these studies also provide an important basis for choosing the right timing for hand pollination in an open farm to improve the yield and breeding efficiency of red-fleshed pitayas, especially in Sabah, East Malaysia.

The study on natural pollinators found that diurnal pollinators mainly include hymenopteran insects such as bumblebees, carpenter bees, wasps, hornets, and honeybees. They were observed to visit pitaya flowers in the field only for 10 to 20 s per flower, mainly between 8.00 in the morning and 12.00 noon. Nocturnal pollinators, including moths (Lepidoptera; Sphingidae), were observed actively visiting the flowers between 7.00 and 8.30 at night. However, only ants were observed visiting the flowers both at night and during the day, which was also reported by Sulistiyowati and Putra (2016) for pitaya farms in Java. Fruit weight, length, and diameter were slightly lower when pollinated naturally than when pollinated by stingless bees (Table 2). It is probably related to the short visit and the lack of adaptation of these natural pollinators to the newly introduced plants, which are flowering for the first time in the area studied (Weiss et al., 1994).

Pollination by natural pollinators such as bees can reduce labour costs for pollination services, as has been observed in other crops, e.g., a kiwi farm in China (Zhang et al., 2022) and strawberry farms in Pakistan (Anees et al., 2022). Then et al. (2020) found that the average weight of individual fruits of a naturally pollinated red-fleshed pitaya ranged from 283.60 to 336.50 g at the Tengi Research Station, Selangor, Malaysia. Other studies by Bellec et al. (2006) and Hoa et al. (2006) reported that the average fruit weight of a naturally pollinated red-fleshed pitaya was 300 and 393 g per fruit, respectively. On the other hand, the higher average fruit weight from natural pollination in this study compared to pitaya fruit reported in previous studies could be due to cross-pollination activity by different hymenopteran insects that visited the flowers studied and brought pollen from many different pitaya flowers, even if it was only a short visit. According to Renfiyeni et al. (2018), a large amount of pollen is the most important requirement to allow better results in the cross-pollination of flowers and for the production of high-quality fruits of red-fleshed pitaya.

Compared to the other treatments, the fruits from pollination by the stingless bee, *T. laeviceps*, had the heaviest (676.50 g), longest (14.87 cm), and thickest (11.22 cm) fruits (Table 2). It indicates that *T. laeviceps* can be successfully used as good pollinators and integrated into commercial pitaya farms as they produce the highest quality and average weight of fruits in the 'AA' class. This study found this species to start foraging early at 6.00 a.m. but to be most active between 7.30 a.m. and 12.30 p.m. when the pitaya flowers are still open. This species was also observed actively foraging at relatively low temperatures (25.17 to 27.67°C) and relatively high humidity (85.67 to 94.17%) between 6.00 a.m. and 12.30 p.m. The duration of flower visitation by *T. laeviceps* was longest at 60 to 70 s per flower and shortest at 30 to 40 s per flower. It was observed that the visiting activity of *T. laeviceps* was related to the availability of pollen, which was abundant in pitaya flowers. This result is also in agreement with studies by Alpionita et al. (2021), who found that *T. laeviceps*

is a species of stingless bees that generally visited strawberry flowers longer (89.15 s/number) than other insect species, such as honeybees (12.64 s/number), resulting in higher fruit quality in both cases.

CONCLUSION

It was observed that anthesis of red-fleshed pitaya lasted 24 hours in all treatments, depending on the local climate, and started at 6.30 p.m. and ended at 6.30 p.m. the next day. The effectiveness of the pollination method determined the quality of the fruit produced by the pitaya. Apart from unsuccessful fruiting by self-pollination, pitaya flowers were successfully pollinated by natural pollination, hand pollination, and stingless bees, and their fruit quality was acceptable in weight, length, and diameter. However, pollination by *T. laeviceps* generally resulted in better fruit quality than natural pollination and hand pollination of the non-native plant of red-fleshed pitaya in Sabah, East Malaysia.

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16S rRNA-based Metagenomic Analysis of Beeswax-coated Saba Banana (*Musa* × *paradisiaca*) Pseudostem

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ABSTRACT

Bananas are one of the most popular fruits, and their production generates significant agricultural waste. Banana pseudostems, a by-product of the banana industry, are being investigated as a renewable and biodegradable alternative to synthetic food packaging materials. However, these pseudostems have the potential to harbor harmful bacteria due to their natural fiber composition. Therefore, this study analyzes the effect of beeswax coating on the microbial communities in banana pseudostems. The microbial community is analyzed through a metagenomics approach that targets the 16S rRNA gene of the Saba banana (*Musa* × *paradisiaca*) pseudostem. Two experimental conditions were considered: pseudostem with beeswax coating and pseudostem without beeswax coating. The findings indicate that the microbial communities in all samples are primarily composed of the phyla Proteobacteria, Cyanobacteria, and Firmicutes. The dominant species found in uncoated banana pseudostem is *Pantoea* sp. At-9b, *Escherichia coli*, *Synechococcus* sp. JA-3-3-Ab, *Pantoea vagans*, and *Klebsiella pneumoniae*. The dominant species found in beeswax-coated banana pseudostem is *Synechococcus* sp. JA-3-3-Ab, *Pseudanabaena* sp. PCC 7367, *Chroococcidiopsis thermalis*, *Priestia megaterium*, and *Ammonifex degensii*. The Chao1, Shannon, Simpson, and Equitability indices indicate that the species richness, diversity, and evenness in the uncoated banana pseudostem are higher than in the

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beeswax-coated banana pseudostem. The degree of similarity between bacterial populations found in uncoated banana pseudostem and beeswax-coated banana pseudostem is around 53.9%.

Keywords: 16S rRNA, banana pseudostem, beeswax, metagenomics

INTRODUCTION

Bananas are extensively grown and are recognized as one of the most widely produced fruits globally. They also play a crucial role as an edible fruit in Indonesia, where various banana studies have been carried out (Dwivany et al., 2014, 2016, 2021; Khairiya et al., 2023). Based on data from the Central Statistics Agency, banana production in Indonesia alone reached 8.74 million tons in 2021 (Badan Pusat Statistik [BSP], 2024). Bananas are perennial plants that have unique characteristics. The crops are harvested singularly and have a brief life cycle lasting one year. When the fruits are harvested, the whole plant must be cut down (Padam et al., 2014; Saraiva et al., 2012). Bananas produce by-products such as rachis, stems, and banana leaves during production (Acevedo et al., 2021). These by-products, which are non-fruit biomass, contribute to a large amount of waste (Saraiva et al., 2012). On average, the waste produced by a single banana plant can reach 80% of its total mass (Padam et al., 2014). Thus, it can be estimated that banana waste produced in Indonesia can reach several tons. Waste produced from bananas can cause various problems in banana production areas, including the growth of banana pathogen deposits and pests (Yoga Milani et al., 2020). The customary practice in banana plantations is to allow banana stems and leaves to undergo decomposition, enriching the soil with nutrients.

However, this practice can interfere with farmers harvesting fruits (Padam et al., 2014). Moreover, after decomposition, banana waste can produce harmful gases like hydrogen sulfide and ammonia (Saraiva et al., 2012). In some cases, open-fire burning is still practiced in disposing of banana waste, further contributing to environmental problems (Padam et al., 2014). Efforts have been made to process banana waste into value-added products to address this issue. Banana stem waste has been utilized to make fertilizer (Padam et al., 2014), animal feed (Yanuartono et al., 2020), paper, and craft materials (Saraiva et al., 2012). Additionally, banana stems have the potential to be an alternative to disposable synthetic packaging, such as styrofoam (Yoga Milani et al., 2020). However, using banana stems as food packaging poses challenges due to their susceptibility to microbial growth in the surface area, leading to odor, discoloration, and potential infection from pathogenic bacteria (Yoga Milani et al., 2020). Beeswax has emerged as a popular coating for natural ingredients, including banana stems, to overcome these challenges. Beeswax possesses antimicrobial properties (Beck et al., 2021), hydrophobic properties, and reduced water vapor permeability (Trevisani et al.,

2017), making it an ideal coating for food packaging. It is also considered generally recognized as safe (GRAS) by the United States Food and Drug Administration (Szulc et al., 2020). Previous studies have shown the potential of using beeswax as a coating for cotton fabrics for packaging purposes (Beck et al., 2021; Pinto et al., 2017). Based on the documentation of its properties, beeswax has the potential to be applied to banana stems and suppress microbial growth. In addition, beeswax can also answer the urgency of the material with a biodegradable, renewable, and non-toxic hydrophobic surface to replace synthetic packaging (Asim et al., 2022).

However, no studies have examined the effect of beeswax coating on the bacterial community of banana stems used as food packaging. Therefore, this study aims to analyze the differences in composition, the diversity index, and the similarity in the microbial communities found in banana stems that are coated and not coated with beeswax.

MATERIALS AND METHODS

Sample Preparation

The sample used in this study was a sample of Saba banana (*Musa* × *paradisiaca*) sourced from the Sumedang region in West Java. Parties from Bandung National Institute of Technology prepared the sample. The part of the Saba banana used is the outermost fourth layer of the pseudostem of the banana. The sample is subjected to two treatments: banana pseudostem samples coated with beeswax and uncoated. The diagram of the samples used in this study is presented in Figure 1.

The beeswax used is obtained from BioPolish and possesses a food-grade certification. The pseudo-stem of the Saba banana is initially dried under the sun. The pseudo-stems that are not too large are then selected and prepared. The pseudo-stems are cut into two equal-sized pieces measuring 4 cm × 4 cm each. A single segment of the pseudo-stem was left untreated to serve as a control sample. Subsequently, the other segment of the pseudo-stem was coated with beeswax thrice within 48 hr to serve as the treated sample. The visual representations of the samples are depicted in Figure 2.

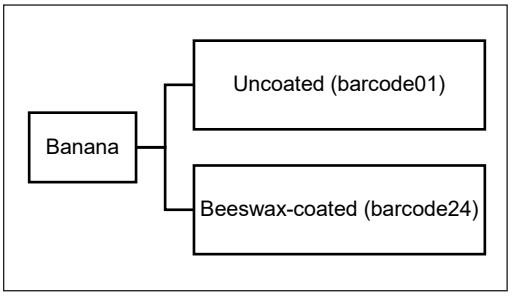
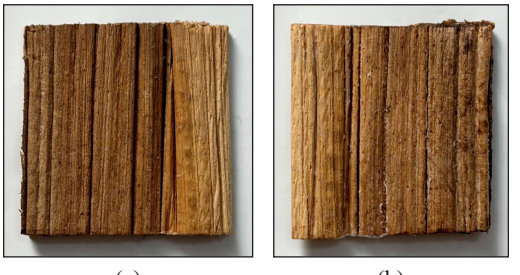


Figure 1. Sample diagram used in this study



(a) (b)

Figure 2. Banana pseudostem samples: (a) uncoated; and (b) beeswax-coated

Metagenomic Analysis

The genomic DNA from each banana pseudostem sample was isolated using Genomic DNA Mini Kit Plant (Geneaid Biotech Ltd., Taiwan) (Karmawan et al., 2009). The concentration of DNA was assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and a Qubit fluorometer (Thermo Fisher Scientific, USA). Library preparation is done utilizing kits provided by Oxford Nanopore Technology (ONT, United Kingdom). The sequencing process is conducted with GridION (ONT, United Kingdom) and operated with MinKNOW software (version 20.06.9). After sequencing, base calling was done using Guppy (version 4.0.11) in high accuration mode (Wick et al., 2019). After obtaining the sequencing results in the form of FASTQ files, the quality of the FASTQ files is visualized with NanoPlot (version 1.41.3) (De Coster et al., 2018).

This study used taxonomic annotations to determine the bacteria in bananas with and without beeswax coating. Classification of reads is carried out with Centrifuge (version 1.0.3). Centrifuge is a fast and sensitive classifier for microbial sequences with low memory requirements but has speeds comparable to the fastest systems (D. Kim et al., 2016). The index used to classify the samples in this study is the index of bacteria and archaea downloaded from the Centrifuge website (https://ccb.jhu.edu/software/centrifuge). The command used in the Centrifuge can also be seen on the Centrifuge website (https://ccb.jhu.edu/software/centrifuge).

After reading the classification, relative abundance is visualized with KronaTools (https://github.com/marbl/Krona). In addition, diversity analysis and data visualization were also carried out with RStudio (version 2022.12.0).

RESULTS AND DISCUSSION

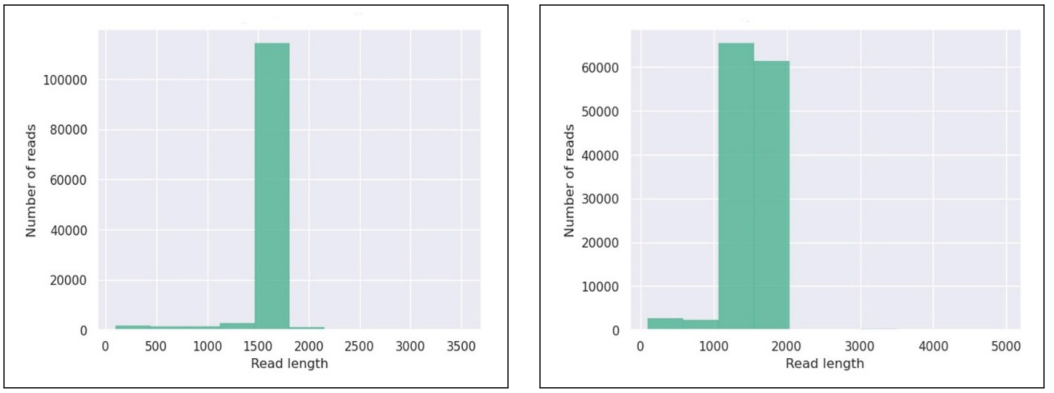
Sequence Statistics

The sequence statistics of the samples are presented in Table 1.

The total base of the uncoated sample was 188,926,678 bases, whereas the beeswax-coated sample was 202,090,361 bases. The total amplicon reads obtained were 122,874 for the uncoated sample and 131,995 for the beeswax-coated sample. The distribution of reading length is presented in Figure 3. The average read length for the uncoated sample was 1,574.6 bp, and for the beeswax-coated sample, it was 1,531.0 bp. The average read quality for both the uncoated and beeswax-coated samples was 11.

Table 1
Banana stem sample sequencing statistics

No.	Sample	Number of reads	Length (bp)	Quality score	Total bases
			mean	mean	
1.	Uncoated sample (barcode01)	122,874	1,574.6	11	188,926,678
2.	Beeswax-coated sample (barcode24)	131,995	1,531.0	11	202,090,361



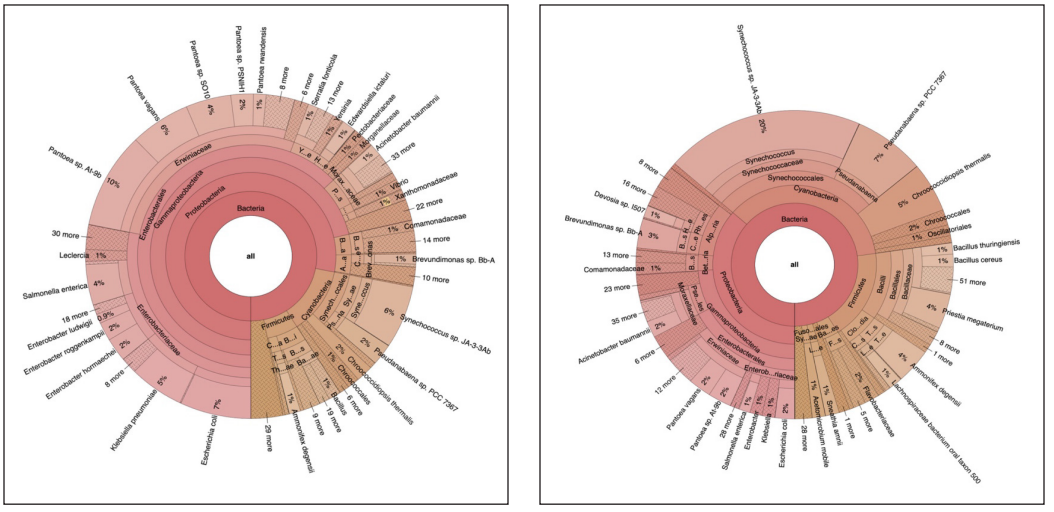
(a) (b)

Figure 3. Distribution of read lengths: (a) Uncoated banana pseudostem sample; and (b) beeswax-coated banana pseudostem sample

Microbial Community Structure

The reads obtained from sequencing, both uncoated and beeswax-coated samples, can be categorized based on taxonomic classification, ranging from the domain level to the species level. The taxonomic annotation results are then represented using a Krona chart, which effectively displays the comparative prevalence of the two samples across various taxonomic levels. Figure 4 displays a graphic representation of the taxonomic composition observed in the two samples.

At the phylum level, the predominant bacterial taxa identified in both samples, namely uncoated banana pseudostem and beeswax-coated, were Proteobacteria, Cyanobacteria, and Firmicutes, but with



(a) (b)

Figure 4. Krona chart: (a) Uncoated banana pseudostem sample; and (b) beeswax-coated banana pseudostem sample

varying proportions. Proteobacteria are the most extensive taxonomic group within the bacterial domain. Numerous human pathogens, such as *Brucella*, *Rickettsia*, *Escherichia*, *Salmonella*, and *Helicobacter*, were also found in the Proteobacteria phylum, encompassing human diseases (Rizzatti et al., 2017). Most organisms belonging to the phylum Proteobacteria exist in a free-living state, which includes a diverse range of nitrogen-fixing bacteria (Sharmin et al., 2013). Firmicutes, which encompasses the genus *Bacillus*, have a significant role within the plant microbiome (Borriss, 2020). Cyanobacteria, regarded as one of the most ancient organisms on the planet, exhibit a remarkable ability to thrive in diverse environments, spanning from arid deserts to scorching hot springs and even arctic regions. Cyanobacteria possess diverse capabilities, including establishing biofilms to protect in diverse environmental circumstances (Kollmen & Strieth, 2022). Furthermore, Cyanobacteria possess a remarkable capacity to fix atmospheric nitrogen and exhibit competitiveness within microflora communities. Cyanobacteria exhibit beneficial biological functions, including their efficacy as biocidal agents against bacteria, fungi, and nematodes, as demonstrated (Elagamey et al., 2023).

The relative abundances of both samples at the phylum level are depicted in Figure 5. Within the uncoated sample, 78% of the total reads are attributed to Proteobacteria. Additionally, the phylum Cyanobacteria comprises 13% of the sample. The phylum Firmicutes comprises 6% of the total reads in the sample. In the beeswax-coated sample, the phylum Proteobacteria comprises 36% of the total reads in the sample. Similarly, Cyanobacteria represents 36% of the total reads in the sample. The phylum Firmicutes comprises 18% of the total reads in the sample.

Other studies in the phyllosphere and other plant species have also reported the dominant phylum found in banana pseudostems. The bacteria identified in banana plants are into four primary phyla: Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Additionally, there are several minor phyla, including Cyanobacteria, Chloroflexi, Verrucomicrobia, Planctomycetes, Acidobacteria, and Spirochaetes (Beltran-Garcia et al., 2021). Actinobacteria, Bacteroidetes, Firmicutes, and especially Proteobacteria are often found in bacterial communities on the surface of plants above the ground (phyllosphere). The main phylum found on banana stems can also be found in other plant species, such as rice,

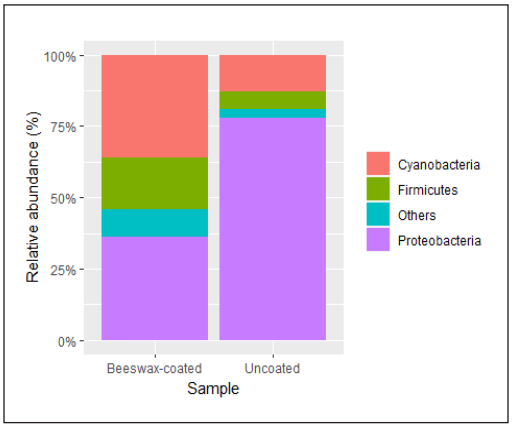


Figure 5. Relative abundance of the four dominant phyla in uncoated and beeswax-coated samples

mustard, spinach, and 56 other tree species (Bulgarelli et al., 2013). In addition to Firmicutes and Proteobacteria (alpha-, beta-, gammaproteobacteria), Cyanobacteria are often found in the phyllosphere environment (Kaewkla & Franco, 2013). Based on a study conducted by Peng et al. (2022), dragon fruit plant stems are also dominated by phyla Proteobacteria and Firmicutes bacteria.

At lower taxonomic levels, differences were also found in samples of banana stems coated with beeswax and not coated with beeswax. Proteobacteria members have the highest relative abundance for samples without beeswax coating, while Cyanobacteria members have the highest relative abundance for beeswaxed banana stems. For the specific genus level in banana stem samples not coated with beeswax, the genus with the highest relative abundance was *Pantoea* (26%). In comparison, other genera had a relative abundance of less than 10%. For the specific genus level in banana stem samples not coated with beeswax, the genus with the highest relative abundance was *Synechococcus* (20%). In comparison, other genera had a relative abundance of less than 10%.

At the species level, there are significant differences in the species that dominate the two samples (Table 2). In the uncoated sample, the five species that exhibit the highest relative abundance were *Pantoea* sp. At-9b (10%), *E. coli* (7%), *Synechococcus* sp. JA-3-3-Ab (6%), *P. vagans* (6%), and *K. pneumoniae* (5%), respectively. In the beeswax-coated sample, the five species that exhibited the highest relative abundance were *Synechococcus* sp. JA-3-3-Ab (20%), *Pseudanabaena* sp. PCC 7367 (7%), *C. thermalis* (5%), *P. megaterium* (4%), and *A. degensii* (4%).

An interesting phenomenon in this study is the difference in bacteria dominating banana stems in both samples at each taxonomic level (Figure 4). This disparity is particularly evident at the species level, where the predominant bacteria found in the uncoated sample had a low relative abundance in the beeswax-coated sample.

Some dominant species in the uncoated sample are classified as opportunistic pathogens, including various species in the genus *Pantoea*, which can cause several infections, although at much lower rates than *E. coli* and *K. pneumoniae* (Cunningham & Leber, 2018). *Klebsiella pneumoniae*, in

Table 2
Species with the highest relative abundance in uncoated and beeswax-coated banana pseudostem samples

Uncoated banana pseudostem samples		Beeswax-coated banana pseudostem samples	
Name	**Relative abundance (%)**	**Name**	**Relative abundance (%)**
Pantoea sp. AT-9b	10	*Synechococcus* sp. JA-3-3-Ab	20
Eschericia coli	7	*Pseudanabaena* sp. PCC 7367	7
Synechococcus sp. JA-3-3-Ab	6	*Chroococcidiopsis thermalis*	5
Pantoea vagans	6	*Priestia megaterium*	4
Klebsiella pneumoniae	5	*Ammonifex degensii*	4

general, is a human pathogen that can cause pneumonia and various infections in the human body (Borkar & Ajayasree, 2021). *Escherichia coli* is also a bacterium involved in various infections, including digestive ones (Jnani & Ray, 2024). Consequently, the application of beeswax coating has the capacity to reduce the presence of pathogenic germs.

This discovery is in line with prior studies conducted in the field. One possible explanation for this phenomenon is attributed to the antimicrobial properties inherent in beeswax, whether independently or in conjunction with other natural substances. Crude beeswax has antimicrobial properties against Gram positive bacteria, such as *Staphylococcus aureus*, *Streptococcus epidermidis*, *Streptococcus pyogenes*, and Gram negative, including *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Aspergillus niger*, and *E. coli* (Fratini et al., 2016). Beeswax has also been found to be antimicrobial against bacteria of the genera *Bacillus*, *Escherichia*, *Listeria*, *Proteus*, *Pseudomonas*, *Salmonella*, *Staphylococcu*s, and various genera of fungi (Szulc et al., 2020). Beck et al. (2021) have also documented the laboratory-based antibacterial properties of beeswax when applied to cotton packing materials.

While the precise mechanism underlying the antibacterial activity of beeswax has not been fully explored, several constituents of beeswax have been documented to possess antimicrobial properties. These include polyphenol components (Anilakumar et al., 2007) and fatty acids (Desbois & Smith, 2010; Fratini et al., 2016). Fatty acids are known to be promising antibacterial agents because they can destabilize bacterial cell membranes. This membrane-destabilizing activity can lead to increased cell permeability and cell lysis. It can inhibit bacterial cell growth (bacteriostatic) or cell death (bactericidal) (Yoon et al., 2018). Additionally, oleic acid, a specific type of fatty acid, can enhance membrane permeability as measured by polarized fluorimetry (Chamberlain et al., 1991). A decreased polarization value indicates an increase in membrane fluidity caused by oleic acid, resulting in cell death. Polyphenols have also been reported to have antimicrobial activity against various types of bacteria. A possible mechanism of action is the aggregatory effect on bacterial cells (Cushnie & Lamb, 2011; Daglia, 2012).

Another phenomenon in this study revealed that the five species that showed comparatively lower abundance in the uncoated sample had higher relative abundance in banana stems treated with beeswax coating. This phenomenon can occur due to the resistance of some species to beeswax. The resilience observed in plants can be attributed to the fertilizers used in the soil, which subsequently move to the pseudostem area, facilitated by precipitation events. Rossmann et al. (2012) have also documented fertilizers derived from animal excrement as a source of antibiotic resistance in bacteria. This microbial community composition data suggests that applying beeswax coating influences the composition of the microbial community

on banana pseudostems. Nevertheless, the bacteria identified as prevalent in the beeswax-coated samples are not categorized as pathogenic microorganisms.

Diversity Estimation

Diversity is one of the most frequently used components in community characterization. Diversity at the habitat level includes species richness and evenness. The species richness index calculates the number of individuals per unit area or sample. In contrast, the evenness index assesses the proportional representation of various species in a community, resulting in a visible distribution. A community with diverse species and equal individuals would exhibit a high evenness index value. In contrast, a community dominated by a particular species in terms of individual count would have a low evenness index value (Thukral, 2017). Alpha diversity is the variability of species in a single sample (Calle, 2019). Alpha diversity can encapsulate the structure of a community depending on its abundance, evenness, or both. In microbial ecology, alpha diversity can determine the difference between environments (Willis, 2019). The alpha diversity indices used in this study include Chao1, Shannon, Simpson, and Equitability, which can be seen in Figure 6.

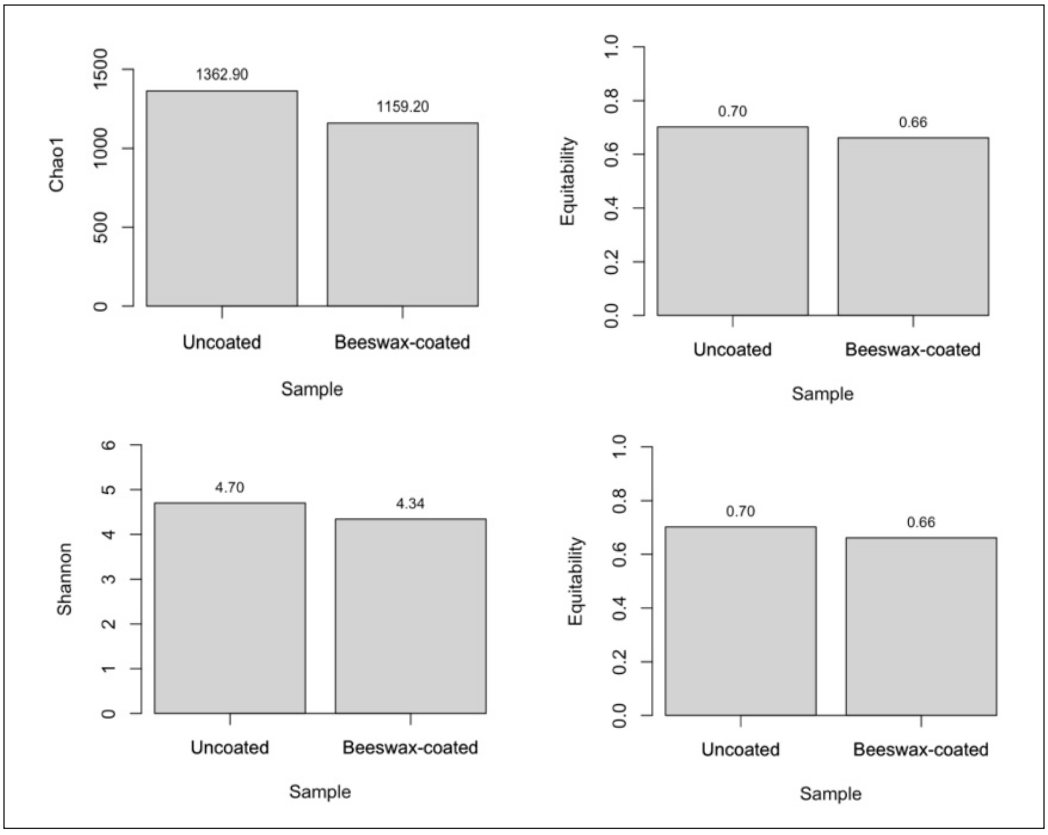


Figure 6. Alpha diversity index of banana stem samples coated and not coated by beeswax

The Chao1 index is a quantitative measure utilized to quantify species richness by considering the relative abundance of different species within a given ecological community. The Shannon and Simpson indices are quantitative measures that can offer insights into the composition of a community and describe population diversity in a sample. Both indices assess diversity by considering both species richness and species evenness. However, their calculations differ in the weight assigned to these two factors. The Shannon index estimates diversity by assigning greater importance to species richness, whereas the Simpson index places greater emphasis on species evenness (B.-R. Kim et al., 2017; Simpson, 1949). The Equitability index is an index that measures the evenness of species in the number of individuals of each species (Thukral, 2017).

Based on the alpha diversity indices in Figure 6, the two samples generally have notable differences. The Chao1 index shows that the uncoated sample has a higher species richness than the beeswax-coated sample. Shannon and Simpson diversity indices show that the uncoated sample is more diverse than the beeswax-coated sample. Based on Shannon index criteria in Ulfah et al. (2019), the Shannon index scores for both samples indicate a high level of diversity ($H' \geq 3$). According to the Simpson index, as the index score approaches 1, the level of diversity increases (Sagar & Sharma, 2012). Based on Simpson index criteria on Wahyuningsih et al. (2019), the Simpson index scores for both samples also indicate a high level of diversity ($0.60 < D \leq 1$). Furthermore, the Equitability index shows that the uncoated sample has a higher species evenness than the beeswax-coated sample. Based on the range of the Equitability index scores from Krebs (1989), both samples are categorized as unstable communities ($0.5 < E \leq 0.75$).

Microbial Community Similarities

Figure 7 depicts a Venn diagram illustrating the similarity of bacterial communities in banana stem samples, comparing the uncoated and beeswax-coated samples.

According to 16S rRNA gene sequencing data, 2,218 operational taxonomic units (OTUs) in the uncoated and beeswax-coated samples were detected overall. The observed similarity between the two samples was 1,196 OTUs (53.9%).

In the uncoated sample, 497 unique OTUs were found unique to the uncoated sample and were not found in the beeswax-coated sample. This number can indicate

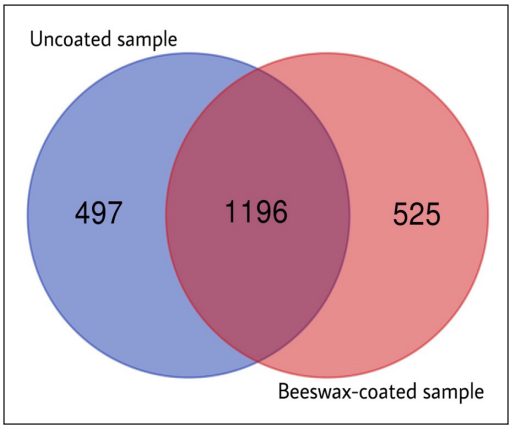


Figure 7. Venn diagram of the uncoated banana pseudostem samples (barcode01) and beeswax-coated banana pseudostem samples (barcode24)

the amount of OTUs lost in banana stems due to beeswax coating. As previously reported, beeswax poses antimicrobial activity due to its composition, including polyphenols (Anilakumar et al., 2007), fatty acids (Desbois & Smith, 2010; Fratini et al., 2016), and propolis (Pinto et al., 2017). Moreover, beeswax has physicochemical properties that can affect the microbial community (Zhang et al., 2023). Beeswax has also been reported to provide a barrier to oxygen, light, and water vapor, potentially impacting microbial activity in water banana stems (Trevisani et al., 2017).

Species uniqueness was also found in the beeswax-coated sample. There were 525 OTUs unique to the beeswax-coated sample that were not found in the uncoated sample. This number can indicate the number of OTUs coming from the beeswax itself. However, it is worth noting that the dominant members of the OTU detected (Table 2) are not pathogenic. Furthermore, these identified OTUs have the potential to exhibit antagonistic activities toward pathogens. As microbial communities in an environment can form interactive networks, this interaction can also occur among fellow species, including antagonistic ones (Hibbing et al., 2010). Therefore, the microbes coming from the beeswax itself may have antagonistic activities toward other bacteria.

CONCLUSION

The research shows that the microbial communities in the uncoated banana pseudostem samples and the beeswax-coated banana pseudostem samples are very different at the species level. The five most dominant species in the uncoated sample are *Panitoea* sp. At-9b, *E. coli*, *Synechococcuis* sp. JA-3-3-Ab, *P. vagans*, and *K. pneumoniae*. The five most dominant species in the beeswax-coated sample are *Synechococcus* sp. JA-3-3-Ab, *Pseudanabaena* sp. PCC 7367, *C. thermalis*, *P. megaterium*, and *A. degensii*. The Chao1, Shannon, Simpson, and Equitability indices for the uncoated sample were 1,362.90; 4.70; 0.97; 0.70, respectively, and for the beeswax-coated sample, they were 1,159.20; 4.34; 0.94; 0.66, respectively. Furthermore, the uncoated sample has higher species richness, diversity, and evenness than the beeswax-coated sample. The similarity of bacterial communities in the beeswax-coated and uncoated samples was 53.9%.

For further research, it is recommended that further examinations be conducted on the bacterial composition of banana stems, comparing those that are coated with beeswax and those that are not. Furthermore, beeswax can be combined with other naturally occurring substances with antimicrobial properties to enhance its antibacterial efficacy, thereby serving as a coating agent.

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Formulation, Physicochemical, and Sensory Evaluation of Cookies Prepared from Sacha Inchi Oil Meal (SIOM)

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ABSTRACT

Due to its substantial amounts of essential amino acids and protein, sacha inchi oil meal (SIOM) is ideal for producing protein-rich food. This study developed the cookies by blending SIOM with wheat flour at 5, 10, 20, and 30% (w/w), respectively. Physical properties, proximate composition, and sensory study were evaluated on the cookies. Data showed that 10% of wheat flour-SIOM cookies had the highest protein content (13.03%) compared to wheat flour cookies (4.89%). Cookies made with 20 and 30% SIOM were also feasible as the crude fiber content was high (48%). The hardness of wheat flour-SIOM incorporated cookies was lower (2.52–3.22 N) than wheat flour cookies (3.30 N). The water activity value of all the cookie samples during the 30-day storage was less than 0.6, indicating that the product was stable. Sensory analysis showed that the panelists preferred 10% SIOM-blend cookies over commercial cookies because of their better color, sweetness, texture, and appearance. Moreover, the overall acceptability of SIOM cookies was greater than 6 on a 9-point hedonic scale. This study concludes that SIOM can be a functional food ingredient that can help develop healthier bakery products.

Keywords: Cookies, flour blends, protein-rich food, sacha inchi oil meal, sensory analysis

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INTRODUCTION

In recent years, there has been a rise in global acceptance and preference for emerging plant-based alternatives like almonds, chickpeas, soybeans, and lupins due to their rich nutrient content, including proteins, essential fatty acids, and fiber. These foods hold the potential for development into functional foods, dietary supplements, and pharmaceutical products, offering preventive benefits for conditions such as metabolic syndrome, diabetes, and lactose intolerance (Kotecka-Majchrzak et al., 2020; Rizzello et al., 2016). Additionally, the use of oil seeds has gained traction in both pharmaceutical and food research, with extracted oils being rich in polyunsaturated fatty acids, bioactive components, and vitamins essential for neuroendocrine system health (Hidalgo & Zamora, 2006; Kotecka-Majchrzak et al., 2020). The byproducts of oil extraction, oil cake/oil meal, are valuable sources of phytochemical compounds, antioxidants, and dietary fibers, making them useful functional ingredients contributing to overall health (Belghith-Fendri et al., 2016; Jeddou et al., 2017). The high protein content in oil meal, resulting from the removal or reduction of fat during oil extraction, presents an opportunity to develop functional foods (Kotecka-Majchrzak et al., 2020).

A few examples of oil meals with health benefits are okara, sesame seed meal, chia seed meal, SIOM, and hemp seed meal (Manikantan et al., 2015; Radočaj et al., 2014; Sánchez et al., 2021; Souza et al., 2015; Swallah et al., 2021). Valorization of agro-industrial byproducts has grown significantly in recent decades due to their presence of essential nutrients while benefiting the environment and economy (de Pilar Sánchez-Camargo et al., 2019).

Sacha inchi seeds (*Plukenetia* spp.) are known globally as a "superfood" due to their excellent nutritional value. The seeds are mainly used to extract the oil as they are enriched with polyunsaturated fatty acids (PUFAs) (α-linolenic acid ~50%, and linoleic acid ~35%) (Gutiérrez et al., 2011, 2019). Due to the presence of PUFAs in the oil, the oil has wide application in nutraceutical, food and pharmaceutical markets (Sánchez et al., 2021). The protein content in the seed varies between 22–30% and has essential amino acids (EAA) higher than other oil seeds (Čepková et al., 2019; Kodahl, 2020). In general, the amino acid composition of the seeds is consistent with dietary guidelines and may represent a valuable source of amino acids, particularly in malnourished and undernourished populations (Sathe et al., 2012).

SIOM, a byproduct of Sacha inchi oil processing, is rich in protein (32 to 62%), and this concentration of protein is generally higher in *Plukenetia volubilis* (59%) as compared to *Plukenetia huayllabambana* (46%) (Ruiz et al., 2013). Nevertheless, regardless of the variety, the protein content in SIOM is similar to the soybean mean (42–50%) (Ibáñez et al., 2020). SIOM also contains important minerals such as potassium, magnesium, calcium, and phosphorus. The investigation by Rawdkuen

et al. (2016) on the mineral content in SIOM showed the presence of calcium (7,616 ppm), magnesium (8,922 ppm), phosphorus (13,125 ppm), and potassium (13,935 ppm), wherein the amount of phosphorus and calcium were similar to soybean meal. SIOM is also rich in amino acids. SIOM is comparable to soy protein in terms of total EAA concentration, making it a rich source of protein. The SIOM has been used as a functional food ingredient and a non-conventional source of protein isolate and hydrolysates (Chirinos et al., 2017; Rawdkuen et al., 2018; Rodríguez et al., 2018).

The development of foods high in fiber, fatty acids, and protein fiber is a prominent trend that meets the current needs of the population and certain consumer groups such as individuals with insufficient dietary fiber intake, weight management and vegans. Due to its high nutritional value, SIOM is suitable for fortifying foods with proteins, fiber, and essential fatty acids (Sánchez et al., 2021).

Cookies are popular bakery products that all age groups prefer. Due to their ease of availability, taste, and longer shelf life, cookies represent a valuable carrier of nutrient supplementation (Machado et al., 2021). Research studies on incorporating oil meal as fortificants have been reported by earlier studies (Shabeer et al., 2016). Thus, this study was undertaken to develop cookies by incorporating SIOM and to investigate the effect of the addition of SIOM on the nutritional and overall acceptability of the formulated cookies.

MATERIALS AND METHODS

Materials

The ingredients, such as whole wheat flour (Pillsbury, India), sugar (CSR Sugar, Malaysia), salt (Double Swallow, Malaysia), margarine (Planta, Malaysia), and vanilla essence (STAR BRAND, Malaysia) were purchased from a local hypermarket (Lulu hypermarket, Malaysia). SIOM was obtained after processing sacha inchi oil seed procured from Myanmar (Wusang Group Sdn. Bhd., Malaysia).

Preparation of Flour Blends

Four flour blends were prepared by blending SIOM with wheat flour at 5, 10, 20, and 30%. The flour blends were designated samples F5, F10, F20, and F30, respectively.

Functional Properties of Flour Blends

Water Absorption Capacity (WAC)

One gram of the sample was mixed with 10 ml distilled water. The solution was centrifuged for 30 min at 1,196 × *g*. The supernatant was discarded, and the sample was weighed again. The results were expressed as g water/g flour.

Oil Absorption Capacity (OAC)

The water was replaced by soybean oil, and the results were expressed as g oil/g flour to determine the OAC (Ghoshal & Kaushik, 2020).

Bulk Density (ρ_B)

A 2 g sample was taken in a measuring cylinder to calculate the bulk density. The

cylinder was gently tapped to dislodge any powder adhering to its inner surface. The bulk density was calculated using Equation 1:

$$\rho_B = \frac{M}{V} \quad [1]$$

where, M = mass of the sample; V = volume of the powder.

Tapped Density (ρ_T)

The measuring cylinder was manually tapped 50 times to ascertain the compact volume (V_T) to calculate the tapped density. The tapped density was then determined using Equation 2:

$$\rho_T = \frac{M}{V_T} \quad [2]$$

where, M = mass of the sample; V_T = compact volume after tapping. The results were expressed as g/ml (Lavanya et al., 2020; Shafi et al., 2016).

Preparation of Cookies

Margarine, powdered sugar, and flavor (vanilla) were creamed in a mixer (Kenwood Chef Classic KM 336, Malaysia) with salt and water. The contents were further mixed for 2 min. The flour blend (5, 10, 20, and 30%) was gradually added, and the contents were kneaded for 2 min to make the dough. The dough sheet was sheeted using a rolling pin and cut into a round shape with a diameter of 4.80 cm using a cookie cutter. The cookies were baked in a baking oven (Elba electric oven, EEO-A2815 (SV), Malaysia) at 160°C for 15–20 min. The products were cooled completely at room temperature (28 ± 1°C). The formulation of the cookies is shown in Table 1.

Proximate Analyses

The proximate analyses of cookies were evaluated following the Association of Official Analytical Chemists (AOAC) method (Horwitz & Latimer, 2005). The calorific value was calculated using 4, 9, and 4 factors for protein, fat, and carbohydrate and was expressed as kcal/100 g (Rai et al., 2014).

Physical Parameters

The physical parameters of the cookies were measured by the method proposed

Table 1
Formulation of cookies

Ingredients	Incorporation of sacha inchi oil meal (SIOM) at different levels in cookies				
	F0	F5	F10	F20	F30
Wheat flour (g)	100	95	90	80	70
SIOM (g)	0	5	10	20	30
Margarine (g)	60	60	60	60	60
Sugar (g)	35	35	35	35	35
Vanilla essence (tsp)	¼	¼	¼	¼	¼
Water (ml)	10	10	10	10	10

Note. F0 = Control; F5 = 5% replacement with SIOM; F10 = 10% replacement with SIOM; F20 = 20% replacement with SIOM; F30 = 30% replacement with SIOM

by Chauhan et al. (2016). The spread ratio was calculated as the diameter-to-thickness ratio, as shown in Equation 3. The cookies' weight was determined using a digital electronic balance (A&D, HR-60, Japan).

$$\text{Spread ratio} = \frac{\text{Diameter (mm)}}{\text{Thickness (mm)}} \quad [3]$$

Color and Hardness of Cookies

The cookies' color was measured using a Chroma Meter CR-400 (Konica Minolta, Inc. Japan). L* indicates lightness, and its values from 0 (black) to 100 (white), a* (±indicates red/green), and b* (± indicates yellow /blue) were used to determine the cookies' color.

The hardness, which represents the maximum force (N) that the cookies could withstand before breaking, was analyzed using a texture analyzer (Stable Micro Systems, United Kingdom) with a shape blade-cutting probe. The analyzer was maintained at pre-test speed =1 mm/s, test speed = 0.5 mm/s, and post-test speed = 10 mm/s.

Microstructural Analysis

Scanning electron microscopy (SEM) (JSM 5800, JEOL Ltd., Japan) was used to investigate the morphology of the cookies. The samples were affixed to a specimen stub with double-sided adhesive tape and then coated with gold using a magnetron sputter coater. The coated samples were examined in SEM at 15 kV at a magnification level of 1,000×.

Moisture Stability of Cookies

The cookies were packed in low-density polyethylene (LDPE) pouches, stored at ambient temperature for a month and analyzed for changes in water activity and moisture content. Moisture content was assessed by calculating the loss on drying at 105°C. The cookies' water activity (a_w) was determined using a water activity meter (AQUALAB Pre Water Activity Analyzer, Decagon Devices Inc., USA). It determines the viable water molecules present in the food system. Lower water activity lowers microbial growth (Theagarajan et al., 2019).

Sensory Analyses

Sixty non-trained panelists over 65 years old performed sensory analyses of the cookies. The panelists were provided with cookie samples, and the objective of the sensory evaluation was presented to them. The sensory attributes of the cookies were evaluated using a 9-point hedonic scale ranging from 1 (strong dislike) to 9 (like to maximum).

Statistical Analysis

A significant difference between the mean values was determined by one-way analysis of variance (ANOVA) and Tukey's range test using Minitab (version 17).

RESULTS AND DISCUSSION

Functional Properties of Flour Blends

Functional properties are the inherent physicochemical characteristics that reflect the intricate interaction between the

composition, structure, physico-chemical properties of proteins, and the type of environment in which these are measured (Kinsella & Melachouris, 1976). These characteristics can ultimately influence the overall quality of the food, specifically concerning its sensory and physicochemical properties. A basic understanding of the functional properties helps to improve processing requirements and optimize their use to develop various food products (Eltayeb et al., 2011).

The effect of incorporating SIOM at various levels on the functional properties such as water holding capacity, oil holding capacity, bulk density, and true density were analyzed, and the results are presented in Table 2.

Water holding capacity is desirable in food processing as it provides the essential organoleptic qualities that make the food acceptable to consumers. In this study, the WAC of the samples varied from 1.45 g/g (F0) to 1.75 g/g (F30). Overall, F0 had the lowest WAC. Germination, fermentation, soaking, or thermal treatments (toasting/autoclaving) considerably improve the water absorption capacity of the meals (Moure et al., 2006). Studies by Iyenagbe et al. (2017) on defatted conophor nuts in raw and toasted oil meal flour showed the highest WAC (1.36 g/g) for toasted flour as compared to untoasted flour (1.18 g/g). The increase in WAC could be due to the partial denaturation of proteins during thermal pre-treatment as during the process, major proteins can break into subunits with more water binding sites than native oligomeric proteins, and this, along with gelatinization of carbohydrates, results in an overall increase in WAC. Variations in WAC between the samples could be attributed to protein structure and various hydrophilic carbohydrates (Sarabhai & Prabhasankar, 2015).

OAC is an important feature for food products, as lipids often improve the flavor and texture of food products. In this study, the OAC decreased with an increase in SIOM addition. Sarabhai and Prabhasankar (2015) reported a similar result when replacing wheat flour with chestnut flour and potato starch. The high OAC value of wheat

Table 2
Functional properties of composite flour blends

Samples	WAC (g water /g flour)	OAC (g oil/g flour)	Bulk density (g/ml)	Tapped density (g/ml)
F0	1.45 ± 0.06^{c}	1.56 ± 0.24^{a}	0.50 ± 0.003^{a}	0.63 ± 0.02^{a}
F5	$1.55 \pm 0.06b^{c}$	1.53 ± 0.05^{a}	0.52 ± 0.006^{a}	0.65 ± 0.01^{a}
F10	1.54 ± 0.03^{bc}	1.53 ± 0.03^{a}	0.53 ± 0.003^{a}	0.66 ± 0.01^{a}
F20	1.61 ± 0.04^{b}	1.53 ± 0.06^{a}	0.54 ± 0.008^{a}	0.66 ± 0.01^{a}
F30	1.75 ± 0.06^{a}	1.53 ± 0.14^{a}	0.54 ± 0.005^{a}	0.67 ± 0.01^{a}

Note. F0 = Control; F5 = 5% replacement with sacha inchi oil meal (SIOM); F10 = 10% replacement with SIOM; F20 = 20% replacement with SIOM; F30 = 30% replacement with SIOM. The values represented by different letters in the same column differ significantly (p<0.05). WAC = Water absorption capacity; OAC = Oil absorption capacity

flour is due to low hydrophobic proteins in wheat flour that exhibit better binding towards lipids (David et al., 2015).

The bulk density of the flour increased with the level of incorporation of SIOM. Similar consistent results were reported by Ndife et al. (2014) on wheat soy flour blends. Likewise, studies by Marak et al. (2019) also reported increased bulk density by replacing wheat flour with foxtail millet and ginger millet powder at increasing proportions. Generally, low bulk density is required for products such as geriatric foods and weaning foods and high bulk density is required for specialty foods to satisfy consumers (Nikitha & Natarajan, 2020). The tapped density measures random dense packing related to the increased bulk density obtained after mechanically tapping the container (Saw et al., 2013). Mechanically tapping a container rearranges the powder particles, lowering the volume of the interparticle gaps, and hence, the tapped density is greater than the bulk density. The tapped density of the samples was between 0.63–0.67 g/ml, and there was no significant difference between the samples.

Proximate Composition of Cookies

The cookies' proximate analyses, including moisture, ash, crude protein, crude fiber, and fat, were studied. The results are shown in Table 3.

The moisture content of cookies with SIOM had higher moisture content as compared to wheat flour cookies, and there was a significant difference ($p<0.05$). The increase in moisture content could be due to the WAC of the composite flours, wherein the WAC was higher in composite flour than wheat flour (Raihan & Saini, 2017). The study results for moisture content agree with cookies developed using carrot pomace, germinated wheat flour, and coffee silver skin, respectively (Baljeet et al., 2014). The protein content of the

Table 3
Proximate composition of cookies

Samples	Moisture (%)	Ash (%)	Crude protein (%)	Crude fat (%)	Crude fiber (%)	Carbohydrate (%)	Calorific value (kcal/100 g)
F0	4.76 ± 0.06^{b}	1.93 ± 0.25^{bc}	4.89 ± 0.67^{c}	28.17 ± 0.04^{a}	46.63 ± 0.29^{b}	13.61 ± 0.56^{a}	327.53
F5	5.34 ± 0.08^{a}	1.82 ± 0.11^{c}	9.88 ± 0.42^{b}	27.33 ± 0.21^{b}	46.82 ± 0.12^{b}	8.82 ± 0.72^{b}	320.77
F10	5.36 ± 0.01^{a}	2.03 ± 0.07^{bc}	13.03 ± 1.79^{a}	28.15 ± 0.21^{a}	47.46 ± 0.18^{b}	3.88 ± 1.92^{c}	320.99
F20	5.38 ± 0.08^{b}	2.21 ± 0.08^{ab}	11.83 ± 0.50^{ab}	27.79 ± 0.13^{ab}	48.05 ± 1.29^{ab}	4.73 ± 0.96^{c}	316.35
F30	5.46 ± 0.15^{c}	2.41 ± 0.12^{a}	11.55 ± 0.72^{ab}	27.75 ± 0.43^{ab}	48.06 ± 0.17^{a}	4.80 ± 1.2^{c}	315.15

Note. F0 = Control; F5 = 5% replacement with sacha inchi oil meal (SIOM); F10 = 10% replacement with SIOM; F20 = 20% replacement with SIOM; F30 = 30% replacement with SIOM. The values represented by different letters in the same column differ significantly ($p<0.05$). WAC = Water absorption capacity; OAC = Oil absorption capacity

cookies decreased as the level of SIOM incorporation increased. A similar result was reported by Arun et al. (2015) on cookies developed by replacing wheat flour with plantain peel flour. A small but statistically significant difference was observed for crude fat. The crude fiber content significantly increased when SIOM replaced wheat flour. Fiber helps in healthy bowel movements and reduces the risk of obesity and colon cancer (Papathanasopoulos & Camilleri, 2010). The data for calorific value shows a reduction in calorific value for cookies. The reduction in calorific value in SIOM cookies could be due to low carbohydrate and high fiber content. Hence, SIOM can be used to develop high-fiber and low-calorie foods to improve health. Yashini et al. (2021) reported a similar reduction in calorific value on cookies developed using defatted tomato seed flour.

Physical Parameters of Cookies

The changes in physical parameters of the cookies, which include weight, diameter, thickness and spread ratio, were analyzed, and the results are presented in Table 4.

The data showed a statistically significant difference ($p<0.05$) in the thickness, diameter, spread ratio, and weight of each sample. The weight of the cookies varied from 13.68 g (F0) to 11.61 g (F30), respectively. The diameter of the cookies varied from 45.43 to 47 mm. The diameter of the cookies developed with SIOM gradually reduced as the level of incorporation of SIOM increased. Studies by Pawde et al. (2020) on biscuits developed using dragon fruit powder reported a similar trend in reduction in diameter of biscuits as the refined wheat flour was replaced by dragon fruit powder (30–60%). Likewise, studies by Ghoshal and Kaushik (2020) also reported a decreasing trend in the diameter of the cookies developed using soy meal flour. The decrease in diameter could be because a higher protein or carbohydrate content in the dough improves its ability to bind water, which promotes the formation of an elastic network and causes the network to shrink after baking (Yashini et al., 2021). However, studies by Guyih et al. (2020) on cookies developed using almond seed and carrot flour reported an increase in

Table 4
Physical parameters of cookies

Samples	Weight (g)	Thickness (mm)	Diameter (mm)	Spread ratio
F0	13.68 ± 1.02^{a}	8.51 ± 0.16^{a}	47.00 ± 0.40^{a}	5.52 ± 0.08^{d}
F5	12.97 ± 0.18^{bc}	8.62 ± 0.06^{b}	46.11 ± 0.52^{b}	5.35 ± 0.11^{bc}
F10	12.57 ± 0.46^{ab}	9.06 ± 0.19^{b}	46.07 ± 0.41^{b}	5.08 ± 0.07^{cd}
F20	11.80 ± 0.63^{c}	9.39 ± 0.09^{c}	45.85 ± 0.39^{b}	4.88 ± 0.10^{a}
F30	11.61 ± 0.28^{c}	9.50 ± 0.12^{c}	45.43 ± 0.40^{b}	4.78 ± 0.06^{ab}

Note. F0 = Control; F5 = 5% replacement with sacha inchi oil meal (SIOM); F10 = 10% replacement with SIOM; F20 = 20% replacement with SIOM; F30 = 30% replacement with SIOM. The values represented by different letters in the same column differ significantly ($p<0.05$). WAC = Water absorption capacity; OAC = Oil absorption capacity

the diameter value as the wheat flour was replaced by almond and carrot flour.

Likewise, the thickness of the cookies gradually increased as the SIOM was incorporated at various levels. Thickness and diameter are inversely related, while the spread ratio depends on the proportion between diameter and thickness (Srivastava et al., 2014). In the present study, the spread ratio of the cookies varied from 5.52 (F0) to 4.78 (F30) respectively. The spread ratio decreased as the level of SIOM addition increased. The decrease in the spread ratio of SIOM cookies can be explained by the fact that wheat and SIOM flour form a higher number of hydrophilic sites for a limited amount of free water in cookie dough. Rapid moments of free water to this hydrophilic region occur during dough blending, which limits the cookie's spread during baking. A similar result was reported by Ghoshal and Kaushik (2020) on soy meal-fortified cookies.

Color and Hardness of Cookies

The effect of the incorporation of SIOM on the color and hardness of cookies was evaluated, and the results are summarized in Table 5. The color values of the freshly baked cookies for lightness (L*) were between 53.05 and 56.00. The value of a* (redness) followed a decreasing pattern with an increase in the level of incorporation of SIOM, and the value of b* for the cookies did not differ much. No significant difference ($p<0.05$) was observed in the L* and b* values. Incorporation of SIOM at the highest level (30%) resulted in a low value of L* (53.05) when compared to the control (53.28), indicating that SIOM cookies are darker in color. Studies by Chauhan et al. (2016) also reported a low value of L* (59.01) on cookies developed by completely replacing wheat flour with amaranth flour. Studies by Ghoshal and Kaushik (2020) on cookies developed using defatted soymeal flour (15–25%) reported that the lightness of cookies (L*) followed an increasing pattern with the level of defatted soymeal flour. During baking, a brown color is formed due to the caramelization of sugar and the Maillard reaction, which are influenced by various factors such as water activity, sugars, and temperature. The lighter color

Table 5
Effect of incorporation of SIOM on color and hardness of cookies

Samples	L*	a*	b*	Hardness (N)
F0	53.28 ± 1.22^{a}	5.68 ± 0.37^{a}	18.88 ± 1.21^{a}	3.30 ± 1.26^{a}
F5	56.00 ± 1.30^{a}	5.67 ± 0.42^{ab}	19.38 ± 1.82^{a}	2.52 ± 0.58^{a}
F10	55.26 ± 1.46^{a}	5.54 ± 0.49^{a}	19.84 ± 1.25^{a}	2.64 ± 1.14^{a}
F20	55.84 ± 0.48^{a}	5.53 ± 0.17^{b}	18.54 ± 0.72^{a}	2.87 ± 0.4^{a}
F30	53.05 ± 1.46^{a}	5.33 ± 0.4^{a}	18.90 ± 1.02^{a}	3.22 ± 0.4^{a}

Note. F0 = Control; F5 = 5% replacement with sacha inchi oil meal (SIOM); F10 = 10% replacement with SIOM; F20 = 20% replacement with SIOM; F30 = 30% replacement with SIOM. The values represented by different letters in the same column differ significantly ($p<0.05$). WAC = Water absorption capacity; OAC = Oil absorption capacity

of cookies may be owing to a lower protein content, which results in less Maillard compound production (Shafi et al., 2016).

Texture, like color, is an important sensory parameter that determines the acceptability of the product. Hardness is one of the texture parameters for cookies (Cheng & Bhat, 2016). The maximum force that the cookies could withstand before breaking was determined using a texture analyzer. Replacement of wheat flour with SIOM to develop cookies changed the breaking strength of the cookies (Table 5). The data reports that the breaking strength of the cookies did not vary significantly (p<0.05). The hardness of cookies is due to the formation of a gluten network, which is caused by the absorption of water molecules by gluten (Aslam et al., 2014). The hardness of the cookies gradually increased with the addition of SIOM (5–30%). The increase in hardness could be due to the fiber and protein content in the SIOM, which has a very good WAC, thus making the dough very sticky. A similar result was reported by Kaur et al. (2019) on cookies developed using flax seed powder. The addition of SIOM at 30% caused an increase in the hardness of cookies, and eventually, a rise in breaking strength was noted. Studies by Ghoshal and Kaushik (2020) also reported that cookies developed with a 25% level of defatted soy meal flour had higher breaking strength when compared to 15 and 20%, respectively. Likewise, studies by Shafi et al. (2016) reported that with an increase in the level of replacement of water chestnut flour (20, 40, 60, 80, and 100%), a gradual increase in the force required to break the cookies was observed.

Microstructural Analysis

The microstructure of the cookies was analyzed using SEM, and the results are shown in Figures 1A-1E. SEM images of control cookies (Figure 1A) showed the presence of large and small gelatinized wheat starch granules embedded in a protein matrix. The matrix is a network enveloping the starch granules (Rao et al., 2022). Figures 1B-1E show the micrograph of wheat flour-SIOM cookies. As the level of SIOM increased from 5 to 30%, the structure appeared loose and open with gaps. This difference was particularly noticeable in the 30% wheat flour- SIOM cookie sample because a lesser amount of starch granules is enclosed in the protein matrix as the gluten in wheat flour is replaced by the proteins in SIOM. Moreover, the presence of fiber and other components of SIOM can affect the starch gluten matrix. It was also evident in the cookie texture, as the spread ratio decreased with the increase in the hardness of cookies (McWatters, 1978).

Moisture Stabilities of Cookies

The cookies prepared using wheat flour and SIOM were stored at ambient temperature for 30 days, and the changes in moisture content and water activity were analyzed; the study results are shown in Figures 2 and 3. The initial moisture content of the cookies was between 4% (F0) and 5.46% (F20). During storage, moisture content increased for all the cookie samples was observed until day 15 and then decreased till day 30 of storage for SIOM cookies. However, an increase in the moisture content of the F0

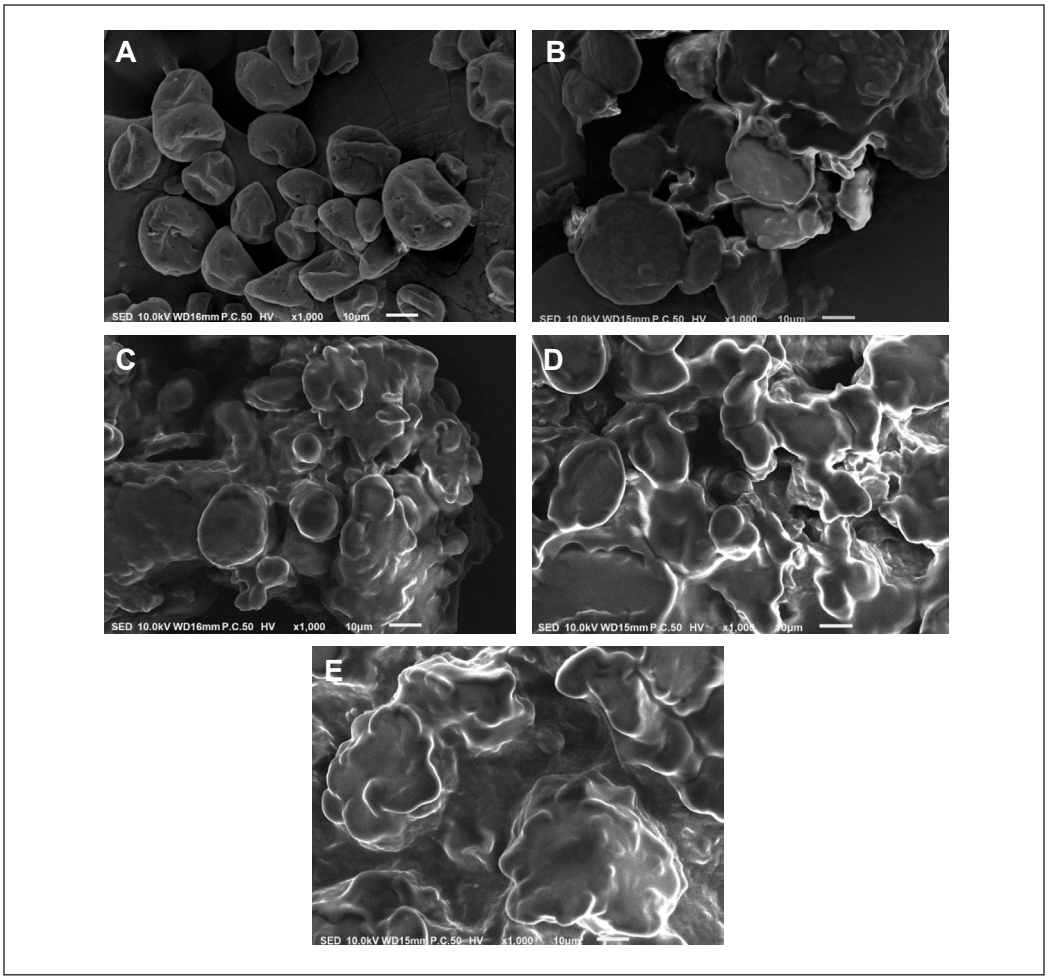

Figure 1. Microstructural analysis of cookies
Note. A = Control; B = 5% replacement with sacha inchi oil meal (SIOM); C = 10% replacement with SIOM; D = 20% replacement with SIOM; E = 30% replacement with SIOM

sample was observed till day 30 of storage, indicating that the F0 sample absorbed moisture from the environment. A similar observation was reported by Chung et al. (2014) on wheat flour cookies during a 7-day storage study.

The loss or gain of moisture in the food component from one place to another continues until thermodynamic equilibrium is attained with the food and the environment (Vasanthakumari & Jaganmohan, 2018). Water activity directly impacts a product's shelf life since most spoilage microbes require specific water activity. In general, the value of water activity close to one indicates that the product is unstable, as most microbes, such as fungi, yeast, and bacteria, require minimum water activity closer to one for their growth. The product's shelf life is acceptable within a water activity range of < 0.6 (Vadukapuram et al., 2014).

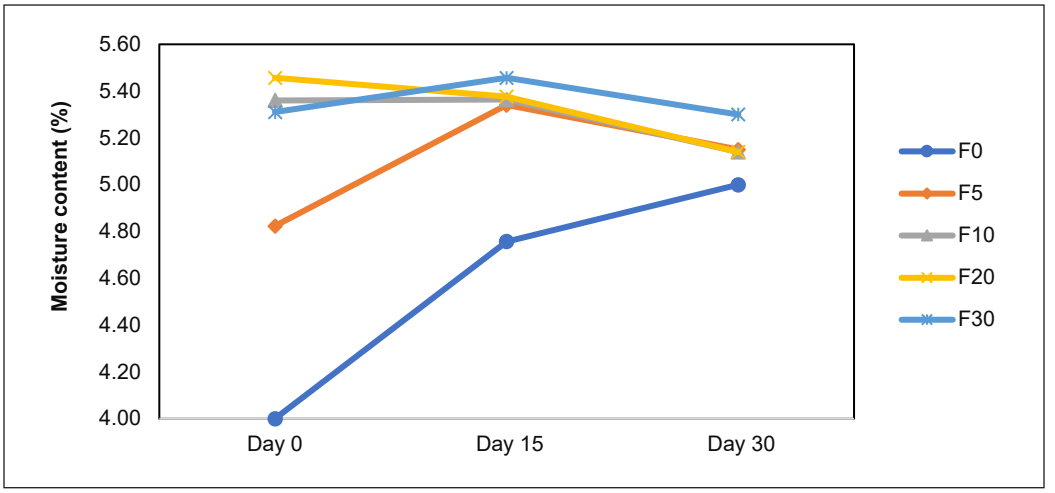

Figure 2. Changes in moisture content of the cookies during 30-day storage
Note. F0 = Control; F5 = 5% replacement with sacha inchi oil meal (SIOM); F10 = 10% replacement with SIOM; F20 = 20% replacement with SIOM; F30 = 30% replacement with SIOM

The water activity of the cookies during the 30-day storage is shown in Figure 3. The F30 cookie sample had the lowest water activity (0.426) compared to the control (0.472) on day 0. The study showed that the water activity of the cookie samples increased during storage. The obtained values were <0.6, indicating that the products were stable during storage. These results were similar to the previously reported studies (Park et al., 2015; Secchi et al., 2011). The increase in water activity during storage is due to the crystallization of sugars (Shafi et al., 2016).

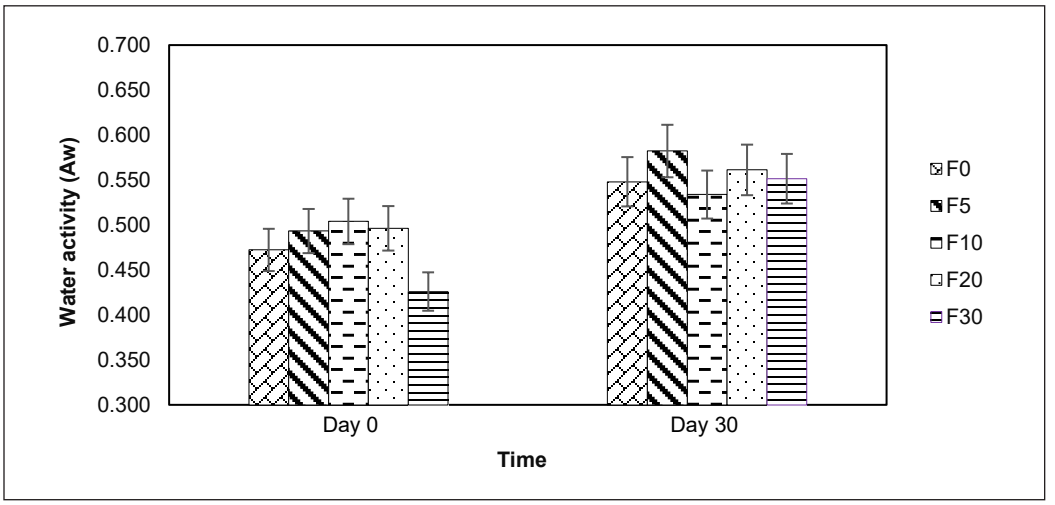

Figure 3. Changes in moisture content of the cookies during 30-day storage
Note. F0 = Control; F5 = 5% replacement with sacha inchi oil meal (SIOM); F10 = 10% replacement with SIOM; F20 = 20% replacement with SIOM; F30 = 30% replacement with SIOM

Sensory Evaluation of Cookies

Sensory evaluation is the simplest and easiest method for consumers to evaluate the acceptability of the product. The results of sensory evaluation are presented in Table 6. The average scores showed a reduction in sensory attributes with increasing levels of SIOM in cookie formulation. Commercial cookies received the lowest score in all the attributes. Among the SIOM cookies, the panelist commented that cookies made from wheat flour blended with 30% SIOM had a nutty taste compared to 5, 10, and 20%. Shafi et al. (2016) reported a similar nutty taste on cookies developed with 100% water chestnut flour. The nutty taste of the wheat flour blended with 30% SIOM reduced the overall acceptability of the cookies. The color of the cookies did not vary across all the cookies. The overall acceptability results showed that SIOM cookies were preferred over commercial cookies. Panelists preferred the sweetness of SIOM cookies more than commercial cookies. Among all the samples, cookies containing 10% SIOM were deemed the best, achieving the highest possible score regarding overall acceptability. Thus, the sensory evaluation results based on the overall acceptability score showed that SIOM could be incorporated as a functional ingredient to develop cookies as healthy snacks for the elderly to promote healthy aging.

CONCLUSION

The study results show that SIOM can be used to develop nutritionally enriched cookies. There was marginal improvement in protein content in SIOM cookies compared to wheat flour cookies. By analyzing the sensory evaluation results, the oil meal can be incorporated up to 10% in the cookies for better acceptability. Further studies can be done to determine the *in-vitro* protein digestibility and minerals in cookies to determine the bio-accessibility of proteins.

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Table 6
Sensory evaluation of cookies

Samples	Color	Texture	Sweetness	Taste	Overall acceptability
A	6.77 ± 1.42^{a}	6.47 ± 1.43^{b}	6.66 ± 1.28^{b}	6.48 ± 1.21^{b}	6.45 ± 1.40^{b}
B	6.87 ± 1.32^{a}	7.17 ± 1.08^{a}	7.26 ± 1.06^{a}	7.05 ± 1.02^{ab}	7.15 ± 0.86^{a}
C	7.05 ± 1.25^{a}	7.35 ± 0.99^{a}	7.16 ± 0.94^{ab}	7.33 ± 0.95^{ab}	7.41 ± 0.85^{a}
D	7.17 ± 1.25^{a}	7.38 ± 1.11^{a}	7.20 ± 1.02^{ab}	7.40 ± 1.06^{ab}	7.48 ± 1.08^{a}
E	7.08 ± 1.31^{a}	7.23 ± 1.13^{a}	7.18 ± 0.97^{ab}	7.03 ± 1.15^{ab}	7.20 ± 1.01^{a}
F	7.17 ± 1.45^{a}	6.95 ± 1.46^{ab}	7.06 ± 1.09^{ab}	6.73 ± 1.33^{b}	7.03 ± 1.30^{a}

Note. A = Commercial cookie; B = Control cookie; C = 5% replacement with sacha inchi oil meal (SIOM); D = 10% replacement with SIOM; E = 20% replacement with SIOM; F = 30% replacement with SIOM. The values represented by different letters in the same column differ significantly ($p<0.05$)

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Identification of Phytochemicals and Mineral Nutrients of Selected Malaysian Plant Extracts and Its Effects on Seed Priming of Maize

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ABSTRACT

Plants contain a variety of phytochemicals, which act as natural bioactive compounds to help plants enhance abiotic tolerance and promote growth. Therefore, plant extracts are considered to have great potential as environmentally friendly biostimulants in sustainable agriculture. This study aimed to identify the phytochemical compounds and quantify nutrients present in three plant extracts, namely *Euphorbia hirta*, *Polygonum minus*, and *Eleusine indica*, as well as to explore the effect on the growth of maize seedlings (*Zea mays* L.). The plant powder was extracted using methanol, followed by a solid-liquid extraction procedure. The phytocompounds were analyzed by liquid chromatography-mass spectrometry, while mineral nutrients were quantified using inductively coupled plasma. Five concentrations of plant extracts, i.e., 5, 15, 25, 50, and 100%, were designed to evaluate seed germination and priming. The result showed that 53, 45, and 39 phytocompounds were identified from *E. hirta*, *P. minus*, and *E. indica*, respectively, and classified into different chemical groups (such as flavonoids and amino acids) and rich nutrients (for example, N, P, and K). Besides, *P. minus* and *E. hirta* extracts with lower concentrations (5 and 15%) showed a positive effect on germination, shoot length and fresh weight, and the Highest concentration (100%) of *E. hirta* and *E. indica* extracts exhibited strong toxicity. This study emphasizes that weed extracts containing abundant secondary metabolites and nutrients can be used as natural biostimulants for maize seed priming.

Keywords: Maize, mineral nutrient, plant extract, phytochemical, seed priming

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INTRODUCTION

Plants can produce a variety of bioactive compounds through metabolism, among which numerous antioxidant compounds have been identified in vegetables and fruits, such as phenolics, flavonoids, tocopherols, and anthocyanins (Ali et al., 2021). Certain medicinal plants have gained widespread use in the pharmaceutical industry for treating related diseases (Hao et al., 2020). Meanwhile, phytochemicals, which accumulate in high concentrations within plants, may protect against damage caused by abiotic stress (Hussein et al., 2015). Additionally, some beneficial phytochemicals serve as natural antioxidants and can supplement the human body's requirements (Boots et al., 2008), thereby increasing immunity and mitigating age-related health issues (Forni et al., 2019). As a result, there has been an increase in the global consumption of medicines and health products derived from plants.

In the world's tropical regions, a wide spread of weeds in agricultural lands affects crop production, and Malaysia is no exception (Dilipkumar et al, 2020). Karim et al. (2004) reported that weed invasion can cause rice yield losses ranging from 5 to 85% in Malaysia. Besides, weeds compete intensively for nutrients, resulting in a yield decrease of 5 to 20% (Sahid et al., 1992). However, farmers and administrators usually ignore the disadvantage of weeds. On the other hand, studies have demonstrated the potential benefits of certain weeds. For instance, *P. minus* is believed to be associated with antioxidant activities, such as flavonoids and phenolic acids (Baharum et al., 2010). *Euphorbia hirta* exhibited high radical scavenging and antioxidant activity, while *E. indica*, native to the tropics and subtropics, also possesses antioxidant properties (Iqbal & Gnanaraj, 2012; Rattanata et al., 2014). Considering that weeds are abundant and inexpensive sources of materials, developing biostimulants from weeds to supplement traditional fertilizers may not only mitigate the negative effects of weeds on crops but also enhance plant growth, development, and the quality of agricultural products.

Several plant extracts are being studied as biostimulants to evaluate their impact on plant growth. Islam et al. (2022) investigated the effects of banana pseudostem sap on sweet corn seedling growth and confirmed the stimulating role of identified phytochemicals and mineral nutrients in banana pseudostem sap. Yasmeen et al. (2013) illustrated that *Moringa oleifera* leaf extract can act as a seed priming agent to effectively enhance seed emergence, seedling vigor, leaf area and yield-related attributes in wheat. Talukder et al. (2015) also examined herbal plant extract and its ability to promote the germination of different vegetable seeds. The foliar application of licorice extract was found to mitigate drought stress in sesame (Pourghasemian et al, 2020), while seaweed extract enhanced stress growth traits and antioxidant contents in *Spiraea* and *Pittosporum* (Elansary et al., 2016). However, there has been limited research on the application of weed extracts for plant growth. Besides, most studies on phytochemicals in weeds have only focused

on specific phytocompounds, with a few observations that are difficult to quantify. Liquid chromatography-mass spectrometry (LC-MS), a methodology-based metabolic profiling technique, has been improved and enables the investigation of the diversity of non-target phytochemicals via a specialized database. Moreover, it provides structural information without extra tandem mass spectrometry (MS/MS) analysis (Matsuda et al., 2009). Thus, the hypothesis of this study is that weed extracts contain abundant phytochemicals and mineral elements, and it may have a beneficial effect on seed priming. Therefore, the objectives of our research were to (1) identify and quantify the phytochemicals in weed extracts, (2) quantify their phytocompounds and mineral nutrients, and (3) evaluate the effects of weed extracts on seedling growth. The findings of this study will contribute to the exploration of weed extracts as natural and cost-effective biostimulants for sustainable agriculture.

MATERIALS AND METHODS

Collection of Selected Plants

Three plants, namely *E. hirta*, *E. indica*, and *P. minus*, were selected for the study (Figure 1). *Polygonum minus* was purchased from a local supplier. In contrast, the others were collected from a local field in the Faculty of Agriculture (2°98' N, 101°73' E) at the Universiti Putra Malaysia in Selangor, Malaysia. The mature and whole plants were washed with fresh tap water to remove mud from the roots and then rinsed twice with distilled water. The washed plants were air-dried for 7 days at room temperature, then pulverized using a blender and stored in sealed plastic bags.

Extraction of Plant Extracts with Methanol Solvent

Extraction of selected weeds was done in Erlenmeyer flasks: 10 g plant materials were mixed with methyl alcohol 99.95% (HPLC grade, Sigma-Aldrich, USA) using

(a) (b) (c)

Figure 1. Selected plants: (a) *Euphorbia hirta*; (b) *Polygonum minus*; and (c) *Elusine indica*

an optimum ratio of 1/8 (w/v) sample weight to solvent volume (Alupului et al., 2012). After that, the mixtures were continuously shaken at 150 rpm for 24 hr via an orbital shaker. The extraction samples were filtered with Whatman No.1 filter paper (diameter 150 mm, Cytiva, USA), and the alcohol and excess water were evaporated under vacuum at 30℃ using a rotary evaporator (CCA-111, EYELA, Japan). Finally, a part of the concentrated sample solutions was collected and preserved in a refrigerator at -20℃ for phytocompounds analysis using liquid chromatography-mass spectrometry (LC-MS), while the other part of the concentrated sample solution was preserved at 4℃ to determine the chemical properties and used for seedling growth.

LC-MS Studies

Separation was performed using a Thermo Scientific™ (USA) C18 column (Acclaim™ Polar Advantage II, 3 mm × 150 mm, 3 µm particle size) on an UltiMate™ 3000 UHPLC systems (Dionex). Gradient elution was performed at a flow rate of 0.4 ml/min and 40°C column temperature using distilled water + 0.1% formic acid (A) and 100% acetonitrile (B) with 22 min total run time. The injection volume of the sample was 1 µl. The gradient started at 5% B (0–3 min), 80% B (3–10 min), 80% B (10–15 min), and 5% B (15–22 min). The sample was diluted in 1:10 methanol. All the chemicals were purchased from Chemiz (Malaysia).

Determination of Chemical Properties in Selected Plant Extracts

The pH and electrical conductivity (EC) were measured from plant extract solution (100 g/L), which concentrated extract solution from 10 g plant materials was dissolved in 100 ml distilled water utilizing a digital pH meter (HI 2211 pH meter,

Table 1
Chemical properties of selected plant extracts

Chemical characters	*Euphorbia hirta* extract	*Polygonum minus* extract	*Elusine indica* extract
pH	4.25	4.79	4.66
Electrical conductivity (µS/cm)	10.83	16.39	16.06
Total C (%)	51.46	49.94	46.10
Total N (%)	0.66	7.10	3.69
Total P (g/kg)	0.67	2.26	0.32
Total K (g/kg)	22.07	38.54	62.08
Total Ca (g/kg)	0.26	0.25	0.39
Total Mg (g/kg)	0.37	0.26	1.31
Fe (mg/kg)	45.79	67.13	52.00
Cu (mg/kg)	5.14	7.17	4.29
Zn (mg/kg)	7.52	20.72	61.82
Mn (mg/kg)	2.36	3.14	6.72
B (mg/kg)	7.24	4.37	3.20

Hanna Instruments, USA) and digital EC meter (Hanna 2300, Hanna Instruments, USA). The plant extract (on a dry weight basis) was for measure the total C and N using TruMac® CNS analyzer (LECO, USA) and characteristics of total mineral nutrients, namely P, K, Ca, Mg, Fe, Cu, Zn, Mn, and B using inductively coupled plasma (ICP)—optical emission spectroscopy (Optima 8300, PerkinElmer, USA), respectively. The chemical properties of weed extract samples are described in Table 1.

Preparation of Selected Plant Extracts for Seedling Growth

The plant extract solution (100 g/L) was diluted with distilled water to prepare 5, 15, 25, and 50% solutions. Meanwhile, plant extracts (100 g/L) and distilled water were considered a 100% concentration solution and controlled to evaluate seedling growth. Maize seeds (*Zea mays* L., Hybrid F1 316, Malaysia) were first soaked for 12 hr in water, and then 10 sprouting seeds were placed in the Petri dish with equal distance between them. According to the treatments, 8 ml of plant extract was poured into a Petri dish with pieces of tissue (material-solution ratio is 1:4) and followed by 5 ml of a similar solution in each Petri dish was added every day to keep humidity, which was continued for all the experimental period (10 days). Each treatment was performed in three repetitions. The experiment was carried out in the Faculty of Agriculture, Universiti Putra Malaysia Lab, where the temperature was 26°C and the humidity 70% conditions. The date of germination (%), shoot length (cm), fresh and dry weight (g), and soil plant analysis development (SPAD) value (SPAD-502Plus, Konica Minolta, Japan) were recorded.

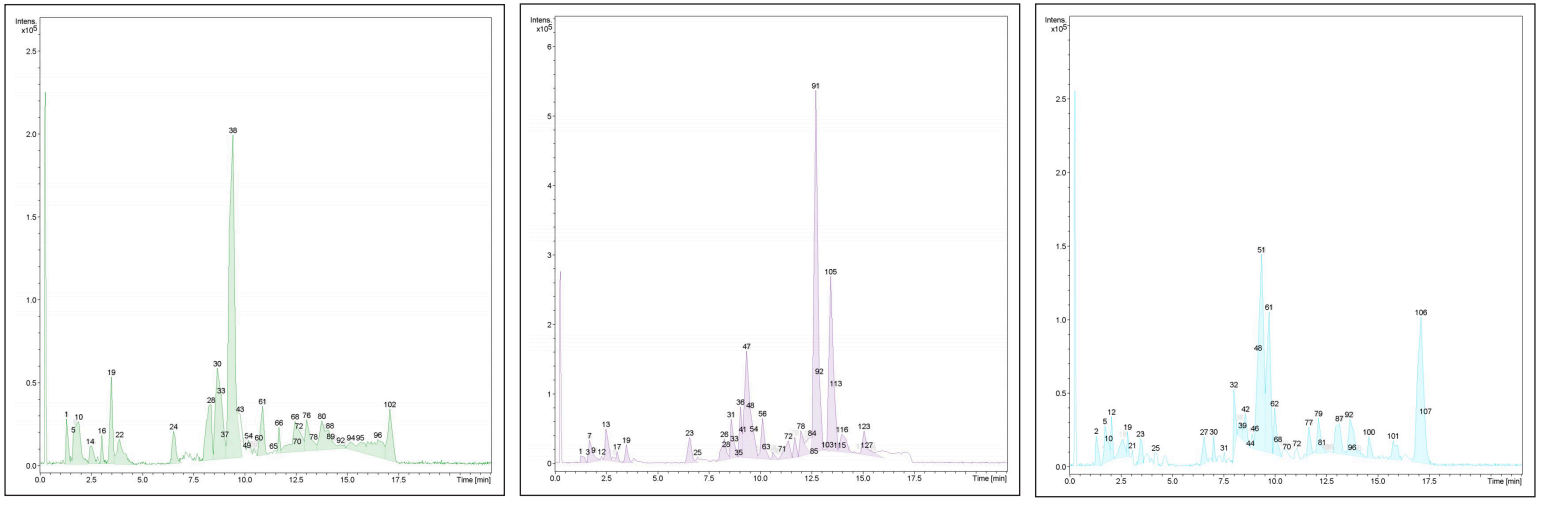
Statistical Analysis

Data were analyzed using the SPSS 25.0 (IBM, USA). Significant differences between treatments were calculated by one-way analysis of variance (ANOVA) with the least significant difference (LSD) test at $p < 0.05$. The shoot lengths of maize seedlings were measured using Image J software (version 1.8).

RESULTS

Characterization of Phytocompounds in Selected Plant Extracts

Phytochemical compounds identified with methanolic extraction of *E. hirta*, *P. minus*, and *E. indica* using LC-MS were shown in Table 2. A total of 45, 53, and 39 phytocompounds were identified by given chromatographic peaks under the retention time of 1.30 to 17.3 min (Figure 2). A total of 112 phytocompounds were classified into different chemical groups, including 19 flavonoids, 14 amino acids, 24 alkaloids, 11 polyketides, 24 terpenoids, 5 phenylpropanoids, 2 carbohydrates, 2 fatty acids, 2 vitamins, as well as 9 others (Table 2). Among the three plant extracts, *P. minus* contains the most phytocompounds, containing 53, followed by *E. hirta*, containing 45; the least is *E. indica*, containing 39. Besides, the three weed extracts contained five identical phytocompounds: lotaustralin, carolinianine,



(a) (b) (c)

Figure 2. Total positive charged ions chromatogram of (a) *Euphorbia hirta*, (b) *Polygonum minus*, and (c) *Elusine indica* obtained by liquid chromatography-mass spectrometry through methanolic extraction

Table 2
Identification of phytochemical compounds in selected plants by liquid chromatography-mass spectrometry

Serial Nr	Compounds name	Chemical group	Chemical formula	Mass	Plant beneficial Yes (Y)/Not (N)	E. H. Y/N	P. M. Y/N	E. I. Y/N
1	Cupressuflavone	Flavonoids	$C_{30}H_{18}O_{10}$	538.09	Y	Y	N	N
2	Quercetin 3, 4', 7-trissulfate		$C_{15}H_{10}O_{16}S_3$	541.91	Y	Y	N	N
3	Azaleatin		$C_{16}H_{12}O_7$	316.05	Y	Y	Y	N
4	Epigallocatechin gallate		$C_{22}H_{18}O_{11}$	458.08	Y	Y	N	Y
5	Hesperidin		$C_{28}H_{34}O_{15}$	610.18	Y	N	Y	N
6	Pachypodol		$C_{18}H_{16}O_7$	344.08	N	N	Y	N
7	Procyanidin B4		$C_{30}H_{26}O_{12}$	578.14	Y	N	Y	N

Table 2 *(continue)*

Serial Nr	Compounds name	Chemical group	Chemical formula	Mass	Plant beneficial Yes (Y)/Not (N)	E. H. Y/N	P. M. Y/N	E. I. Y/N
8	Piperaduncin B		$C_{29}H_{30}O_8$	506.54	N	Y	N	N
9	Myricitrin		$C_{21}H_{20}O_{12}$	464.09	Y	N	Y	Y
10	Myricetin		$C_{15}H_{10}O_8$	318.03	Y	N	N	Y
11	Morin		$C_{15}H_{10}O_7$	302.04	Y	N	N	Y
12	Isovitexin		$C_{21}H_{20}O_{10}$	432.10	Y	Y	N	Y
13	Isoterchebin		$C_{41}H_{30}O_{27}$	954.09	Y	N	Y	N
14	Tricin		$C_{17}H_{14}O_7$	330.07	Y	Y	N	N
15	Vicenin-2		$C_{27}H_{30}O_{15}$	594.15	Y	N	Y	N
16	Ptaerochromenol		$C_{15}H_{14}O_5$	274.08	Y	Y	N	Y
17	Pelargonidin 3-O-3, 6-O-dimalonylglucoside		$C_{27}H_{25}O_{16}$	605.11	Y	N	Y	N
18	Silychristin		$C_{25}H_{22}O_{10}$	482.12	Y	Y	Y	N
19	Isoscutellarein		$C_{15}H_{10}O_6$	286.04	Y	N	Y	N
20	Pidolic acid	Amino acid	$C_5H_7NO_3$	129.04	Y	N	Y	N
21	Amaranthin		$C_{30}H_{34}N_2O_{19}$	726.17	Y	Y	N	N
22	N-Succinyl-LL-2,6-diaminoheptanedioate		$C_{11}H_{18}N_2O_7$	290.11	N	N	Y	N
23	Homocapsaicin		$C_{19}H_{29}NO_3$	319.21	Y	N	Y	N
24	Indicaxanthin		$C_{14}H_{16}N_2O_6$	308.10	Y	N	Y	N
25	Lotaustralin		$C_{11}H_{19}NO_6$	261.12	Y	Y	Y	Y
26	L-Tyrosine		$C_9H_{11}NO_3$	181.07	Y	N	N	Y
27	L-Valine		$C_5H_{11}NO_2$	117.07	Y	Y	N	N
28	L-Phenylalanine		$C_9H_{11}NO_2$	165.07	Y	Y	N	N
29	Glucobrassicin		$C_{16}H_{20}N_2O_9S_2$	448.06	Y	N	Y	N
30	N-Carbamoylputrescine		$C_6H_{13}N_3O$	131.10	N	N	N	Y
31	Neoglucobrassicin		$C_{17}H_{22}N_2O_{10}S_2$	478.07	Y	N	Y	N
32	L-Homoserine		$C_4H_9NO_3$	119.05	Y	Y	N	N
33	Methylthio-2-oxobutanoic acid		$C_5H_8O_3S$	148.01	N	Y	N	N
34	Alangiside	Alkaloids	$C_{25}H_{31}NO_{10}$	505.19	Y	N	N	Y

Table 2 *(continue)*

Serial Nr	Compounds name	Chemical group	Chemical formula	Mass	Plant beneficial Yes (Y)/Not (N)	E. H. Y/N	P. M. Y/N	E. I. Y/N
35	Casimiroin		$C_{12}H_{11}NO_4$	233.06	N	N	N	Y
36	Cinnamoylcocaine		$C_{19}H_{23}NO_4$	329.16	Y	Y	N	N
37	Carolinianine		$C_{16}H_{24}N_2O_2$	276.37	Y	Y	Y	Y
38	Echitovenine		$C_{23}H_{28}N_2O_4$	396.20	N	N	Y	N
39	Deacetylisoipecoside		$C_{25}H_{33}NO_{11}$	523.20	Y	Y	N	N
40	Finaconitine		$C_{33}H_{46}N_2O_{10}$	630.31	N	N	Y	N
41	Heliotrine		$C_{16}H_{27}NO_5$	313.18	N	N	Y	N
42	Leiokinine A		$C_{14}H_{17}NO_2$	231.12	N	N	Y	N
43	Lophocerine		$C_{15}H_{23}NO_2$	249.17	N	N	Y	N
44	Lunamarine		$C_{18}H_{15}NO_4$	309.10	N	Y	N	N
45	Norhyoscyamine		$C_{16}H_{21}NO_3$	275.15	Y	Y	N	N
46	N-Methyltyramine		$C_9H_{13}NO$	151.09	Y	N	Y	N
47	Melicopine		$C_{17}H_{15}NO_5$	313.09	N	N	N	Y
48	Mesembrinol		$C_{17}H_{25}NO_3$	291.18	N	N	Y	N
49	Clivoline		$C_{21}H_{27}NO_7$	405.17	N	N	N	Y
50	Lobelanidine		$C_{22}H_{29}NO_2$	339.21	N	Y	Y	Y
51	Terpendole K		$C_{32}H_{39}NO_5$	517.28	N	Y	N	N
52	Tyramine		$C_8H_{11}NO$	137.08	Y	N	Y	N
53	Reserpine		$C_{33}H_{40}N_2O_9$	608.27	N	N	N	Y
54	Salutaridine		$C_{19}H_{21}NO_4$	327.14	Y	N	Y	N
55	Vasicinol		$C_{11}H_{12}N_2O_2$	204.08	Y	N	Y	N
56	21, 22-Diprenylpaxilline		$C_{36}H_{49}NO_4$	571.36	N	N	Y	N
57	(6s)-Hydroxyhyoscyamine		$C_{17}H_{23}NO_4$	305.16	Y	N	Y	N
58	Hamaudol	Polyketides	$C_{15}H_{16}O_5$	276.09	N	Y	N	N
59	Aclacinomycin S		$C_{36}H_{45}NO_{13}$	699.28	Y	N	N	Y
60	Ansamitocinoside P-3		$C_{37}H_{51}ClN_2O_{14}$	783.06	Y	N	N	Y
61	dTDP-D-glucuronate		$C_{18}H_{16}O_7$	344.08	N	N	Y	N

Table 2 *(continue)*

Serial Nr	Compounds name	Chemical group	Chemical formula	Mass	Plant beneficial Yes (Y)/Not (N)	E. H. Y/N	P. M. Y/N	E. I. Y/N
62	5-O-Methylalloptaeroxylin		$C_{16}H_{16}O_4$	272.10	Y	N	Y	N
63	Glucofrangulin B		$C_{26}H_{28}O_{14}$	564.14	Y	Y	N	Y
64	Leucomycin A7		$C_{38}H_{63}NO_{14}$	757.42	N	N	N	Y
65	Leucomycin A8		$C_{39}H_{63}NO_{15}$	785.41	N	Y	Y	N
66	Niddamycin		$C_{39}H_{63}NO_{14}$	769.42	N	Y	N	N
67	Troleandomycin		$C_{41}H_{67}NO_{15}$	813.45	N	Y	N	Y
68	Rifamycin		$C_{37}H_{47}NO_{12}$	697.30	N	N	Y	N
69	Frullanolide	Terpenoids	$C_{15}H_{20}O_2$	232.14	Y	N	Y	N
70	Tutin		$C_{15}H_{18}O_6$	294.11	N	Y	N	N
71	Thevetin B		$C_{42}H_{66}O_{18}$	858.42	N	Y	N	N
72	Taxine B		$C_{33}H_{45}NO_8$	583.31	N	N	N	Y
73	Terpenoid EA-T		$C_{30}H_{40}O_8$	528.27	N	Y	N	N
74	Petasin		$C_{20}H_{28}O_3$	316.20	Y	Y	N	N
75	Phorbol 12-tiglate 13-decanoate		$C_{35}H_{52}O_8$	600.36	Y	Y	N	N
76	Picrasin C		$C_{23}H_{34}O_7$	422.23	N	N	N	Y
77	Baliospermin		$C_{32}H_{50}O_8$	562.35	N	N	Y	Y
78	Diterpenoid EF-D		$C_{27}H_{38}O_7$	474.26	Y	Y	N	N
79	Dehydron gaione		$C_{15}H_{20}O_3$	248.14	Y	N	Y	N
80	Deltonin		$C_{45}H_{72}O_{17}$	884.47	Y	Y	N	N
81	Diterpenoid SP-II		$C_{20}H_{32}O_4$	336.23	Y	Y	N	N
82	Erioflorin acetate		$C_{21}H_{26}O_7$	390.16	N	N	Y	N
83	Germacrene		$C_{15}H_{22}O_2$	234.16	Y	N	Y	N
84	Lathyrol		$C_{20}H_{30}O_4$	334.21	Y	N	N	Y
85	Lycoxanthin		$C_{40}H_5O_6$	552.43	Y	Y	N	Y
86	Alpha-Irone		$C_{14}H_{22}O$	206.16	Y	N	N	Y
87	Alpha-Zeacarotene		$C_{40}H_{58}$	538.45	Y	N	Y	N
88	Alpha-Vetivone		$C_{15}H_{22}O$	218.16	Y	N	Y	N

Table 2 *(continue)*

Serial Nr	Compounds name	Chemical group	Chemical formula	Mass	Plant beneficial Yes (Y)/Not (N)	E. H. Y/N	P. M. Y/N	E. I. Y/N
89	Arbusculin A		$C_{15}H_{22}O_3$	250.15	Y	N	Y	N
90	Agavoside A		$C_{33}H_{52}O_9$	592.36	Y	N	Y	N
91	7-Deoxyloganate		$C_{16}H_{24}O_9$	360.14	N	N	N	Y
92	12-O-Tetradecanoylphorbol 13-acetate		$C_{36}H_{56}O_8$	616.39	N	N	Y	N
93	Coniferyl alcohol	Phenylpropanoids	$C_{10}H_{12}O_3$	180.07	Y	N	Y	N
94	Cleistanthin A		$C_{28}H_{28}O_{11}$	540.16	Y	N	Y	N
95	Eudesobovatol		$C_{33}H_{44}O_4$	504.32	Y	Y	N	N
96	Peucedanin		$C_{15}H_{14}O_4$	258.08	Y	Y	N	N
97	Umbelliferone		$C_9H_6O_3$	162.03	Y	N	N	Y
98	D-Glucose	Carbohydrate	$C_6H_{12}O_6$	180.06	Y	N	N	Y
99	Neuraminic		$C_9H_{17}NO_8$	267.09	N	Y	N	Y
100	Gamolenic acid	Fatty acid	$C_{18}H_{30}O_2$	278.22	Y	Y	N	Y
101	3-Indoleacrylate		$C_{11}H_9NO_2$	187.06	Y	Y	Y	Y
102	Phylloquinol	Vitamin	$C_{31}H_{48}O_2$	452.36	Y	Y	N	Y
103	5, 6, 7, 8-Tetrahydromonapterin		$C_9H_{15}N_5O_4$	257.11	N	N	N	Y
104	Actinorhodin	Others	$C_{32}H_{26}O_{14}$	634.13	N	N	Y	N
105	Butirosin A		$C_{21}H_{41}N_5O_{12}$	555.27	N	N	Y	N
106	4-Deoxy-4-thio-alpha-D-digitoxosyl-calicheamicin T0		$C_{30}H_{38}N_2O_{11}S_4$	730.13	N	N	Y	N
107	Pedunculagin		$C_{34}H_{24}O_{22}$	784.07	Y	N	N	Y
108	Pyrogallic acid		$C_6H_6O_3$	126.03	N	Y	N	N
109	Prostaglandin E3		$C_{20}H_{30}O_5$	350.20	N	Y	N	N
110	Uroporphyrin I		$C_{40}H_{38}N_4O_{16}$	830.22	Y	Y	Y	Y
111	7,8-Dihydroneopterin3"-triphosphate		$C_{17}H_{22}N_2O_{10}S_2$	494.99	N	N	Y	Y
112	Glucosyl-4, 4'-diaponeurosporenoate		$C_{36}H_{50}O_7$	594.35	N	N	N	Y

Note. *E. H.*, *P. M.*, and *E. I.* represent *Euphorbia hirta*, *Polygonum minus*, and *Elusine indica* extracts, respectively

lobelanidine, 3-indoleacrylate, and uroporphyin I. The overall study found that 68 molecules were beneficial to the plant, of which *E. hirta*, *P. minus*, and *E. indica* contained 29, 33, and 23, respectively.

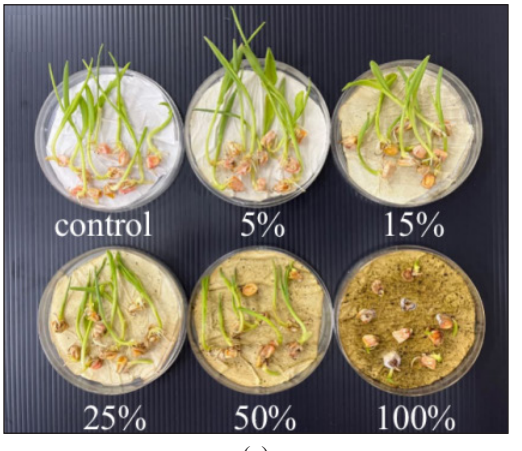
Effects of Selected Plant Extracts on Seedling Growth of Maize

The germination of maize seed was strongly affected by plant extract application, and the effects differed with different concentrations (Table 3, Figure 3). Overall, low concentrations (5 and 15%) of plant extracts can promote seed germination, while seed germination was significantly inhibited by high concentrations (p<0.05). Correspondingly, 100% *E. hirta* and *E. indica* extract treatments significantly decreased germination compared with control (p<0.05). Results also showed that the germination of *E. indica* extract treatments was higher than that of *E. hirta* and *P. minus* extract treatments at the same concentration except for 100% concentration.

The change in shoot length after applying weed extract was the same as germination (Table 3, Figure 3); the data on root length were not shown because that was not evident. Especially compared to the control, 100% *E. hirta* and *E. indica* extract treatments significantly decreased by 78.1 and 80.8%, respectively (p<0.05). In addition, 5% *E. hirta* and *E. indica* extract treatments were significantly higher than other concentrations (p<0.05). A similar trend was found in fresh weight, in which treatments with the highest concentration

(a)

(b)



(c)

Figure 3. Effect of different concentrations: (a) *Euphorbia hirta*; (b) *Polygonum minus*; and (c) *Elusine indica* extracts on seedling growth, respectively

Table 3
Effect of different concentrations of selected plant extracts on seedling growth of maize

Treatments	Germination (%)	Shoot length (cm)	Fresh weight (g)	Dry weight (g)	Soil plant analysis development value
Control	86.67 ± 5.77	7.55 ± 1.78	0.38 ± 0.04	0.10 ± 0.03	21.20 ± 2.01
5% *Euphorbia hirta*	90.00 ± 10.00a	6.80 ± 0.47a	0.35 ± 0.08a	0.11 ± 0.01a	19.23 ± 0.57a
15% *Euphorbia hirta*	80.00 ± 0.00ab	4.99 ± 0.75b	0.26 ± 0.04ab	0.07 ± 0.01b	15.00 ± 1.30b*
25% *Euphorbia hirta*	76.67 ± 11.55ab	4.76 ± 1.22b	0.26 ± 0.07ab	0.09 ± 0.02ab	14.60 ± 1.92b*
50% *Euphorbia hirta*	70.00 ± 10.00b	4.42 ± 0.82b	0.31 ± 0.04ab	0.10 ± 0.02a	14.80 ± 0.60b*
100% *Euphorbia hirta*	50.00 ± 10.00c*	1.65 ± 0.22c*	0.21 ± 0.05b*	0.09 ± 0.02ab	9.47 ± 0.85c*
5% *Polygonum minus*	93.33 ± 5.77a	7.62 ± 0.77a	0.41 ± 0.02a	0.10 ± 0.01ab	19.27 ± 0.65a
15% *Polygonum minus*	96.67 ± 5.77ab	7.76 ± 0.95a	0.35 ± 0.06ab	0.08 ± 0.03b	20.47 ± 1.78a
25% *Polygonum minus*	73.33 ± 15.28bc	3.54 ± 0.54c	0.29 ± 0.06b	0.12 ± 0.03a	18.87 ± 0.86a
50% *Polygonum minus*	76.67 ± 5.77bc	7.56 ± 0.51a	0.37 ± 0.06ab	0.08 ± 0.01b	20.67 ± 2.15a
100% *Polygonum minus*	76.67 ± 12.58c	5.33 ± 0.26b	0.28 ± 0.06b*	0.07 ± 0.03b	18.37 ± 2.05a
5% *Elusine indica*	96.67 ± 5.77a	9.47 ± 0.93a	0.44 ± 0.10a	0.11 ± 0.02a	19.40 ± 1.76b
15% *Elusine indica*	100.00 ± 0.00a	6.77 ± 0.81b	0.37 ± 0.05ab	0.10 ± 0.03a	23.03 ± 1.56a
25% *Elusine indica*	80.00 ± 20.00a	5.93 ± 0.35b	0.35 ± 0.03ab	0.11 ± 0.01a	13.67 ± 2.32c*
50% *Elusine indica*	90.00 ± 10.00a	4.03 ± 0.79c	0.25 ± 0.05bc*	0.08 ± 0.03a	14.47 ± 1.29c*
100% *Elusine indica*	50.00 ±17.32b*	1.45 ± 0.32d*	0.22 ± 0.08c*	0.08 ± 0.01a	-

Note. Values mean ± standard error of three replicates. Different letters represent significant differences between the same plant extract with different concentrations; "*" represents a significant difference between the treatment with control (p<0.05)

(100%) significantly reduced fresh weight (*E. hirta*: 44.7%; *P. minus*: 26.3%; *E. indica*: 42.1%), whereas it was not significant effect on dry weight ($p<0.05$).

The SPAD value in the seedling varied due to different weed extracts and concentrations. All *E. hirta* extract treatments, except for 5% concentration, were significantly lower than that of the control. Besides, 25 and 50% *E. indica* extract treatments significantly decreased by 35.5 and 31.7% compared to control, respectively, while the effect of *P. minus* extract treatments was not significant ($p<0.05$). Notably, the visual fungal colonies were found in the Petri dish with 100% *E. indica* extract treatment (Figure 2), while the other treatments had no external growth.

DISCUSSION

Phytochemicals and Chemical Properties in Selected Plant Extracts

Phytochemicals play crucial roles in the plant's secondary metabolism, including pest repellence and growth regulation (da Silva et al., 2016). Plants that contain a large number of bioactive compounds have the potential to improve human health (Forbes-Hernández et al., 2014). In our study, 45, 53, and 39 secondary metabolites were identified in *E. hirta*, *P. minus*, and *E. indica*, respectively, which can be categorized into a variety of groups (Table 2). Additionally, diverse important flavonoid compounds were identified and quantified in plant extracts compared with previous studies, such as tricin, myricitrin, and isovitexin. Flavonoids are considered to have antioxidant and anti-stress effects, which can usually help plants cope with drought, salinization and other environmental stresses (Stolarzewicz et al., 2013), and have beneficial functions, including regulation of plant respiration and photosynthesis (Cushnie et al., 2005), drivers of symbiosis between rhizobacteria and plants (Weston & Mathesius, 2013), furthermore promoting the growth and development of plants, which may help to increase yield and improve quality.

Results also revealed the presence of various amino acids, such as methylthio-2-oxobutanoic acid, pidolic acid, and lotaustralin, in plant extracts (Table 2). Amino acids are known to act as chelators of metal ions in agricultural products and interact with trace elements to form small, electrically neutral molecules that facilitate their absorption and transport in plants (Paleckiene et al., 2007) and promote growth and development (Qiu et al., 2020). In addition, alkaloids are another group of vital secondary metabolites related to plants and humans (War et al., 2012). In our investigation, a total of 24 alkaloids were discovered. It is worth noting that only 10 are beneficial to the plant because alkaloids usually play a role in plants' defense against pests and diseases, so they may be toxic and even have a negative effect on plants and the human body. Moreover, in our study, 4 polyketides, 15 terpenoids, 5 phenylpropanoids, 2 carbohydrates, 2 fatty acids, and one vitamin benefit plants. Accumulation of these bioactive compounds in plants can inhibit reactive oxygen species (ROS) inside cells through ROS scavenging

and decreasing ROS-related enzyme activity. Thus, the balance of redox reactions in the cell is maintained. Additionally, our study showed that all three weed extracts were rich in nutrient elements, such as N, P, and K. Among them, *P. minus* extracts contained the highest content of N and P (Table 1). Based on the above information, the identified phytochemical compounds in weed extracts have the potential to serve as natural biostimulants in terms of plant protection, growth, and development in sustainable agriculture.

Application of Selected Plant Extracts on Maize Seeding Growth

The study showed that three different plant extracts greatly influence the growth and development of sweet corn (Table 3, Figure 3). Results illustrated that high concentration (100%) plant extracts severely restricted seed germination and decreased the shoot height and fresh weight. These findings are in accordance with Ghodake et al. (2012), who found that the allelopathic effect of *Euphorbia* species caused inhibition in germination percentage and shoot-root length on wheat. Agarwal et al. (2002) and Gella et al. (2013) have also shown that weed extracts reduce the seed germination, plumule length, radicle, and weight of wheat. Besides, a high concentration of banana pseudostem sap inhibited any expansion in germination on sweet corn (Islam et al., 2022). It might be because some unknown compounds and pathogenic microorganisms cause negative effects on the germination rate of seeds (Talukder et al., 2015). On the other hand, weed extracts of *P. minus* and *E. indica* with low concentrations (5 and 15%) exhibited a slight promoting effect on germination and shoot length; this finding corroborates the results illustrated by Aslam et al. (2016), who reported that plant extract with low concentrations promoted seed germination. However, all *E. hirta* extract treatments in this study reduced shoot length (9.9~78.1%) compared with control. Therefore, different plant species and extract concentrations have diverse effects on seeding growth.

In this study, there was a negative trend of SPAD value as the concentrations of *E. hirta* and *E. indica* extracts increased, which is similar to the result of Joshi and Joshi (2016), who revealed the total chlorophyll accumulation in seedlings of wheat after being treated with six different weed extracts. Besides, Oyerinde et al. (2009) have also shown that allelochemicals in weed extract may affect chlorophyll content and photosynthesis in plants. It may be because weed extracts contain some toxic metabolites, such as alkaloids, that cause adverse effects on crop growth (Qasem, 2002). On the other hand, easier development of fungal colonies at higher concentrations also inhibits seedling growth. However, there was no significant effect on the SPAD value under all concentrations of *P. minus* extract treatments compared with the control (Table 3). It may be because *P. minus* extract contains fewer toxic metabolites and more beneficial phytochemicals and nutrients such as N, P, and K (Tables 1 and 2); thus, *P. minus*

extract has great potential for improving crop production.

CONCLUSION

The study identified phytochemical compounds in extracts of *E. hirta*, *P. minus*, and *E. indica* extracts, classifying them into 11 categories, including flavonoids, amino acids, alkaloids, polyketides, terpenoids, phenylpropanoids, carbohydrate, fatty acid, vitamin and others. Besides, *P. minus* extracts have the highest content of nitrogen and phosphorus. These phytocompounds and soluble nutrients are related to the growth and development of plants. The application of weed extracts has a significant impact on maize seed priming. In particular, *E. hirta* and *E. indica* extracts exhibited inhibitory effects at higher concentrations, while *P. minu*s extract maintained a higher germination rate, indicating lower toxicity. This finding emphasizes the importance of phytochemicals in seed germination and plant development. However, there is still limited knowledge regarding the specific effects of molecules on plants and human health. Future quantitative studies of beneficial phytocompounds will help to better understand how the application of weed extracts in agriculture can be economical and environmentally friendly.

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Physicochemical Characterisation of White Pepper: A Comparative Study Between Traditional Sun Drying and Convective Rotary Drum Drying Methods

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ABSTRACT

Drying is a crucial process in preserving the physicochemical qualities of white pepper. This study investigates the impact of two drying processes, namely traditional sun drying (TSD) and rotary drum drying (RDD), on the quality of white pepper. TSD requires three consecutive sunny days for drying, whereas RDD achieves the target moisture content of 12% within a rapid drying time of 120 min. The research employs thermo-gravimetric analysis (TGA), Fourier transform infrared (FTIR) spectroscopy analysis, and scanning electron microscopy (SEM) to analyse the dimensions, thermo-physical profiles, chemical constituents, and microstructure of the pepper samples. RDD, with a drying temperature of 55°C and centrifugation force of 129.7 × *g*, ensures fast and uniform drying while preserving the physicochemical qualities of white pepper. In terms of physical characteristics, RDD results in larger dried pepper dimensions, measuring 4.56 mm on average, compared to TSD, which measures 4.35 mm. SEM observations reveal varying pore sizes and cracks in both drying methods. Additionally, quality validation conducted by the Malaysian Pepper Board demonstrates that RDD exhibits superior quality compared to TSD. The RDD samples show moisture content, piperine, volatile, and ash percentages of

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11.83, 8.18, 2.53, and 0.82, respectively, while the TSD samples show 10.37, 7.16, 2.43, and 0.74. All samples complied with Standard Malaysian White Pepper No. 1 and International Pepper Community Grade 1. Future studies should focus on enhancing different drying methods to achieve efficient white pepper drying while preserving its quality.

Keywords: Agrotechnology, FTIR, SEM, TGA

INTRODUCTION

White pepper has extensive applications in the food, pharmaceutical, perfumery, and cosmetics industries (Megat et al., 2020; Olalere et al., 2017; Tiwari et al., 2020). It is derived from dried, skinless mature pepper berries, scientifically known as *Piper nigrum* L. (Salehi et al., 2019). There are various types of *Piper nigrum*, including Semongok Emas, Semongok Aman, Kuching, Semongok Perak, and India (Azman et al., 2020; Chen & Tawan, 2020). The Kuching variety is particularly suitable for white pepper production due to its thinner pericarp, facilitating the extraction of smooth and ivory-coloured white pepper (Azman et al., 2020; Malaysian Pepper Board [MPB], 2017a; Megat et al., 2020).

White pepper is produced from yellowish-green mature berries and red, fully ripe pepper berries (Azman et al., 2020; Singh et al., 2013). The berries undergo a soaking process to remove their pericarp (Azman et al., 2020). The physical structure of pepper berries consists of a fleshy outer part surrounding a single shell of hardened endocarp with a single seed inside. The pericarp comprises three sections: exocarp (outer layer), mesocarp (middle layer flesh), and endocarp (innermost layer). Following soaking, the berries must undergo immediate drying (Aziz et al., 2019).

Pepper drying is a crucial process for moisture removal while preserving the chemical properties, which can easily deteriorate during drying. External factors such as temperature, humidity, airflow, drying volume, and pressure influence the drying process (Abidin et al., 2020; Mühlbauer & Müller, 2020). Increasing air temperature accelerates moisture removal, thereby shortening drying time, but excessively high temperatures can degrade the chemical properties (Abidin et al., 2020; Rigit et al., 2013). Generally, pepper drying methods can be categorized as traditional sun-drying and modern methods such as drum drying, convective drying, infrared drying, and vacuum drying.

Sun drying is widely practised today for its simplicity, convenience, and low cost (Lamidi et al., 2019; Saha et al., 2022). However, it is time-consuming, unhygienic, and reliant on weather conditions (Abidin et al., 2020). Direct exposure to sunlight affects product quality, leading to vitamin loss, aroma degradation, and uneven drying (Lamidi et al., 2019; Saha et al., 2022). Therefore, RDD has been developed to improve pepper drying and energy efficiency (Friso, 2023). RDD is a very suitable and effectively dry grainy biomass material as white pepper berries (Kaveh & Abbaspour-Gilandeh, 2020; Rezaei & Sokhansanj,

2021). RDD operates by tumbling the drying material in a rotating drum with hot air to expedite moisture removal. The horizontal position of the drum allows gravity to assist the material's movement inside (Trojosky, 2019; Wae-hayee et al., 2021). Flights or fins within the drums improve the material contact with hot air, which enhances thermal and moisture diffusivity (Wae-hayee et al., 2021). Air velocity and drum rotation influence the material's drying capacity, drying time, and final moisture content (Kerr, 2019). RDD has significantly improved the drying time of black pepper from 4–7 days of sun drying to a few hours (Abidin et al., 2020). Rotary drum drying offers rapid, hygienic and uniform batch drying.

The quality of white pepper can be assessed through chemical analysis, including moisture content, oleoresin content, volatile oil, piperine, and starch (Aziz et al., 2019; Chithra et al., 2011). Physical quality is determined by surface appearance, colour, bulk density, and extraneous matter in the white pepper sample (Aziz et al., 2019). The MPB and the International Pepper Community (IPC) have established standard specifications for the chemical and physical quality of white pepper products (Tables 1 and 2). Compliance with these standards is crucial for white pepper products to enter the international market. Therefore, ensuring that the proposed drying process can produce white pepper that meets the specified chemical and physical quality standards is essential.

Pepper berries are rich in chemical components such as minerals, lignans, flavonoids, aromatic compounds, amides, and primary alkaloids or lipids. When it comes to white pepper, there are eight important chemical parameters to consider: piperine, volatile oil, oleoresin, β-caryophyllene, sphericity, α-pinene, limonene, and moisture content (Chithra et al., 2011; Olalere et al., 2018; Salehi et al., 2019). Piperine ($C_{17}H_{19}NO_3$), an amide alkaloid, is a valuable nutrient

Table 1
Malaysian Pepper Board (MPB) standard for white pepper (MPB, 2017b)

Characteristic	Grade				
	Standard Malaysian White Pepper No. 1	Sarawak Special White	Sarawak FAQ White	Sarawak Field White	Sarawak Coarse Field White
Moisture, percentage by weight, maximum (%)	12.00	15.00	16.00	16.00	16.00
Light berries, per cent per weight, maximum (%)	0.20	0.50	1.00	1.50	-
Extraneous matter, percent by weight, maximum (%)	0.25	0.25	0.50	1.00	3.00
Amount of black/dark grey berries in white pepper, per cent by weight, maximum (%)	1.00	1.00	3.00	3.00	5.00

Note. MPB mentions FAQ as the third-class white pepper without any definition of abbreviation (MPB, 2017b)

Table 2
International Pepper Community standard specifications for white pepper international market (CAC, 2021)

Parameters	Grade		
	I	II	III
Physical properties			
Bulk density (g/L), min	600.0	600.0	550.0
Light berries/Corn (m/m)%, max	1.0	2.0	2.0
Extraneous matter (m/m)%, max	0.8	1.5	2.0
Black coloured berries/Corn (m/m)%, max	2.0	3.0	10.0
Mouldy berries/Corn (m/m)%, max	1.0	3.0	3.0
Insect spoiled berries (% by wt.), max	1.0	2.0	2.0
Broken berries (m/m)%, max	2.0	3.0	3.0
Chemical properties			
Moisture content (m/m)%, max	12.0	13.0	14.0
Total ash (m/m)% max, on dry basis	3.5	4.0	4.0
Non-volatile matter using ether extract (m/m)% min, on a dry basis	6.0	6.0	6.0
Volatile matter (oil) (ml/100 g)%, min, on dry basis	1.5	1.5	1.0
Piperine (m/m)%, min	4.0	3.5	3.0

in pepper but is susceptible to damage during heating processes (Singh et al., 2013). Pepper berries contain piperine in concentrations ranging from 2.4 to 7.4%, co-existing with five other alkaloids and four isomeric forms of piperine (Tiwari et al., 2020). Volatile or essential oils are secondary metabolites in plants widely used in fragrance, cosmetics, medicine, and food for their scent and flavour (Nikolić et al., 2015). In white pepper, β-caryophyllene is the primary component of the essential oils, while piperine is present in the oleoresin (Singh et al., 2013). White pepper contains essential oils (1-2.5%) and alkaloids (5-9%), with the major constituents being piperine (1.7-7.4%), chavicine, and piperidine (Kusumorini et al., 2021).

The structure of piperine ($C_{17}H_{19}NO_3$), also known as 1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl], consists of three functional groups: aromatic, aliphatic, and amide (Tiwari et al., 2020). Piperine is a weak base compound with a distinct pungent flavour, smell, and hot effect. Its functional groups can be detected using FTIR include C-H (peaks at 2,800-3,000), O=C-N and C=C (diene) (peak at 1,635), C=C (benzene) (peaks at 1,495-1,589), =C-O-C (peaks at 1,030-1,257), and C-O-C (peak at 1,134) (Kusumorini et al., 2021; Mohammed et al., 2016). According to Sarifudin et al. (2021), piperine functional groups such as C-N aliphatic amines, C-O stretch ethers, C-N stretch aromatic amines, C-C stretch in ring aromatics, and C=C stretch alkenes are observed as peaks at wavenumbers of 1,134, 1,193, 1,251, 1,492, and 1,633 per cm, respectively. White pepper also contains minerals and trace elements, including sodium, magnesium, potassium, and calcium, which exhibit wide variability in

white pepper extracts (oleoresins) (Olalere et al., 2019). Thus, the chemical properties of white pepper, such as moisture content, volatile matter, piperine, non-volatile matter, and ash, justify the quality standards used to assess its quality.

TGA provides high sensitivity, reproducibility, and response to even minor mass variations. The sample's temperature range and thermal stability help identify peak profiles (Picolotto et al., 2020). Thermal profiling of white pepper's physical stability using TGA shows weight loss peaks in the temperature range of 270–350°C, indicating the decomposition of crude protein, lipid, and starch (H. Liu et al., 2018). At the same time, FTIR spectroscopy analysis is a reliable, cost-efficient, non-destructive analytical technique for physicochemical study that allows for the accurate, rapid, and direct assessment of various functional group properties (Candoğan et al., 2021; Wang, 2012). Studies have demonstrated that FTIR and TGA can provide valuable data for identifying herbs and spices (L. Liu et al., 2020; Laouni et al., 2022; Wang, 2012). Therefore, FTIR and TGA are convenient techniques for investigating the structural and compositional changes of macromolecules found in spices and herbs, including white pepper.

The drying process involves mass and energy transfer, with moisture vaporized by heat and diffused through pores, affecting the pepper berries' microstructure (Surendhar et al., 2019). The drying method employed significantly influences microstructural changes in the berries, as well as their moisture diffusivity and drying rate. Scanning electron microscopy (SEM) is commonly used to observe microstructural changes in the drying process of various agricultural products, including black pepper, red pepper, sweet potato, Chinese angelica, *Astragalus*, and banana (Abidin et al., 2023).

Sun drying is convenient for Sarawak local producers, although it is ineffective and unhygienic. Extensive research has been conducted on drying agricultural products, but limited studies focus on white pepper drying. Concerns have been raised regarding the physicochemical quality of the white pepper produced using rapid modern drying methods. Therefore, the main objective of this study is to characterize the white pepper produced through two different drying methods, TSD and RDD. The thermo-physical profiles and chemical constituents of the results were compared.

MATERIALS AND METHODS

White Pepper Sample Preparation

Fresh Kuching variety pepper was obtained from a Simunjan, Sarawak, Malaysia farmer. The pepper berries underwent a traditional soaking process. The soaked berries were then divided into two groups for drying using two different methods: TSD and RDD. Detailed schematic diagrams and drying procedures of RDD have been explained and adapted from Abidin et al. (2020). For the RDD method, a constant drum speed with centrifugation force of 129.7 × *g* and a chamber temperature of 55°C were maintained. The samples were dried until they reached the desired

moisture content. The drying process of 200 g sample for TSD took three consecutive sunny days, and RDD required 120 min of drying time. A portion of the dried pepper berries from both methods was sent to the MPB for quality validation. Another portion of the samples was prepared for further characterization using analytical techniques, namely FTIR spectroscopy, TGA, and SEM. These techniques were employed to analyse the chemical and physical properties of the white pepper samples.

Quality Validation

The quality of the samples obtained from both drying methods was validated at the MPB Central Testing Laboratory. MPB is an accredited institution that follows the MS ISO/IEC 17025:2017 standard. MPB is responsible for conducting analyses to determine the natural chemical content in pepper samples, including piperine, volatile oil, non-volatile ether extract, total ash, acid insoluble ash, and aroma profile. The PerkinElmer Spectrum One Near-infrared Testing System Fourier Transform Near Infrared (NTS FT-NIR) Spectrometer (USA) was employed for the analysis. The FT-NIR machine has been synchronized to the database of laboratory analysis results on the chemical content of white pepper. The sample was analysed in its berry form. The chemical content percentage provided by the FTNIR machine has errors up to 1% (max).

Sizing and Dimensions

The white pepper berries obtained from RDD and TSD methods were measured using a vernier calliper. Specifically, the Pro'sKit model PD-153 (Taiwan) standard vernier calliper with a sensitivity of 0.02 mm and made of AISI 430 stainless steel was utilized for the measurements. A total of 100 white pepper berries from each drying method were selected for measurement. For each berry, three readings were taken using the vernier calliper, recording the measurements for the first axis (a), second axis (b), and third axis (c) of the diameter. The average of the three axes was then calculated, and the diameter measurement for the respective white pepper berry sample was considered, as Megat et al. (2020) described.

Thermo-Gravimetric Analysis

TGA was performed using Shimadzu DTG-60H simultaneous DTA-TG (USA) apparatus capable of high-temperature measurements up to 1,500°C and an inert gas atmosphere or air (oxygen). The heating rate, atmosphere, gain or loss of mass, temperature, and heat flow were applied to identify the mass profile for white pepper berries. For the analysis, reference alumina (Al_2O_3) crucibles were used. The sample was prepared by placing one white pepper berry, weighing approximately 100 mg, into the crucible. The TGA experiment involved heating the sample from room temperature (25°C) to 700°C at 10°C/min while maintaining a streaming nitrogen (N_2) atmosphere of 100 ml/min. The TGA equipment was calibrated using a calibration set/manual provided by Shimadzu (USA). Each TGA analysis was repeated four times

to ensure reliable and consistent results. By monitoring the variations in mass as a function of time and temperature under a dynamic atmosphere, TGA enabled the examination of the thermal behaviour of the white pepper berries.

Fourier Transforms Infrared Spectroscopy

This study performed infrared-spectra (IR-spectra) analysis to determine the chemical bonds and functional groups in the sample constituents within the 4,000-400 per cm range. The analysis was conducted using attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy on a Shimadzu Fourier Transform Infrared Spectrophotometer (Model IRAffinity-1S, USA). The FTIR analysis for both samples was performed according to the method by Chumroenphat et al. (2021) with modification to the standard procedure introduced by MPB that utilized white pepper samples in berry form. The analysis was repeated five times using duplicate samples to ensure reliable results.

Surface Morphology by Scanning Electron Microscopy

Five white pepper berries were randomly selected from each sample group for surface structure observation. Hitachi scanning electron microscopy TM4000Plus (Japan), equipped with the TM 4000plus program, was employed for this purpose. The selected pepper berry from each sample was mounted on a sample stub and coated with a thin layer of conductive material, gold, using a sputter coater (Abidin et al., 2023). The surface of the sample was then observed under the scanning electron microscope at magnifications of 150× (300 µm) and 600× (50 µm). It allowed for a detailed examination of the surface characteristics and microstructure of the white pepper berries.

Statistical Analysis

A one-way analysis of variance (ANOVA) analysis was conducted using Microsoft Excel software to assess the significant differences between the mean values of the parameters related to berry size. The data were presented as the mean of 100 determinations with the corresponding standard deviation. A p-value of 0.05 or less was considered statistically significant, indicating a meaningful difference between the mean values of the compared parameters.

RESULTS AND DISCUSSION

Quality Validation

Samples from both drying methods were taken to the MPB laboratory for FT-NIR analysis validation. The average percentages of moisture, piperine, volatile oil, and ash content for the samples from both drying methods are presented in Table 3. The analysis was conducted in five duplications. The test was conducted using whole forms of white pepper berries. Based on the MPB and IPC standards (Tables 1 and 2), it can be observed that the samples from both drying methods were below the maximum limit of 12% moisture content,

Table 3
Comparison of chemical quality analysis for white pepper samples from rotary drum drying (RDD) and traditional sun drying (TSD) methods with the International Pepper Community (IPC) and Malaysian Pepper Board (MPB) standards

Chemical quality	IPC Grade 1 White pepper	MPB	RDD	TSD
Moisture (%)	≤12.00	≤12.00	11.83	10.37
Piperine (%)	≥4.00	≥4.50	8.18	7.16
Volatile (%)	≥1.50	≥0.80	2.53	2.43
Ash (%)	≤3.50	≤2.00	0.82	0.74

which is in line with the quality standards. However, the TSD sample had a moisture content of 10.37%, indicating that it was over-dried compared to the RDD sample, which had a moisture content of 11.83%. Over-drying the berries can reduce the farmer's income, as the price is typically based on the product's weight.

On the other hand, the piperine content in both drying methods exceeded the minimum standard requirement, with the RDD sample containing 4.18% more piperine compared to the standard and the TSD sample containing 3.16% more piperine. Similarly, the volatile oil content for both samples met the standard requirements and exhibited a minimal difference of 0.1% between the two methods.

The ash content in both samples was significantly lower than the standard requirements, with the RDD sample showing a reduction of 2.68% and the TSD sample showing a reduction of 2.76%. It is important to note that the ash content was nearly identical for both samples, as they originated from the same source.

In summary, the results indicate that the RDD method yielded better quality white pepper compared to the TSD method, as it retained higher levels of piperine and volatile oil content, essential factors contributing to the overall quality of white pepper.

Sizing and Dimensions

The dimensions of 100 white pepper berries dried using the RDD and TSD methods were analysed through ANOVA statistical analysis using Microsoft Excel. The analysis revealed a p-value of less than 0.05, indicating significant differences in berry size between the two drying methods. Tables 4 and 5 summarize the dimension data obtained from the analysis. The average berry size for RDD was 4.56±0.29 mm, while for TSD, it was 4.35±0.23 mm. Both average sizes meet the standard specifications for white pepper, with the IPC standard requiring a size range of 2–6 mm (Codex Alimentarius Commission [CAC], 2017) and the MPB specifying creamy white pepper to have a size greater than 4 mm (MPB, 2017b). The results reveal that the RDD sample exhibited a slightly larger size, approximately 0.2 mm larger, compared to the TSD sample. It suggests that the RDD method resulted in minimal shrinkage during the drying process, as the size of the berries remained closer to their original dimensions.

Table 4
The average size of rotary drum drying white pepper berries for 100 samples

Axis dimension	Average (mm)	Variance (mm^2)	Standard deviation (mm)
Diameter	4.56	0.086	±0.290
a	4.53	0.083	±0.290
b	4.57	0.089	±0.300
c	4.56	0.086	±0.290

Table 5
The average size of traditional sun drying white pepper berries for 100 samples

Axis dimension	Average (mm)	Variance (mm^2)	Standard deviation (mm)
Diameter	4.35	0.054	±0.23
a	4.35	0.049	±0.22
b	4.36	0.063	±0.25
c	4.35	0.052	±0.23

Thermo-Gravimetric Analysis

The TGA results for samples obtained from the RDD and TSD methods are depicted in Figure 1. The graphs illustrate a consistent trend in thermal profiling, with a narrowing towards the end. The initial region of the graphs represents the loss of water content from the samples' moisture content, with temperatures ranging from room temperature up to 100–110°C, similar to previous findings by H. Liu et al. (2018) and Simonovska et al. (2016). The weight loss represents the evaporated water and other low molecular weight components. Subsequently, a mass break into volatiles occurs between 100 and 170°C, indicating the thermal decomposition of these components, including moisture. The mass loss until 170°C may not be apparent, but it is aligned with the moisture content measured in the corresponding samples. The RDD samples exhibit slightly higher moisture content compared to TSD samples (Table 3).

Notably, the RDD samples exhibit a significant mass dropped approximately 34–40.4% between the temperature range of 280 to 350°C. In contrast, the TSD samples demonstrate different mass drops (40.71–46.97%) within the temperature range of 270 to 350°C. Similar results were observed by H. Liu et al. (2018) in the temperature range of 270–350°C, with a mass drop of 48.07 to 51.16%, representing the decomposition of crude protein, lipid, and starch in the white pepper samples (H. Liu et al., 2018). According to Simonovska et al. (2016), the degradation of cellulose occurs between 240 to 350°C. It is worth noting that slight differences were observed between the results of this research and the literature, which could be attributed to the different forms of the sample used during the TGA analysis. In this study, whole white pepper berries were utilized, whereas other researchers, such as H. Liu et al. (2018), pulverized the samples before conducting the analysis.

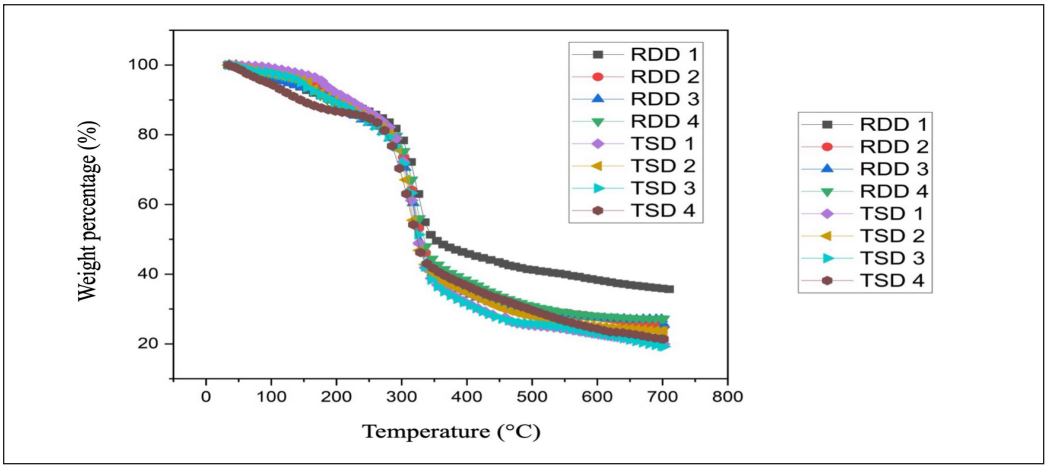


Figure 1. Thermo-gravimetric analysis graph for white pepper samples from the rotary drum drying (RDD) and traditional sun drying (TSD) methods, respectively

Fourier Transform Infrared Spectroscopy

Figure 2 shows FTIR transmittance spectra for white pepper samples dried using RDD and TSD methods. In the spectra, characteristic peaks were observed for both drying methods, exhibiting identical peak patterns. In Figure 2, the peaks appear at approximately 3,340-3,290, 2924, and 1,651-1,620 per cm in both samples. It shows that FTIR detects the same functional groups for both TSD and RDD. The results (3,340-3,290 per cm) indicate O-H vibration, which is harmonised with the findings of Simonovska et al. (2016). The sharp peaks at 2,960-2,920 and 2,880-2,860 per cm (Huynh et al., 2020; Simonovska et al., 2016) were assigned to the asymmetrical and symmetrical stretching vibrations of aliphatic C-H methylene groups and olefinic chains, which are typical bands for alkanes and aligned to the result (2,924 per cm). The peak 1,651-1,620 per cm correspond to alkenyl C=C stretch and O=C-N symmetrical stretching similar to (Kusumorini et al., 2021; Mohammed et al., 2016; Sarifudin et al., 2021) findings.

In the fingerprint region of both spectra shown in Figure 2, peaks were observed at 1,442-1,438, 1,249-1,238, and 1,053-1,018

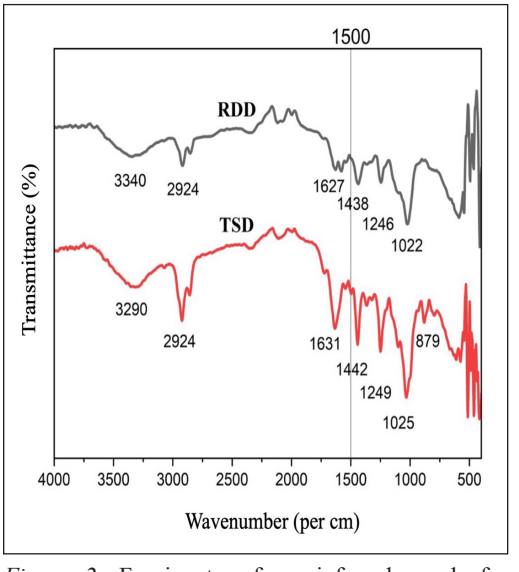


Figure 2. Fourier transform infrared graph for white pepper samples from the rotary drum drying (RDD) and traditional sun drying (TSD) methods, respectively

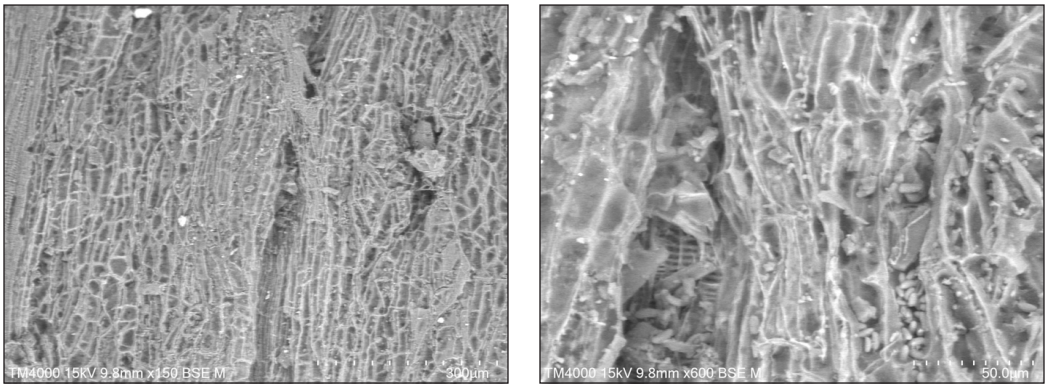
per cm, with only the TSD sample displaying an additional peak at 883-879 per cm. A peak at 1,600 per cm indicates the vibration of the aromatic skeletal structure, typically associated with lignin peaks, while a peak at 1,450 per cm represents a less intense deformation vibration of C-H in the aromatic ring of lignin moieties (Simonovska et al., 2016). Intense bands at 1,640, 1,440, and 885 per cm are characteristic of *b*-caryophyllene, *d*-limonene, 3-carene, *a*-pinene, *b*-pinene, and other hydrocarbons found in oils, representing stretching and bending vibrations of the C-C, C=C, and C-H bonds, respectively (Huynh et al., 2020). The peak around 1,251 per cm is associated with the C-O groups, esters of fatty acids or ketones (Huynh et al., 2020). In contrast, the symmetrical stretching of =C-O-C occurs around 1033 cm^{-1} (Kusumorini et al., 2021; Mohammed et al., 2016; Sarifudin et al., 2021).

Piperine is the main active compound of interest in this study and plays a crucial role in determining the chemical quality of the white pepper samples. Piperine consists of aromatic, aliphatic, and amide functional groups (Tiwari et al., 2020). These functional groups manifest as distinct peaks in FTIR analysis. Specifically, piperine exhibits functional groups of C-H peaks at 3,000–2,800 per cm, O=C-N and C=C (diene) peaks at 1,635 per cm, C=C (benzene) peaks at 1,589–1,495 per cm, =C-O-C peaks at 1,257–1,030 per cm, and C-O-C peaks at 1,134 per cm (Kusumorini et al., 2021; Mohammed et al., 2016). Additionally, Sarifudin et al. (2021), who used pulverized samples, claimed that piperine functional groups include C-N aliphatic amines, C-O stretch ethers, C-N stretch aromatic amines, C-C stretch in ring aromatics, and C=C stretch alkenes, which are observed as peaks at specific wavenumbers of 1,134, 1,193, 1,251, 1,492, and 1,633 per cm, respectively. In contrast, RDD and TSD analysis only show three peaks of piperine at wavenumbers 1,251, 1,492, and 1,633 per cm, indicating low piperine intensity. However, as shown in Table 3, the piperine content is considerably higher compared to (1.7 to 7.4%) (Kusumorini et al., 2021). Moreover, the RDD and TSD samples prepared for the analysis were in berry form instead of pulverized.

Surface Morphology by Scanning Electron Microscopy

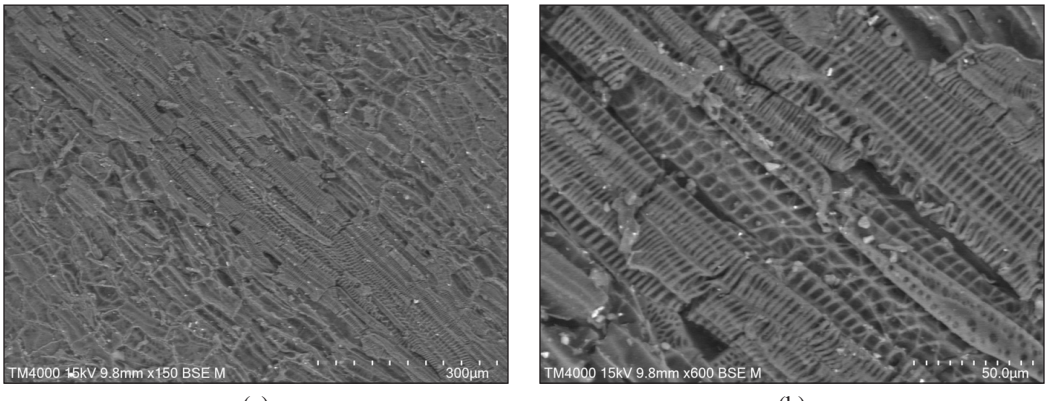
Figures 3 and 4 present the microstructure of white pepper samples dried using the RDD and TSD methods, respectively. The micrographs clearly show distinct differences in the microstructure between the two samples. The RDD sample exhibits well-defined pores and cracks, while the TSD sample demonstrates a finer, more organised microstructure.

The soaking process of the pepper berries before drying contributes to morphological changes, similar to pre-treatment effects (Abidin et al., 2023). Additionally, pore collapse occurs during drying (Kang et al., 2018). The presence of pores and cracks in the microstructure facilitates moisture diffusion. However, the diffusion of water vapour during the



(a) (b)

Figure 3. Microstructure of rotary drum drying white pepper at a magnification of: (a) 150′; and (b) 600′



(a) (b)

Figure 4. Microstructure of traditional sun drying white pepper at a magnification: (a) 150′; and (b) 600′

rapid drying process leads to the disruption of the solid structure, resulting in notable changes and the formation of cracks in the microstructure of the RDD sample. This phenomenon is attributed to the rapid release of water vapour and the generation of high-water vapour pressure within the sample due to the supplied heat energy during drying (Surendhar et al., 2019). The formation of pores and cracks in the microstructure results from the heat and moisture gradients that occur during the drying process, causing deterioration, deformation, and folding (Chumroenphat et al., 2021). The SEM images significantly illustrate the impact of the drying method on the microstructure of the white pepper surfaces. These observations provide further insights into how microstructural damage can affect the drying time, as well as the chemical and physical quality of the samples.

CONCLUSION

This study investigated the physicochemical characterization of white peppers dried using two different methods, RDD and

TSD. TGA, FTIR, and SEM analyses were performed to assess the drying method's impact on the samples' thermo-physical profiles and chemical constituents. The results provide valuable insights into the impact of drying methods on white pepper's chemical and physical properties.

RDD and TSD samples met the IPC white pepper standard and MPB creamy white pepper specification, indicating their compliance with quality standards. However, the samples from RDD exhibited better chemical properties, as revealed by TGA and FTIR analysis, compared to those from TSD. It can be attributed to the improved drying time achieved through RDD, which had a significant effect on the microstructure of the samples. SEM observations clearly showed the presence of pores and cracks in the RDD samples. Furthermore, the dimension of white pepper berries from RDD surpassed that of TSD samples. It suggests that the controlled drying method accelerates the drying rate and enhances the thermo-physical and chemical properties of white pepper.

In conclusion, this study's findings demonstrate that employing controlled drying methods can expedite the drying rate and improve the thermo-physical and chemical properties of white pepper. The results from TGA, FTIR, and the validation by the MPB laboratory support these findings. These findings open opportunities for further research to explore different drying methods that not only focus on drying efficiency but also aim to enhance the quality of white pepper.

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Enhancing Leafy Vegetable Growth and Yield with Goat Urine, *Moringa* Leaf, and Banana Stem-based Liquid Organic Fertiliser

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ABSTRACT

Pak choy and mustard greens are traditionally grown with many inorganic fertilisers, which can reduce soil fertility when applied frequently. The adoption of organic fertilisers offers a sustainable solution to this challenge. This study investigates the impact of liquid organic fertiliser (LOF) derived from goat urine, *Moringa* leaves, and banana stems on the growth and yield of pak choy and mustard greens. The research design employed a randomised complete block design with four treatments and ten replications. These treatments included a control group, 100% nitrogen, phosphorus, potassium (NPK, inorganic fertiliser), 100% LOF, and a 50% NPK + 50% LOF blend. The application of LOF, sourced from goat urine, *Moringa* leaves, and banana stems, demonstrated a significant influence on nearly all plant parameters. Notably, the 100% LOF treatment yielded the highest results for fresh leaf weight (26.46 g), fresh stalk weight (39.64 g), dry leaf weight (4.57 g), stem diameter (48.27 mm), Soil Plant Analysis Development value (SPAD, 40.73 units), plant height (31.59 cm), leaf width (8.45 cm), and leaf length (13.33 cm) for pak choy. Additionally, 100% LOF also produced the highest results for fresh leaf weight (21.34 g), dry root weight (0.31 g), stem diameter (21.53 mm), SPAD value (32.40 units), plant height (31.59 cm), leaf width (10.40 cm), and leaf length (15.65 cm) for mustard greens. This study demonstrates that a blend of goat urine, *Moringa* leaves, and banana stems can be used as a LOF that can replace inorganic NPK fertilisers in growing pak choy and mustard. It not

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only addresses issues with soil fertility but also contributes to environmentally friendly farming practices.

Keywords: Dry weight, fresh weight, mustard greens, NPK, organic fertilisers, pak choy, relative agronomic efficiency

INTRODUCTION

Leafy vegetables are important sources of dietary fibre, critical vitamins, and minerals, all crucial for human nutrition. Leaf vegetables like pak choy and mustard greens are popular choices among consumers. Because it has a great flavour and is simple to prepare, pak choy (*Brassica rapa* L.) is a vegetable plant with significant economic worth. From a climatological, technological, and economic perspective, pak choy plants are excellent candidates for growing in Indonesia. The vegetable plant that produces mustard greens (*B. juncea* L.) may thrive in subtropical and tropical regions. Plants that are part of the Brassicaceae family include mustard greens. Because of the different nutrients in mustard greens and the growing global population, they are needed (Rosalina et al., 2019).

In Indonesia, inorganic fertilisers like NPK continue to be used mostly in growing vegetables, especially pak choy and mustard greens. Such inorganic fertilisers should not be used for an extended period or in excess since this can result in several problems, such as reduced soil nutrient availability, soil water pollution, soil acidification, and a drop in beneficial soil microbes (Abebe et al., 2022). Additionally, the limited availability of subsidised fertilisers and fluctuating market prices have increased fertiliser costs, prompting the investigation of affordable and readily available alternatives for farmers. In this situation, switching to an organic agricultural system appears to be a workable approach. Organic farming is characterised by its commitment to sustainable practices, prioritising natural resource conservation by eschewing agrochemicals (da Costa Stuart et al., 2018). One such strategy is utilising LOF, which demonstrates the implementation of organic agricultural principles and provides a solution to the issue of limited access to subsidised fertilisers.

A practical substitute for conventional fertiliser for agricultural usage is LOF, produced from a mixture of goat urine, *Moringa* leaves, and banana stems. These components have a wide range of nutrients that can be used to enhance plants' nutritional needs. According to Abdullah et al. (2011), goat urine has a high nutrient content, with N at 1.35%, P at 0.13%, and K at 2.10%. *Moringa* leaves make a considerable contribution with 4.02% nitrogen, 1.17% phosphorus, 1.80% potassium, 12.3% calcium, 0.10% magnesium, and 1.16% sodium. These dietary elements are ideal for improving soil fertility and promoting healthy plant growth (Adiaha, 2017). Furthermore, adding additional nutrients required for the best plant growth and productivity is greatly facilitated by P in banana stem extract in concentrations ranging from 0.2 to 0.5% (Saraiva et al., 2012). These resources make excellent candidates for LOF because of

their availability, simplicity in cultivation, and affordability, allowing farmers to create organic fertilisers.

Farmers widely use NPK fertiliser because of its reliable composition and simplicity. To maximise plant growth, there is an increasing demand for organic fertilisers with formulations similar to NPK fertilisers. This work investigates goat urine, *Moringa* leaves, and banana stems-based LOF. Several crops, including tomatoes (Culver et al., 2012), corn (Biswas et al., 2016), lettuce, and spinach (Chanthanousone et al., 2022) have shown improved growth and production when *Moringa* leaf extract is applied. Similar to this, using goat urine LOF for a variety of crops, such as vegetables (Syahputra, 2022), maise (Nwite, 2015), and legumes like *Mucuna bracteata* (Sitinjak & Pratomo, 2019), shows significant potential. Additionally, it has been discovered that LOF made from banana stems has a large impact on the growth of tomatoes (Kilo et al., 2023), soybeans (Faozi et al., 2018), and sweet corn (Pangaribuan et al., 2019) among other plants. Notably, little research or information on combining these three organic elements for green vegetables is available. Despite separate studies highlighting the beneficial effects of these materials on various crops, the specific combination of goat urine, *Moringa* leaves, and banana stems into a LOF remains an area under investigation, particularly in terms of its impact on plants such as pak choy and mustard greens. This study addresses this gap by evaluating the impact of this unique blended organic fertiliser, presenting a promising avenue for enhancing plant growth and yield.

MATERIALS AND METHODS

Study Area

This study was conducted from February to March 2023 in the integrated field of the Faculty of Agriculture at the Universitas Lampung, Indonesia. The geographic coordinates are situated between 5° 22' 11.38" S and 105° 14' 25.96" E to 5° 21' 58.35" S and 105° 14' 43.83" E. The field is 120 meters above sea level, with annual rainfall ranging from 1,164 to 2,737 mm. The soil characteristics in the study area are classified as Ultisols with a pH level of 6.83, organic C (1.59%), nitrogen (0.04%), available P_2O_5 (188.85 ppm), potential P (159.25 mg phosphorus pentoxide [P_2O_5]/100 g), potential K (38.80 mg potassium oxide [K_2O]/ 100 g).

Research Methods

The experimental design employed in this study followed a randomised complete block design (RCBD) comprising four treatments with ten replications each. Mean separation was accomplished by utilising the least significant difference (LSD) test at a significance level of 5%. The research involved the application of four treatments, as follows:

P0: Control
P1: 100% NPK
P2: 100% LOF derived from a blend of goat urine, *Moringa* leaves, and banana stems
P3: 50% NPK + 50% LOF

LOF Production

LOF preparation involves a systematic procedure and specific tools and materials. The essential tools included drums, scales, hoses, and plastic bottles. At the same time, the materials consisted of 20 L of goat urine, 5 kg of *Moringa* leaves, 5 kg of banana stems, 10 L of water, 300 ml of molasses, and 30 ml of Effective Microorganisms 4 (EM-4®, Indonesia). The process commenced with preparing the plant materials, wherein the *Moringa* leaves were meticulously blended, and the banana stems were cut into small pieces. Subsequently, 20 L of goat urine were introduced, adding 5 kg of blended *Moringa* leaves and 5 kg of finely cut banana stems. This mixture incorporated 30 ml of EM-4®, 300 ml of molasses solution, and 10 L of water. Thorough mixing ensued until homogeneity was achieved, after which the container was securely sealed. A hose was connected to the container's outlet, facilitating the connection to a water bottle. The fermentation process was allowed to proceed for 30 days. Prior to application, the fermented extract underwent a filtration process at a ratio of 1 L of organic nutrients to 4 L of water, as illustrated in Figure 1. According to laboratory tests, each LOF's nutrient composition is N at 0.18%, P_2O_5 at 0.06%, and K_2O at 0.77%, with a pH of 5.78.

Research Implementation

Soil aeration on the selected site is the first step in preparing the planting medium. After that, the loosening soil is mixed with 1 kg of dolomite per square metre and let to rest for a week. The soil then goes through a laborious sifting procedure to achieve a fine-grain consistency. Once the soil has the desired texture, it is carefully packed into 40 polybags, each measuring 40 cm × 40 cm, for 40 polybags.

Figure 1. Production workflow for making liquid organic fertiliser

During the seeding phase, pak choy seeds of the Nauli F1 variety and Tosakan mustard greens are sown onto the ready-planting medium using soil. Fourteen (14) days of seeding activities are completed when the sprouting plants have four to five leaves. The pak choy and mustard green seedlings are now moved into the polybag media, with two plants per polybag, and are prepared for transplantation.

Fertiliser NPK 16:16:16 is applied at a rate of 100%, or 3 g per polybag, once when the plants reach one week after planting (WAP), according to a predetermined schedule for fertilisation. Applications of LOF are made nine times, once every three days, by soil drenching with a flush volume of 148.30 ml per polybag for the full dose of LOF. Alternatively, a 50% NPK dose of 1.5 g per polybag and a 50% LOF dose of 73.80 ml are given once during the first week following planting. The outline scheme for making and applying LOF on pak choy and mustard greens can be seen in Figure 2.

Both morning and evening are regularly used for watering. Manual labour is used to carefully remove weeds by hand as part of weed management. Mechanical methods are used to perform pest and disease control procedures, which comprise the direct removal of pests that harm pak choy and mustard greens plants. Plants with disease infestations are quickly segregated from healthy plants to stop the spread of the disease.

Observational Variables

Variables observed in this study were the fresh weight of leaves, fresh weight of stalk,

Figure 2. Application scheme of liquid organic fertiliser

fresh weight of root, dry weight of leaves, dry weight of stalk, dry weight of root, stem diameter, number of leaves, green level of leaves (SPAD readings), plant height, leaf width, leaf length, and stalk length. The agronomic effectiveness of fertiliser is determined by the relative agronomic effectiveness (RAE) with the following formula:

$$RAE = \frac{\text{Results of the tested LOF} - \text{Control}}{\text{Results of NPK fertiliser} - \text{Control}} \times 100\%$$

If the RAE value ≥ 100%, then the tested fertiliser is effective compared to standard treatment.

RESULTS AND DISCUSSION

The plants under consideration, pak choy and mustard greens showed strong growth and overall great health. Feeding these plants 100% LOF made from goat urine, *Moringa* leaves, and banana stems produced outcomes comparable to those in the 100% NPK and 50% NPK + 50% LOF treatments. Pest incursions were noted during the pak choy plant's growth stages, mostly attributable to green locusts (*Valanga nigricornis*) and caterpillars (*Plutella xylostella*). Despite being below the threshold for intervention, these insect encounters caused damage to the leaves, enabling the implementation of efficient control measures. Both pak choy and mustard greens plants began to experience caterpillar infestations in the latter growth phases, notably around the 4 WAP stage. Most pest control was done manually, carefully removing leaf-eating caterpillars and locusts. Protective paranets were judiciously placed around the production area in addition to manual control efforts to reduce pest pressures and protect the crop.

The statistical analysis revealed that the LOF treatment of goat urine, *Moringa* leaves, and banana stems in both vegetables significantly affected the fresh weight of leaves (Tables 1 and 2), fresh weight of stalks (Tables 1 and 2), fresh weight of roots (Tables 1 and 2), dry weight of leaves (Tables 3 and 4), dry weight of stalks (Tables 3 and 4), dry weight of roots (Tables 3 and 4), stem diameter (Tables 5 and 6), number of leaves (Tables 5 and 6), and level of greenness of leaves (Tables 5 and 6). The analysis also revealed significant effects on plant height (Tables 7 and 8), leaf width (Tables 7 and 8), leaf width (Tables 7 and 8), leaf length (Tables 7 and 8), and stalk length (Tables 7 and 8).

Effect on Growth Parameters

Both the fresh weight of the leaves and roots of pak choy (Table 1) and the fresh weight of the stalks and roots of mustard greens (Table 2) did not differ substantially after treatment with 100% NPK, 100% LOF, or 50% NPK + 50% LOF. For all variables, the treatments of 100% NPK, 100% LOF, and 50% NPK + 50% LOF produced substantially different findings from the controls (Tables 1 and 2). The 100% LOF treatment considerably outperformed both the 50% NPK + 50% LOF and 100% NPK treatments on the fresh weight of pak choy stalks, while the 50% NPK + 50% LOF treatment did not

significantly vary from 100% NPK (Table 1). The fresh weight of mustard greens' leaves in the 100% NPK treatment (Table 2) differed noticeably from that of the 100% LOF treatment and the 50% NPK + 50% LOF treatments. However, the 50% NPK + 50% LOF treatment and the 100% LOF treatment did not substantially differ in terms of fresh weight of leaves (Table 2). On the fresh weight of pak choy leaves and stalks (Table 1) and mustard greens leaves (Table 2), the 100% LOF treatment produced the best results. According to the RAE approach, the RAE value of a 100% LOF treatment in pak choy plants is 154.52% (Table 1) and in mustard greens plants is 128.35% (Table 2). It demonstrates that the 100% LOF treatment is superior to the 100% NPK treatment in terms of efficacy.

Fresh biomass yields of leaves, stalks, and roots were comparable to those seen in the 100% NPK treatment after applying 100% LOF (Tables 1 and 2). The similarity in biomass production is probably due to the high K concentration in the 100% LOF treatment. Banana stems, a key ingredient in the production of LOF, are an important source of K minerals. It is important to note

Table 1
The effect of treatments on the parameters of fresh weight of leaves, fresh weight of stalks, and fresh weight of roots of pak choy plants

Treatment	Fresh weight of leaves (g)	RAE (%)	Fresh weight of stalk (g)	Fresh weight of root (g)
Control	14.67 b	-	17.70 c	1.16 b
100% NPK	22.30 a	100.00	31.01 b	1.77 a
100% LOF	26.46 a	154.52	39.64 a	1.96 a
50% NPK + 50% LOF	26.44 a	154.26	37.00 ab	2.12 a
LSD 5%	5.36	-	0.12	0.18

Note. Numbers followed by the same letter in the same column are not significantly different based on the least significant difference (LSD) test at the 5% level; RAE = Relative agronomic effectiveness; NPK = Nitrogen, phosphorus, potassium; LOF = Liquid organic fertiliser

Table 2
The effect of treatments on the parameters of fresh weight of leaves, fresh weight of stalks, and fresh weight of roots of mustard green plants

Treatment	Fresh weight of leaves (g)	RAE (%)	Fresh weight of stalk (g)	Fresh weight of root (g)
Control	14.46 c	-	12.29 b	1.22 b
100% NPK	19.82 b	100.00	18.81 a	1.95 a
100% LOF	21.34 a	128,35	17.96 a	2.05 a
50% NPK + 50% LOF	20.72 a	116,79	18.41 a	2.05 a
LSD 5%	0.74	-	1.04	0.10

Note. Numbers followed by the same letter in the same column are not significantly different based on the least significant difference (LSD) test at the 5% level; RAE = Relative agronomic effectiveness; NPK = Nitrogen, phosphorus, potassium; LOF = Liquid organic fertiliser

that banana stems, sometimes regarded as agricultural waste, contain various possible chemicals, including K, P, Fe, and N (Riyandani et al., 2021). A crucial nutrient called potassium has a variety of functions in plant physiology. According to Grzebisz et al. (2013), it is essential for many functions, including photosynthesis, osmotic control, cell proliferation, stomatal organisation, plant water movement, and nitrogen transfer. Additionally, potassium makes it possible for water and nutrients to be transported effectively throughout the plant. According to Prajapati and Modi (2012), a lack of potassium may hinder nitrogen fixation and translocation as well as the absorption of phosphate, calcium, magnesium, and amino acids. Along with potassium, nitrogen is recognised as a crucial factor in higher crop yields. Chlorophyll, proteins, amino acids, different enzymes, nucleic acids, and many other crucial molecules found in plant cells all contain nitrogen as a key component (Reeza & Azman, 2022). Its presence is essential for maximising plant performance and growth.

In the 100% NPK, 100% LOF, and 50% NPK + 50% LOF treatments, the dry weight of pak choy leaves (Table 3) and dry weight of mustard greens roots (Table 4) did not significantly differ, although the three treatments produced various results when compared to the control. The dry weight of the pak choy stalks and roots in the 100% NPK, 100% LOF, and 50% NPK + 50% LOF treatments all revealed results that were not significantly different. On the variable dry weight of the pak choy stalks and roots, the 50% NPK + 50% LOF and 100% NPK treatments significantly outperformed the control (Table 3). The dry weight of leaves mustard green of the 100% LOF treatment was not significantly different from those of the 100% NPK treatment or the 50% NPK + 50% LOF treatment. On the dry weight of mustard green leaves, the treatments of 100% NPK, 100% LOF, and 50% NPK + 50% LOF produced significantly different outcomes from the control (Table 4). The 100% LOF treatment demonstrated significantly different results from the 100% NPK treatment in the mustard greens dry weight of stalk variable. On the mustard greens dry weight of stalk variable, the treatments of 100% NPK, 100% LOF, and 50% NPK + 50% LOF produced significantly different results from the control (Table 4).

Compared to the 100% NPK treatment, the 100% LOF treatment showed a remarkable capacity to match the dry weight of leaves and roots in pak choy and mustard greens plants. An essential measure known as dry weight is crucial in evaluating photosynthetic activities. In fact, according to Anjarwati et al. (2017), measures of the dry weight of plant roots and leaves closely reflect photosynthesis results. According to Sarif et al. (2015), a plant's dry weight is a crucial measure of its capacity to effectively collect nutrients from its growing medium and support strong growth. The ideal photosynthesis that emerges from satisfying a plant's nutritional needs produces increased dry weight values for both the leaves and the roots. Because

Table 3
The effect of treatments on the parameters of dry weight of leaves, dry weight of stalks, and dry weight of roots of pak choy plants

Treatment	Dry weight of leaves (g)	Dry weight of stalks (g)	Dry weight of roots (g)
Control	2.67 b	2.13 b	0.36 b
100% NPK	3.94 a	3.57 a	0.74 a
100% LOF	4.57 a	3.25 ab	0.63 ab
50% NPK + 50% LOF	3.96 a	3.71 a	0.68 a
LSD 5%	1.14	1.18	0.30

Note. Numbers followed by the same letter in the same column are not significantly different based on the least significant difference (LSD) test at the 5% level; NPK = Nitrogen, phosphorus, potassium; LOF = Liquid organic fertiliser

Table 4
The effect of treatments on the parameters of dry weight of leaves, dry weight of stalks, and dry weight of roots of mustard green plants

Treatment	Dry weight of leaves (g)	Dry weight of stalks (g)	Dry weight of roots (g)
Control	1.36 c	0.44 c	0.18 b
100% NPK	1.67 b	1.25 a	0.27 a
100% LOF	1.90 ab	1.08 b	0.31 a
50% NPK + 50% LOF	2.06 a	1.17 ab	0.28 a
LSD 5%	0.32	0.13	0.06

Note. Numbers followed by the same letter in the same column are not significantly different based on the least significant difference (LSD) test at the 5% level; NPK = Nitrogen, phosphorus, potassium; LOF = Liquid organic fertiliser

of the nutrient-rich content of LOF, plants thrive and achieve high dry-weight values in their leaves and roots, which considerably boosts photosynthesis.

The 100% LOF treatment demonstrated significantly different results from the control, 100% NPK, and 50% NPK + 50% LOF treatments in the stem diameter of pak choy (Table 5). However, the results of the 50% NPK + 50% LOF treatment were significantly different from those of the 100% NPK and control treatments. The stem diameter of the pak choy did not significantly differ between the 100% NPK treatment and the control (Table 5). In contrast, the stem diameter of mustard green plants treated with 100% LOF and 50% NPK + 50% LOF did not differ significantly (Table 6). The number of pak choy and mustard green leaves in the 100% NPK, 100% LOF, and 50% NPK + 50% LOF treatments did not differ significantly from one another, but the results from the three treatments were very different from the control. The SPAD values of pak choy leaves under 100% LOF and 50% NPK + 50% LOF treatments (Table 5) did not differ significantly. In contrast, the SPAD value under 100% NPK treatment differed significantly from the control. The SPAD values in the mustard green leaves (Table 6) did not differ significantly from the 100% LOF and 100% NPK

treatments. Additionally, there was no noticeable distinction between the 50% NPK + 50% LOF treatment and the 100% NPK treatment's level of green in the leaves. Table 6 shows that the 100% NPK, 100% LOF, and 50% NPK + 50% LOF treatments significantly differed from the control in the SPAD values in the mustard green leaves.

In both pak choy and mustard greens plants, the use of 100% LOF demonstrated a surprising ability to parallel the results seen in the 100% NPK treatment across a spectrum of critical traits, including stem diameter, leaf number, and the SPAD unit. In pak choy and mustard greens, the stem diameter is linked to the number of leaves; a higher number always equates to a bigger stem diameter. It is impossible to understate the importance of nitrogen and phosphorus in *Moringa* leaves and banana stems in encouraging leaf growth. These two vital nutrients are crucial for promoting cell division and acting as the building blocks for synthesising organic compounds in plants, significantly impacting vegetative growth, especially leaf number. As amino acids, proteins, nucleic acids, enzymes, nucleoproteins, and alkaloids are all essential for promoting vegetative growth and enhancing leaf

Table 5
The effect of treatments on the parameters of stem diameter, number of leaves, and Soil Plant Analysis Development (SPAD) of pak choy plants

Treatment	Stem diameter (mm)	Number of leaves (blade)	SPAD (units)
Control	36.37 c	14.70 b	36.19 c
100% NPK	41.03 bc	16.75 a	39.08 b
100% LOF	48.27 a	17.55 a	40.73 a
50% NPK + 50% LOF	42.96 b	17.60 a	40.37 a
LSD 5%	4.71	1.91	0.68

Note. Numbers followed by the same letter in the same column are not significantly different based on the least significant difference (LSD) test at the 5% level; NPK = Nitrogen, phosphorus, potassium; LOF = Liquid organic fertiliser

Table 6
The effect of treatments on the parameters of stem diameter, number of leaves, and Soil Plant Analysis Development (SPAD) of mustard green plants

Treatment	Stem diameter (mm)	Number of leaves (blade)	SPAD (units)
Control	13.98 c	6.15 b	24.92 c
100% NPK	19.88 b	8.45 a	30.97 ab
100% LOF	21.53 a	8.25 a	32.40 a
50% NPK + 50% LOF	21.47 a	8.40 a	30.31 b
LSD 5%	1.14	0.48	1.43

Note. Numbers followed by the same letter in the same column are not significantly different based on the least significant difference (LSD) test at the 5% level; NPK = Nitrogen, phosphorus, potassium; LOF = Liquid organic fertiliser

pigmentation, their formation is directly facilitated by adding nitrogen to plants (Mokhele et al., 2012). Notably, the 100% LOF treatment's significantly increased levels of leaf greenness highlight the LOF's significant nitrogen content obtained from goat urine and *Moringa* leaves. Nitrogen not only aids in plant growth but also gives leaves a vivid green tint. The degree of green pigmentation in the leaves of mustard and pak choy is directly connected to the amount of nitrogen taken up by the plants (Nugroho, 2015; Shorna et al., 2020).

The results of the 100% NPK, 100% LOF, and 50% NPK + 50% LOF treatments were not statistically different in the pak choy plant's variable height (Table 7). On factors related to plant height, the 50% NPK + 50% LOF treatment and 100% NPK revealed results that were not significantly different from the control (Table 7). In the 100% NPK, 100% LOF, and 50% NPK + 50% LOF treatments, pak choy leaf width (Table 7) and mustard greens plant height (Table 8) revealed results that were not significantly different. However, the three treatments revealed significantly different results from the control. With 100% LOF and 50% NPK + 50% LOF treatments, the leaf width and length of mustard greens leaves (Table 8) showed findings that were not significantly different, and the two treatments were significantly different with 100% NPK and control. The 100% LOF treatment for pak choy leaf length (Table 7) produced significantly distinct results from those of the other three treatments. The 100% NPK and 50% NPK + 50% LOF treatments did not significantly differ in terms of pak choy leaf length (Table 7). Although there was no statistically significant difference between the 100% LOF and 50% NPK + 50% LOF treatments for pak choy (Table 7), the stalk was much longer than the control. In the 100% LOF and 50% NPK + 50% LOF treatments (Table 8), the length of the mustard greens stalks was not significantly different, but they were significantly longer than the control.

Pak choy and mustard greens' vigorous leaf growth is supported by the 100% LOF, which contains a significant amount of nitrogen. During their vegetative phase, these green vegetables, mostly used for their stems and leaves, have a high demand for nitrogen. Nitrogen, which is frequently regarded as the main force behind plant growth, significantly impacts how plants develop, their general quality, and their ability to produce (Gebeyaw & Belete, 2020). The production of chlorophyll pigment, a vital element in photosynthesis, is another function of nitrogen. It is also crucial to develop proteins, amino acids, plant cells, tissues, and organs, with the vegetative phase of growth being of special significance (Liang et al., 2023; Pernitiani et al., 2018). Plants allocate a significant percentage of their carbohydrates to the growth of their leaves, stems, and roots during the vegetative phase (Rizal, 2017). As a result, nitrogen becomes a key nutrient that has an important effect on plant development, growth, and output (Imran et al., 2021). The adequate nitrogen supply greatly enhances the productive capacity of leafy crops like pak choy and mustard greens.

Table 7
The effect of treatments on the parameters of plant height, leaf width, leaf length, and stalk length of pak choy plants

Treatment	Plant height (cm)	Leaf width (cm)	Leaf length (cm)	Stalk length (cm)
Control	20.34 b	7.01 b	11.62 c	6.36 b
100% NPK	22.16 ab	7.92 a	12.60 b	6.70 b
100% LOF	23.91 a	8.45 a	13.33 a	7.12 a
50% NPK + 50% LOF	22.42 ab	8.13 a	12.62 b	7.14 a
LSD 5%	2.23	0.61	0.64	0.41

Note. Numbers followed by the same letter in the same column are not significantly different based on the least significant difference (LSD) test at the 5% level; NPK = Nitrogen, phosphorus, potassium; LOF = Liquid organic fertiliser

Table 8
The effect of treatments on the parameters of plant height, leaf width, leaf length, and stalk length of mustard greens

Treatment	Plant height (cm)	Leaf width (cm)	Leaf length (cm)	Stalk length (cm)
Control	22.29 b	7.41 c	12.12 c	10.44 c
100% NPK	30.96 a	9.19 b	13.90 b	15.21 a
100% LOF	31.59 a	10.40 a	15.65 a	14.16 b
50% NPK + 50% LOF	31.39 a	10.20 a	15.32 a	14.27 b
LSD 5%	1.39	0.77	0.83	0.80

Note. Numbers followed by the same letter in the same column are not significantly different based on the least significant difference (LSD) test at the 5% level; NPK = Nitrogen, phosphorus, potassium; LOF = Liquid organic fertiliser

Correlation Among Parameters

The results of the correlation study performed on pak choy (Table 9) and mustard greens (Table 10) have shown important insights into the relationships among numerous growth-related variables. Notably, the fresh weight of leaves emerges as a central variable that displays strong correlations with a variety of other growth parameters, including stem diameter, number of leaves, level of leaf greenness, plant height, leaf width, leaf length, and stalk length. However, the fresh weight of leaves did not show a significant correlation with the fresh weight of stalks and roots in the case of pak choy plants (Table 9). On the other hand, mustard greens plants (Table 10) showed a significant association between the fresh weight of leaves and the fresh weight of stalks and roots. This association highlights that the increase in new leaf weight during the vegetative growth's final stages is closely related to the plant's early stages of development. The availability of vital plant nutrients significantly impacts the plant's overall fresh weight, which in turn affects vegetative growth. In contrast, the primary factor affecting dry weight is photosynthesis within the plant (Sarif et al., 2015). These findings are notably consistent

with the composition of the LOF, which is derived from goat urine, *Moringa* leaves, and banana stems and has a considerable amount of nitrogen and potassium. Nitrogen stimulates the growth of stems, branches, and leaves, crucial components of overall plant growth. Additionally, according to Adi et al. (2020), green pigmentation plays a substantial part in producing the essential for photosynthesis. In essence, nitrogen plays a significant role in leaf formation. Potassium is one of the three nutrients, along with nitrogen and phosphorus, necessary for plant growth (Muthu et al., 2023). Additionally, potassium controls how photosynthetic products are distributed, causing an increase in leaf width in response to its availability.

In comparison to the 100% NPK treatment, the 100% LOF treatment has demonstrated superior performance. This trend is evident in the average fresh weight of leaves for pak choy (Table 1) and mustard

Table 9
Correlation among growth parameters of pak choy plants

Parameters	2	3	4	5	6	7	8	9	10	11	12	13
1) FWL	X	X	0.62 *	0.67 *	0.53 *	0.85 *	0.84 *	0.60 *	0.59 *	0.72 *	0.68*	0.34 *
2) FWS		0.50 *	X	X	X	X	X	X	X	X	X	0.34 *
3) FWR			X	X	X	X	X	X	X	X	X	0,37 *
4) DWL				0.65 *	0.57 *	0.58 *	0.59 *	0.49 *	0.48 *	0.45 *	0.60 *	0.44 *
5) DWS					0.54 *	0.54 *	0.58 *	0.47 *	0.45 *	0.47 *	0.54 *	0.35 *
6) DWR						0.43 *	0.54 *	0.28 *	0.32 *	0.46 *	0.37 *	X
7) SD							0.74 *	0.66 *	0.49 *	0.75 *	0.74*	0.37 *
8) NL								0.64 *	0.73 *	0.72 *	0.72*	0.48*
9) SPAD									0.65 *	0.71 *	0.76*	0.67 *
10) PH										0.64 *	0.74 *	0.57 *
11) LW											0.81 *	0.48 *
12) LL												0.67 *
13) SL												

Note. FWL = Fresh weight of leaves; FWS = Fresh weight of stalk; FWR = Fresh weight of root; DWL = Dry weight of leaves; DWS = Dry weight of stalk; DWR = Dry weight of root; SD = Stem diameter; NL = Number of leaves; SPAD = Soil Plant Analysis Development; PH = Plant height; LW = Leaf width; LL = Leaf length; SL = Stalk length. * = Correlation; x = No correlation

Table 10
Correlation among growth parameters of mustard greens plants

Parameters	2	3	4	5	6	7	8	9	10	11	12	13
1) FWL	0.88 *	0.79 *	0.59 *	0.82 *	0.55 *	0.88 *	0.81 *	0.78 *	0.87 *	0.79 *	0.81 *	0.81 *
2) FWS		0.77 *	0.58 *	0.84 *	0.43 *	0.86 *	0.82 *	0.78 *	0.87 *	0.67 *	0.69 *	0.87 *
3) FWR			0.57 *	0.67 *	X	0.78 *	0.72 *	0.75 *	0.76 *	0.74 *	0.72 *	0.68 *
4) DWL				0.46 *	X	0.64 *	0.66 *	0.52 *	0.60 *	0.72 *	0.65 *	0.48 *
5) DWS					0.50 *	0.82 *	0.83 *	0.70 *	0.88 *	0.71 *	0.70 *	0.83 *
6) DWR						0.55 *	0.53 *	0.38 *	0.55 *	0.42 *	0.41 *	0.55 *
7) SD							0.85 *	0.75 *	0.91 *	0.79 *	0.80 *	0.77 *
8) NL								0.72 *	0.89 *	0.78 *	0.68 *	0.84 *
9) SPAD									0.79 *	0.74 *	0.76 *	0.75 *
10) PH										0.80 *	0.80 *	0.89 *
11) LW											0.91 *	0.64 *
12) LL												0.59 *
13) SL												

Note. FWL = Fresh weight of leaves; FWS = Fresh weight of stalk; FWR = Fresh weight of root; DWL = Dry weight of leaves; DWS = Dry weight of stalk; DWR = Dry weight of root; SD = Stem diameter; NL = Number of leaves; SPAD = Soil Plant Analysis Development; PH = Plant height; LW = Leaf width; LL = Leaf length; SL = Stalk length. * = Correlation; x = No correlation

greens (Table 2), which are the primary focus of this investigation. When compared to traditional NPK fertiliser, LOF has a more comprehensive nutritional composition, which can be attributed to its remarkable efficiency. Organic fertilisers are a common practice in organic vegetable production systems to maintain the quality of the land resources (Fahrurrozi et al., 2023). According to Mohamad et al. (2022), adding organic matter to the soil results in a number of positive effects, including improved soil structure stability, increased fertility, a decrease in nutrient leaching, an increase in soil biological activity, improved water retention, and a decrease in greenhouse gas emissions. According to Muarif et al. (2002), organic fertilisers also offer a number of advantages, such as an increase in cation metabolism, improved nutrient availability, and the steady, slow release of a variety of nutrients. The ability of

organic fertilisers to enhance soil chemical characteristics through increased cation exchange capacity is one of its unique benefits. Soils with higher cation capacities can give plants more nutrients than soils with lower capacities. Additionally, because they contain microorganisms that increase the already-existing soil microbiota, organic fertilisers help improve soil's biological qualities. According to Situmeang et al. (2017), the addition of LOF that have been microorganism-enhanced has the ability to alter soil structure and make it more porous. Additionally, organic fertilisers are crucial in reducing pollution problems brought on by inorganic fertilisers, helping promote a more environmentally friendly and sustainable agricultural paradigm (Siavoshi et al., 2011).

This study shows that LOF based on goat urine, *Moringa* leaves, and banana stems can provide plants with a complete nutrient supply because these materials contain both macro and micronutrients as well as the growth hormones gibberellins, cytokinins, and ındole-3-acetic acid (IAA) that plants require. In contrast to inorganic fertilisers like NPK, which only contain a few types of nutrients, LOF can make up for the lack of nutrients in inorganic fertilisers.

The significance of organic fertilisers in transforming agricultural practises, particularly in cultivating leafy vegetables such as pak choy and mustard greens, cannot be overstated. It presents a viable alternative to farmers' common practice of using inorganic fertilisers on a wide range of plant commodities or, at the very least, a way to reduce that use. Since inorganic fertilisers only give a single element to address plant nutrient needs and frequently fall short of fully satisfying them, their limitations are readily apparent. Additionally, using inorganic fertilisers can negatively affect the environment and the crops, contrary to their intended use. The adoption of LOF made from plant-based materials offers an alternative and environmentally friendly method of fertilisation that is conducive to the development of organic farming. However, several interrelated factors, including the source of organic waste, the length of fermentation, and the storage conditions of these extracts, affect the nutritional profile of plant-derived extracts (Pangaribuan et al., 2019). In organic farming, notably in the development of leafy vegetables and a variety of other plant commodities, applying LOF is an efficient and very cost-effective technology. In this line, LOF made from goat urine, *Moringa* leaves, and banana stems emerges as a promising substitute that adheres to organic farming's core values. These studies will surely help to improve organic agricultural techniques and increase their long-term viability.

CONCLUSION

In terms of fresh weight of leaves, fresh weight of stalks, dry weight of leaves, stem diameter, greenness level of leaves, plant height, leaf width, and leaf length of pak choy plants, treatment with 100% LOF blend of goat urine, *Moringa* leaves, and banana stems produced the highest results. On mustard greens plants, the

100% LOF treatment with a mixture of goat urine, *Moringa* leaves, and banana stems had the highest results in terms of fresh leaf weight, dry root weight, stem diameter, greenness level of leaves, plant height, leaf width, leaf length. It is known that 100% of LOF treatment is >100% due to the RAE calculation. These findings highlight the potential of LOF made from goat urine, *Moringa* leaves, and banana stems as an alternative to standard inorganic NPK fertilisers when growing leafy vegetable greens. This research contributes significantly to the knowledge of sustainable farming practises by demonstrating superior results in critical growth parameters for both pak choy and mustard greens, offering a promising avenue for increasing crop yield and quality while reducing reliance on synthetic fertilisers.

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Evaluation of Probiotics Ability to Enhance Population Density, Growth Rate, and Neonates Production of *Moina micrura* in Different Environmental Parameters

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ABSTRACT

Salinity, light intensity, and oxygen concentration are key environmental factors that significantly affect biological processes and the composition and dispersion of *Moina* biomass. Evaluating the effectiveness of probiotic enrichment in improving population density, growth rate, and neonate production can provide valuable details on the effectiveness of probiotics in enhancing the resilience and viability of *Moina micrura* under suboptimal circumstances. The purpose of this research project is to assess the efficacy of two probiotics, *Bacillus pocheonensis* strain S2 and *Lysinibacillus fusiformis* strain A1, in improving the population density, growth rate, and reproductive output in *M. micrura* across various environmental conditions. *Moina micrura* were treated with each probiotic at a volume of 5 x 10^5 CFU/ml under different levels of salinity (0, 2, 4, and 6 ppt), light intensity (800, 1,000, 1,500, and 2,000 lux), and oxygen concentration (80, 70, 60, and 50%). The results indicated that *M. micrura* treated with *L. fusiformis* A1 at 0 ppt attained the highest population density (6 ± 0.90 Ind./ml), growth rate (0.355 ± 0.030 µ), and number of offspring production (5 ± 0.75 Ind./ml). The highest point of population density (5 ± 0.07 Ind./ml), growth rate (0.381 ± 0.002 µ) and

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number of offspring (7 ± 0.41 Ind./ml) of *M. micrura* were obtained while treated with *B. pocheonensis* S2 at light intensity of 1,500 lux. Similarly, the highest population density (5 ± 0.60 Ind./ml), growth rate (0.365 ± 0.190 μ), and offspring production (2 ± 0.25 Ind./ml) of *M. micrura* were observed during enrichment with *B. pocheonensis* S2 at 70% oxygen concentration. Therefore, these results suggested that the optimum conditions for enriching *M. micrura* with *B. pocheonensis* S2 are salinity of 0 ppt, 70% oxygen concentration, and a light intensity level of 1,500 lux.

Keywords: *Bacillus pocheonensis*, enrichment, environmental parameters, growth rate, *Lysinibacillus fusiformis*, *Moina micrura*

INTRODUCTION

Many species in aquaculture can be effectively nourished with live food organisms such as phytoplankton and zooplankton as their initial feed source. These individuals are often called "living capsules of nutrition" due to their rich content of essential macro and micronutrients. Live feed can freely move within the water column and remains consistently accessible to fish and shellfish larvae, whose jerking movements stimulate larval feeding responses (Wikfors, 2004). Live feeds are easily ingested and digested (Rasdi et al., 2020), have no negative effect on water quality (Watanabe et al., 1978) and consist of crucial elements that facilitate growth. Conventional live feed such as copepods, freshwater cladocerans, and rotifers exhibit a significant capacity for reproduction, a capacity to reproduce rapidly, and the ability to live in harsh environments (Neelakantan et al., 1988). Most fish larvae favor cladocerans and have been utilized effectively as starter food in fish farming, hence contributing to the successful production of high-quality larvae (Coronado & Camacho, 2014; Taghavi et al., 2013). *Moina* is one of the largest genera of Cladocera found in North and South America, Australia, China, tropical Asia as well as Eurasia waters (Alonso et al., 2019; Bekker et al., 2016; Wang et al., 2010). *Moina* possesses the capability to adapt to numerous environmental parameters (Rizo et al., 2017) and has been used effectively in inland aquaculture as a live diet for larval fish and shrimp (Gogoi et al., 2016; Saini et al., 2013).

Moreover, *Moina* has been used as a substitute for *Artemia* in the production of red sea bream (Kotani et al., 2016). *Moina* is small, has a short embryonic stage, easy to handle, reproduces easily (Rottmann et al., 2003) and has abundant energy storage (Okunsebor & Ayuma, 2010; Sipaúba-Tavares & Bachion, 2002). *Moina*'s nutritional content varies considerably depending on the stage of their life cycle and the kind of nourishment they get (Gogoi et al., 2016). On average, *Moina* has 50% dry protein content, whereas adults often have a greater fat content (20–27%) than juveniles (4–6%) and their fatty acid content varies depending on the types of media used (Rottmann et al., 2003). However, *Moina*

does not meet the larval fish and crustaceans' requirement for highly unsaturated fatty acids (HUFA) (Kamrunnahar et al., 2019). Hence, enrichment of *Moina* is necessary to further enhance their nutritional values and obtain the optimum level needed for fish growth and survival. However, *Moina* plays a crucial role in the diet of finfish larvae and larger crustaceans. However, the inconsistent availability of *Moina* has been a significant obstacle in hatcheries, leading to the limited ability to produce high-quality fish offspring for the advancement of aquaculture (Kagali et al., 2022). Manipulating microbial community composition has received much attention to improve culture stability and diminish the spreading of detrimental bacteria (Bentzon-Tilia et al., 2016). The use of probiotics has been proven to successfully improve the nutritional value of live feed (Carter, 2015) and subsequently increase survival, stress tolerance (Singh et al., 2019), and growth performance (Pratiwy et al., 2021) of larvae.

Salinity, light, and oxygen are essential ecological elements influencing zooplankton development and reproduction (Nandini & Sarma, 2000; Rose et al., 2002; Sarma et al., 2003). These factors influence zooplankton differently (Peck et al., 2008; Zhang & Baer, 2000). In some cases, light could be a more important factor than salinity (Boolootian, 1963) since it serves as the primary recurring motion of numerous crustaceans, influencing the development, maturity, reproductive processes, and feeding behaviors of aquatic invertebrates (Buikema Jr., 1973; Miliou, 1992). The richness and variety of zooplankton and phytoplankton in the environment are influenced by salinity and diets. As a result, changes in water salinity can manipulate the primary taxonomic composition and ecological activities such as primary productivity, decomposition, nutrient cycles, and functioning of the food web (Yuslan et al., 2021). Different salinity concentrations also directly affect cladocerans' survival, growth, reproduction rate, and fatty acid levels (Rasdi et al., 2019). Oxygen concentration is one of the main limiting factors influencing the growth and reproduction of live feed (Svetlichny & Hubareva, 2002). The metabolic rate of cladocerans is lowered by oxygen deficiency (Wong, 2011) thereby reducing detoxification (Cairns Jr. et al., 1975). Anthropogenic activity has changed the aquatic environment (El-Gamal et al., 2014) and significantly affects the diversity of freshwater species, specifically aquatic organisms that cannot migrate (Rizo et al., 2017).

Thus, understanding these conditions would be beneficial for consistently ensuring the production of high-quality live foods in a controlled environment. This study aims to gauge how probiotics could positively impact the population density, specific growth rate, and production of neonates in *M. micrura* under varying environmental challenges.

MATERIALS AND METHODS

Culture of Probiotic Strains

Two probiotic strains derived from microalgae, specifically *Amphora* sp. and *Spirulina* sp., which were previously isolated, were received from the Fish Health Laboratory, Faculty of Agriculture, Universiti Putra Malaysia (Nur Natasya Ain, 2018). The two strains were identified as *Lysinibacillus fusiformis* strain A1 (GenBank accession number: MK764897) and *Bacillus pocheonensis* strain S2 (GenBank accession number: MK764898). These strains were selected based on their probiotic attributes and strong antagonistic activities toward two marine disease agents, *Vibrio harveyi* and *Vibrio parahaemolyticus* (Rosland et al., 2021) and two freshwater disease agents, *Aeromonas hydrophila* and *Streptococcus agalactiae* (Samat et al., 2021). These probiotic strains were initially cultured on tryptic soy agar (TSA, Millipore®, Germany). After overnight incubation, pure colonies of *L. fusiformis* and *B. pocheonensis* were inserted individually inside a 30 ml inoculum tube of 10 ml tryptic soy broth (TSB, Millipore®, Germany). The cultures were left to incubate for 24 hr at ambient temperature with constant agitation at 250 rpm. The probiotics were harvested through centrifugation at 3,074 x *g* for 10 min. The residue was discharged, and the settling was suspended in sterile distilled water. The probiotics' concentration was measured individually using a UV-1800 spectrophotometer (Eppendorf, Germany) at 550 nm. For experimental use, the end-state proportions of *L. fusiformis* A1 and *B. pocheonensis* S2 were altered to 5×10^5 CFU/ml (Samat, Yusoff, Chong, et al., 2020) within the experimental receptacles.

Chlorella vulgaris Culture

Chlorella vulgaris was cultivated in 1 L conical flasks containing Bold's Basal Medium, which was prepared by referring to Natrah et al. (2007)'s study. Mild aeration was supplied continuously using an air pump. The *C. vulgaris* concentration was ascertained with the aid of an enhanced 0.1 mm Neubauer chamber and viewed using a light microscope as per the subsequent formula:

$$\text{Density, d } \left(\frac{\text{cells}}{\text{ml}}\right) = \frac{\text{Average number of cells per square}}{4 \times 10^{-6}}$$

where, 4×10^{-6} is the sample volume from across the small square area, equal to 0.004 mm^3 (0.2 x 0.2 x 0.1) measured in cm^3 (ml).

Moina micrura Culture

Moina micrura was cultivated in pond water that had been filtered and sterilized (0.45 µm Whatman® fiber glass filters, United Kingdom). The starting culture was kept in a plastic aquarium with a capacity of 2 L and provided with daily feeding of *C. vulgaris* at a concentration of 5×10^4 cells/ml (Munirasu et al., 2016) for two weeks prior to the commencement of the experiment. The concentration of *C. vulgaris* was determined by applying the method and formula described in *C. vulgaris* culture.

Experimental Design

The impacts of different salinities (0, 2, 4, and 6 ppt) (Sarma et al., 2006), light intensities (800, 1,000, 1,500, and 2,000 lux) (Serra et al., 2019), and oxygen concentrations (80, 70, 60, and 50%) (Svetlichny & Hubareva, 2002) on *M. micrura* with probiotics enrichment were observed. Two separate experimental setups were designed. The first experiment determined the population growth of *M. micrura*, while the second experiment evaluated the production of neonates throughout their lifetime. All treatments were run with triplicates.

Salinity

For experiment one, ten one-day-old *M. micrura* were allocated 50 ml of Falcon tube comprising 40 ml of sterilized freshwater. On day one, a probiotic was added at a concentration of 10^5 CFU/ml, and *Moina* was then fed with *C. vulgaris* once a day at 10^4 CFU/ml. The cultures were maintained for 12 days at the desired salinity level (0, 2, 4, 6, and 8 ppt) at 25°C and were exposed to 12 hr light and 12 hr dark. The salinity level was adjusted using sodium chloride (NaCl, Millipore®, Denmark). The concentrations of NaCl were determined using a refractometer (ATAGO, Japan). Water exchange and re-supplementation of probiotics were done once every two-day interval. The *M. micrura* population density was measured daily by extracting 1 ml of the well-mixed culture and analyzing the sample within a Petri dish. The formula below was employed to compute the population growth rate:

$$\text{Population growth rate, } \mu = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1}$$

where, X_1 = the number of *M. micrura* at the beginning of the selected time interval; X_2 = the number of *M. micrura* at the end of the selected time interval; t_1–t_2 = the selected time (in days).

For experiment two, five one-day-old *M. micrura* were allocated in a 50 ml Falcon tube with 40 ml of purified sterile freshwater. Similar conditions were applied to the first experiment. The presence of neonates was monitored in the morning every day and discharged after the count. The experiment continued until the natural death of all *M. micrura* occurred.

Light Intensity

For experiment one, ten newly hatched *M. micrura* were allocated into a 50 ml Falcon tube containing 40 ml of sterilized freshwater. On day one, each probiotic was added individually at 10^5 CFU/ml, and *M. micrura* was given microalgae *C. vulgaris* once a day at 10^4 CFU/ml. The cultures were maintained for 12 days at different light intensities (600, 800, 1,000, 1,500, and 2,000 lux) at 25°C and experienced a cycle of 12 hr of light followed by 12 hr of darkness. The light intensity levels were adjusted using a digital lux meter (LX1020B, Redmark Industry, Malaysia). The light-intensity experiment was carried out in a dark room, and an adjustable lamp was used as the light source. Water exchange and re-supplementation of probiotics were

done every two days. The daily evaluation of *M. micrura* population density involved extracting 1 ml from the thoroughly mixed culture and scrutinizing the sample within a Petri dish. The population's growth rate was determined using the identical section salinity formula.

For experiment two, just one day old, five newly hatched *M. micrura* were placed into a 50 ml Falcon tube containing 40 ml of sterilized freshwater. Similar conditions were applied to the first experiment. The presence of neonates was monitored in the morning every day and discharged after the count. The experiment continued until the natural demise of all *M. micrura*.

Oxygen Concentration

For experiment one, ten *M. micrura* that were one day old, were allocated in a 50 ml Falcon tube consisting of 40 ml of sterilized freshwater. On day one, each probiotic was added individually at 10^5 CFU/ml, and *M. micrura* was nourished with microalgae of the species *C. vulgaris* once a day at 10^4 cells/ml. The cultures were sustained for 12 days at different oxygen concentrations (80, 60, 50, 40, and 30%) at 25°C and subjected to a 12-hr light and 12-hr dark cycle. The oxygen concentrations were determined using a dissolved oxygen meter (YSI 550A, USA). Water exchange and re-supplementation of probiotics were done every two days. Each day, the population density of *M. micrura* was assessed by extracting 1 ml from the well-mixed culture and analyzing the specimen within a Petri dish. The population growth rate was determined using the identical formula under section salinity.

For experiment two, five one-day-old *M. micrura* were allocated in a 50 ml Falcon tube containing 40 ml of sterilized freshwater. Similar conditions were applied to the first experiment. The presence of neonates was monitored in the morning every day and discharged after the count. The experiment continued until all the *M. micrura* died naturally.

Statistical Analysis

All the data was analyzed using a one-way analysis of variance (ANOVA). Multiple comparison tests (Tukey's test) at the 0.05 probability level were performed to identify a significant difference between treatments. All statistical tests were performed using IBM SPSS statistic V27.0 software.

RESULTS

Salinity

The effects of different salinities on population density, population growth rate, and neonates' production of *M. micrura* throughout the culture period were presented in Figures 1, 2A, and 3A, respectively. Results showed that *M. micrura* treated with *L. fusiformis* A1 at 0 ppt obtained the maximum population density (6 ± 0.90 Ind./ml) on day eleven of culture (Figure 1A). Similarly, *M. micrura* treated with *L. fusiformis* A1 at 0 ppt reached the peak growth rate (0.355 ± 0.030 µ), significantly higher ($p < 0.05$) contrasted with the control group having akin salinity concentration (Figure 2A). Additionally, treatment with *L. fusiformis* at 0 ppt produced the highest number of offspring (5 ± 0.75 Ind./ml, Figure 3A) compared to other treatments

at all salinity concentrations (Figure 3A). Meanwhile, *M. micrura* was treated with both probiotic strains at 6 ppt and died on the 5th day of the experiment (Figure 1D), whereby no population growth and neonate production were observed. Regardless of salinity concentrations, enrichment of *M. micrura* with either *L. fusiformis* A1 or *B. pocheonensis* S2 showed a higher growth rate and neonate production compared to the control group. However, the data were not statistically significant for some treatments ($p > 0.05$) (Figures 2A and 3A).

Light Intensity

Results of the effects on different light intensity levels for population density, growth rate, and neonate production of *M. micrura* were presented in Figures 2B, 3B, and 4 respectively. The ultimate peak population density of *M. micrura* was obtained at 1,500 lux when treated with *B. pocheonensis* S2 (5 ± 0.07 Ind./ml) on day ten of culture (Figure 4C). Similarly, the highest growth rate (0.381 ± 0.002 µ) was obtained at 1,500 lux when enhanced with *B. pocheonensis* S2 (Figure 2B). Although the utmost neonate's production (7 ± 0.41 Ind./ml) was observed during enrichment with *B. pocheonensis* S2 at 1,000 lux, the data showed no significant difference compared to other treatments ($p > 0.05$) (Figure 3B).

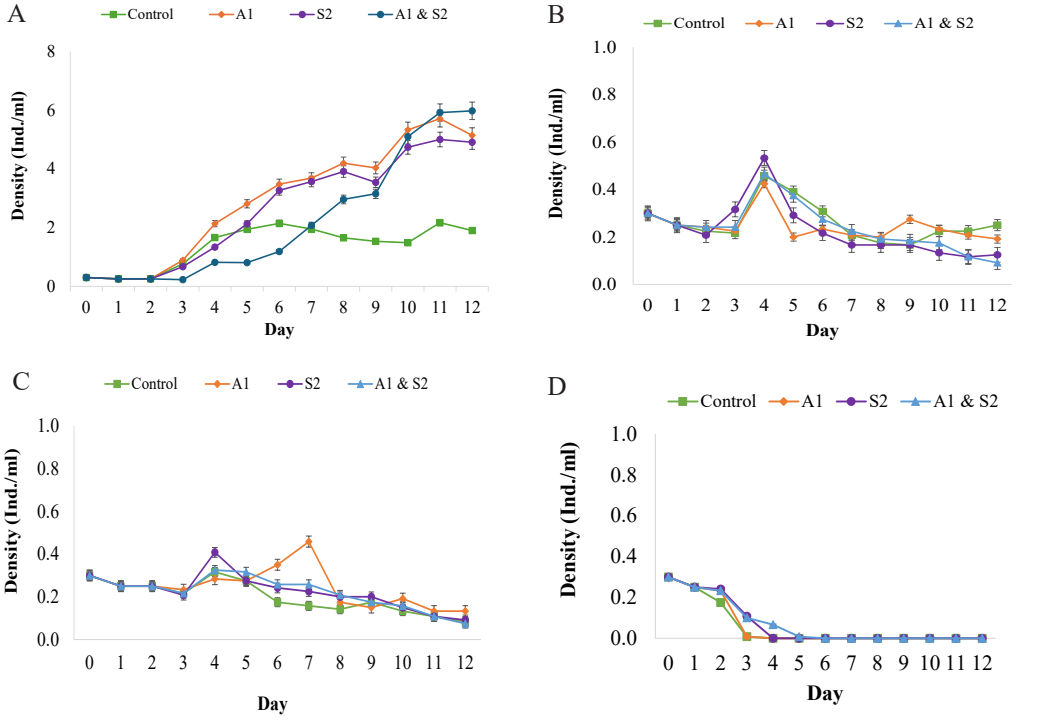
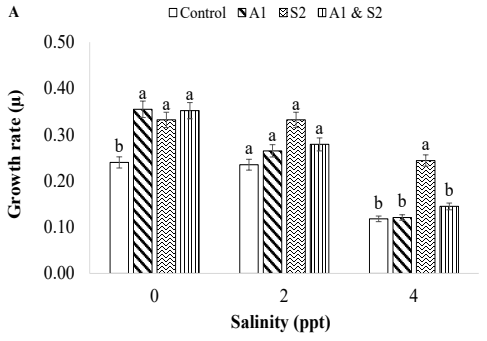
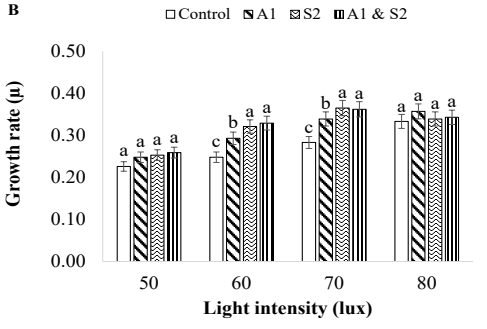


Figure 1. Differences in *Moina micrura* population density under varying salinity levels: (A) = 0 ppt, (B) = 2 ppt, (C) = 4 ppt, and (D) = 6 ppt. *Note*. Four different treatments have been done in each salinity level: Control (no probiotic added), A1 (*Lysinibacillus fusiformis*), S2 (*Bacillus pocheonensis*), A1 and S2 (*Lysinibacillus fusiformis* combined with *Bacillus pocheonensis*); Vertical bars = Standard errors of the means (n = 3)





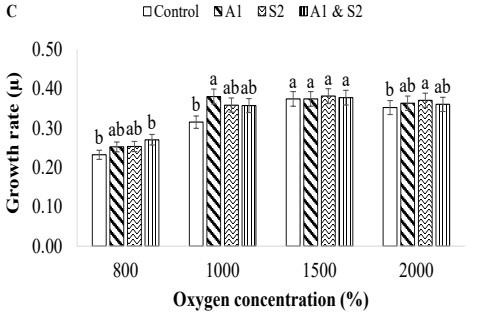
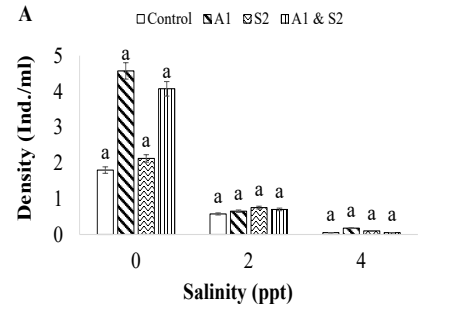
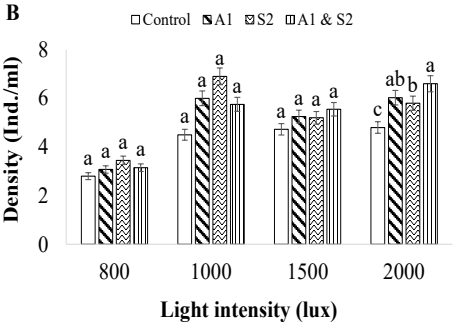


Figure 2. Specific growth rate (µ) of *Moina micrura* in different environmental parameters: (A) salinity (ppt), (B) light intensity (lux), and (C) oxygen concentration (%)

Note. Four different treatments have been done in each environmental parameter: Control (no probiotic added), A1 (*Lysinibacillus fusiformis*), S2 (*Bacillus pocheonensis*), A1 and S2 (*Lysinibacillus fusiformis* combined with *Bacillus pocheonensis*); Vertical bars = Standard errors of the means (n = 3); Different letters show significant differences among treatments ($p < 0.05$)





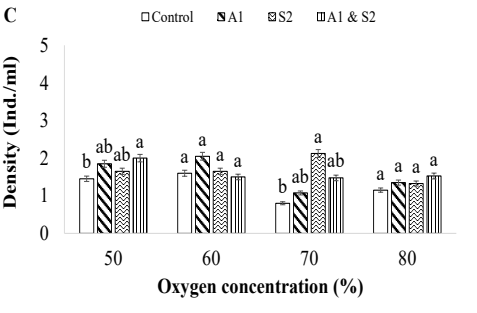


Figure 3. The number of neonates produced by *Moina micrura* in different environmental parameters: (A) salinity (ppt), (B) light intensity (lux), and (C) oxygen concentration (%)

Note. Four different treatments have been done in each environmental parameters level: Control (no probiotic added), A1 (*Lysinibacillus fusiformis*), S2 (*Bacillus pocheonensis*), A1 and S2 (*Lysinibacillus fusiformis* combined with *Bacillus pocheonensis*); Vertical bars = Standard errors of the means (n = 3); Different letters show significant differences among treatments ($p < 0.05$)

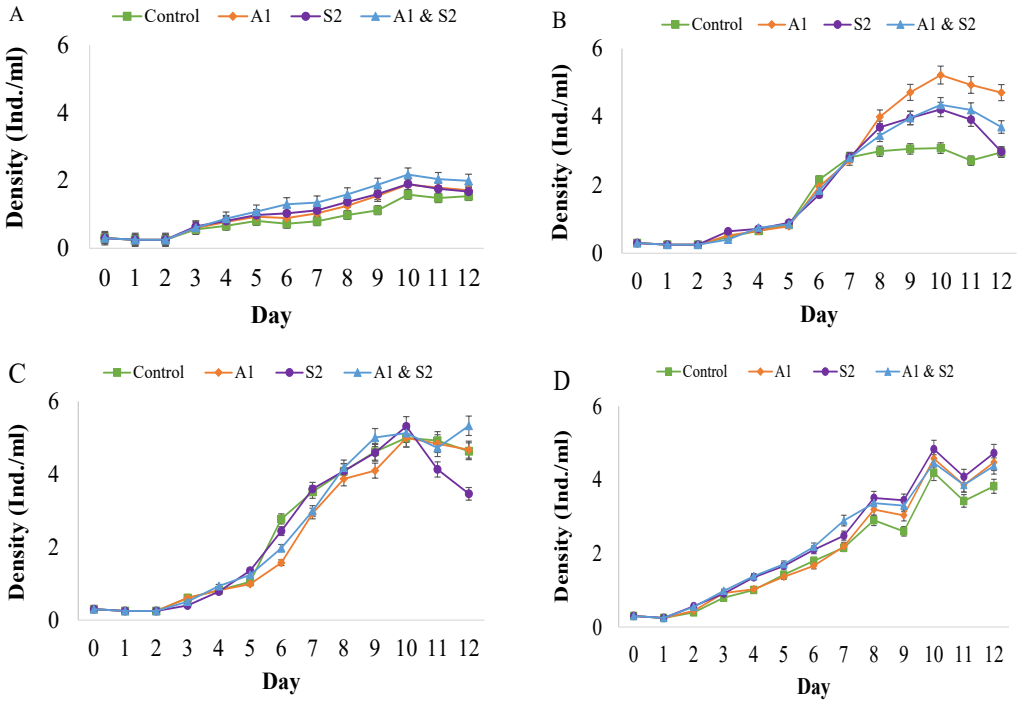


Figure 4. Shifts in the population density of *Moina micrura* in response to diverse light intensity levels: (A) = 800 lux, (B) = 1,000 lux, (C) = 1,500 lux, and (D) = 2,000 lux

Note. Four different treatments have been done in each intensity level: Control (no probiotic added), A1 (*Lysinibacillus fusiformis*), S2 (*Bacillus pocheonensis*), A1 and S2 (*Lysinibacillus fusiformis* combined with *Bacillus pocheonensis*); Vertical bars = Standard errors of the means (n = 3)

Oxygen Concentration

The effects of different oxygen concentrations on the population density, growth rate, and neonate production of *M. micrura* throughout the process of enrichment with probiotics were presented in Figures 2C, 3C, and 5, respectively. Results showed that the enrichment with *B. pocheonensis* S2 at 70% oxygen concentration had a significant outcome. The top maximum population density (5 ± 0.60 Ind./ml, Figure 5C), growth rate (0.365 ± 0.190 µ, Figure 2C), and offspring production (2 ± 0.25 Ind./ml, Figure 3C) of *M. micrura* were observed in the same treatment. Moreover, the growth rate and offspring production of *M. micrura* enriched with *B. pocheonensis* S2 at 70% oxygen concentration exhibited a notable increase compared to the control group (Figures 2C and 3C).

DISCUSSION

Although *Moina* is considered an important live feed for the larval rearing of most freshwater species, they are not readily obtainable from natural habitats in commercial-scale quantities (Loh et al., 2009). The larvae of red sea bream (*Pagrus major*) and kuruma shrimp (*Marsupenaeus*

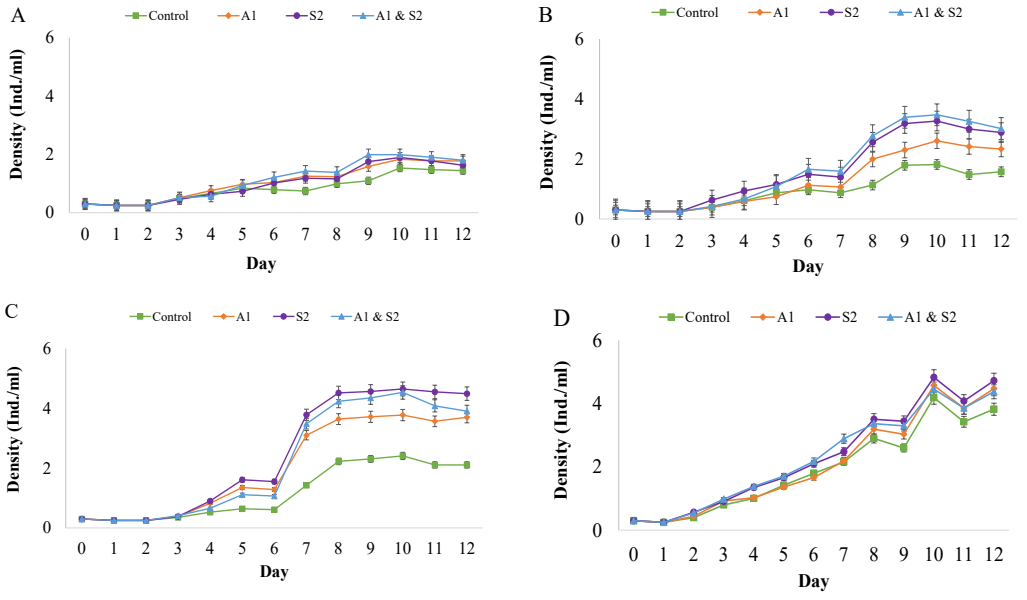


Figure 5. Changes in population densities of *Moina micrura* in varied oxygen concentrations: (A) = 50%, (B) = 60%, (C) = 70%, and (D) = 80%

Note. Four different treatments have been done in each oxygen concentration: Control (no probiotic added), A1 (*Lysinibacillus fusiformis*), S2 (*Bacillus pocheonensis*), A1 and S2 (*Lysinibacillus fusiformis* combined with *Bacillus pocheonensis*; Vertical bars = Standard errors of the means (n = 3)

japonicus) were reared successfully using *Moina* in small-scale experiments (Kamrunnahar et al., 2019; Nakamoto et al., 2008). However, mass production of *Moina* is unstable and often results in culture crashes. Although organic waste such as animal manure has been used as the source of nutrients in *Moina* culture, the risk of pathogen transfer is greater with these diets (Kagali et al., 2022; Kamrunnahar et al., 2019). Moreover, the nutritional content of *Moina* is unfavorable for small fish and shellfish. Therefore, introducing probiotics may be a potential solution to address these limitations by stabilizing the culture conditions and improving the reproductive capacity of *Moina*.

This study indicated that each of the probiotics, *L. fusiformis* and *B. pocheonensis*, had the ability to enhance the growth and production of *Moina*. The influence of a diet on the population growth of zooplankton can be evaluated based on its population density (Peña-Aguado et al., 2005). By improving the quality and quantity of the feed, the reproductive capacity and duration can be manipulated to enhance *Moina*'s population density and growth (Damayanti et al., 2020). The nutritional content of *Moina* can be altered through enrichment by taking advantage of primitive feeding characteristics (Samat, Yusoff, Rasdi, et al., 2020; Singh et al., 2019). Probiotics have been proven to increase the variety of microbial organisms present in live

feed (Jiang et al., 2019). The population and dispersion of *Moina* are affected by seasonal change, the number and size of the eggs, sexual development, changes in temperature, salinity, and light, as well as other environmental parameters (Ramírez-Merlano et al., 2013; Samat et al., 2022).

The focus of this research was to analyze the potential of probiotics to tolerate high salinity stress. Increased salinity can cause osmotic stress, reducing somatic development, reproduction, and survival and delaying sexual maturation in cladocerans (Santangelo et al., 2008). This study showed that *M. micrura* enriched with 10^5 CFU/ml of *L. fusiformis* have higher population density, growth rate, and neonates' production at 0 ppt salinity. As indicated by (Yuslan et al., 2021), for another species of *Moina*, *M. macrocopa* had achieved the highest survival and growth rate ever recorded for an environmental salinity of 0 ppt. Moreover, according to (Santangelo et al., 2008), salinity above 2 ppt negatively affected the reproduction of *M. micrura*, which could be due to osmoregulation pressure whereby the organism was unable to adapt its physiology as the salinity level increased. A study also showed that increased salinity concentration towards *Daphnia magna* and *Daphnia longispina* can affect their production performance (Toruan, 2012). Changes in water salinity may cause osmotic disequilibrium in zooplankton dwelling in freshwater-marine interface zones (El-Gamal et al., 2014; Harris et al., 2000). These findings suggested that the growth and production of *Moina* are undoubtedly affected by the increase in salinity levels. Enrichment with probiotics was unable to enhance *Moina* tolerance in a high salinity environment.

Although several studies on cladocerans have been published, most information was limited to the genus *Daphnia*. Comparatively limited studies have so far investigated the impact of light intensity on *Moina* culture. In recent research, *M. micrura* enriched with *B. pocheonensis* has greater population density, growth rate and neonate production at 1,500 lux of light intensity. For *D. magna*, the amount of neonate production was negatively affected by increasing light intensity, while it was positively correlated to survival rate (Wonkwon et al., 2019). Additionally, since the effectivity of probiotics is marginally influenced by light intensity, photosynthetic microalga probably was the main cause of reduced population growth of *M. micrura* in our experiment, as lower light intensity conditions reduced food availability (Vijverberg, 1989).

Because of its fast potential growth, *Moina* is being used for live feed in larvae and juvenile finfish worldwide (*Moina* is much smaller compared to *Daphnia* and contains 70% higher protein) (He et al., 2001). *Moina* is frequently utilized as a substitute meal for *Artemia* in both larval fish and shrimp cultures. *Moina micrura* has also been discovered to be an intriguing alternative to *Artemia* for increasing *Macrobrachium rosenbergii* production (Kang et al., 2006). It seems necessary to enrich the nutritional content of *Moina* as live feed to enhance their

nutritional value, particularly in terms of important fatty acids. *Moina* has nourished various aquatic species, including catfish *Clarias macrocephalus*, different types of catfish, shrimp, and larvae of red sea bream (Nakamoto et al., 2008). Enrichment of live food with probiotics aids their survival and proliferation within the components of the live food, allowing them to be successfully transferred into the hosts (Hai, 2015). Probiotic bacteria have the dual potential to improve the nutrient content of live food by delivering vital nutrients like vitamins or non-organic components that are absent from the diet. Additionally, they can increase the live food's population density while inhibiting the growth of harmful pathogens (Douillet, 2000).

Moreover, introducing probiotics directly into the cultivation water carries a risk due to their susceptibility to contamination by microorganisms (Sun et al., 2013). Moreover, using live food as a carrier is an optimal approach, as probiotics in seawater have a short survival period (Gatesupe, 2008). Live food persists within the rearing water for multiple hours prior to consumption. Thus, encapsulated bacteria must possess the ability to endure within the live food for a duration sufficient for the larvae to engage in feeding (Pintado et al., 2010). The multiplication of bacteria during the initial growth phase of fish larvae's gut microflora is intricately linked to the bacteria in the live food. As a result, the bioencapsulation approach of enriching live food with probiotics enables the controlling of the microbial community in the live food. It may result in improved development and viability of fish and crustacean larvae (Olafsen, 2002). The traditional application of antibiotics for minimizing bacterial diseases is debatable, and in certain circumstances, it has lost its effectiveness in addressing such infections (Defoirdt et al., 2011).

In recent times, the popularity of using dietary intake of nutritional supplements like probiotics for the management or treatment of illnesses has increased (Hoseinifar et al., 2018). In certain circumstances, administering probiotics to the target host's gut via probiotic enrichment through a bioencapsulation technique of using zooplankton as a live food is an intriguing initiative (Gomez-Gil & Roque, 2000). Studies have indicated that encapsulating probiotics within live food can enhance zooplankton's growth, population density, and reproductive rate (Le et al., 2017; Planas et al., 2004).

Even though most of the probiotics treated in *M. micrura* were not significantly different among the treatments, they remained higher compared to the control group. *M. micrura* enriched with probiotic *B. pocheonensis* S2 has maximum population density, growth rate and neonates' production in light intensity and oxygen concentration parameters, while *M. micrura* enriched with *L. fusiformis* A1 has the highest population density, growth rate and neonates' production in 0 ppt salinity. *Moina micrura* enriched with *L. fusiformis* shows the finest result than control treatment in population density, growth rate and neonate

production at pH 8 (Babitha Rani et al., 2006). Synbiotic enrichment of *Artemia* (*Pediococcus acidlactici* at 700 mg/L and fructooligosaccharide at 100 mg/L) substantially enhanced fish development, diversity of microorganisms, ability to handle stress, and immune responses (Lobo et al., 2018). Studies have shown that feeding fish larvae with *Artemia* enriched with *Shewanella putrefaciens* can increase n-3 HUFA levels (Sun et al., 2013). Copepod enrichment with any one of lyophilized *Bacillus clausii* or *Bacillus pumilus* at 10^6 CFU/ml for 3 hr increased fish larval development, survival, and favorable gut microbiota (Green, 1956). It provides strong evidence that the probiotics were successfully incorporated into the live feed culture, thus validating their efficacy in enhancing the experimental conditions.

In this research endeavor, the enrichment of *M. micrura* with *B. pocheonensis* S2 at 70% oxygen concentration showed the highest population density, growth rate, and offspring production. Similarly, Svetlichny and Hubareva (2002) found that low oxygen level negatively affects the growth rate and reproduction of *M. micrura*, and the study suggested that low oxygen levels reduced the locomotion efficiency of *M. micrura*, which consequently reduced their filtration rate. Furthermore, a study reported a reduction in growth and egg production, increased egg abortion, alteration of feeding behavior, and reduction in feeding rate of cladoceran *Daphnia* spp. when exposed to a lower oxygen concentration of less than 0.002% (Jiang et al., 2019). Therefore, the enrichment of *M. micrura* with probiotics in 70% oxygen concentration assisted in improving the population density, growth rate, and neonates' production of *M. micrura*.

CONCLUSION

The population density, population growth rate, and neonates' production of *M. micrura* enriched with probiotics surpass those in the control treatments. Both probiotics have proved to be partially effective in conferring benefits to *M. micrura*. The current study unveiled that the most effective performance was observed with *B. pocheonensis* S2 at improving the population density, population growth rate, and neonates' production of *M. micrura* under diverse environmental parameters. This study's findings demonstrated that probiotics could play a role in enhancing *M. micrura*'s ability to withstand conditions slightly higher or lower than the ideal range. Furthermore, the results suggested that the most effective enrichment for *M. micrura* is achieved with *B. pocheonensis* S2 at 0 ppt, with 70% oxygen concentration, and at the light intensity level of 1,500 lux. This study gathered important data that would be valuable for the mass production of *M. micrura* through probiotic enrichment since this species holds promise as a viable alternative source of live food during the larviculture of many fish and crustacean species.

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