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 second 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 16 articles; two short communications; and the rest are regular articles. The authors of these articles come from different countries namely Indonesia, Malaysia, Nigeria, Thailand and United States of America.

A selected regular article entitled "Effect of Leaf Pruning and Additional Fertilizer on Growth and Young Pods Yield of Winged Beans" determined the effect of leaf pruning and additional fertilizer on the growth and yield parameter of winged beans. A randomized complete block design was used with two factors and three replications, namely leaf pruning intensities (0, 15, and 30% leaf pruning) and rates of additional fertilizer (0, 6.25, 12.50, and 18.50 g NPK 16-16-16/plant). The observed variables were plant height, leaf number, root length, leaf nutrient, auxin content, nutrient uptake, and young pod yield. The findings revealed that the interaction of pruning intensities and additional fertilizer rates significantly influenced leaf number and root length. Full information of this study is presented on page 323.

A short communication entitled "Spore Germination of *Diplazium simplicivenium* Holtt. (Athyriaceae) in Peninsular Malaysia" reported an efficient method *for D. simplicivenium* spore sterilisation and the effect of plant growth regulators via green globular bodies. The outcomes showed that this is an efficient spore culture approach that would allow for large-scale production of *D. simplicivenium*, which might contribute to conserving species on the verge of extinction and be applied to propagate other fern species. The detailed information of this article is available on page 411.

Azizah Mahmood and her teammates from Universiti Malaysia Terengganu explored the potential of inulin as a fat substitute to produce low-fat muffins. There are in total of five batches of muffins, each with varying levels of inulin replacing oil (ranging from 0 to 100%), were prepared to examine how this substitution would impact the physicochemical and sensory properties. The results proved that incorporating inulin into muffin recipes yields notable fat and calorie content reductions while enhancing fibre. The further details of this study are found on page 495.

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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Economically Imperative *Ananas comosus* Diseases, Status, and Its Control Measures Documented in Producing Countries

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ABSTRACT

Ananas comosus, commonly known as pineapple, is a fruit with a large potential market as a commodity and commercial fruit. Numerous pests and diseases affect pineapple, directly or indirectly, by lowering the quality and quantity. The fungal causative agents, namely *Fusarium ananatum* and *Thalaromyces stolii* (previously named *Penicillium funiculosum*), cause fruitlet core rot (FCR) and fusariosis by *Fusarium guttiforme*. Bacteria heart rot (BHR) is an infection by *Erwinia chrysanthemi*, newly known as *Dickeya zeae*. Nevertheless, the mealybug wilt of pineapple (MWP) is another pineapple treat to susceptible pineapple varieties caused by pineapple mealybug wilt-associated viruses (PMWaVs). Other diseases include destruction caused by pathogenic nematodes. This review discusses the status of these diseases and the control measures that greatly affect the economy of pineapple-producing countries due to the economic significance of these crops. Growers need up-to-date information on the identity of the diseases that affect pineapple crops in the various countries that produce them to effectively manage the diseases in the field.

Keywords: Economy, pineapple, plant disease, plant pathogens

INTRODUCTION

Pineapple (*Ananas comosus*), a member of the Bromeliaceae family, is an important fruit crop with over 2,000 species of pineapple. This fruit is an important source of carbohydrates, minerals, fiber, organic acids, and vitamins for humans (Chaudhary et al., 2019). In addition, pineapple has antioxidants such as flavonoids, ascorbic acid, and carotenoids. The chemical makeup of this fruit varies depending on the variety. One fresh pineapple contains 17% of the daily recommended value for vitamin C

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and is high in B-complex vitamins like pyridoxine, folate, riboflavin, and niacin. Pineapple contains bromelain, which has anti-clotting, anti-cancer, and anti-inflammatory properties (Ajayi et al., 2022; Habotta et al., 2022).

The pineapple, the second-highest tropical crop after mango, is one of the most economically significant fruits worldwide, with Asia, South Central America, and Africa being the world's top fruit producers. The negative effects of the COVID-19 pandemic in 2020 appear to have significantly influenced pineapple exports. According to the most recent data, exports totaled 3.1 million tons in 2020, representing a 7.9% decrease from 2019. Costa Rica and the Philippines, the world's two largest pineapple exporters, saw a 7.7 and 5.8% drop in their shipments, respectively (Food and Agriculture Organization of the United Nations [FAO], 2021). The most important fact about pineapple is that it is a commercial fruit grown in tropical and subtropical areas. Tropical countries such as Malaysia, Thailand, Indonesia, India, Kenya, the Philippines, and China are among the world's leading pineapple growers (Sukri et al., 2023). In 2020, Malaysia had 17,228 ha of pineapple farming, accounting for 9.5% of the nation's total fruit-growing area ("Malaysia's pineapple export," 2022). Malaysia's pineapple-producing states are Johor, Kedah, Negeri Sembilan, Pahang, Selangor, Sabah, Sarawak, and Terengganu.

Production of pineapples is expected to increase to 31 million tons globally by 2028, growing at a 1.9% annual rate with more than 100 cultivars worldwide (Muhamad et al., 2022). There are currently 6 major pineapple varieties grown in producing countries by small- and large-scale producers, including MD2, Sweet Cayenne, Española, Pernambuco, Perolera, and Queen Victoria (Alejo Jeronimo et al., 2023). The susceptibility of pineapple varieties to pathogens could vary significantly by impacting disease incidence and management. For example, the susceptibility of various pineapple cultivars to BHR disease may vary. According to Oculi et al. (2020), different pineapple cultivars respond to BHR disease differently, with some being more resistant than others. In some cases reported by Dey et al. (2018), MWP disease is more susceptible, especially on smooth Cayenne, while some pineapple cultivars may be more resistant. Plant diseases are one of many factors that could affect the number and quality of pineapple output (Sapak et al., 2021). Fungi, bacteria, viruses, and nematodes are always associated with pineapple diseases. These disease infections can potentially destroy the entire pineapple plant or significant portions of it. Pineapple-producing countries economically affected by pineapple diseases worldwide are listed in Table 1. This article reviews the prevalent and destructive pineapple diseases affecting the producing countries' crops.

MEALYBUG WILT OF PINEAPPLE (MWP)

Mealybug wilt of pineapple (MWP) is one of the most significant diseases affecting pineapple production globally (Hernández-

Table 1
List of pineapple-producing countries that were economically affected by pineapple diseases

Disease	Country	References
Mealybug wilt of pineapple (MWP)	Cuba	Hernández-Rodrıguez et al. (2019)
	Ghana	Asare-Bediako et al. (2020)
	Hawaii	Hernández-Rodrıguez et al. (2019)
Bacteria heart rot (BHR)	Costa Rica Malaysia Brazil Philippine Hawaii	Cano-Reinoso et al. (2021)
	Uganda	Oculi et al. (2020)
Fruitlet core rot (FCR)	Reunion Island	Vignassa et al. (2021)
	South Africa	Chillet et al. (2020)
Fusariosis	Central America Africa Asia Brazil	Carnielli-Queiroz et al. (2019)
Causal agent by nematodes	Philippine	Benzonan et al. (2021)
	Nigeria	Tanimola et al. (2021)
	Brazil	Norwegian Institute of Bioeconomy Research (2021)
	France	Soler et al. (2021)

Rodrıguez et al., 2019; Larrea-Sarmiento et al., 2021). The primary symptoms of MWP are wilting, reddish-yellow leaves beginning with the outer leaves and undersized fruit. When the symptoms are severe, the plant will likely die, the leaf tips curl and dry up, and plant growth slows down (Hutahayan et al., 2022). Although the exact cause of MWP is unknown, it is thought to be linked to specific viruses, mealybugs as vectors, ants that guard mealybugs and facilitate their spread, and environmental factors. The MWP symptoms in Hawaii, Columbia, and Cuba established the presence of a Closterovirus as the causative agent (Hernández-Rodrıguez et al., 2019; Larrea-Sarmiento et al., 2021; Moreno et al., 2023). To date, three recognized members known as pineapple mealybug wilt-associated virus-1 (PMWaV-1), -2 (PMWaV-2), and -3 (PMWaV-3), as well as one putatively postulated member known as PMWaV-5, have been identified as Ampeloviruses that infect pineapple and have been linked to MWP. It suggests that a virus previously known as PMWaV-4 is a strain of PMWaV-1 (Green et al., 2020). Recently, PMWaV-6 has been suggested as a potential new *Ampelovirus* species member (Larrea-Sarmiento et al., 2021).

In Hawaii, PMWaV-2 has been demonstrated to contribute to the etiology of MWP, whereas pineapple plants infected with PMWaV-1, PMWaV-2, or both without

visible mealybug feeding do not appear to exhibit symptoms (Green et al., 2020; Larrea-Sarmiento et al., 2021).

The transmission of five pineapple mealybug-associated viruses (PMWaVs) requires using vectors rather than being physically possible. Thus, vector insects play a crucial role. Mealybugs, which can act as PMWaV vectors, also claimed that the disease's symptoms were caused by an interaction between the PMWaV virus and mealybugs' feeding behavior (Hutahayan et al., 2021). All these viruses are primarily spread semi-regularly by two *Dysmicoccus* species, the pink (*Dysmicoccus brevipes*) and grey mealybugs (*Dysmicoccus neobrevipes*). The vegetative propagation of infected pineapple materials is the primary source of viral dissemination during the establishment of new planting areas (Hernández-Rodrıguez et al., 2019).

According to our research in Malaysia (unpublished data), MWP can be common in Malaysian pineapple farms due to the widespread distribution of suckers among pineapple growers. According to the observations, the prevalence of MWP in the field area is approximately 100%. In the case of the MD2 variety, the symptoms include chlorotic patches along the lamina, necrotic leaf tips, and leaves that curled downward and dried out (Figure 1 A). In other varieties, such as Sarawak, Josephine, and Crystal Honey, the symptoms include wilting, reddening leaves starting with the outer leaves, curling down, and drying up of the leaf tips (Figure 1 B-D).

In pineapple fields, the development of MWP causes significant yield losses, which are greater in plants infected earlier by mealybugs in their life cycles. Mealybugs cause immediate damage to pineapple, resulting in chlorotic areas, and they also weaken the plant by feeding on the stem and roots, making it more vulnerable to other pests and diseases, which reduces the fruit's market value (Araya, 2019). In plants with MWP symptoms, the pineapple tips curve down, the leaves change bronze-red and lose turgidity, and the fruits are unmarketable because the flesh is fibrous and sour (Araya, 2019). MWP contributes 35 to 55% of pineapple crop yield losses in Hawaii. Yield losses in the pineapple-producing countries

Figure 1. Occurrence of mealybug wilt of pineapple (MWP) on different varieties in Melaka, Johor, and Negeri Sembilan, Malaysia: (A) MD2, (B) Sarawak, (C) Josaphine, and (D) Crystal Honey.

of Cuba were estimated to be more than 50% (Hernández-Rodrıguez et al., 2019). In other producing countries, such as Ghana, the yield loss caused by MWP is estimated to be around USD 248 per hectare (Asare-Bediako et al., 2020). When combined with abiotic stress, ampelovirus infection may also affect pineapple crop losses. In Hawaii, asymptomatic infection by PMWaV-1 causes a 6.7% output decline, with losses increasing up to 13.3% in plantations under water stress (Hernández-Rodrıguez et al., 2019).

For the management of MWP, an integrated strategy that incorporates cultural practices, biological control, and chemical control measures is required. Mealybugs and their associated ants are reduced by good soil preparation and a year-long fallow period or intercycle before planting (Araya, 2019). Pineapple growers must consider the risks of using their plants for subsequent production cycles or exchanging plants with neighbors. The continued use of infected material may encourage virus species mutations and the emergence of new virus strains and species, which could have an unpredictable effect on production (Moreno et al., 2023).

According to Gungoosingh-Bunwaree et al. (2021), despite the fact that Mauritius' pineapple growers frequently trade suckers, the failure to properly control insect vectors during the 2020 COVID-19 pandemic's forced quarantine of people may be to blame for the spread of PMWaV-1 and -2 to new pineapple-growing regions. Careful selection of disease-free suckers and the adoption of effective vector control measures must be resumed immediately to protect Mauritius' pineapple production from MWP. According to a study by Araya (2019), the control of mealybugs on pineapple stems and roots was successful when Nemacur® 40EC (fenamiphos-AMVAC) was applied to the foliage of pineapple trees, and it worked best at 10 L/ha. Synthetic insecticides are frequently applied after sowing to kill or prevent mealybugs. Since the pest likes to feed on the roots or the stem above the roots, most products used to control it are non-systemic and have limited effectiveness. A biological control method like a sugar feeder can work by managing mealybugs through ants because mealybugs and ants have a mutualistic relationship. Sugar supply can disrupt the mutualistic interaction between ants and mealybugs in citrus. Ant activity in the orange tree canopy decreased as sugar was introduced, and ant attendance at mealybug populations decreased as a result, leading to an increase in parasitism (Pérez-Rodríguez et al., 2021).

BACTERIA HEART ROT (BHR) DISEASE

BHR disease is still a major economic issue for pineapple producers in many countries, such as the Philippines, Indonesia, Costa Rica, Brazil, and Hawaii (Cano-Reinoso et al., 2021). The bacterium *Erwinia chrysanthemi*, newly known as *Dickeya zeae*, infects the plant through wounds and causes decay in the heart of the plant. The bacterial pathogen, which affects a wide range of plant species, causes soft rot

diseases by attacking various plant parts at various development stages (Young et al., 2022). The bacteria with the following morphological traits were circular, convex, cream-whitish, milk-colored, and colonies with a 1–2 mm diameter were found (Aeny et al., 2020).

The disease manifests as water-soaked lesions on the interior leaves, which then coalesce into blisters as the bacteria ferment plant matter. These will eventually exude a contagious bacterial ooze. As the fruit matured, the pineapple's center crown of leaves finally collapsed, killing the apex and stopping the fruit development. Gas accumulates because of the fermented fruit. The hissing sound that the gas makes as it leaves can be heard, particularly in the stillness of the night. It was dubbed "ghost rot" because it terrified many field laborers as they went home at night (Veléz-Negrón et al., 2023; Young et al., 2022).

The symptoms of fungal heart rots can be differentiated from those of bacterial heart rots because the infection is not spread to the greener areas of older leaves (Cano-Reinoso et al., 2021). The main inoculum source for bacterial heart rot is thought to be exuded juice from previously diseased plants, and the leaves are easily detached from the plants (Figure 2). Instead, because the bacteria do not survive long on leaf surfaces, infected seed material such as suckers, slips, or crowns are not a significant source of infection. The stomata become infected, and the bacteria are primarily spread by insects, such as big-headed ants (*Pheidole megacephala*) or Argentine ants (*Linepithema humile*), as well as by wind and rain. Four- to eight-month-old plants are more susceptible to this disease. Additionally, it is believed that common pineapple plantations are more vulnerable than ratooning (Cano-Reinoso et al., 2021).

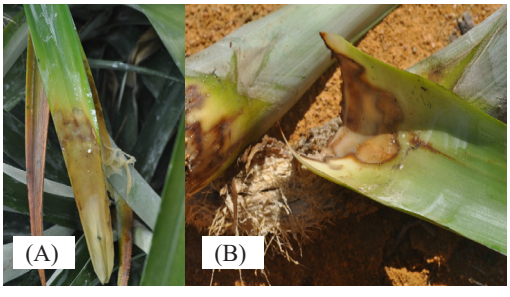

Figure 2. Signs and symptoms of bacterial heart rot (BHR) disease appear as (A) water-soaked, rotten tissue with a bad odor and (B) the leaves effortlessly detaching from the plant

BHR may reduce crop yields and quality by reducing fruit size, premature fruit drop, and reduced sugar content, resulting in economic losses for growers. In the meantime, bacterial heart rot has been reported in Malaysia, Costa Rica, Brazil, the Philippines, and Hawaii (Cano-Reinoso et al., 2021). According to Young et al. (2022), BHR disease can cause yield losses of up to 40%, which were quite significant in Malaysia in 1979. The pineapple is one of the fruits chosen for export diversification and sustainable family income growth in Uganda's fruit subsector. If not properly controlled, BHR disease can result in a 100% yield loss in Uganda (Oculi et al., 2020).

The BHR disease field study revealed an aggregated pattern of disease dissemination. The initial inoculum source site was found to be the main driver of disease onset and

spread. Pineapple growers have used a variety of techniques to combat the disease, including disease monitoring, early detection, removal, and destruction of infected plants (Nor et al., 2019). Nevertheless, when disease symptoms appear, the growers tend to treat the planting materials with chemical pesticides such as benomyl (50% a.i.) and malathion (57% a.i.), which could lead to several environmental issues (Sidik & Sapak, 2021). Therefore, the disease must be controlled using alternative techniques that reduce heavy dependence on synthetic chemical pesticides. The biological control method is one of the alternative methods being investigated for controlling BHR. The potential for biological control agents (BCAs) derived from beneficial microbes isolated from healthy plants to effectively combat the disease is enormous (Sidik & Sapak, 2021). According to Sidik and Sapak (2021), *Bacillus cereus*, an isolate from asymptomatic MD2 pineapple leaves, inhibited the growth of the BHR pathogen.

Chrystal Honey, Maspine, and Sarawak types were recommended based on their BHR tolerance. However, regular disease surveillance is crucial for preventing infection for producers who plant other varieties. This discovery is crucial, particularly for plant breeders who will use it to develop new varieties, identify potential varieties, and search for resistance genes to increase varietal diversity in the future (Nor et al., 2019).

FRUITLET CORE ROT (FCR) DISEASE

FCR is one of the most common postharvest diseases of pineapples. FCR causes damage to pineapple depending on the variety. FCR is a major concern for the "Queen" cultivar but is virtually non-existent in the "MD2" cultivar. Notably, numerous pre- and postharvest diseases could also affect the MD2 cultivar. *Fusarium ananatum* and *Thalaromyces stolii* (previously known as *Penicillium funiculosum*) are the two fungal pathogens that typically cause this aggressive disease (Barral et al., 2019). For effective management, FCR disease should be managed during early postharvest stages. Typically, the appearance of the formed rot lesion can be used to distinguish between *Talaromyces* and *Fusarium* infection. *Talaromyces* typically causes dark to medium brown rot lesions with a moist, grey region in the middle. In comparison, *Fusarium* infection causes rot lesions that range in color from light to dark brown and affect the fruitlet core (Figure 3). *Fusarium* rot spots are also typical of the dry rot variety (Zakaria, 2023). According to Sapak et al. (2021), there is no proof that *F. ananatum* causes fruit rot in Malaysia. However, the authors discovered several *Fusarium* species connected to fruit rot in pineapple, including *Fusarium proliferatum*, *Fusarium sacchari*, *Fusarium verticillioides*, and *Fusarium* spp. *Fusarium proliferatum* is the most common species discovered in the infected tissues of fruit rot. In relation to the information on wind direction and the location of these crops, FCR may be caused by similar causal

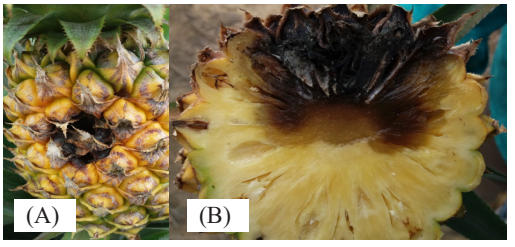

Figure 3. Fruitlet core rot (FCR) disease symptoms demonstrate (A) affected fruitlet with brown to dark brown and sunken external symptoms as the fruit ripens; (B) internal symptoms of a dark browning fruitlet from the edge to the core

pathogens as the adjacent cultivated crops (Vignassa et al., 2021).

The pathogen enters the plant through the nectary ducts and stylar canals during flowering. Once the fungus enters the floral chamber, it does not reactivate throughout fruit development. There are physiological changes after a pathogen attack, and the pineapple stimulates the phenylpropanoid pathway. Following *F. ananatum* infection, there is a buildup of free coumaroyl isocitrate and caffeoyl isocitrate in fruitless pineapples. These hydroxycinnamic acids (HCA) are involved in lignin biosynthesis and are essential in plant-pathogen interactions due to their antifungal properties (Chillet et al., 2020). The fungus is dormant during fruit development; once the fruit matures, it spreads out (Barral et al., 2019). These situations cause browning of the internal tissues (flesh), which only affects the fruitlet and has no effect on the core or exterior of the pineapple fruit. These symptoms, known as "black spots," are normal indicators of fruitlessness with the FCR infection. However, due to the lack of external symptoms, the disease incidence could not be properly assessed during or after pineapple cultivation. The complexity of the FCR pathosystem is dependent on the coexistence of healthy and diseased fruitlets within a single pineapple fruit, indicating that a particular microbiota may be responsible for pathogenesis (Vignassa et al., 2021). Because of these internal damages, determining the FCR is difficult for producers and consumers. The etiology of FCR is complicated because of the climate, fungi variety, and pineapples' physiological makeup (Barral et al., 2019).

The postharvest quality of pineapples, as well as local and international markets, are impacted when FCR occurs in tropical and subtropical regions. Brazil, Japan, China, Reunion Island, India, Hawaii, and Malaysia are the top pineapple-producing where fruitlet core rot has been reported. On Reunion Island, however, thorough investigations into the disease and its causing microbes have been carried out (Chillet et al., 2020; Vignassa et al., 2021). Disease incidence and severity have increased in the producing areas, resulting in a significant decline in fruit quality and consequent economic concerns (Vignassa et al., 2021). The output of this crop on Reunion Island is declining due to several diseases, including FCR, a postharvest disease that appears in mature pineapple. Because there are no visible external symptoms during harvest, FCR disease significantly impacts domestic and international markets (Chillet et al., 2020). In South Africa, FCR-related losses are far more severe than those caused by any other postharvest disease (Barral et al., 2019).

As a result of the economic effects of FCR, researchers and pineapple farmers started looking into ways to control FCR. Petty et al. (2005) noticed a significant decrease in black spots per fruit after spraying a mixture of two fungicides at flower induction. The application of miticide increased the frequency of black spots, contrary to expectations, in another program designed to control the mite vector (Barral et al., 2019). According to Chillet et al. (2020), essential oils and other naturally occurring antifungal substances have the potential to be an alternative control method for postharvest infections. The physiological mechanisms of fruit resistance can be stimulated by essential oils, which also have antimicrobial qualities. New alternative approaches to disease management, including the use of plant extracts with medicinal properties, have been proposed.

FUSARIOSIS DISEASE

Fusariosis, a serious disease that also affects pineapples, primarily infects the fruit but also affects all other parts of the plant. *Fusarium guttiforme*, which causes fusariosis, is the most serious fungus that affects pineapples. According to Zakaria (2023), Brazil was the world's top producer of pineapples until the discovery of fusariosis caused a serious infection. Fusariosis was later discovered in other pineapple-producing countries such as Cuba, South Africa, India, and Malaysia. Fusariosis infection typically begins in the early stages of flowering and continues through all phases of fruit growth. The first signs of fusariosis manifest in the fruitlets because the disease's pathogens penetrate the inflorescence through wounds. The lesion becomes brown discoloration and rotten fruit skin, a common disease symptom, discolored flesh (Figure 4). Contaminated planting materials could also transmit the disease. Finally, secondary infections that form on growing suckers and slips may result from an infection of the developing fruit (Zakaria, 2023).

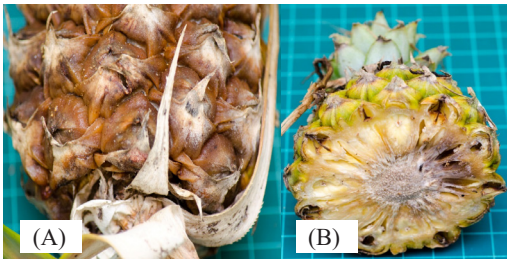

Figure 4. Fusariosis disease symptoms show (A) external brown discoloration and rotten fruit skin; (B) internal symptoms depicting discolored flesh, pinkish to brownish lesion

Zakaria (2023) also mentioned that although the symptoms of fruitlet core rot and fusariosis are identical, there are slight distinctions between the two diseases. Fruitlet core rot, which is mild, results in dry-type rotting. Contrarily, the plant-rot phase frequently connected to fruitlet core rot is absent from pineapple fusariosis.

Pathogenicity studies with susceptible pineapple varieties and morphological features have been used to identify *F. guttiforme*. The ability to identify *Fusarium* by microscopy is time-consuming and needs highly skilled personnel. Furthermore, identifying the fungus in plant cells, which

may contain other *Fusarium* species, can be challenging. The *Fusarium fujikuroi* species complex (FFSC), which includes *F. guttiforme*, has a controversial taxonomy based on the genus' conventional categorization system (Carnielli-Queiroz et al., 2019). Developing novel methods for quick, accurate, and sensitive identification of this fungus is critical. In comparison to other molecular diagnostic methods and even conventional pathogen isolation techniques from infected plant tissues, real-time polymerase chain reaction represents a low-cost, high sensitivity, and high-specificity method for the diagnosis of pineapple fusariosis caused by *F. guttiforme* (Carnielli-Queiroz et al., 2019). *Fusarium semitectum* and *F. fujikuroi* were found in Malaysian pineapple plants that exhibited symptoms of fusariosis. Both species exhibited symptoms through fruit and leaf pathogenicity testing that matched those seen in the field. Unsurprisingly, both *F. semitectum* (syn. *Fusarium incarnatum*) and *F. fujikuroi* were involved in pineapple fusariosis because they are common species throughout the tropics and can infect different plant hosts. Although *F. guttiforme* and *F. ananatum* are the main species linked to pineapple fusariosis, other *Fusarium* species could also be responsible for the disease (Zakaria, 2023).

Mycotoxigenic *Fusarium* species may generate mycotoxins on pineapple plants during critical growth stages in the field, which accumulate in colonized pineapple tissues. Mycotoxin production on infected plants in the field may begin during preharvest, continue through postharvest, and begin during storage. As a result, mycotoxin contamination began in the field and continued after collection. This condition may also cause severe problems for pineapple plants when fresh pineapple fruits are consumed (Ibrahim et al., 2020). *Fusarium* species that infect cereal grains are the primary source of these mycotoxins, but mycotoxigenic species can also negatively impact tropical fruit yields; losses of up to 50% have been recorded, including pineapple (Zakaria, 2023).

Fusariosis of the pineapple is a serious fungal disease that affects both the plant and the fruit economically. There are significant phytosanitary issues with this crop, which result in financial losses and restrict pineapple exports. It is estimated that 30–40% of the fruit and up to 20% of the vegetative propagation material were lost. Fusariosis is a significant limiting factor for pineapple output in Brazil due to phytosanitary restrictions by countries in Central America, Africa, and Asia. These countries are presently free of *F. guttiforme*, but they must abide by phytosanitary laws and regulations that can be informed by quick diagnostics to streamline logistics for quarantine, prevent the introduction of the pathogen, and reduce potential economic losses (Carnielli-Queiroz et al., 2019).

Most pineapple farmers have implemented sanitation measures to reduce disease incidence on farms and treated planting materials with fungicides like benomyl or captafol. Hot water treatment at 54°C for 90 min is also recommended

to reduce disease spread on planting materials, as discussed by Sapak et al. (2021). Goncalves et al. (2021) found that using garlic, neem, and ginger extracts to treat fusariosis disease in a pineapple in the state of Tocantins was ineffective, necessitating additional research for new doses and/or collaboration with chemical products intended for this purpose. In many pineapple plantations, chemical fungicides are still the primary means of disease management. For instance, various concentrations of fungicide combinations, such as azoxystrobin, cyproconazole, carboxy, thiram, tebuconazole, and methyl thiophanate, have been tried to control fusariosis in a pineapple field in Brazil (Nogueira) (Sapak et al., 2021).

According to Soler (2019), pineapple cropping methods such as mycorrhizal fungi to increase nutrient availability from the soil, bacteria for direct action on pathogens with toxins or chitinolytic enzymes, nitrogen-fixing bacteria to reduce nitrogen application, fast multiplication of disease-free planting material through *in vitro* propagation, and non-pathogenic fungi inoculation are currently integrated by utilizing biotechnologies (*Trichoderma* spp.). This presentation demonstrates the possible integration of a few of these biotechnologies and their influence on developing ecologically friendly pineapple cropping systems.

DISEASE CAUSED BY NEMATODE

Plant parasitic nematodes (PPNs) are one of the most important crop issues affecting crop production worldwide, and pineapple is one of the most economically significant crops infected by these plant pathogens. Approximately 10% of all nematode species are plant parasites, making nematodes the most diverse group of multicellular organisms on Earth. Plant parasitic nematodes are tiny organisms that reside underground (Benzonan et al., 2021). Despite reports of PPNs infecting pineapple, little research has been conducted on the fruit. Numerous species of parasitic nematodes, including root-knot (*Meloidogyne javanica* and *Meloidogyne incognita*), lesion (*Pratylenchus brachyurus*), and reniform (*Rotylenchulus reniformis*), have been observed in pineapple plants (Rabie, 2017).

In commercial pineapple plantations in Malaysia, there have been reports of the high prevalence of *Paratylenchus* sp. and a low population of *Aphelenchoides* sp. and *Pratylenchus* sp. The decrease in pineapple yield on peat soil was most likely caused by the nematode *Paratylenchus* sp. This nematode was discovered throughout the crop's development stages, with the oldest plants exhibiting the greatest population, particularly in peat soil with high acidity levels (Masdek et al., 2007). Based on a study by Tanimola et al. (2021), *Helicotylenchus* was the most prevalent nematode pest in the Obio-Akpor Local Government Area (LGA) of the five nematode pest genera discovered in both soil and pineapple roots, *Scutellonema* and *Paratylenchus* had the lowest percentage of any nematode taxa. However, the most significant nematode parasite connected to pineapple in Nigeria

was the *Pratylenchus* species, with a relative importance value (RIV) of 33.8%.

In pineapple, PPNs typically feed by entering the soil through the roots. Most infections include lesions in the vascular tissues and piths that the plant relies on to transport water and nutrients. When root and vascular tissues are damaged, chlorosis on the foliage begins (Benzonan et al., 2021). As the infection progresses, the leaves will promote necrosis, and the roots will easily be removed from the infected soil. These infections can potentially spread to nearby plants and permanently harm the soil, resulting in a yearly loss of production (Benzonan et al., 2021). Due to the nature and symptoms of the disease by these nematodes, their destructive potential is often underestimated and confused with that of other plant pathogens like fungi and bacteria. Most of the time, growers lack the knowledge and awareness to address their crops (Tanimola et al., 2021).

Each year, the Philippines loses an average of 10-15% of crop yield, amounting to an estimated $78 billion in crop losses globally. Today, many farmers, particularly those in developing countries, are unaware of plant parasitic nematodes (Benzonan et al., 2021). As of 2019, Nigeria is the eighth-largest pineapple producer globally and the top producer in Africa. Pineapple production contributes significantly to household nutrition and health and improves the quality of rural life for most farmers in Nigeria. PPNs, on the other hand, have been linked to substantial yield losses in pineapple, making them one of the banes of Nigeria's pineapple industry (Tanimola et al., 2021). According to some reports, PPNs can decrease yield in Brazil by 60% for the plant crop and 40% for the second harvest (Norwegian Institute of Bioeconomy Research, 2021). The pineapple suffers significant yield losses in France due to an increasing infestation of the nematode *R. reniformis* and other "soil-borne pathogens" (Soler et al., 2021).

In their recent review article, Sapak et al. (2021) stated that cultural practices, nematicides, biological control, and the integration of all available control methods are some of the methods used by pineapple producers to handle nematode attacks. The most effective way to reduce the likelihood of subsequent sowing is to remove infected plants from the fields. According to Soler et al. (2021), the most popular short-term treatment method has remained using nematicides. Following decades of intensive monoculture, in which the natural balance between beneficial and harmful communities of soil organisms has vanished, French authorities recently prohibited using pesticides to manage "soil-borne pathogens" on pineapple. Increased infestation of the nematode *R. reniformis* and other "soil-borne pathogens" causes significant crop damage in pineapple. New cropping systems with creative ecological nematode control are needed. First, a sustainable cropping system based on pineapple and sunn hemp (*Crotalaria juncea*) rotation reduces the initial biotic stress on pineapple plants (inoculum of nematodes) (Soler et al., 2021). In addition, systemic resistance could be induced by using a chemical elicitor or

pineapple endophytic bacteria to sustain low nematode populations in pineapple plants. Thus, integrated disease management could be a solution for curative and preventive measures.

CONCLUSION

This review on the predominant and destructive pineapple diseases (MWP, BHR, FCR, fusariosis, and parasitic nematode damages) affecting pineapple crops in producing countries highlights the critical importance of disease management strategies. The impact of yield losses caused by these prevalent and economically imperative pineapple diseases may vary from one producing country to another, depending on the agronomic practices, environmental factors, and farming systems that influence the pathogen populations. Strict quarantine measures are required to reduce the impact of the disease in pineapple on the international fruit trade, which affects the socioeconomic situation of some producing countries. Overall, expanding the knowledge of the diseases that affect pineapple plants will improve the effectiveness of management measures relating to causal pathogens. Since pre- and postharvest phases may alter a crop's susceptibility to pathogens, identifying pathogenic pineapple disease provides a foundation for correct handling during preharvest and postharvest storage.

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REFERENCES

Aeny, T. N., Suharjo, R., Ginting, C., Hapsoro, D., & Niswati, A. (2020). Characterization and host range assessment of *Dickeya zeae* associated with pineapple soft rot disease in east Lampung, Indonesia. *Biodiversitas*, *21*(2), 587–595. https://doi.org/10.13057/biodiv/d210221

Ajayi, A. M., Coker, A. I., Oyebanjo, O. T., Adebanjo, I. M., & Ademowo, O. G. (2022). *Ananas comosus* (L) Merrill (pineapple) fruit peel extract demonstrates antimalarial, anti-nociceptive and anti-inflammatory activities in experimental models. *Journal of Ethnopharmacology*, *282*, 114576. https://doi.org/10.1016/J.JEP.2021.114576

Alejo Jeronimo, M., Manuel Arevalo de la Cruz, E., Brito-Vega, H., Gomez-Vazquez, A., Manuel Salaya-Dominguez, J., & Gomez-Mendez, E. (2023). The production and marketing issues of pineapple (*Ananas comosus*) under humid tropical conditions in the state of Tabasco and Way-out. In M. S. Khan (Ed.), *Tropical plant species and technological interventions for improvement*. IntechOpen. https://doi.org/10.5772/intechopen.106499

Araya, M. (2019). Chemical control of mealybugs on pineapples. *Acta Horticulturae*, *1239*, 147–152. https://doi.org/10.17660/ActaHortic.2019.1239.18

Asare-Bediako, E., Nyarko, J., & van der Puije, G. C. (2020). First report of *Pineapple mealybug wilt associated virus-2* infecting pineapple in Ghana. *New Disease Reports*, *41*(1), 9. https://doi.org/10.5197/j.2044-0588.2020.041.009

Barral, B., Chillet, M., Léchaudel, M., Lartaud, M., Verdeil, J. L., Conéjéro, G., & Schorr-Galindo, S. (2019). An imaging approach to identify mechanisms of resistance to pineapple fruitlet core rot. *Frontiers in Plant Science*, *10*, 1065. https://doi.org/10.3389/fpls.2019.01065

Benzonan, N. C., Dalisay, L. C. S., Reponte, K. C. C., Mapanao, C. P., Alvarez, L. V, Rendon, A. O., & Zurbano, L. Y. (2021). Plant-parasitic nematodes associated with pineapple (*Ananas comosus*) in selected provinces in Luzon, Philippines. *European Journal of Molecular and Clinical Medicine*, *8*(2), 945–957.

Cano-Reinoso, D. M., Soesanto, L., Kharisun., & Wibowo, C. (2021). Fruit collapse and heart rot disease in pineapple: Pathogen characterization, ultrastructure infections of plant and cell mechanism resistance. *Biodiversitas*, *22*(5), 2477–2488. https://doi.org/10.13057/biodiv/d220504

Carnielli-Queiroz, L., Fernandes, P. M. B., Fernandes, A. A. R., & Ventura, J. A. (2019). A rapid and reliable method for molecular detection of *Fusarium guttiforme*, the etiological agent of pineapple fusariosis. *Brazilian Archives of Biology and Technology*, *62*, e19180591. https://doi.org/10.1590/1678-4324-2019180591

Chaudhary, V., Kumar, V., Sunil., Vaishali., Singh, K., Kumar, R., & Kumar, V. (2019). Pineapple (*Ananas cosmosus*) product processing: A review. *Journal of Pharmacognosy and Phytochemistry*, *8*(3), 4645–4645.

Chillet, M., Hoareau, A., Hoarau, M., & Minier, J. (2020). Potential use of essentials oils to control fruitlet core rot (FCR) in pineapple (Queen Victoria variety) in Reunion Island. *American Journal of Plant Sciences*, *11*, 1671–1681. https://doi.org/10.4236/ajps.2020.1111119

Dey, K. K., Green, J. C., Melzer, M., Borth, W., & Hu, J. S. (2018). Mealybug wilt of pineapple and associated viruses. *Horticulturae*, *4*(4), 52. https://doi.org/10.3390/horticulturae4040052

Food and Agriculture Organization of the United Nations. (2021). Major tropical fruits: Market review 2020. FAO.

Goncalves, M. V., Ferreira, L. L., Pereira, A. I. A., & Curvelo, C. R. D. S. (2021). Management of *Fusarium subglutinans* in pineapple using garlic, ginger and denim extract. *Revista Brasileira de Plantas Medicinais*, *23*(1), 12–19.

Green, J. C., Rwahnih, M. A., Olmedo-Velarde, A., Melzer, M. J., Hamim, I., Borth, W. B., Brower, T. M., Wall, M., & Hu, J. S. (2020). Further genomic characterization of pineapple mealybug wilt-associated viruses using high-throughput sequencing. *Tropical Plant Pathology*, *45*, 64-72. https://doi.org/10.1007/s40858-019-00330-y

Gungoosingh-Bunwaree, A., Maudarbaccus, F., Knierim, D., Margaria, P., Winter, S., & Menzel, W. (2021). First report of *Pineapple mealybug wilt-associated virus-1* and *-2* associated with mealybug wilt disease of pineapple in Mauritius. *New Disease Reports*, *44*(1), e12037. https://doi.org/10.1002/ndr2.12037

Habotta, O. A., Dawood, M. A. O., Kari, Z. A., Tapingkae, W., & Doan, H. V. (2022). Antioxidative and immunostimulant potential of fruit derived biomolecules in aquaculture. *Fish and Shellfish Immunology*, *130*, 317–322. https://doi.org/10.1016/j.fsi.2022.09.029

Hernández-Rodrıguez, L., Ramos-González, P. L., Sistachs-Vega, V., Zamora-Rodrıguez, V., Batista-Le Riverend, L., Ramos-Leal, M., Peña-Bárzaga, I., & Llanes-Alvarez, Y. (2019). The viral complex associated with mealybug wilt disease of pineapple in Cuba. *Acta Horticulturae*, *1239*, 203–212. https://doi.org/10.17660/ActaHortic.2019.1239.25

Hutahayan, A. J., Tantawi, A. R., Tobing, M. C., & Lisnawita. (2021). *Pineapple mealybug wilt-associated virus* (PMWaV) on Sipahutar pineapple, in North Tapanuli, Indonesia. In *IOP Conference Series: Earth and Environmental*

Science (Vol. 782, No. 4, p. 042062). IOP Publishing. https://doi.org/10.1088/1755-1315/782/4/042062

Hutahayan, A. J., Tantawi, A. R., Tobing, M. C., & Lisnawita. (2022). Survey and distribution of *Pineapple Wilt Mealybug Wilt-associated Virus* (PMWaV) on pineapple plants in North Tapanuli, Indonesia. In *IOP Conference Series: Earth and Environmental Science* (Vol. 977, No. 1, p. 012037). IOP Publishing. https://doi.org/10.1088/1755-1315/977/1/012037

Ibrahim, N. F., Mohd, M. H., Nor, N. M. I. M., & Zakaria, L. (2020). Mycotoxigenic potential of *Fusarium* species associated with pineapple diseases. *Archives of Phytopathology and Plant Protection*, *53*(5–6), 217–229. https://doi.org/10.1080/03235408.2020.1736971

Larrea-Sarmiento, A., Olmedo-Velarde, A., Wang, X., Borth, W., Matsumoto, T. K., Suzuki, J. Y., Wall, M. M., Melzer, M., & Hu, J. (2021). A novel ampelovirus associated with mealybug wilt of pineapple (*Ananas comosus*). *Virus Genes*, *57*, 464–468. https://doi.org/10.1007/s11262-021-01852-x

Malaysia's pineapple export shows an upward trend from 2016-2020. (2022, April). *Bernama*. https://www.bernama.com/en/business/news.php?id=2069936

Masdek, H. N., Ismail, A. B., Zulkifli, M., & Malip, M. (2007). *Paratylenchus* sp. associated with pineapple yield decline. *Journal of Tropical Agriculture and Food Science*, *35*(1), 191-199.

Moreno, I., Rodríguez-Arévalo, K. A., Tarazona-Velásquez, R., & Kondo, T. (2023). Occurrence and distribution of pineapple mealybug wilt-associated viruses (PMWaVs) in MD2 pineapple fields in the Valle del Cauca Department, Colombia. *Tropical Plant Pathology*, *48*, 217–225. https://doi.org/10.1007/s40858-023-00559-8

Muhamad, M. Z., Shamsudin, M. N., Kamarulzaman, N. H., Nawi, N. M., & Laham, J. (2022). Investigating yield variability and technical efficiency of smallholders pineapple production in Johor. *Sustainability*, *14*(22), 15410. https://doi.org/10.3390/su142215410

Nor, A. A. M., Zainol, R., Abdullah, R., Jaffar, N. S., Rasid, M. Z. A., Laboh, R., Shafawi, N. A., & Aziz, N. B. A. (2019). Dissemination pattern of bacterial heart rot (BHR) disease and screening of the disease resistance among commercial pineapple varieties in Malaysia. *Malaysian Journal of Microbiology*, *15*(4), 346–350. https://doi.org/10.21161/mjm.1915412

Norwegian Institute of Bioeconomy Research. (2021, May 10). Plant parasitic nematodes harm pineapple crop yields in Kenya. *Phys.org*. https://phys.org/news/2021-05-parasitic-nematodes-pineapple-crop-yields.html

Oculi, J., Bua, B., & Ocwa, A. (2020). Reactions of pineapple cultivars to pineapple heart rot disease in central Uganda. *Crop Protection*, *135*, 105213. https://doi.org/10.1016/j.cropro.2020.105213

Pérez-Rodríguez, J., Pekas, A., Tena, A., & Wäckers, F. L. (2021). Sugar provisioning for ants enhances biological control of mealybugs in citrus. *Biological Control*, *157*, 104573. https://doi.org/10.1016/j.biocontrol.2021.104573

Petty, G. J., Tustin, H. A., & Dicks, H. M. (2005). Control of black spot disease fruitlet core rot in queen pineapple with integrated mealybug, pineapple fruit mite and fungus control programmes. *Acta Horticulturae*, *702*, 143-149. https://doi.org/10.17660/ActaHortic.2006.702.17

Rabie, E. C. (2017). Nematode pests of pineapple. In H. Fourie, V. Spaull, R. Jones, M. Daneel, & D. De Waele (Eds.), *Nematology in South Africa: A view from the 21st century* (pp. 395-407). Springer. https://doi.org/10.1007/978-3-319-44210-5_18

Sapak, Z., Mohd Faisol Mahadeven, A. N., Nurul Farhana, M. H., Norsahira, S., & Mohd Zafri, A. W. (2021). A review of common diseases of pineapple: The causal pathogens, disease symptoms, and available control measures. *Food Research*, *5*(S4), 1–14. https://doi.org/10.26656/fr.2017.5(s4).004

Sidik, S., & Sapak, Z. (2021). Evaluation of selected chemical pesticides for controlling bacterial heart rot disease in pineapples variety MD2. In *IOP Conference Series: Earth and Environmental Science* (Vol. 757, No. 1, p. 012072). IOP Publishing. https://doi.org/10.1088/1755-1315/757/1/012072

Soler, A. (2019). Pineapple cultivation under agro-ecological management with biotechnology approaches. *Acta Horticulturae*, *1239*, 65–75. https://doi.org/10.17660/ActaHortic.2019.1239.8

Soler, A., Marie-Alphonsine, P.-A., Quénéhervé, P., Prin, Y., Sanguin, H., Tisseyre, P., Daumur, R., Pochat, C., Dorey, E., Gonzalez Rodriguez, R., Portal, N., & Smith-Ravin, J. (2021). Field management of *Rotylenchulus reniformis* on pineapple combining crop rotation, chemical-mediated induced resistance and endophytic bacterial inoculation. *Crop Protection*, *141*, 105446. https://doi.org/10.1016/j.cropro.2020.105446

Sukri, S. A. M., Andu, Y., Sarijan, S., Khalid, H.-N. M., Kari, Z. A., Harun, H. C., Rusli, N. D., Mat, K., Khalif, R. I. A. R., Wei, L. S., Rahman, M. M., Hakim, A. H., Lokman, N. H. N., Hamid, N. K. A., Khoo, M. I., & Doan, H. V. (2023). Pineapple waste in animal feed: A review of nutritional potential, impact and prospects. *Annals of Animal Science*, *23*(2), 339-352. https://doi.org/10.2478/aoas-2022-0080

Tanimola, A. A., Olotu, O., & Asimiea, A. O. (2021). Occurrence, diversity and abundance of nematode pests of pineapple (*Ananas comosus*) in two local government areas of Rivers State, Nigeria. *Journal of Applied Sciences and Environmental Management*, *25*(4), 665-675. https://doi.org/10.4314/jasem.v25i4.29

Veléz-Negrón, Y. I., Simbaña-Carrera, L. L., Soto-Ramos, C. M., Medina, O. H., Dinkel, E., Hardy, C., Rivera-Vargas, L. I., & Ramos-Sepúlved, L. (2023). First report of bacterial pineapple heart rot caused by *Dickeya zeae* in Puerto Rico. *Plant Disease*, *107*(1), 210. https://doi.org/10.1094/pdis-01-22-0174-pdn

Vignassa, M., Meile, J. C., Chiroleu, F., Soria, C., Leneveu-Jenvrin, C., Schorr-Galindo, S., & Chillet, M. (2021). Pineapple mycobiome related to fruitlet core rot occurrence and the influence of fungal species dispersion patterns. *Journal of Fungi*, *7*(3), 175. https://doi.org/10.3390/jof7030175

Young, A. J., Pathania, N., Manners, A., & Pegg, K. G. (2022). Heart rot of Australian pineapples caused by *Dickeya zeae*. *Australasian Plant Pathology*, *51*, 525–533. https://doi.org/10.1007/s13313-022-00880-x

Zakaria, L. (2023). Fusarium species associated with diseases of major tropical fruit crops. *Horticulturae*, *9*(3), 322. https://doi.org/10.3390/horticulturae9030322

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Effect of Leaf Pruning and Additional Fertilizer on Growth and Young Pods Yield of Winged Beans

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ABSTRACT

Winged bean (*Psophocarpus tetragonolobus* L.) exhibits luxuriant foliage, making leaf pruning essential to enhance sunlight interception. Additionally, supplementing with additional fertilizer helps offset the impact of gradual harvesting. Therefore, this research aimed to determine the effect of leaf pruning and additional fertilizer on the growth and yield parameter of winged beans in the Institut Pertanian Bogor (IPB) experimental station at Leuwikopo, IPB University, Bogor, Indonesia. A randomized complete block design was used with two factors and three replications, namely leaf pruning intensities (0, 15, and 30% leaf pruning) and rates of additional fertilizer (0, 6.25, 12.5, and 18.5 g NPK 16-16-16/plant). The observed variables were plant height, leaf number, root length, leaf nutrient, auxin content, nutrient uptake, and young pods yield. The findings revealed that the interaction of pruning intensities and additional fertilizer rates significantly influenced leaf number and root length. Specifically, plants receiving a treatment combination without pruning and 6.25 g of additional fertilizer/plant exhibited the highest leaf number. In contrast, those subjected to 15% leaf pruning showed the greatest root length. Leaf nutrient levels, auxin content, and nutrient uptake exhibited noticeable improved with the addition of fertilizer. Meanwhile, a higher phosphorus and organic carbon content was observed in the 15% pruning treatment, and the young pods yield were not affected because the Fairuz variety reached its potential yield. Based on the results, the most recommended treatment is a combination without leaf pruning, supplemented with 6.25 g of additional fertilizer/plant.

Keywords: Leaf nutrient, legumes, root length, phosphorus, vegetables

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INTRODUCTION

Psophocarpus tetragonolobus L., winged beans, are legume species frequently consumed as a vegetable (Calvindi et al., 2020; Eagleton, 2020). The young pod, leaves, and tuber of winged beans are suitable for vegetable consumption (Bassal et al., 2020). Furthermore, 100 g of the young pod contains 1.9–4.3 g of protein, 0.1–3.4 g of fat, and 0.9–3.1 g of fiber, with 300–900 IU of vitamin A and 205–381 mg of potassium (Mohanty et al., 2020). The winged beans boast 157.6 and 107.8 mg of gallic acid equivalent (GAE) total phenolic and flavonoid compounds, respectively (Calvindi et al., 2020). In Southeast Asia, the young pod is consumed as a main dish or appetizer by eating fresh, boiled, or steamed (Mohanty et al., 2020). Winged beans can also treat diseases like diabetes and asthma, and strengthen the immune system. The species can boost the immune system by having 140–167 µmol antioxidant capacity (TE)/100 g FW (Calvindi et al., 2020). The nutritional quality and production make winged beans a potential tropical commodity candidate to be developed (Bassal et al., 2020) through the improvement of the techniques related to production systems (Anjos et al., 2021).

The winged bean plant is made of vines (Calvindi et al., 2020) and has lush leaves (Eagleton, 2020). Plants with lush leaves have high leaf humidity, which accelerates the development of pest and disease growth. Several pests and diseases pose a threat to these plants, including *Mylabris pustulata*, the black bean aphid (*Aphis craccivora*), necrotic mosaic virus, and the false rust disease caused by *Synchytrium psophocarpi*. Plants affected by leaf rust exhibit orange pustules on their leaves, pods, or stems. In the case of plants infected by *Synchytrium psophocarpi*, the size of the sporangia measures 20.69 µm, with a diameter of 2.02 µm and a length of flagella at 10.75 µm. The necrotic mosaic virus of winged beans infects approximately 9% of young plants and is responsible for causing yield losses ranging from 10 to 20% (Bassal et al., 2020).

Genetic and environmental conditions can affect a plant's growth and production (Asefa et al., 2021). Fairuz IPB variety is one of the winged beans developed by IPB University. The productivity of Fairuz IPB up to 23 weeks after transplanting (WAT) can reach 2.53 tons of dry seeds/ha (44.31 g of dry seeds/plant) and 5.06 tons of young pods/ha (88.63 g of young pods/plant). Fairuz IPB has a green-colored pod and starts at 65 days after transplanting (Laia, 2019). One limitation of this variety is its dense foliage, making it highly vulnerable to leaf rust attacks, ultimately reducing production (Susanti et al., 2022). In plants exhibiting abundant foliage, it becomes imperative to optimize sunlight absorption and humidity reduction by employing pruning techniques to trim the lush leaves (Samira et al., 2014). Pruning is needed to reduce humidity, risk of pests, disease attacks (Tsegaye & Struik, 2000), and transpiration (K.-T. Li et al., 2016), as well as strengthen growth and increase crop production (Maudu et al., 2010).

Leaf pruning can inhibit the generative phase (Susanto et al., 2013); additional fertilization may be needed to cope with this problem. One of the fundamental environmental conditions conducive to plant growth is the availability of complete nutrients, which can be supplied through fertilization (Nahed et al., 2010), primarily through inorganic fertilizers (Owolabi et al., 2016). There are two phases of fertilizer application in plants, namely basic and additional fertilizer. Basic fertilizers are given at the end of land preparation, while additional fertilizers are administered during plant growth. This additional fertilization is essential, specifically for plants with long growth phases, such as tomatoes (Maillard et al., 2015), tobacco (Iskandar et al., 2020), cauliflower (Sofian & Susila, 2018), and winged bean (Ishthifaiyyah et al., 2021).

A continuous supply of nutrients is crucial to sustain its growth, fruit quality, and productivity (Kueklang et al., 2021). The winged bean plant can be harvested more than seven times in six months (Ishthifaiyyah et al., 2021); sufficient nutrients are needed for its development and growth. Essential macronutrients for plant growth and development, such as nitrogen, phosphorus, and potassium, can be supplied by compound fertilizers (Prajapati & Modi, 2018). Applying these nutrients increases soil nutrient levels, fostering enhanced plant growth and productivity (Owolabi et al., 2016). Additionally, it can positively impact various aspects, such as sucrose content or carbon-nitrogen ratio (Kamhun et al., 2022), morphological and physiological traits of crops (Mahmoodi et al., 2020), and overall yield quality (Cano-Reinoso et al., 2022). Compound fertilizers are inorganic fertilizers that can increase plant growth and are more efficient than single fertilizers (Betty et al., 2021).

Leaf pruning serves the purpose of mitigating plant transpiration and curtailing the proliferation of pests and diseases. However, it impedes generative growth, necessitating the application of additional fertilization to expedite the generative phase. The basic fertilizer recommendations for winged bean plants are 50 kg urea/ha, 90 kg SP-36/ha, and 150 kg MOP/ha (Laia, 2019). Combining leaf pruning and additional fertilization will increase young-winged beans' growth and production. Therefore, this research was conducted to determine the effect of leaf pruning intensities and additional fertilizer rates on winged beans' growth and yield parameters.

MATERIALS AND METHODS

Field Condition and Materials

The experiment was carried out on Latosol soil at IPB Leuwikopo experimental station (latitude 6° 33' 45.2" S, longitude 106° 43' 11.7" E), IPB University, Bogor, West Java, Indonesia from June to December 2021. Fairuz IPB variety was used as plant material. Compound fertilizers (NPK 16-16-16, NPK Mutiara, Indonesia), dolomite, chicken manure, and bamboo stalks were used as nutrients, ameliorants, and plant support sources as used as the material of this study.

Climate Data

The recorded data for the given period indicates an average temperature between 25 and 26°C, with minimum and maximum values of 21 and 32°C, as shown in Table 1. Additionally, the average relative humidity fluctuated between 77 and 86%, and the sun intensity measurements show values varying between 383 and 553 Cal/cm^2.

Table 1
Climate data in Darmaga station in Bogor, Indonesia

Month	Temperature (°C)			Rainfall (mm)		Rainy days	8 hours of sunshine (%)		Relative humidity (%)	Light intensity (Cal/cm^2)
	Average	Max	Min	Average	Total		Average	Total		
June	25.8	31.7	22.0	12.4	311.1	18.0	47.6	1429.1	86.2	383.1
July	26.0	32.1	21.3	11.5	115.6	3.0	76.5	2371.8	79.7	459.9
August	25.9	32.0	21.7	13.3	399.5	16.0	74.0	2294.5	81.8	459.7
September	26.3	32.3	22.0	10.5	317.3	18.0	75.1	2254.0	81.0	520.9
October	26.4	32.6	22.1	18.2	566.5	18.0	71.0	2202.0	77.1	553.7
November	26.4	31.6	22.7	6.1	183.6	17.0	42.4	1359.0	83.7	381.8
December	26.1	31.5	22.2	9.0	279.1	20.0	45.3	1316.0	85.2	399.7

Experimental Design

The research was elaborated using a randomized complete block design (RCBD) with two factors and three replications. The first factor was leaf pruning intensities (0, 15, and 30%), while the second was rates of additional fertilizer (0, 6.25, 12.5, and 18.5 g NPK fertilizer (16-16-16) per plant applied five times (7, 9, 11, 13, and 15 WAT). This fertilizer was administered by pouring 250 ml of the solution of NPK fertilizer (16-16-16) plants at 7, 9, 11, 13, and 15 WAT, and leaf pruning was carried out in 11 WAT, as shown in Table 2.

Table 2
The treatment details employed in the experiment

Factor	Description
Factor 1 (Pruning)	1. 0% leaf pruning
	2. 15% leaf pruning (at 11 WAT)
	3. 30% leaf pruning (at 11 WAT)
Factor 2 (Additional fertilizer)	1. 0 g/plant
	2. 6.25 g/plant (from 7, 9, 11, 13, and 15 WAT)
	3. 12.5 g/plant (from 7, 9, 11, 13, and 15 WAT)
	4. 18.75 g/plant (from 7, 9, 11, 13, and 15 WAT)

WAT = Weeks after transplanting

Land Preparation, Planting, and Harvest

After land preparation, each plot size was 1 m x 5.7 m, and the land was applied with 2 t lime/ha and 10 t chicken manure/ha as an ameliorant three weeks before planting. All plots were added with 50 kg urea/ha (PT Pupuk Indonesia, Indonesia), 90 kg SP-36/ha (Petrokimia Gresik, Indonesia), and 150 kg KCl/ha (MerokeMOP®, Indonesia) (Laia, 2019) at 2 weeks before planting. The winged bean seeds were soaked in warm water overnight to ease seed germination. Furthermore, they were planted in a seedling tray for two weeks, then transplanted in an experimental plot with two plants/hole and (a 50 cm x 30 cm planting distance). The winged beans were harvested eight days after anthesis when Fairuz IPB was considered to achieve its optimal physicochemical characteristics for future consumption (Susanti et al., 2022). The harvesting time was carried out every week until 24 WAT.

Assessments and Measures

Measurement of Plant Growth Parameter. The plant growth parameter includes plant height (cm), leaf number, and root length (cm), measured at 5, 10, and 15 WAT. Each plant height sample was observed from the ground (root base) to the highest shoot. The leaf number was calculated from the number of open trifoliate leaves in one plant, while the root length was observed from the root base to the tip of the longest root in the sample plant taken. Because the leaf pruning was conducted at 11 WAT, the intensity's effect was statistically analyzed for the data of 15 WAT onwards. Meanwhile, the effect of rates of additional fertilizer was evaluated on the variables observed of plants since 5 WAT.

Measurement of Leaf Nutrient and Auxin Content in the Shoot. Fresh leaf samples were taken at 11 WAT, dried overnight in the air, and at 80°C for 48 hr. The dry samples were ground with a blender to pass a sieve with 40 mesh. The measurements on leaf tissue were organic carbon (spectrophotometry method), nitrogen (Kjeldahl method), phosphorus (spectrophotometry method), and potassium (atomic absorption spectrophotometry [AAS] method) (Bhandari, 2018). Auxin analysis on shoots was conducted by taking plant shoots after one week of pruning (12 WAT), drying them overnight in the air, and then dried with an oven at 80°C for 48 hr. The dry samples were ground to pass a sieve with 40 mesh, and CAMAG® thin layer chromatography (TLC) Visualizer 3 (Switzerland) was used to analyze auxin (Porfírio et al., 2016). The nutritional analysis of leaves and shoot auxin was conducted in the Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI) laboratory.

Measurement of Yield Parameter. Young pods were harvested eight days after anthesis (Susanti et al., 2022), and the period was 13–24 WAT. Harvesting was done by cutting the ready-to-harvest pods and measuring the weight/plot of 5.7 m^2 weekly until the plants were 24 WAT.

Statistical Analysis

The data analysis was conducted using the analysis of variance (ANOVA) method, and statistically significant differences were determined by applying Duncan's multiple range test (DMRT) at a significance level of 5%. The statistical software utilized for this analysis was Statistical Analysis System (SAS, version 9.4), and principal component analysis (PCA) was performed using the *R* software.

RESULTS AND DISCUSSION

Plant Growth

In this research, there was an interaction between leaf pruning intensities and additional fertilizer rates on plant growth variables such as leaf number and root length at 15 WAT (Tables 3 and 4). The results showed that the combination treatment without leaf pruning and 6.25 g additional fertilizer/plant was the highest leaf number but did not differ from the combination of 15% pruning and additional fertilizer (6.25 and 18.75 g), as well as 30% pruning and additional fertilizer (0, 6.25, 12.5, and 18.75 g). Furthermore, implementing a 30% leaf pruning approach led to an increase in leaf number, which increased by 20-44% compared to the control group.

Pruning aids the plant in eliminating deceased and unproductive branches, enhancing its vigor and overall growth (Dufour et al., 2019). Additionally, it fosters the development of new branches and vegetative growth (Shashi et al., 2022), leading to increased productivity compared to plants subjected to pruning (Valdes-Rodriguez et al., 2020). Pruning increases shoots and biomass in subsequent growth (Mediene et al., 2002), reduces transpiration (K.-T. Li et al., 2016), and increases the root-shot ratio (Carrillo et al., 2011). The application of additional fertilizer at a rate of 6.25 g/plant resulted in a significant increase in the number of leaves at 15 WAT (weeks after treatment) and root length at 10 WAT. Therefore, the rate of 6.25 g of fertilizer/plant effectively enhanced the leaf number.

The interaction between pruning intensities and additional fertilizer rates significantly influenced the root length at 15 WAT. The longest root length was observed in plants treated with a combination of 15% pruning and 6.25 g of additional fertilizer, and this result was not significantly different from the combination of 30% and 0 g. On the other hand, the shortest root length was recorded in plants subjected to a combination of 15% pruning and 18.75 g of additional fertilizer. The result showed that higher additional fertilizer rates treatment could decrease a root length (Table 4). In conditions of low soil nutrition, roots take longer to acquire the necessary nutrients from the soil. Roots are crucial in plant growth and absorb nutrients and water for metabolism. Roots need to spread widely in the field to enhance nutrient absorption, ensuring a well-distributed supply of nutrients to all plant parts. Therefore, a well-developed root system is vital for optimal plant health and growth (Nugroho et al., 2017).

Table 3
The effect of pruning and additional fertilizer on plant height and leaf numbers of winged bean plant

Treatment	Plant age (WAT)			Plant age (WAT)		
	5	10	15	5	10	15
	Plant height (cm)			Leaf numbers		
Pruning (P)						
0%	24.16 ± 1.37	169.58 ± 16.15	199.00 ± 8.44	10.0 ± 0.71	37.5 ± 3.18	50.3 ± 3.79
15%	26.33 ± 1.58	150.33 ± 12.98	202.75 ± 10.68	9.9 ± 0.93	35.0 ± 2.41	52.2 ± 3.19
30%	23.33 ± 1.01	180.58 ± 14.52	278.00 ± 8.01	9.5 ± 0.57	38.7 ± 4.14	58.2 ± 2.31
p-value	0.30	0.36	0.42	0.87	0.72	0.08
Additional fertilizer (F)						
0 g/plant	25.05 ± 1.62	180.89 ± 20.14	195.11 ± 13.50	10.2 ± 0.92	32.7 ± 427	49.6 ± 4.19
6.25 g/plant	25.27 ± 1.17	143.22 ± 14.70	201.78 ± 11.09	10.3 ± 1.12	33.3 ± 2.31	59.5 ± 2.85
12.5 g/plant	24.66 ± 1.98	155.00 ± 16.42	200.33 ± 8.50	9.4 ± 0.67	40.5 ± 4.93	51.6 ± 4.01
18.75 g/plant	23.44 ± 1.59	188.22 ± 13.83	309.11 ± 11.47	9.1 ± 0.47	41.7 ± 2.39	53.5 ± 3.46
p-value	0.85	0.23	0.40	0.70	0.23	0.11
P×F						
0%, 0 g	27.16 ± 3.98	108.00 ± 39.71	180.7 ± 12.19	9.00 ± 1.25	39.00 ± 11.71	43.00 ± 8.62 c
0%, 6.25 g	23.00 ± 2.46	127.33 ± 15.01	212.3 ± 25.46	11.50 ± 2.25	38.67 ± 4.66	67.00 ± 1.52 a
0%, 12.5 g	25.33 ± 2.60	154.00 ± 35.94	220.3 ± 10.91	10.33 ± 1.42	33.33 ± 6.35	43.00 ± 4.35 c
0%, 18.75 g	21.16 ± 1.58	189.00 ± 26.63	182.7 ± 5.78	9.33 ± 0.83	39.00± 3.51	48.33 ± 5.20 bc
15%, 0 g	24.33 ± 2.72	129.67 ± 26.99	216.0 ± 36.8	11.16 ± 2.45	32.33 ± 5.04	48.67 ± 6.76 bc
15%, 6.25 g	27.83 ± 1.48	162.67 ± 43.02	206.0 ± 24.17	8.83 ± 2.94	32.00 ± 4.04	50.67 ± 4.91 abc
15%, 12.5 g	27.33 ± 5.34	148.33 ± 26.02	195.7 ± 17.90	8.16 ± 1.20	38.67 ± 7.44	49.33 ± 7.42 bc
15%, 18.75 g	25.83 ± 3.65	160.67 ± 10.20	193.3 ± 9.20	9.83 ± 0.60	37.33 ± 3.75	60.33 ± 7.42 ab
30%, 0 g	23.66 ± 2.24	185.00 ± 24.33	188.7 ± 18.35	10.66 ± 1.16	27.00 ± 4.50	57.33 ± 6.22 abc
30%, 6.25 g	25.00 ± 1.52	139.67 ± 13.33	187.0 ± 8.32	10.83 ± 1.42	29.33 ± 3.75	61.00 ± 1.73 ab
30%, 12.5 g	21.33 ± 1.64	162.67 ± 34.89	185.00 ± 10	9.83 ± 0.88	39.66 ± 0.67	62.67 ± 4.25 ab
30%, 18.75 g	23.33 ± 3.03	215.00 ± 27.31	251.3 ± 32.23	8.33 ± 1.01	39.00 ± 3.05	52.00 ± 4.93 abc
p-value	0.69	0.23	0.26	0.66	0.34	0.04

Note. Means ± SE values with different letters indicate significant ($p < 0.05$) differences by Duncan's multiple range test; WAT = Weeks after transplanting

Table 4
The effect of pruning and additional fertilizer on root length of winged bean plant

Treatment	Plant age (WAT)		
	5	10	15
	Root length (cm)		
Pruning (P)			
0%	8.16 ± 0.38	15.91 ± 2.71	18.50 ± 1.54
15%	7.95 ± 0.46	19.16 ± 0.12	24.08 ± 3.20
30%	7.79 ± 0.62	16.83 ± 1.68	23.08 ± 2.78
p-value	0.88	0.45	0.21
Additional fertilizer (F)			
0 g/plant	7.55 ± 0.51	20.55 ± 2.65	23.88 ± 3.28
6.25 g/plant	8.88 ± 0.61	13.44 ± 1.72	24.66 ± 3.90
12.5 g/plant	7.33 ± 0.57	19.77 ± 2.24	20.33 ± 2.53
18.75 g/plant	8.11 ± 0.48	15.44 ± 1.59	18.66 ± 2.19
p-value	0.30	0.08	0.35
P×F			
0%, 0 g	6.67 ± 0.44	19.33 ± 7.53	18.67 ± 3.48 bc
0%, 6.25 g	9.00 ± 0.57	19.33 ± 2.84	18.00 ± 1.00 bc
0%, 12.5 g	8.16 ± 0.83	21.67 ± 6.33	18.00 ± 3.60 bc
0%, 18.75 g	8.83 ± 0.60	13.33 ± 3.17	21.33 ± 4.37 bc
15%, 0 g	8.66 ± 1.09	16.33 ± 2.40	22.33 ± 4.05 bc
15%, 6.25 g	9.00 ± 11.54	20.33 ± 0.33	37.67 ± 4.97 a
15%, 12.5 g	6.50 ± 0.28	22.00 ± 2.64	22.33 ± 6.17 bc
15%, 18.75 g	7.67 ± 0.44	18.00 ± 2.30	14.00 ± 1.00 c
30%, 0 g	7.33 ± 0.88	22.00 ± 4.50	30.67 ± 7.96 ab
30%, 6.25 g	8.67 ± 1.69	14.67 ± 3.75	20.33 ± 5.04 bc
30%, 12.5 g	7.33 ± 1.58	15.66 ± 0.66	20.67 ± 4.63 bc
30%, 18.75 g	7.83 ± 1.36	15.00 ± 3.05	20.67 ± 4.63 bc
p-value	0.72	0.77	0.05

Note. Means ± SE values with different letters indicate significant ($p < 0.05$) differences by Duncan's multiple range test; WAT = Weeks after transplanting

Nutrient Leaf and Auxin Value

The interaction of leaf pruning intensities and additional fertilizer rates had no significant effect on nutrient leaf, auxin value (Table 5), and nutrient uptake (Table 6). Leaf pruning affected leaf nutrients phosphorus and C organic concentration, as shown in Table 5. The leaf pruning intensity is directly related to the phosphorus content. A 30% leaf pruning treatment gave the highest phosphorus content (0.67 ppm), although it was not significantly different from 15% pruning (0.61 ppm), and the control treatment was 0.42 ppm of leaf P content. It was in line with previous research that pruning increased nutrient P in mango plants

Table 5
The effect of pruning and additional fertilizer on the leaf nutrient contents at 11 WAT and auxin concentration in the shoot of winged beans at 12 WAT

Treatment	Nitrogen (%)	Phosphorus (ppm)	Potassium (ppm)	C-organic (%)	C/N ratio	Auxin (%)
Pruning (P)						
0%	6.50 ± 0.15	0.42 ± 0.02 b	2.16 ± 0.17	29.43 ± 0.81 b	4.54 ± 0.81	0.023 ± 0.02
15%	6.14 ± 0.21	0.61 ± 0.01 a	1.63 ± 0.16	36.35 ± 1.00 a	6.01 ± 1.00	0.021 ± 0.02
30%	6.10 ± 0.24	0.67 ± 0.02 a	1.96 ± 0.11	31.66 ± 1.12 b	5.28 ± 1.12	0.022 ± 0.02
p-value	0.29	<0.001	0.06	<0.001	0.007	0.83
Additional fertilizer (F)						
0 g/plant	5.75 ± 0.20	0.52 ± 0.05	1.71 ± 0.13	33.71 ± 1.43	5.94 ± 0.31 a	0.017 ± 0.02 b
6.25 g/plant	6.30 ± 0.22	0.54 ± 0.04	1.86 ± 0.24	32.84 ± 1.69	5.30 ± 0.41 ab	0.018 ± 0.02 b
12.5 g/plant	6.31 ± 0.23	0.58 ± 0.05	2.18 ± 0.19	33.20 ± 1.19	5.27 ± 0.11 ab	0.025 ± 0.02 ab
18.75 g/plant	6.63 ± 0.23	0.62 ± 0.03	1.90 ± 0.14	30.17 ± 0.79	4.60 ± 0.23 b	0.027 ± 0.03 a
p-value	0.07	0.13	0.31	0.09	0.01	0.02
P×F						
0%, 0 g	5.97 ± 0.05	0.34 ± 0.01	1.61 ± 0.34	28.11 ± 0.65	4.70 ± 0.10	0.016 ± 0.003
0%, 6.25 g	6.81 ± 0.09	0.42 ± 0.00	2.37 ± 0.28	27.87 ± 2.08	4.08 ± 0.25	0.020 ± 0.006
0%, 12.5 g	6.40 ± 0.48	0.39 ± 0.01	2.46 ± 0.46	32.07 ± 1.08	5.04 ± 0.23	0.027 ± 0.005
0%, 18.75 g	6.84 ± 0.04	0.53 ± 0.09	2.19 ± 0.07	29.67 ± 1.79	4.33 ± 0.27	0.027 ± 0.006
15%, 0 g	5.74 ± 0.65	0.61 ± 0.05	1.48 ± 0.25	29.27 ± 0.55	7.05 ± 0.94	0.019 ± 0.004
15%, 6.25 g	6.19 ± 0.27	0.56 ± 0.03	1.21 ± 0.42	37.89 ± 1.38	6.16 ± 0.47	0.016 ± 0.001
15%, 12.5 g	6.64 ± 0.17	0.64 ± 0.02	2.07 ± 0.31	36.53 ± 1.28	5.50 ± 0.11	0.026 ± 0.002
15%, 18.75 g	5.98 ± 0.53	0.63 ± 0.01	1.75 ± 0.25	31.71 ± 1.42	5.35 ± 0.28	0.022 ± 0.005
30%, 0 g	5.54 ± 0.27	0.63 ± 0.07	2.03 ± 0.13	33.76 ± 3.51	6.07 ± 0.49	0.017 ± 0.001
30%, 6.25 g	5.91 ± 0.53	0.65 ± 0.08	2.01 ± 0.28	32.76 ± 1.78	5.68 ± 0.77	0.017 ± 0.002
30%, 12.5 g	5.89 ± 0.49	0.71 ± 0.02	2.01 ± 0.25	30.99 ± 2.39	5.27 ± 0.18	0.021 ± 0.003
30%, 18.75 g	7.07 ± 0.15	0.70 ± 0.00	1.78 ± 0.31	29.14 ± 0.72	4.12 ± 0.16	0.032 ± 0.009
p-value	0.36	0.59	0.46	0.14	0.26	0.72

Note. Means ± SE values with different letters indicate significant ($p < 0.05$) differences by Duncan's multiple range test; WAT = Weeks after transplanting

Table 6
The effect of pruning and additional fertilizer on nutrition uptake of winged beans

Treatment	Nitrogen (g/kg)	Phosphorus (mg/kg)	Potassium (mg/kg)	C-organic (g/kg)	C/N ratio
Pruning (P)					
0%	84.56 ± 12.59	5.41 ± 0.77	28.24 ± 5.28 a	390.03 ± 61.33	60.54 ± 9.76
15%	54.61 ± 6.98	5.48 ± 0.68	14.87 ± 2.47 b	328.51 ± 48.07	55.40 ± 9.67
30%	72.62 ± 11.15	8.03 ± 1.17	23.07 ± 3.38 ab	367.39 ± 51.55	59.89 ± 7.61
p-value	0.12	0.63	0.04	0.68	0.89
Additional fertilizer (F)					
0 g/plant	65.97 ± 13.55	5.70 ± 0.99 ab	18.36 ± 2.79 b	376.73 ± 60.92	67.21 ± 10.24
6.25 g/plant	49.77 ± 9.49	4.17 ± 0.73 b	15.14 ± 3.41 b	254.71 ± 50.45	40.38 ± 8.02
12.5 g/plant	90.83 ± 12.79	8.25 ± 1.11 a	33.18± 6.65 a	474.67 ± 61.68	75.09 ± 8.69
18.75 g/plant	75.82 ± 10.47	7.11 ± 1.08 ab	21.57 ± 2.88 ab	341.81 ± 40.23	51.76 ± 5.69
p-value	0.11	0.03	0.02	0.09	0.08
P×F					
0%, 0 g	89.25 ± 39.52	4.96 ± 1.99	21.41 ± 7.32	414.6 ± 176.47	69.78 ± 29.89
0%, 6.25 g	64.35 ± 20.52	3.96 ± 1.20	22.37 ± 7.90	272.7 ± 101.58	39.74 ± 14.46
0%, 12.5 g	106.63 ± 31.07	6.59 ± 1.80	44.36 ± 17.86	532.7 ± 143.47	82.97 ± 19.44
0%, 18.75 g	78.00 ± 7.79	6.14 ± 1.53	24.82 ± 1.70	340.2 ± 46.09	49.68±6.72
15%, 0 g	47.41 ± 15.38	5.14 ± 1.77	11.29 ± 2.89	342.4 ± 144.52	66.75 ± 34.73
15%, 6.25 g	47.42 ± 19.28	4.33 ± 1.76	10.03 ± 4.39	293.2 ± 130.83	47.88 ± 22.46
15%, 12.5 g	69.07 ± 17.38	6.61 ± 1.54	22.37 ± 7.72	381.3 ± 96.84	56.78 ± 13.43
15%, 18.75 g	54.56 ± 2.55	5.85 ± 0.56	15.80 ± 1.00	292.2 ± 24.45	50.21 ± 7.88
30%, 0 g	61.25 ± 15.98	6.99 ± 2.13	22.38 ± 5.87	318.2 ± 99.33	65.09 ± 14.22
30%, 6.25 g	37.54 ± 10.23	4.21 ± 1.38	13.01 ± 4.10	198.3 ± 24.97	33.53 ± 2.56
30%, 12.5 g	98.78 ± 17.84	1.56 ± 1.04	32.81 ± 5.56	510.0 ± 95.07	85.55 ± 9.75
30%, 18.75 g	94.89 ± 28.95	9.35 ± 2.78	24.09 ± 8.41	393.0 ± 118.99	55.39 ± 16.48
p-value	0.84	0.78	0.93	0.93	0.91

Note. Means ± SE values with different letters indicate significant ($p < 0.05$) differences by Duncan's multiple range test

(Singh et al., 2010). Phosphorus is mobile in the phloem and can be transported from source to sink, from old to young leaves, to grow and develop young leaves. Therefore, pruning can affect the plant's transport and distribution of mineral nutrients. The current study shows that leaf P concentrations increase significantly due to pruning. Y. Liu et al. (2022) state that leaf pruning in tea (young leaf pruning) prevents phosphorus distribution from older to younger leaves via phloem connections because the young leaf has been removed.

Meanwhile, in the current study, old (mature) leaves were pruned, and other plant organs covered the leaves, so the distribution of P leaf can be transported from old to young leaf. Furthermore, the treatment of 15% leaf pruning increased the C-organic by 23%. The leaf pruning treatment was conducted in the 11 WAT or flowering stage, so the nutrient in the leaf was increasing at peak nutrient status. As growth progressed, nutrients increased from the vegetative stage to the peak generative stage and gradually declined from pod production to the harvesting stage, with significant variation between each other (Hari Prasath et al., 2017). In this study, the leaf nutrients increased organic C and P because pruning was carried out when the plants were flowering (Yan et al., 2021).

The additional fertilizer treatment affected the C/N ratio and auxin levels (Table 5) and phosphorus and potassium uptake (Table 6). The result showed that the increased rate of additional fertilizer decreased the C/N ratio. It is similar to previous research, where fertilization reduces the C/N ratio in the flag leaves of lowland rice plants. It was possible because adding fertilizer increased leaf nutrient content such as N, P, and potassium and decreased the C/N ratio (Yin et al., 2020). The results indicated that the additional fertilizer significantly increased the plant growth regulators (auxin level). The fertilizer was rich in nutrients, specifically phosphorus and nitrogen. The elements entered the protein synthesis enzymes, nucleic acids, DNA, and RNA, which stimulated the formation of cytokines, as well as containing humic acid in the potassium hydroxide, preventing indole acetic acid (IAA) from breaking down IAA-oxidase (Toman et al., 2020). Table 5 shows that the additional fertilizer did not have a statistically significant effect on nitrogen, phosphorus, and potassium levels. It was possible because of the application of inorganic and organic fertilizers (chicken manure) before planting as basic fertilizers, which were enough for plant growth.

Increasing rates of additional fertilizer increase the phosphorus and potassium nutrition uptake significantly at 12.5 g fertilizer/plant. Therefore, the plants responded well to the additional fertilizer by uptaking the soil nutrients. Suitable climates supported the availability of soil nutrients during plant growth when there was enough rainfall and sunlight from August to October 2021, as shown in Table 1. Climate conditions affected ecosystems, specifically plants and soils (G. J. Kim et al., 2023). During vegetative growth,

many nutrients are needed (Nahed et al., 2010; Owino & Sigunga, 2012; Prajapati & Modi, 2018). Macronutrients are also essential for plant growth when absorbed by the roots in the soil (Feng et al., 2020). Fertilizer application improved the soil nutrients, and the increased levels also enhanced plant growth (Owolabi et al., 2016) with a carbon-nitrogen ratio (Kamhun et al., 2022). Furthermore, plants conducted physiological processes properly with sufficient nutrient conditions (Yong et al., 2010). The additional fertilizer did not affect the nitrogen level (Table 5) and the uptake (Table 6) because legume plants formed a symbiosis with rhizobium to bind N_2 from the atmosphere, which served as a source of nutrition essential for plant growth and development (Zahran, 1999). Therefore, nitrogen absorption becomes less significant in legume plants, relying on the nitrogen-fixing ability of rhizobium to meet their requirements.

Yield

This research harvested winged beans until 24 WAT, equivalent to 13 harvests. The interaction between leaf pruning and additional fertilizer did not significantly affect the yield, as shown in Table 7. Even though the treatments did not show significant differences, a 30% leaf pruning resulted in a 9% higher yield than the control (Table 7). The application of higher-intensity leaf pruning is expected to provide advantages for the plant, particularly considering the dense canopy and abundant leaves of Fairuz IPB. The dense canopy can impede light distribution within the plant, and leaf pruning emerges as a suitable method to improve the distribution. The positive effects of pruning on fruit trees have been previously reported in the context of peach cultivation (Samira et al., 2014).

Additionally, pruning offers the benefit of reducing air humidity around the plant canopy, lowering the risk of plant disease incidence, such as leaf rust. Consequently, this can lead to an increase in bean production, and this finding aligns with Bassal et al. (2020), who also reported similar outcomes. Previous research elucidated that pruning serves the purpose of controlling the size and shape of the plant, promoting accelerated and robust growth, and enhancing the quality and quantity of production. The young leaves at the top can absorb the most solar radiation to have a high rate of carbon dioxide (CO_2) assimilation and translocate most assimilated to other plant parts. Furthermore, leaves positioned at the lowermost stratum and overshadowed by those above will exhibit a diminished rate of CO_2 assimilation due to reduced sunlight exposure. Consequently, they contribute insignificantly to assimilating other plant parts (Santanoo et al., 2020; T. Liu et al., 2011).

The pod yield was not significantly different among pruning intensities and additional fertilizer rates treatments, probably because the pod yield (10.83–13.99 young pods/plant) was above the potential yield (8.35–10.42 young pods/plant) according to the Indonesian Ministry of Agriculture (Kementerian Pertanian

Table 7
The effect of pruning and additional fertilizer on the total young pod yield of winged beans

Treatment	Yield per plant		Yield per plot (5.7m^2)		Yield per ha
	Number of pods	Weight of pod (g)	Number of pods	Weight of pod (kg)	Weight of pod (kg)
Pruning (P)					
0%	11.9 ± 0.85	139.33 ± 13.18	416.50 ± 29.83	4.87 ± 0.46	8555 ± 809.76
15%	12.9 ± 1.01	144.46 ± 10.52	453.25 ± 35.49	5.05 ± 0.36	8870 ± 464.00
30%	12.7 ± 0.75	148.85 ± 11.20	447.00 ± 26.58	5.20 ± 0.39	9140 ± 687.98
p-value	0.63	0.85	0.63	0.85	0.85
Additional fertilizer (F)					
0 g/plant	12.65 ± 0.80	144.83 ± 11.05	442.78 ± 28.25	5.06 ± 0.42	8893 ± 730.13
6.25 g/plant	11.98 ± 0.75	141.09 ± 13.42	419.33 ± 26.29	4.93 ± 0.46	8663 ± 824.15
12.5 g/plant	13.44 ± 1.36	150.27 ± 18.62	470.44 ± 47.77	5.25 ± 0.65	9227 ± 1143.71
18.75 g/plant	12.08 ± 0.76	140.66 ± 8.92	423.11 ± 26.74	4.92 ± 0.31	8637 ± 548.08
p-value	0.69	0.95	0.69	0.95	0.95
P×F					
0%, 0 g	10.83 ± 0.69	122.98 ± 13.64	379.33 ± 24.36	4.30 ± 0.47	7552 ± 837.53
0%, 6.25 g	11.72 ± 1.49	143.27 ± 28.06	410.33 ± 52.41	5.01 ± 0.98	8798 ± 1723.1
0%, 12.5 g	13.76 ± 3.56	161.30 ± 48.57	481.67 ± 12.46	5.64 ± 1.70	9904 ± 2982.6
0%, 18.75 g	11.27 ± 0.87	129.75 ± 8.04	394.67 ± 30.60	4.54 ± 0.28	7967 ± 493.73
15%, 0 g	13.12 ± 1.84	137.44 ± 22.21	459.33 ± 64.67	4.81 ± 0.77	8439 ± 1363.8
15%, 6.25 g	11.67 ± 1.77	130.77 ± 32.68	408.33 ± 62.14	4.57 ± 1.14	8030 ± 2006.7
15%, 12.5 g	13.58 ± 1.29	155.52 ± 17.86	475.33 ± 45.17	5.44 ± 0.62	9549 ± 1097
15%, 18.75 g	13.42 ± 2.00	154.11 ± 18.12	470.00 ± 70.16	5.39 ± 0.63	9463 ± 1113
30%, 0 g	13.99 ± 1.12	174.06 ± 19.26	489.67 ± 39.47	6.09 ± 0.67	10688 ± 1182.8
30%, 6.25 g	12.55 ± 1.08	149.22 ± 14.75	439.33 ± 38.11	5.22 ± 0.51	9162 ± 905.89
30%, 12.5 g	12.98 ± 2.80	133.99 ± 35.74	454.33 ± 98.09	4.69 ± 1.25	8228 ± 2194.8
30%, 18.75 g	11.56 ± 0.92	138.12 ± 20.24	404.67 ± 32.35	4.83 ± 0.70	8481 ± 1242.8
p-value	0.87	0.72	0.87	0.72	0.72

Note. Means ± SE values

Republik Indonesia [Kementan RI], 2020). The climatology environment of this study was similar to the previous research, where the winged beans grow well in environmental conditions with sufficient sunlight, nutrients, and water (Eagleton, 2020; Ishthifaiyyah et al., 2021). The growth of plants during the rainy season is facilitated by a sufficient water supply, thereby leading to higher yields compared to the sunny season (Feng et al., 2020; Prajapati & Modi, 2018).

There are several reasons why the additional fertilizer rates did not significantly affect the yield, as shown in Table 7. The experiment benefited from favorable climate conditions with exceptionally high sunlight intensity, elevated air humidity, and rainfall. These conditions facilitated robust plant growth, which is evident from the increasing leaf nutrient concentrations (Table 5) and uptake (Table 6). Despite these favorable factors, a substantial increase in the pod yield was not reported. Firstly, before planting, a soil analysis showed that the soil possessed relatively favorable properties such as pH 4.56, 1.86 ppm C-organic, 0.22% N, 61.7 ppm P, and 334.2 ppm potassium. Secondly, adding 10 t chicken manure/ha as a basic fertilizer for all experiments improved soil structure, added nutrients, and enhanced availability. Thirdly, inorganic fertilizer was also applied as a basic fertilizer.

The initial presumption that the winged bean plant, with its non-simultaneous flowering and susceptibility to pruning effects, would necessitate additional fertilizer has not been carried out. However, this level was insufficient for proper physiological plant processes, and the fertilizer treatment did not lead to an increased yield. It was similar to the previous study stating that an adequate fertilizer application rate enhanced nutrient levels, promoting plant growth and yield, as supported by previous research (Nahed et al., 2010; Prajapati & Modi, 2018). Adequate fertilizer rates are essential to ensure the proper execution of physiological processes in plants (Taiz & Zeiger, 2002). The fourth reason was that the plant in this study produced young pods similar to the yield potential stated in the description of the Fairuz variety, so the plant cannot increase its production beyond its potential yield.

Based on the PCA analysis of several observed variables, PC1 (28.9%) and PC2 (22.2%) can explain 50.1% of the total variance. Figure 1 shows that some observed variables can be divided into four quadrants. The first quadrant consists of nutrient uptake root length and yield (number and weight of pods). The second quadrant comprises plant height, auxin, N content, and leaf potassium. The third quadrant comprises the number of leaf variables, while the fourth comprises organic C and C/N leaves. Variables adjacent and exhibiting positive correlation share a common description, while those in opposing positions or showing negative correlation possess distinct explanations. The PCA can determine the correlation between the observed variables, which is substantial when the value is $r \geq 0.75$ (Limpawattana & Shewfelt, 2010).

The correlation matrix shows a significant positive correlation between

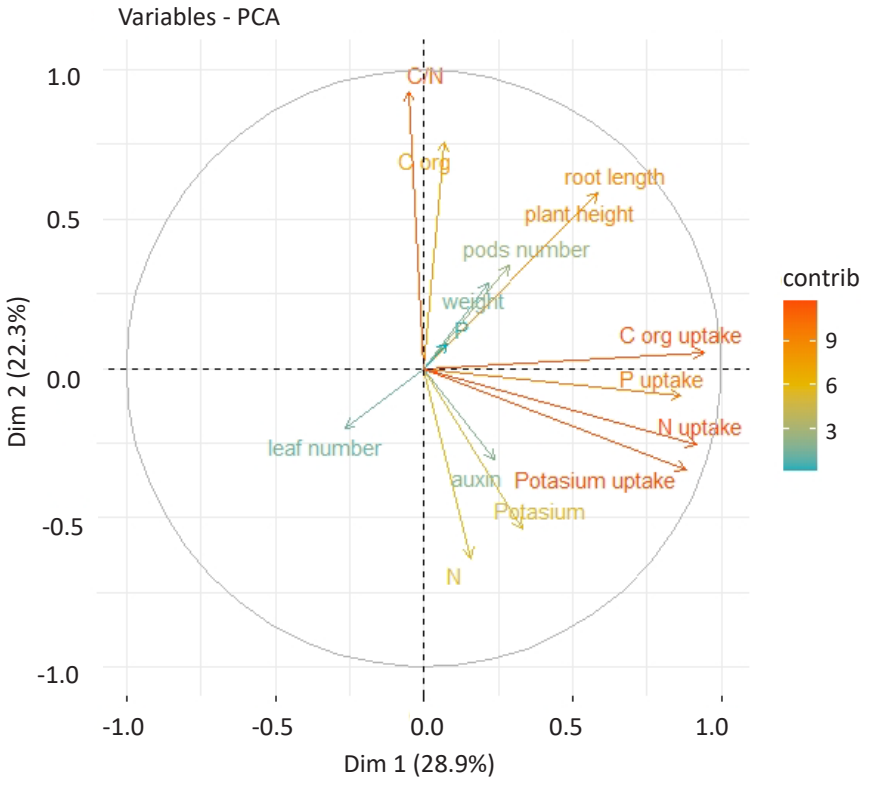

Figure 1. Principal component analysis of variables observed in winged bean

auxin and potassium values, phosphorus value and root length, and pod number and weight (yield). The analysis reported a positive correlation between potassium and auxin levels, indicating that the level increases with the auxin in the leaves. This observation aligns with prior research indicating that the expression of potassium transporter plays a role in regulating auxin levels (Tenorio-Berrío et al., 2018), and the potassium channel AKT1 is involved in auxin-related processes (J. Li et al., 2017).

A positive correlation was also shown by phosphorus nutrient leaf and root length. Previous research has shown that P deficiency enhanced root length in wheat (Shen et al., 2018). An increase in P concentrations can improve the biomass of both roots and flowers. This results in a higher root-to-shoot ratio, with P-deficient plants exhibiting the longest root length when grown in lower P concentrations. Root biomass has been found to correlate positively with the P level, affecting the number of storage roots in cassava (Omondi et al., 2019) and *Lantana* (H.-J. Kim; K.-T. Li, 2016). P deficiency has been shown to enhance root length, a phenomenon co-regulated by DNA replication, transcription, protein synthesis, degradation, and cell growth, as shown by Shen et al. (2018). This increase in root length enables the roots to reach nutrients more effectively, supporting overall plant growth (Qazizadah et al., 2023).

Furthermore, a positive correlation exists between the number of pods and their weight (yield), indicating that an increase in pods leads to a higher yield. This finding aligns with previous research by Bakal et al. (2020), where an increase in peanut pods directly corresponds to a higher peanut plant yield. Figure 1 shows the first quadrant, encompassing nutrient uptake, root length, and yield variables. Some variables exhibit significant relationships, particularly between adjacent lines, such as weight, number of pods, P content, and root length variables. There is a corresponding augmentation in the plant yield, encompassing the weight and number of pods when the P content and root length experience an increase. This observation underscores the significance of elongated plant roots in enhancing nutrient absorption from the soil, promoting optimal photosynthesis, and increasing pod yield and weight. Moreover, previous research reported a positive correlation between P value and favorable root conditions, encompassing root density and length (He et al., 2021).

CONCLUSION

Pruning and additional fertilizer treatment interaction significantly affected the winged bean's leaf number and root length. Without pruning and 6.25 g, additional fertilizer/plant treatment had increased leaf number. The P and C organic content increased with 15% leaf pruning intensity. The effects of additional fertilizer rates were found in auxin content, potassium, and phosphorus uptake, showing the increase of those variables with the additional fertilizer. Still, the additional fertilizer rates did not affect the yield. The young pods yield of this study reached the potential yield of the Fairuz variety.

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REFERENCES

Anjos, R. A., Lisboa, C., Silva, G., Vaz, V., Teixeira, I., Eduardo, M., Corrêa, P. C., Mota, J. H., Silva, A., & Lourenço, A. (2021). Yield of common bean cultivars and castor hybrids intercropped in two cultivation sites with oxisol in the midwest region of Brazil. *Australian Journal of Crop Science*, *15*(10), 1307–1313. https://doi.org/10.21475/ajcs.21.15.10.p3255

Asefa, G., Mohammed, H., & Ayano, A. (2021). Hybrid vigor of yield and yield related characters on Limmu Coffee (*Coffea arabica* L .) genotypes at South-Western Ethiopia. *Journal of Tropical Crop Science*, *8*(2), 89–109. https://doi.org/10.29244/jtcs.8.02.89-100

Bakal, H., Kenetli, A., & Arioglu, H. (2020). The effect of plant density on pod yield and some agronomic characteristics of different growth-type peanut varieties (*Arachis hypogaea* l.) grown as a main crop. *Turkish Journal of Field Crops*, *25*(1), 92–99. https://doi.org/10.17557/tjfc.748671

Bassal, H., Merah, O., Ali, A. M., Hijazi, A., & Omar, F. E. (2020). *Psophocarpus tetragonolobus*: An

underused species with multiple potential uses. *Plants*, *9*(12), 1730. https://doi.org/10.3390/plants9121730

Betty, B., Aziz, S. A., & Suketi, K. (2021). The effects of different rates of chicken manure and harvest intervals on the bioactive compounds of bitter leaf (*Vernonia amygdalina* Del.). *Journal of Tropical Crop Science*, *8*(2), 80–88. https://doi.org/10.29244/jtcs.8.02.80-88

Bhandari, N. (2018). Review: Techniques used in plant tissue analysis for essential elements on horticultural plants and correlate with nutrient requirement. *North American Academic Research*, *1*(2), 94–113.

Calvindi, J., Syukur, M., & Nurcholis, W. (2020). Investigation of biochemical characters and antioxidant properties of different winged bean (*Psophocarpus tetragonolobus*) genotypes grown in Indonesia. *Biodiversitas*, *21*(6), 2420–2424. https://doi.org/10.13057/biodiv/d210612

Cano-Reinoso, D. M., Soesanto, L., Kharisun, & Wibowo, C. (2022). Effect of pre-harvest foliar calcium and silicon fertilization on pineapple quality and fruit collapse incidence. *AGRIVITA Journal of Agricultural Science*, *44*(3), 405–418. https://doi.org/10.17503/agrivita.v41i0.3635

Carrillo, Y., Jordan, C. F., Jacobsen, K. L., Mitchell, K. G., & Raber, P. (2011). Shoot pruning of a hedgerow perennial legume alters root-derived nitrogen's availability and temporal dynamics in a subtropical setting. *Plant Soil*, *345*, 59–68. https://doi.org/10.1007/s11104-011-0760-8

Dufour, B. P., Kerana, I. W., & Ribeyre, F. (2019). Effect of coffee tree pruning on berry production and coffee berry borer infestation in the Toba Highlands (North Sumatra). *Crop Protection*, *122*, 151–158. https://doi.org/10.1016/j.cropro.2019.05.003

Eagleton, G. E. (2020). Review: Winged bean (*Psophocarpus tetragonolobus*) cropping systems. *Biodiversitas Journal of Biological Diversity*, *21*(12), 5927–5946. https://doi.org/10.13057/biodiv/d211258

Feng, J., Hussain, H. A., Hussain, S., Shi, C., Cholidah, L., Men, S., Ke, J., & Wang, L. (2020). Optimum water and fertilizer management for better growth and resource use efficiency of rapeseed in rainy and drought seasons. *Sustainability*, *12*(2), 703. https://doi.org/10.3390/su12020703

Hari, P. C. N., Balasubramanian, A., & Radhakhishnan, S. (2017). Effect of pruning intensities on leaf nutrient status and fruit yield in tamarind plantation at Dharmapuri District of Tamil Nadu, India. *The Bioscan*, *12*(1), 615–618.

He, J., Jin, Y., Siddique, K. H. M., & Li, F.-M. (2021). The trade-off between root efficiency and root size is associated with the ith yield performance of soybean under different water and phosphorus levels. *Agriculture*, *11*(6), 481. https://doi.org/10.3390/agriculture11060481

Ishthifaiyyah, S. A., Syukur, M., Trikoesoemaningtyas., & Maharijaya, A. (2021). Agro-morphological traits and harvest period assessment of winged bean (*Psophocarpus tetragonolobus*) genotypes for pod production. *Biodiversitas Journal of Biological Diversity*, *22*(2), 1069–1075. https://doi.org/10.13057/BIODIV/D220264

Iskandar, H. M., Suhandono, S., Pambudi, J., Kristianti, T., Putranto, R. A., Mose, W., & Sustiprijatno, S. (2020). Characterization of a drought-inducible dehydrin promoter from sugarcane (*Saccharum officinarum* L.) in tobacco (*Nicotiana tabacum* L.). *Journal of Tropical Crop Science*, *7*(1), 28–36. https://doi.org/10.29244/jtcs.7.01.28-36

Kamhun, W., Pheng-am, S., Uppananchai, T., Ratanasut, K., & Rungrat, T. (2022). Effects of nitrogen levels on sucrose content, disease severity of *Xanthomonas oryzae* pv. oryzae and yield of hybrid rice (BC4F5). *Agriculture and Natural Resources*, *56*(5), 909–916.

Kementerian Pertanian Republik Indonesia. (2020). *Pengumuman permohonan hak PVT* [Announcement of PVP rights application]. Kementan RI. https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&ved=2ahUKEwjC8-uvouSEAxXo1jgGHVOtBhAQFnoECA4QAQ&url=https%3A%2F%2Fcdn.ipb.ac.id%2Fkekayaanintelektual%2FDokumenPerlindunganVT_006__Kecipir_Fairuz_IPB_7d23268c-42fc-4a9a-a0c0-aa09d6f0900b.pdf&usg=AOvVaw0mblA_D_wEOZ7CK3snXi76&opi=89978449

Kim, G. J., Jo, H., Kim, H., Cho, M. S., Noh, N.-J., Chang, H., Kim, H.-S., & Son, Y. (2023). Experimental design of open-field temperature and precipitation manipulation system to simulate summer extreme climate events for plants and soils. *Turkish Journal of Agriculture and Forestry*, *47*(1), 132–142. https://doi.org/10.55730/1300-011X.3070

Kim, H.-J., & Li, X. (2016). Effects of phosphorus on shoot and root growth, partitioning, and phosphorus utilization efficiency in Lantana. *HortScience*, *51*(8), 1001–1009. https://doi.org/10.21273/hortsci.51.8.1001

Kueklang, M., Krisanapook, K., Havananda, T., Phavaphutanon, L., & Luengwilai, K. (2021). Seasonal variation of fruit yield and leaf macronutrient concentrations of Thai aromatic coconut. *Agriculture and Natural Resources*, *55*(5), 854–862. https://doi.org/10.34044/j.anres.2021.55.5.16

Laia, K. (2019). *Perbedaan keragaan, daya hasil, dan kandungan proksimat kecipir (*Psophocarpus tetragonolobus *L.)* [Differences in performance, yield, and proximate content of chipper (*Psophocarpus tetragonolobus* L.)] [Unpublish Bachelor's thesis]. Institut Pertanian Bogor.

Li, J., Wu, W.-H., & Wang, Y. (2017). Potassium channel AKT1 is involved in the auxin-mediated root growth inhibition in *Arabidopsis* response to low K^+ stress. *Journal of Integrative Plant Biology*, *59*(12), 895–909. https://doi.org/10.1111/jipb.12575

Li, K.-T., Lakso, A. N., Piccioni, R., & Robinson, T. (2016). Summer pruning reduces whole-canopy carbon fixation and transpiration in apple trees. *The Journal of Horticultural Science and Biotechnology*, *78*(6), 749–754. https://doi.org/10.1080/14620316.2003.11511694

Limpawattana, M., & Shewfelt, R. L. (2010). Flavor lexicon for sensory descriptive profiling of different rice types. *Journal of Food Science*, *75*(4), S199–S205. https://doi.org/10.1111/j.1750-3841.2010.01577.x

Liu, T., Song, F., Liu, S., & Zhu, X. (2011). Canopy structure, light interception, and photosynthetic characteristics under different narrow-wide planting patterns in maize at silking stage. *Spanish Journal of Agricultural Research*, *9*(4), 1249–1261. https://doi.org/10.5424/sjar/20110904-050-11

Liu, Y., Tian, J., Liu, B., Zhuo, Z., Shi, C., Xu, R., Xu, M., Liu, B., Ye, J., Sun, L., & Liao, H. (2022). Effects of pruning on mineral nutrients and untargeted metabolites in fresh leaves of *Camellia sinensis* cv. Shuixian. *Frontiers in Plant Science*, *13*, 1016511. https://doi.org/10.3389/fpls.2022.1016511

Mahmoodi, B., Moballeghi, M., Eftekhari, A., & Neshaie-Mogadam, M. (2020). Effects of foliar application of liquid fertilizer on agronomical and physiological traits of rice (*Oryza sativa* L.). *Acta Agrobotanica*, *73*(3), 7332. https://doi.org/10.5586/AA.7332

Maillard, A., Diquélou, S., Billard, V., Laîné, P., Garnica, M., Prudent, M., Garcia-Mina, J.-M., Yvin, J.-C., & Ourry, A. (2015). Leaf mineral nutrient remobilization during leaf senescence and modulation by nutrient deficiency. *Frontiers in Plant Science*, *6*, 317. https://doi.org/10.3389/fpls.2015.00317

Mediene, S., Jordan, M. O., Pages, L., Lebot, J., & Adamowicz, S. (2002). The influence of severe shoot pruning on growth, carbon and nitrogen status in young peach trees (*Prunus persica*). *Tree Physiology*, *22*(18), 1289–1296. https://doi.org/10.1093/treephys/22.18.1289

Mohanty, C. S., Singh, V., & Chapman, M. A. (2020). Winged bean: An underutilized tropical legume on the path of improvement, to help mitigate food and nutrition security. *Scientia Horticulturae*, *260*, 108789. https://doi.org/10.1016/j.scienta.2019.108789

Maudu, M., Mudau, F. N., & Mariga, I. K. (2010). The effect of pruning on growth and chemical composition of cultivated bush tea (*Athrixia phylicoides* D.C). *Journal of Medicinal Plant Research*, *4*(22), 2353–2358. https://doi.org/10.5897/JMPR10.486

Nahed., Aziz, G. A, Azza., Mazher, A. M., & Farahat, M. M. (2010). Response of vegetative growth and chemical constituents of *Thuja orientalis* L. plant to foliar application of different amino acids at Nubaria. *Journal of American Science*, *6*(3), 295–301.

Nugroho, P. A., Widyastuti, Y., Samanhudi., & Yunus, A. (2017). Growth response of *Artemisia annua* by the effect of types and composition of organic fertilizer in the lowland. *Journal of Agricultural Science and Technology*, *7*, 339–345. https://doi.org/10.17265/2161-6264/2017.05.006

Omondi, J. O., Lazarovitch, N., Rachmilevitch, S., & Yermiyahu, U. (2019). Phosphorus affects the storage root yield of cassava through root numbers. *Journal of Plant Nutrition*, *42*(17), 2070–2079. https://doi.org/10.1080/01904167.2019.1655033

Owino, C., & Sigunga, D. O. (2012). Effects of rainfall pattern and fertilizer nitrogen on nitrogen loss in bypass flow in vertisols at the onset of rain season under tropical environments. *Journal of Environmental Science and Water Resources*, *1*(9), 207–215.

Owolabi, I. O., Ashaolu, J. T., & Abayomi, Y. A. (2016). Effects of inorganic fertilizer application on plant growth and dry matter yield of physic nut (*Jatropha curcas* L.). *International Journal of Agricultural and Food Research*, *5*(1), 14-21. https://doi.org/10.24102/ijafr.v5i1.587

Porfírio, S., da Silva, M. D. R. G, Peixe, A., Cabrita, M. J., & Azadi, P. (2016). Current analytical methods for plant auxin quantification - A review. *Analytica Chimica Acta*, *902*, 8–21. https://doi.org/10.1016/j.aca.2015.10.035

Prajapati, K., & Modi, H. A. (2018). The importance of potassium in plant growth - A review. *Indian Journal of Plant Sciences*, *1*(2-3), 177-186.

Qazizadah, A. Z., Nakasha, J. J., Sinniah, U. R., & Wahab, P. E. M. (2023). Improvement of growth and development of sweet basil (*Ocimum basilicum* L .) through the application of chitosan at different plant maturity stages. *Pertanika Tropical Agricultural Science*, *46*(2), 647–670. https://doi.org/10.47836/pjtas.46.2.16

Samira., Mohamed, M., Fayed, T. A., Hussein, A. M., & Maged, S. M. (2014). Effect of some pruning applications on the leaf-to-fruit ratio, yield, and fruit quality Florida "Prince" peach trees. *Journal of Horticultural Science and Ornamental Plants*, *6*(1), 18–26. https://doi.org/10.5829/idosi.jhsop.2014.6.1.1134

Santanoo, S., Vongcharoen, K., Banterng, P., Vorasoot, N., Jogloy, S., Roytrakul, S., & Treerakulpisut, P. (2020). Canopy structure and photosynthetic performance of irrigated cassava genotypes growing in different seasons in a tropical savanna climate. *Agronomy*, *10*(12), 2018. https://doi.org/10.3390/agronomy10122018

Shashi., Garhwal, O. P., Choudhary, M. R., Bairwa, L. N., Kumawat, K. L., Kumar, P., Basile, B., Corrado, G., Rouphael, Y., & Gora, J. S. (2022). Effects of time of pruning and plant bio-regulators on the growth, yield, fruit quality, and post-harvest losses of ber (*Ziziphus mauritiana*). *Horticulturae*, *8*(9), 809. https://doi.org/10.3390/horticulturae8090809

Shen, Q., Wen, Z., Dong, Y., Li, H., Miao, Y., & Shen, J. (2018). The responses of root morphology and phosphorus-mobilizing exudations in wheat to increasing shoot phosphorus concentration. *AoB PLANTS*, *1*(5), ply054. https://doi.org/10.1093/aobpla/ply054

Singh, S. K., Singh, S. K., Sharma, R. R., & Patel, V. B. (2010). Influence of pruning intensity on flowering, fruit yields and floral malformation in three mango cultivars planted under high density. *Indian Journal Horticulture*, *67*, 84-89.

Sofian, M. E., & Susila, A. D. (2018). Increasing cauliflower yields through fertigation. *Journal of Tropical Crop Science*, *5*(1), 1–5. https://doi.org/10.29244/jtcs.5.1.1-5

Susanti, D., Melati, M., & Kurniawati, A. (2022). Identification of secondary metabolite compounds in two varieties of young winged beans (*Psophocarpus tetragonolobus* L .) at two harvest ages. *Journal of Tropical Crop Science*, *9*(1), 52–67.

Susanto, S., Aji, T. G., & Rahayu, A. (2013). The effect of apex pruning on vegetative and generative growth of roselle (*Hibiscus sabdariffa* L.). *Jurnal Hortikultura Indonesia*, *4*(3), 150–156. https://doi.org/10.29244/jhi.4.3.150-156

Taiz, L., & Zeiger, E. (2002). *Plant physiology* (3rd ed.). Sinauer Associates.

Tenorio-Berrío, R., Pérez-Alonso, M. M., Vicente-Carbajosa, J., Martín-Torres, L., Dreyer, I., & Pollmann, S. (2018). Identification of two auxin-regulated potassium transporters involved in seed maturation. *International Journal of Molecular Sciences*, *19*(7), 2132. https://doi.org/10.3390/ijms19072132

Toman, S. S., Al-Taey, D. K. A., Al-Tawaha, A. R., Sirajuddin, S. N., Rasyid, I., & Hassan, A. A. A.-H. (2020). Effect of foliar application and mineral fertilizer on growth parameters and content auxins, GA and CK in cucumber leaves. In *IOP Conference Series: Earth Environonment Science* (Vol. 492 No. 1, p. 012009). IOP Publishing. https://doi.org/10.1088/1755-1315/492/1/012009

Tsegaye, A., & Struik, P. C. (2000). Influence of repetitive transplanting and leaf pruning on dry matter and food production of enset (*Ensete ventricosum* Welw. (Cheesman)). *Field Crops Research*, *68*(1), 61–74. https://doi.org/10.1016/S0378-4290(00)00111-8

Valdes-Rodriguez, O. A., Gomez-Zuniga, I., Palacios-Wassenaar, O. M., Quintas, G. S., & Perez-Vazquez, A. (2020). Effect of pruning and organic fertilization over productive parameters of four Mexican provenances of *Jatropha curcas* L. *Emirates Journal of Food and Agriculture*, *32*(8), 567–576. https://doi.org/10.9755/ejfa.2020.v32.i8.2133

Yan, B., Zhang, Y., Zhang, X., Wang, S., Cui, J., Sun, K., Wang, T., Kang, C., Sun, J., Ge, Y., Guo, L., & Wang, W. (2021). Effects of carbon, nitrogen, phosphorus, and potassium on flowering and fruiting of *Glycyrrhiza uralensis*. *bioRxiv*, 2021-01. https://doi.org/10.1101/2021.01.23.427937

Yin, J.-D., Hou, H.-Z., Zhang, X.-C., Wang, H.-L., Yu, X.-F., Fang, Y.-J., Ma, Y.-F., Zhang, G.-P., & Lei, K.-N. (2020). Effects of organic fertilizer application on flag leaf C/N ratio, photosynthetic characteristics and yield of spring wheat with full plastic film mulching. *Chinese Journal of Applied Ecology*, *31*(11), 3749–3757. https://doi.org/10.13287/j.1001-9332.202011.029

Yong, J. W. H., Ng, Y. F., Tan, S. N., & Chew, A. Y. L. (2010). Effect of fertilizer application on photosynthesis and oil yield of *Jatropha curcas* L. *Photosynthetica*, *48*(2), 208–218. https://doi.org/10.1007/s11099-010-0026-3

Zahran, H. H. (1999). *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol Molecular Biology Rev*iew, *63*(4), 968–989. https://doi.org/10.1128/mmbr.63.4.968-989.1999

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Evaluation of Fermented Plant Extracts as Bioinsecticides in Controlling *Phenacoccus solenopsis* Colonies on *Hibiscus rosa-sinensis* under Laboratory Conditions

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ABSTRACT

The mealybug, *Phenacoccus solenopsis*, is a serious pest of *Hibiscus rosa-sinensis*. The waxy coating on its body may hinder pesticide penetration, and the extensive use of pesticides is risky to humans and the environment. Considering these drawbacks, fermented plant extracts (FPEs) were explored for their potential use in controlling this pest in a more user and environmentally-friendly manner. FPEs derived from eleven plant materials were evaluated against *P. solenopsis* for their insecticidal activity, mealybug wax removal potential and phytotoxicity effect on *H. rosa-sinensis*. Five concentrations of FPE [5, 10, 15, 20, and 25% (w/v)] were prepared. Among the 11 FPEs, FPE derived from ficus, kaffir lime, and turmeric were effective in suppressing *P. solenopsis* with lethal concentration at 50% (LC_{50} value) less than 20% concentration. Although ficus FPE was the top performer in the insecticidal assay, it induced medium to very high levels of leaf damage after being treated with 15–25% concentration at 24 and 72 hr post-treatment. A low level of leaf damage was observed in treatment with turmeric and kaffir lime FPEs at 72 hr post-treatment. FPEs could remove wax from the body of *P. solenopsis* with no significant difference among them. In conclusion, the FPE of ficus, kaffir lime, and turmeric showed promising insecticidal effects against *P. solenopsis*.

Keywords: Fermented plant extract, *Hibiscus rosa-sinensis*, *Phenacoccus solenopsis*, phytotoxicity

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INTRODUCTION

Phenacoccus solenopsis is a destructive polyphagous pest that has infested over 154 plant species belonging to 53 families, including 25 shrubs and trees, 20 field and horticultural crops, 45 ornamentals, and 64 weeds (Alam et al., 2011). *Phenacoccus solenopsis* reproduces sexually and completes many generations in a year. It has been reported as a serious pest of *Hibiscus rosa-sinensis* in Pakistan, India, Nigeria, and Malaysia (Al-obaidy et al., 2017). It was recorded for the first time on *H. rosa-sinensis* in Malaysia in 2016 (Sartiami et al., 2016). The infested *H. rosa-sinensis* showed malformation of buds, shortened twigs and branches, stunted shoots, or wrinkled young leaves. *Phenacoccus solenopsis* did not cause severe economic loss in the country. However, it incurred 1.3 million bales of losses in cotton production in Pakistan (Abdullah, 2009) and 14.9–53.6% yield losses in India (Kumar et al., 2014). It is a threat to agricultural and horticultural industries. Synthetic chemicals are effective for the control of *P. solenopsis*. However, the frequent and indiscriminate application of chemical insecticides has led to a resurgence of new pests, insects resistant to insecticides (Omarini et al., 2020), destruction of beneficial non-target organisms (parasitoids and predators), hazardous effects on human beings, food and water contamination, and negative impact on biological diversity (Khaliq et al., 2012). The waxy coating on their body may hinder the penetration of insecticides and make it challenging to manage the mealybugs efficiently (Badshah et al., 2015). Considering these drawbacks, alternate options need to be explored to control this pest in a more user-friendly and environmentally friendly manner.

Fermented plant extract (FPE) has been evaluated on aphids, whiteflies (Nzanza & Mashela, 2012), 28-spotted beetles (Baloc & Bulong, 2015), cutworm (Sahayaraj et al., 2011), mosquitoes, flies, rats, cockroaches (Neupane & Khadka, 2019), and citrus mealybug (Khadem et al., 2022). In India, FPEs were used to control the pests of onion, brinjal, and sugarcane occasionally (Sahayaraj et al., 2011). They are produced by natural fermentation of plant materials with sugar and water. It is believed that the byproducts of FPE, which consist of acetic acid, "vinegar", alcohol, and propionic acid, contain insecticidal potentiality (Nazim & Meera, 2017; Neupane & Khadka, 2019). These byproducts seem to provide a commercial option to replace conventional chemical control and could overcome insecticide resistance. FPE has been proven to cause mortality in *Planococcus citri* under laboratory conditions and to be able to remove wax from the body of *P. citri* (Khadem et al., 2022). However, Nazim and Meera (2017) reported that the byproducts of FPE, such as acetic acid, "vinegar", alcohol, and propionic acid, could induce a phytotoxicity effect on plant leaves. The severity of the phytotoxicity effect of a particular FPE depends on the plant material used in preparing the FPE. There is no report on the potential use of FPE against *P. solenopsis*. Therefore, this study was conducted to evaluate the

insecticidal potential of different FPEs against *P. solenopsis* as well as their wax removal efficiency on *P. solenopsis*. Since the byproduct of FPEs may cause certain degrees of leaf damage to the test plant, the phytotoxicity level of different FPEs was conducted on *H. rosa-sinensis* to ensure optimum use of FPEs for effective control of *P. solenopsis*.

MATERIALS AND METHODS

Insect

Phenacoccus solenopsis were collected from infested *H. rosa-sinensis* in the surroundings of Universiti Putra Malaysia (UPM, latitude of 03°00 N′, longitude 101°72′ E and altitude of 64 m a.s.l.), Selangor, Malaysia. Mealybug specimens were collected under field conditions at 28–32°C and 60–65% relative humidity. Infested twigs and leaves were brought to the Laboratory of Insect Pathology, UPM, for mass rearing. Green okra B501 and sprouted Granola potatoes were used for rearing *P. solenopsis* (Badshah et al., 2015). They were purchased from the local markets and dipped into 0.1% (v/v) sodium hypochlorite (Clorox®, Malaysia) for 10 min, followed by washing with distilled water (dH_2O) and air drying at room temperature. Okra and potatoes were put into plastic aquariums after air drying. The potatoes were sprinkled with water daily to encourage sprouting. When the sprouts reached 5–7 cm in length, they were used for rearing *P. solenopsis*. The females of *P. solenopsis* were collected gently from the infested plant parts of *H. rosa-sinensis* with a camel hairbrush and then released onto the sprouted potatoes and green okra to generate an F_1 population (Figure 1). The mealybugs were maintained at room temperature of 25±2°C and relative humidity (RH) of 65±5%.

(A) (B) (C)

Figure 1. *Phenacoccus solenopsis* sampled from infested (A) *Hibiscus rosa-sinensis*, (B) reared on green okra and (C) sprouted potatoes

Plant Materials and Fermentation

A total of 11 plant species, namely peppermint (*Mentha piperita*), Mexican mint (*Plectranthus amboinicus*), variegated mint (*Plectranthus madagascariensis*), onion bulb (*Allium cepa*), turmeric rhizome (*Curcuma longa*), aromatic ginger (*Kaempferia galanga*), kaffir lime (*Citrus hystrix*), lime (*Citrus aurantiifolia*), garlic (*Allium sativum*), mahogany (*Swietenia*

mahagoni), and ficus (*Ficus hispida*) were used in this study (Table 1). These plant materials were selected based on their insecticidal activity and availability in the study area. Peppermint, onion bulb, turmeric rhizome, kaffir lime, lime, and garlic were purchased from the local markets located nearby UPM. Other plants were cultivated in UPM. Plant materials were subjected to a 5 min surface sterilisation with 0.1% (v/v) sodium hypochlorite (Clorox®, Malaysia), followed by rinsing twice with dH_2O and air-drying at room temperature. The plant materials were chopped with a sterile sharp knife on a chopping board, placed into airtight plastic containers, and mixed with molasses and dH_2O at 3:1:10 (w:v:v). A quarter of the container was kept vacant, and the lid was closed tightly. The containers were stored in a dry, cool, and shady place. The lid of the containers was untightened once a week to release the air generated in the containers during fermentation. The containers were shaken gently every week. After three months of fermentation, the FPEs were filtered with a Whatman No. 1 filter paper placed on a Falcon® 50 ml high-clarity polypropylene centrifuge tube. The filtrate was kept at -80°C overnight prior to freeze-drying at -110°C for approximately 3 days. The filtrate was then stored at -20°C.

Table 1
List of plant materials used in the preparation of fermented plant extracts

Common name	Scientific name	Family	Part	References
Peppermint	*Mentha piperita*	Lamiaceae	Leaf	Karamaouna et al. (2013)
Variegated mint	*P lectranthus madagascariensis*	Lamiaceae	Leaf	Matias et al. (2019)
Mexican mint	*Plectranthus amboinicus*	Lamiaceae	Leaf	Arumugam et al. (2016)
Onion	*Allium cepa*	Lilliaceae	Bulb	Gharsan et al. (2018)
Turmeric	*Curcuma longa*	Zingiberaceae	Rhizome	de Souza Tavares et al. (2016)
Aromatic ginger	*Kaempferia galanga*	Zingiberaceae	Leaf	Liu et al. (2014)
Lime	*Citrus aurantiifolia*	Rutaceae	Fruits	Bilal et al. (2012); Karamaouna et al. (2013)
Kaffir lime	*Citrus hystrix*	Rutaceae	Fruits	Loh et al. (2011)
Garlic	*Allium sativum*	Lilliaceae	Clove	Okolle et al. (2018)
Mahogany	*Swietenia mahagoni*	Meliaceae	Leaf	Yasmin et al. (2017)
Ficus	*Ficus hispida*	Moraceae	Leaf	Ahmadi et al. (2012)

Insecticidal Assay

The insecticidal effect of FPEs was tested against the third instar nymph of *P. solenopsis* at the Laboratory of Insect Pathology, UPM, following the method of Mostafa et al. (2018) with some modifications. Petri dishes of 9 cm diameter were used in this experiment. The chemical-free, disease-free, and non-infested leaves of *H. rosa-sinensis* were collected and disinfected prior to the experiment. Leaves with a diameter of 9 cm were selected, and the petiole of each leaf was wrapped with wet cotton wool and aluminium foil to keep the leaves turgid for a longer period. A single leaf was placed in a Petri dish. A moist filter paper was put under the leaf in each Petri dish. A total of 10 *P. solenopsis* (third instar) were released onto each leaf with a soft camel hairbrush. The FPE was diluted with distilled water into 5, 10, 15, 20, and 25% (w/v) using the $C_1V_1 = C_2V_2$ formula where C_1 and C_2 are the concentration of the first and second solution, and V_1 and V_2 are the volume of the first and second solution, respectively (Khadem et al., 2022).

The FPEs were applied to the mealybugs and left with a hand atomiser (250 µl/replication). Treatment with dH_2O served as the negative control. A commercial botanical insecticide (A-Force, Malaysia) containing 0.1% citrus extract and aqua solvent was used as the positive control. After spraying, the Petri dishes were covered with a fine muslin cloth to provide ventilation to *P. solenopsis* and prevent them from escaping. The petri dishes were then covered with a lid. The bioassay was performed under a completely randomised design (CRD) with 10 replications, maintained under temperature at 25±2°C and relative humidity of 65±5%. The mortality of *P. solenopsis* was recorded at 24, 48, 72, 96, and 120 hr post-treatment. *Phenacoccus solenopsis* was considered dead if they did not move their legs after gentle probing with a camel hairbrush for 2 s under Dino-Lite eye microscope 2.0 (Ganjisaffar et al., 2019). The percentage of mortality was calculated using the following formula (Henderson & Tilton, 1955):

$$\% \text{ Corrected mortality} = (1 - T_a \times C_b / T_b \times C_a) \times 100\% \quad [1]$$

where T_a is the number of alive insects after treatment in the treated area, T_b is the number of alive insects before treatment in the treated area, C_a is the number of alive insects after treatment in the control area, and C_b is the number of alive insects before treatment in the control area.

Phytotoxicity Assay

The phytotoxicity test used chemical-free, disease-free, and non-infested *H. rosa-sinensis* leaves. Leaves were rinsed with 0.1% (v/v) sodium hypochlorite (Clorox®, Malaysia) for 5 min, followed by rinsing with dH_2O twice and then air-dried prior to the experiment. Each leaf petiole was wrapped with wet cotton wool and aluminium foil to keep the leaf turgid during the experiment. The leaves were dipped in five different concentrations [5, 10, 15, 20, and 25% (w/v)] of FPEs for 30 s, and the negative control

was treated with distilled water only. For each concentration, ten replications were used, and the treated leaves were observed for the presence of leaf discolouration or formation of necrotic spots on the leaf at 24 and 72 hr post-treatment. Equation 2 calculated the percentage of damaged leaves (Sreerag & Jayaprakas, 2014). The severity of the leaf damage was graded using the rating scale in Table 2.

Table 2
Phytotoxicity rating scale

Level	% Leaf damage*	Severity
0	<1	No
1	1–10	Low
2	11–20	Moderate
3	21–30	High
4	31–40	Very high
5	41–50	Severe
6	>50	Very severe

Note. *The leaf damage percentage was calculated based on the damaged area over the total surface leaf area x 100%

Mealybug Wax Removal Test

The experiment was carried out in CRD with ten replications under laboratory conditions. At first, 10 adults of *P. solenopsis* of similar body size were weighed (weight before treatment = W_0) for each treatment. A hand atomiser sprayed an equal volume of FPEs (250 µl/replication) on the adults. Mealybugs sprayed with dH_2O served as a negative control, while mealybugs sprayed with 100% (v/v) chloroform (Sigma-Aldrich, USA) served as a positive control. At 72 hr post-treatment, the body weight of adults (W_T) was taken. The adults were then dipped in 100% (v/v) chloroform (Sigma-Aldrich, USA) for 60 s in a beaker to harvest the remaining undetached wax on the body of *P. solenopsis*. A syringe filter removed the dirt in the dissolved wax. The dissolved wax was harvested by drying at 37°C for 24 hr in an incubator (Salunkhe et al., 2013). The weight of the dissolved wax (W_{DW}) was measured. The amount of detached mealybug wax was calculated using Equation 3.

where W_0 is the weight of mealybugs before treatment, W_T is the weight of mealybugs after treatment, and W_{DW} is the weight of dissolved wax extracted from FPE-treated mealybugs by chloroform.

Statistical Analysis

Recorded data were subjected to analysis of variance using the statistical program SAS® software (version 9.4) and means comparison using Tukey's studentised range test. The LC_{50} value of each FPE was determined using POLO-Plus (version 0.03).

RESULTS AND DISCUSSION

Insecticidal Assay

The present study demonstrated the insecticidal activity of 11 FPEs derived from peppermint (*M. piperita*), Mexican mint (*P. amboinicus*), variegated mint (*P. madagascariensis*), onion bulb (*A. cepa*), turmeric rhizome (*C. longa*), aromatic ginger (*K. galanga*), kaffir lime (*C. hystrix*), lime (*C. aurantiifolia*), garlic (*A. sativum*),

mahogany (*S. mahagoni*), and ficus (*F. hispida*) against *P. solenopsis* (Table 3). The result revealed that the mortality of *P. solenopsis* was dependent on FPE concentration and exposure time. There was a gradual increment in the mortality rate of *P. solenopsis* with an increase in the FPE concentration and exposure time. The mortality rate of *P. solenopsis* induced by FPEs was lower than that of A-Force at 5% concentration at 72 hr post-treatment. When the concentration of FPE was increased to 10%, only kaffir lime FPE (42% mortality) was comparable to A-Force treatment (45% mortality). The highest mortality of *P. solenopsis* was observed with ficus FPE (61–74%), while the lowest mortality was observed with garlic FPE (16–28%) at 15–25% FPE concentrations. The onion, turmeric, and kaffir lime FPEs exhibited a higher mortality rate than A-Force at 72 hr post-treatment. The exposure time was further increased to 120 hr, and higher mortality of *P. solenopsis* was recorded in all FPE treatments. Other FPEs, such as mahogany, onion, Mexican mint, and variegated mint, performed better than A-Force at 120 hr post-treatment. FPEs such as peppermint, garlic, lime, and aromatic ginger were not comparable to the A-Force in the *P. solenopsis* control.

The LC_{50} value towards *P. solenopsis* was 12.89% for ficus FPE, followed by 12.87% for kaffir lime FPE, 18.12% for turmeric FPE, 22.23% for onion FPE, 23.99% for mahogany FPE, 30.78% for Mexican mint FPE, 31.38% for variegated mint FPE, 57.85% for peppermint FPE, 68.46% for aromatic ginger FPE, 87.40% for lime FPE, and the least efficacious FPE was garlic (93.82%) recorded at 72 hr post-treatment (Table 4). Similar results were recorded at 120 hr post-treatment, except the onion and peppermint FPEs were less effective when exposed for a longer time to *P. solenopsis*. Among the FPEs tested, ficus FPE exhibited the highest insecticidal action against *P. solenopsis* throughout the exposure time compared to other FPEs. *Ficus* sp. has a broader spectrum of insecticidal and acaricidal properties (Romeh, 2013). It has been proven effective against the fourth instar larvae of *Aedes albopictus* (Wang et al., 2011). The fermented form of ficus showed a very strong and promising insecticidal action to *P. solenopsis* in the present study.

Kaffir lime and lime belong to the Rutaceae family and genus *Citrus*. The essential oil of citrus contains insecticidal properties against *P. ficus* (Karamaouna et al., 2013) and *Spodoptera litura* (Loh et al., 2011). Besides citrus fruit, citrus seed extracts, such as *C. sinensis* and *C. reticulata* (L.), were also effective against *Ae. albopictus* (Bilal et al., 2012). The fermented form of kaffir lime caused significant mortality to *P. solenopsis* in the present study. The results are consistent with those reported by Khadem et al. (2022) that *P. citri* exposed to kaffir lime FPE exhibited a high mortality rate. Although lime belongs to the genus *Citrus*, the fermented form of lime did not show promising control on *P. solenopsis* in the present study. It required more than 6 times higher FPE concentration

than kaffir lime FPE to cause 50% mortality in *P. solenopsis* at 72 hr post-treatment. Khadem et al. (2022) also reported poor insecticidal activity of fermented lime extract against *P. citri*.

The essential oil of turmeric contains pesticidal activity against insects and weeds (de Souza Tavares et al., 2016). Natural compounds such as arturmerone could induce insect mortality (de Souza Tavares et al., 2016). In the present study, the insecticidal activity of turmeric FPE is comparable with those of ficus and kaffir lime which required less than 20% FPE concentration to kill 50% mealybugs at 72 hr post-treatment.

In the present study, onion FPE revealed its capability of inducing high mortality in *P. solenopsis*. Other mealybugs, such as *P. citri*, were also reported to be susceptible to onion FPE (Khadem et al., 2022). Onion, in the form of essential oil, had shown its potential insecticidal activity against *Ae. aegypti* (Susheela et al., 2016) and *Oryzaephilus surinamensis* (Gharsan et al., 2018). Some previous studies have confirmed that 10% aqueous solution of onion oil could inhibit the hatching of embryonated eggs and induce acaricidal activity in all stages of *Boophilus annulatus* at more than 5% (v/v) concentrations (Aboelhadid et al., 2013).

Mahogany has been proven effective in controlling aphids in essential oil (Yasmin et al., 2017) and termites in the form of leaves, seeds, and bark extract (Cruz et al., 2018). The mahogany FPE tested in the present study also demonstrated good insecticidal activity against *P. solenopsis*. Other FPEs such as Mexican mint, variegated mint, and peppermint demonstrated 31–46% mortality to *P. solenopsis* at 72 hr post-treatment. They are members of the *Mentha* family. The results of this *Mentha* spp. are consistent with those reported previously that *Mentha* spp. could inhibit the growth and reproduction of some important pests, namely *Planococcus ficus* (Karamaouna et al., 2013), *Ferrisia virgata* (El-Ashram et al., 2020), and *P. citri* (Khadem et al., 2022). Among the *Mentha* spp. tested, Mexican mint and variegated mint FPEs exhibited less than 50% mortality, whereas peppermint FPE caused less than 35% mortality on *P. solenopsis* at 72 hr post-treatment. Khadem et al. (2022) reported that the Mexican mint FPE had the best FPE (LC_{50} = 1.83%) against *P. citri*; however, the Mexican mint FPE was not promising against *P. solenopsis* in the present study. Different insects may have different hydrophilic-hydrophobic cuticle structures that could influence the penetration of an FPE into an insect's body (Alotaibi et al., 2022). Although garlic extract has been demonstrated to be toxic to the long-tailed mealybugs on banana plants (Okolle et al., 2018), the garlic FPE showed the least insecticidal activity to *P. solenopsis* (LC_{50} value higher than 60%) in the present study.

Phytotoxicity Effect of FPEs on *H. rosa-sinensis* Leaves

Results presented in Table 5 indicated different levels of phytotoxicity of FPEs on *H. rosa-sinensis* leaves. Turmeric, kaffir lime, and ficus FPEs were selected for

Table 3
Mortality of Phenacoccus solenopsis *after treatment with fermented plant extracts (FPEs) at 72 and 120 hr post-treatment. Values are expressed in mean ± S.E*

FPE	% Mean mortality of *P. solenopsis*									
	72 hr					120 hr				
	5%	10%	15%	20%	25%	5%	10%	15%	20%	25%
Peppermint	5 ± 1.67^{ef}	15 ± 1.67^{ef}	16 ± 1.63^{g}	23 ± 2.13^{g}	31 ± 2.77^{gh}	11 ± 2.33^{ef}	33 ± 2.60^{cd}	40 ± 2.11^{e}	45 ± 1.67^{e}	53 ± 3.00^{e}
Mexican mint	15 ± 2.23^{de}	21 ± 2.77^{def}	25 ± 2.24^{efg}	45 ± 3.41^{bcd}	46 ± 3.39^{def}	29 ± 3.48^{bcd}	33 ± 3.00^{cd}	44 ± 3.05^{de}	67 ± 3.00^{b}	71 ± 3.14^{c}
Variegated mint	6 ± 2.21^{ef}	20 ± 1.49^{def}	32 ± 1.33^{def}	36 ± 2.21^{def}	40 ± 3.65^{efgh}	12 ± 2.49^{ef}	33 ± 2.13^{cd}	56 ± 2.67^{bcd}	65 ± 2.23^{b}	69 ± 1.79^{cd}
Onion	8 ± 2.00^{ef}	22 ± 2.00^{cdef}	38 ± 2.49^{bcd}	40 ± 2.58^{de}	58 ± 2.91^{bcd}	17 ± 2.13^{de}	29 ± 3.48^{d}	58 ± 2.49^{bc}	62 ± 2.00^{bcd}	71 ± 2.77^{c}
Garlic	9 ± 1.79^{def}	12 ± 1.33^{f}	16 ± 2.21^{g}	22 ± 3.59^{g}	28 ± 3.89^{h}	26 ± 2.21^{cd}	29 ± 1.79^{d}	40 ± 2.98^{e}	42 ± 2.49^{e}	51 ± 3.14^{e}
Turmeric	19 ± 1.79^{cd}	21 ± 2.33^{def}	47 ± 4.23^{bc}	53 ± 3.00^{abc}	63 ± 3.67^{abc}	25 ± 2.68^{cd}	32 ± 4.16^{cd}	59 ± 3.48^{bc}	69 ± 2.33^{b}	72 ± 2.00^{c}
Kaffir lime	32 ± 2.00^{b}	42 ± 2.91^{ab}	48 ± 3.26^{b}	56 ± 2.21^{ab}	66 ± 2.21^{ab}	35 ± 2.23^{bc}	49 ± 3.48^{ab}	62 ± 2.49^{b}	74 ± 3.05^{a}	86 ± 3.39^{ab}
Lime	10 ± 1.49^{def}	22 ± 1.33^{cdef}	22 ± 1.33^{fg}	23 ± 1.52^{g}	28 ± 2.00^{h}	28 ± 3.26^{bcd}	38 ± 2.49^{bcd}	43 ± 2.60^{e}	50 ± 2.11^{de}	57 ± 1.53^{de}
Aromatic ginger	12 ± 2.00^{de}	16 ± 2.21^{ef}	23 ± 2.13^{fg}	25 ± 1.67^{fg}	39 ± 5.04^{efgh}	22 ± 3.59^{de}	35 ± 2.68^{bcd}	43 ± 2.60^{e}	51 ± 3.14^{cde}	57 ± 3.35^{de}
Mahogany	11 ± 3.14^{de}	32 ± 2.49^{bc}	36 ± 2.67^{cde}	43 ± 3.00^{cd}	51 ± 2.33^{cde}	26 ± 3.39^{cd}	55 ± 3.07^{a}	63 ± 3.00^{b}	64 ± 2.21^{b}	75 ± 1.67^{bc}
Ficus	26 ± 2.20^{bc}	30 ± 2.10^{dc}	61 ± 2.33^{a}	62 ± 2.00^{a}	74 ± 1.63^{a}	40 ± 1.49^{ab}	46 ± 2.67^{abc}	76 ± 3.05^{a}	86 ± 1.63^{a}	90 ± 2.11^{a}
A-Force (Standard)	45 ± 3.07^{a}	45 ± 3.07^{a}	45 ± 3.07^{bc}	45 ± 3.07^{bcd}	45 ± 3.07^{defg}	48 ± 2.00^{a}	48 ± 2.00^{ab}	48 ± 2.00^{cde}	48 ± 2.00^{e}	48 ± 2.00^{e}
Water (control)	0 ± 0^{f}	0 ± 0^{g}	0 ± 0^{h}	0 ± 0^{h}	0 ± 0^{i}	0 ± 0^{f}	0 ± 0^{e}	0 ± 0^{f}	0 ± 0^{f}	0 ± 0^{f}

Note. Mean of ten replications. Original values given in a column mean followed by the same letter are not significantly different at $P < 0.001$ as per Tukey's studentised range test

Table 4
Median lethal concentration (LC_{50}) of fermented plant extracts (FPEs) against third-instar nymphs of Phenacoccus solenopsis

FPE	72 hr post-treatment		120 hr post-treatment	
	LC_{50}* (95% C. L.) (%)	χ^2	LC_{50}* (95% C. L.) (%)	χ^2
Ficus	12.89 (11.175–14.813)	17.87	7.75 (6.49–8.87)	25.17
Kaffir lime	13.87 (11.037–17.832)	13.89	9.02 (7.47–10.42)	24.60
Turmeric	18.12 (15.686–21.769)	26.66	10.16 (8.95–14.37)	24.67
Onion	22.23 (19.279–27.103)	20.04	14.28 (12.68–16.14)	19.20
Mahogany	23.99 (19.770–32.635)	20.65	10.35 (8.56–12.06	18.50
Mexican mint	30.78 (23.789–49.306)	23.67	13.69 (11.68–16.13)	30.16
Variegated mint	31.38 (24.972–46.641)	15.98	14.43 (12.92–16.18)	14.38
Peppermint	57.85 (37.910–149.881)	15.73	21.87 (18.38–28.31)	18.86
Aromatic ginger	68.46 (38.852– 96.331)	17.56	19.20 (15.72–25.88)	19.95
Lime	87.40 (45.201–158.303)	7.57	19.31 (15.0–29.756)	12.67
Garlic	93.82 (47.713–172.101)	23.28	28.73 (20.39–66.11)	14.67

Note. * Lethal concentration killing 50% of the exposed adult population; C. L. = Confidence Level

phytotoxicity study based on their low LC_{50} value in the insecticidal assay. The severity of the leaf damage increased with exposure time and concentration of FPE. Among the FPEs tested, the turmeric and kaffir lime showed low levels of phytotoxicity to *H. rosa-sinensis* leaves after treatment with 20–25% FPE at 72 hr post-treatment. Although ficus FPE showed the highest potential in the insecticidal assay, it induced medium to very high leaf damage after treatment with 15–25% FPE at 24 and 72 hr post-treatment. A lower concentration of ficus and kaffir lime at the proposed LC_{50} value showed a very low percentage of leaf damage (<5%) at 72 hr post-treatment. De Martino et al. (2010) and Vokou et al. (2003) reported that monoterpenes and oxygenated compounds (ketones, alcohols, aldehydes, and phenols) accredited to ficus seem to be responsible for phytotoxicity activity. Khadem et al. (2022) also documented different levels of leaf damage caused by different fermented plant materials on citrus leaves. According to Mousavi et al. (2021), the phytotoxic potential of a plant varies with plant species and the concentration of an extract. The level of leaf damage could be induced by chemical, botanical, or biological pesticides; therefore, a phytotoxicity assessment is needed prior to registering a plant material as a pesticide (Karamaouna et al., 2013). The phytotoxic effect of FPEs on *H. rosa-sinensis* leaves is shown in Figure 2.

Table 5
Phytotoxic effect of fermented plant extracts (FPEs) on Hibiscus rosa-sinensis *leaves*

Treatment	% Leaf damage									
	24 hr					72 hr				
	5%	10%	15%	20%	25%	5%	10%	15%	20%	25%
Turmeric FPE	0^{a} (N)	0^{b} (N)	0^{b} (N)	0^{b} (N)	0^{b} (N)	0^{a} (N)	0^{b} (N)	0.5^{b} (N)	1^{b} (L)	1^{b} (L)
Kaffir lime FPE	0^{a} (N)	0^{b} (N)	0^{b} (N)	0^{b} (N)	0^{b} (N)	0^{a} (N)	0^{b} (N)	0^{b} (N)	0.7^{b} (N)	1^{b} (L)
Ficus FPE	1^{a} (L)	2^{a} (L)	7^{a} (L)	12^{a} (M)	22^{a} (H)	1^{a} (L)	5.5^{a} (L)	12^{a} (M)	32^{a} (VH)	34^{a} (VH)
Water	0^{a} (N)	0^{b} (N)	0^{b} (N)	0^{b} (N)	0^{b} (N)	0^{a} (N)	0^{b} (N)	0^{b} (N)	0^{b} (N)	0^{b} (N)

Note. Mean of ten replications. Original values given in a column mean followed by the same letter are not significantly different at $P < 0.001$ as per Tukey's studentised range test. The value in parenthesis indicates grade of leaf damage: N = No; L = Low; M = Moderate; H = High; VH = Very high

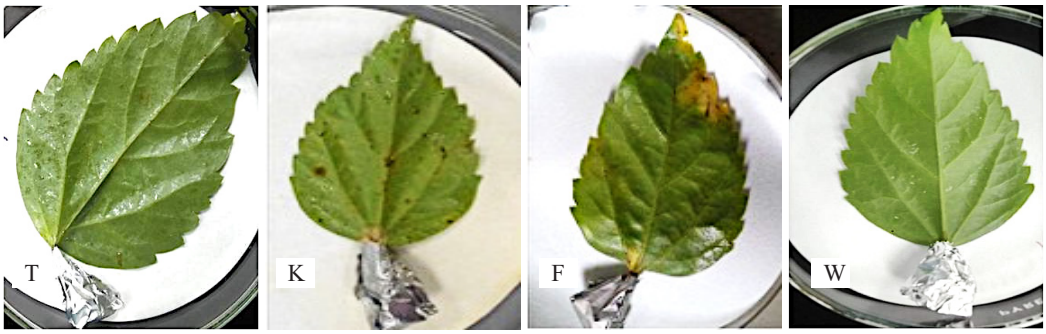

Figure 2. Phytotoxicity effect of fermented plant extract (FPE) on *Hibiscus rosa-sinensis* leaves at 72 hr post-treatment

Note. T = Turmeric FPE, K = Kaffir lime FPE, F = Ficus FPE, W = Distilled water

Effect of FPEs on Mealybug Wax

The turmeric, ficus, and kaffir lime FPEs tested in the present study were able to remove the wax from the body of *P. solenopsis*, along with their insecticidal potential (Figure 3). The percentage of wax removal increased with the concentration of FPEs. There was no significant difference among the test FPEs except the FPE of kaffir lime at 20% concentration. An ascending order of the percentage of mealybug wax removal was observed when higher concentrations of FPE were applied. Overall, FPE of turmeric, kaffir lime, and ficus below 20% could remove less than 50% of mealybug wax (44.16, 33.48, and 41.95%, respectively) compared to 96.58% of mealybug wax removed by chloroform. Chloroform has been reported to dissolve plants' epicuticular wax (Loneman et al., 2017) and mealybug wax (Salunkhe et al., 2013). The FPEs tested in the present study had shown their wax-removing potential in the mealybug wax of *P. solenopsis* (Figure

4). Mealybugs have a waxy layer on their body that protects them from the penetration of insecticides. Removing wax from the insect cuticle could cause dehydration of the membrane cells, which may result in the death of the insects (Regnault-Roger et al., 2012). Consequently, removing wax from their body will allow better penetration of the FPEs and cause death to the mealybugs. Similar findings have been reported by Khadem et al. (2022), who had proven the capability of Mexican mint FPE to remove the wax from *P. citri*.

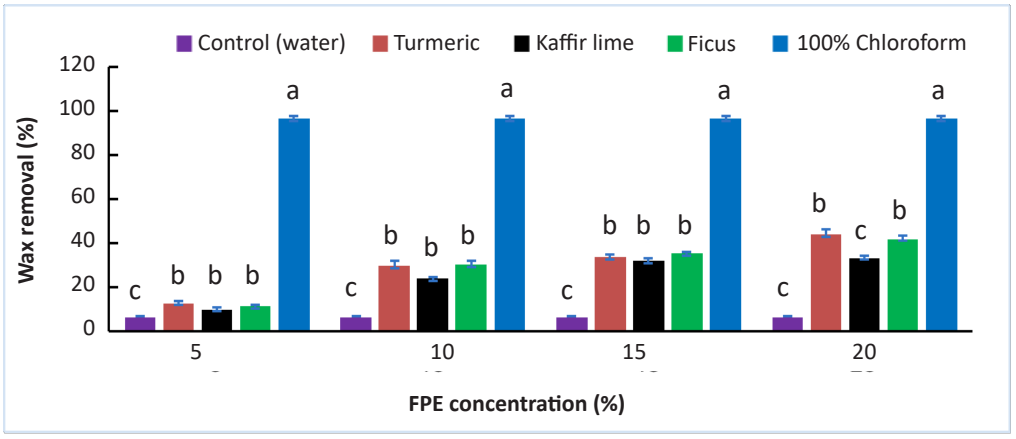

Figure 3. Percentage of *Phenacoccus solenopsis* wax removal (%) by different fermented plant extract (FPE) concentrations (%) at 72 hr post-treatment. Similar letters on bars corresponding to the similar response indicate no significant variation among the FPEs according to Tukey's test ($P < 0.001$). The vertical line on top of the bars corresponds to the standard error

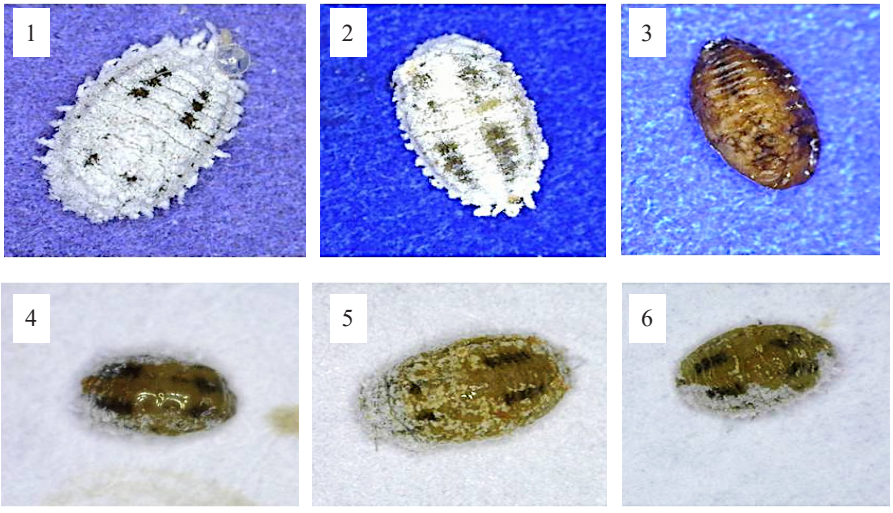

Figure 4. Effect of fermented plant extracts (FPEs) on the mealybug wax. 1) Untreated mealybug, 2) water-treated mealybug, 3) chloroform-treated mealybug, 4) turmeric FPE-treated mealybug, 5) ficus FPE-treated mealybug, and 6) kaffir lime FPE-treated mealybug

CONCLUSION

The present findings revealed the potentiality of FPEs in the control of *P. solenopsis*. There was variation in the mortality of *P. solenopsis* in response to different FPEs. The FPEs derived from ficus, kaffir lime and turmeric were effective in suppressing the *P. solenopsis* colonies on *H. rosa-sinensis* with an LC_{50} value of less than 20% (w/v). FPEs were also able to remove less than 50% of the mealybug wax, exposing the mealybugs to greater FPE penetration and resulting in the death of the mealybug. Selecting FPE with appropriate concentration for controlling *P. solenopsis* is crucial to avoid phytotoxic impact on plant growth. This study was conducted on a laboratory scale; thus, further studies may be required to evaluate the efficacy of FPEs under field conditions.

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REFERENCES

Aboelhadid, S. M., Kamel, A. A., Arafa, W. M., & Shokier, K. A. (2013). Effect of *Allium sativum* and *Allium cepa* oils on different stages of *Boophilus annulatus*. *Parasitology Research*, *112*, 1883–1890. https://doi.org/10.1007/s00436-013-3344-0

Abdullah, A. (2009). Analysis of mealybug incidence on the cotton crop using ADSS-OLAP (Online Analytical Processing) tool. *Computers and Electronics in Agriculture*, *69*(1), 59-72. https://doi.org/10.1016/j.compag.2009.07.003

Ahmadi, M., Amiri-Besheli, B., & Hosieni, S. Z. (2012). Evaluating the effect of some botanical insecticides on the citrus mealybug *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae). *African Journal of Biotechnology*, *11*(53), 11620–11624. https://doi.org/10.5897/AJB11.4226

Al-Obaidy, S. H., Abdul-Rassoul, M. S., & Jaafar, N. A. (2017). Study of some biological parameters of mealybug *Phenacoccuc solenopsis* (Tinsley) (Hemiptera: Pseudococcidae) as an exotic pest on *Hibiscus rosa-sinensis* in Iraq. *IOSR Journal of Agriculture and Veterinary Science*, *10*(10), 25-28.

Alam, S. K. F., Seni, A., & Sahoo, A. K. (2011). Biology of the mealybug, *Phenacoccus solenopsis* Tinsley (Pseudococcidae: Hemiptera) on sprouted potato and brinjal plant. *The Journal of Plant Protection Sciences*, *3*(2), 32–36.

Alotaibi, S. S., Darwish, H., Alzahrani, A. K., Alharthi, S., Alghamdi, A. S., Al-Barty, A. M., Helal, M., Maghrabi, A., Baazeem, A., Alamari, H. A., & Noureldeen, A. (2022). Environment-friendly control potential of two citrus essential oils against *Aphis punicae* and *Aphis illinoisensis* (Hemiptera: Aphididae). *Agronomy*, *12*(9), 2040. https://doi.org/10.3390/agronomy12092040

Arumugam, G., Swamy, M. K., & Sinniah, U. R. (2016). *Plectranthus amboinicus* (Lour.) Spreng: Botanical, phytochemical, pharmacological and nutritional significance. *Molecules*, *21*(4), 369. https://doi.org/10.3390/molecules21040369

Badshah, H., Ullah, F., Farid, A., Calatayud, P. A., & Crickmore, N. (2015). Toxicity of neem seed *Azadirachta indica* Juss (Meliaceae) different solvents extracts against cotton mealybug *Phenacoccus solenopsis* Tinsley

(Sternorrhyncha: Pseudococcidae) under laboratory conditions. *Journal of Entomology and Zoology Studies*, *3*(4), 45-49.

Baloc, H. A., & Bulong, M. P. (2015). Efficacy of fermented botanical plant extracts in the management of white flies and 28-spotted beetles in tomato. *International Journal of Science and Research*, *4*(7), 2566–2569.

Bilal, H., Akram, W., Khan, H. A. A., Hassan, S. A., & Khan, I. A. (2012). Toxicity of selected indigenous plant extracts against *Aedes albopictus* (Diptera: Culicidae): A potential dengue vector in dengue positive areas. *Pakistan Journal of Zoology*, *44*(2), 371–375.

Cruz, P. H. G., Nunez, R. L., Anulacion, C. A., Gregorio, N. A., Iniwan, K., Nolasco, E. J., Pallarca, R. M., & Waing, K. G. D. (2018). Insecticidal property and phytochemical screening of mahogany (*Swietenia macrophylla* King) leaves, barks and seeds as an alternative insecticide against fungi growing termites [*Macrotermes gilvus* (HAGEN, 1858)]. *International Journal of Biology, Pharmacy and Allied Sciences*, *7*(8), 1521–1528. https://doi.org/10.31032/IJBPAS/2018/7.8.4504

de Martino, L., Formisano, C., Mancini, E., de Feo, V., Piozzi, F., Rigano, D., & Senatore, F. (2010). Chemical composition and phytotoxic effects of essential oils from four *Teucrium* species. *Natural Product Communications*, *5*(12), 1969-1976.

de Souza Tavares, W., Akhtar, Y., Gonçalves, G. L. P., Zanuncio, J. C., & Isman, M. B. (2016). Turmeric powder and its derivatives from *Curcuma longa* rhizomes: Insecticidal effects on cabbage looper and the role of synergists. *Scientific Reports*, *6*, 34093. https://doi.org/10.1038/srep34093

El-Ashram, D., Abd El-Mageed, S. A. M., & Shaaban, A. R. (2020). Potential toxicity of some essential oils on mealybug, *Maconellicoccus hirsutus* (Hemiptera: Pseudococcidae). *Egyptian Journal of Plant Protection Research Institute*, *3*(2), 794–803.

Ganjisaffar, F., Andreason, S. A., & Perring, T. M. (2019). Lethal and sub-lethal effects of insecticides on the pink hibiscus mealybug, *Maconellicoccus hirsutus* (Hemiptera: Pseudococcidae). *Insects*, *10*(1), 31. https://doi.org/10.3390/insects10010031

Gharsan, F., Jubara, N., Alghamdi, L., Almakady, Z., & Basndwh, E. (2018). Toxicity of five plant oils to adult *Tribolium castaneum* (Coleoptera: Tenebrionidae) and *Oryzaephilus surinamensis* (Coleoptera: Silvanidae). *Florida Entomologist*, *101*(4), 592–596. https://doi.org/10.1653/024.101.0420

Henderson, C. F., & Tilton, E. W. (1955). Tests with acaricides against the brown wheat mite. *Journal of Economic Entomology*, *48*(2), 157–161. https://doi.org/10.1093/jee/48.2.157

Karamaouna, F., Kimbaris, A., Michaelakis, A., Papachristos, D., Polissiou, M., Papatsakona, P., & Tsora, E. (2013). Insecticidal activity of plant essential oils against the vine mealybug, *Planococcus ficus*. *Journal of Insect Science*, *13*, 142. https://doi.org/10.1673%2F031.013.14201

Khadem, A. G., Chin, N. L., Mokhtar, A. S., & Lau, W. H. (2022). Potential use of fermented plant extracts as biological control agents for citrus mealy bugs, *Planococcus citri*. *Annals of Agri-Bio Research*, *27*(1), 79–86.

Khaliq, A., Matloob, A., & Riaz, Y. (2012). Bio-economic and qualitative impact of reduced herbicide use in direct seeded fine rice through multipurpose tree water extracts. *Chilean Journal of Agricultural Research*, *72*(3), 350-357.

Kumar, R., Nagrare, V. S., Nitharwal, M., Swami, D., & Prasad, Y. G. (2014). Within-plant distribution of an invasive mealybug, *Phenacoccus solenopsis*, and associated losses in cotton. *Phytoparasitica*, *42*, 311-316. https://doi.org/10.1007/s12600-013-0361-6

Liu, X. C., Liang, Y., Shi, W. P., Liu, Q. Z., Zhou, L., & Liu, Z. L. (2014). Repellent and insecticidal effects of the essential oil of *Kaempferia galanga* rhizomes to *Liposcelis bostrychophila* (Psocoptera: Liposcelidae). *Journal of Economic Entomology*, *107*(4), 1706–1712. https://doi.org/10.1603/EC13491

Loh, F. S., Awang, R. M., Omar, D., & Rahmani, M. (2011). Insecticidal properties of *Citrus hystrix* DC leaves essential oil against *Spodoptera litura* fabricius. *Journal of Medicinal Plants Research*, *5*(16), 3739–3744.

Loneman, D. M., Peddicord, L., Al-Rashid, A., Nikolau, B. J., Lauter, N., & Yandeau-Nelson, M. D. (2017). A robust and efficient method for the extraction of plant extracellular surface lipids as applied to the analysis of silks and seedling leaves of maize. *PLOS One*, *12*(7), e0180850. https://doi.org/10.1371/journal.pone.0180850

Matias, D., Nicolai, M., Fernandes, A. S., Saraiva, N., Almeida, J., Saraiva, L., Faustino, C., Díaz-Lanza, A. M., Reis, C. P., & Rijo, P. (2019). Comparison study of different extracts of *Plectranthus madagascariensis*, *P. neochilus* and the rare *P. porcatus* (Lamiaceae): Chemical characterization, antioxidant, antimicrobial and cytotoxic activities. *Biomolecules*, *9*(5), 179. https://doi.org/10.3390/biom9050179

Mostafa, M. E., Youssef, N. M., & Abaza, A. M. (2018). Insecticidal activity and chemical composition of plant essential oils against cotton mealybug, *Phenacoccus solenopsis* (Tinsley) (Hemiptera: Pseudococcidae). *Journal of Entomology and Zoology Studies*, *6*(2), 539–543.

Mousavi, S. S., Karami, A., Haghighi, T. M., Alizadeh, S., & Maggi, F. (2021). Phytotoxic potential and phenolic profile of extracts from *Scrophularia striata*. *Plants*, *10*(1), 135. https://doi.org/10.3390/plants10010135

Nazim, F., & Meera, V. (2017). Comparison of treatment of greywater using garbage and citrus enzymes. *International Journal of Innovative Research in Science, Engineering and Technology*, *6*(4), 49-54.

Neupane, K., & Khadka, R. (2019). Production of garbage enzyme from different fruit and vegetable wastes and evaluation of its enzymatic and antimicrobial efficacy. *Tribhuvan University Journal of Microbiology*, *6*, 113–118. https://doi.org/10.3126/tujm.v6i0.26594

Nzanza, B., & Mashela, P. W. (2012). Control of whiteflies and aphids in tomato (*Solanum lycopersicum* L.) by fermented plant extracts of neem leaf and wild garlic. *African Journal of Biotechnology*, *11*(94), 16077–16082. https://doi.org/10.5897/AJB12.775

Okolle, J. N., Lum, N. M. C., Ngosong, C., Nanganoa, L. T., Clovis, T., Sakwe, N. P., & Mih, A. M. (2018). Efficacy of four plant emulsions for managing long tail mealybugs (*Pseudococcus longispinus*) infesting banana plantations in Cameroon. *Journal of Entomology and Zoology Studies*, *6*(3), 837–843.

Omarini, A. B., Achimón, F., Brito, V. D., & Zygadlo, J. A. (2020). Fermentation as an alternative process for the development of bioinsecticides. *Fermentation*, *6*(4), 120. https://doi.org/10.3390/fermentation6040120

Regnault-Roger, C., Vincent, C., & Arnason, J. T. (2012). Essential oils in insect control: Low-risk products in a high-stakes world. *Annual Review of Entomology*, *57*, 405–424. https://doi.org/10.1146/annurev-ento-120710-100554

Romeh, A. A. (2013). Phytochemicals from *Ficus sycomorus* L. leaves act as insecticides and acaricides. *African Journal of Agricultural Research*, *8*(27), 3571–3579. https://doi.org/10.5897/AJAR2013.7243

Sahayaraj, K., Kombiah, P., & Kumar, D. S. (2011). Evaluation of insecticidal activity of fermented plant products on *Spodoptera litura* (Fab.). *Indian Journal of Agricultural Research*, *45*(1), 77–82.

Salunkhe, R. B., Patil, C. D., Salunke, B. K., Rosas-García, N. M., & Patil, S. V. (2013). Effect of wax degrading bacteria on life cycle of the pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green) (Hemiptera: Pseudococcidae). *BioControl*, *58*, 535–542. https://doi.org/10.1007/s10526-013-9513-3

Sartiami, D., Watson, G. W., Mohamad Roff, M., & Idris, A. B. (2016). Mealybugs (Hemiptera: Coccomorpha: Pseudococcidae) attacking *Hibiscus rosa-sinensis* L. in Malaysia, with two new country records. In *AIP Conference Proceedings* (Vol. 1784, No. 1, p. 060007). AIP Publishing. https://doi.org/10.1063/1.4966845

Sreerag, R. S., & Jayaprakas, C. A. (2014). Management of two major sucking pests using neem oil formulation. *Journal of Biofertilizers and Biopesticides*, *6*(1), 1000147. https://doi.org/10.4172/2155-6202.1000147

Susheela, P., Radha, R., & Padmapriyanga, S. (2016). Evaluation of larvicidal action of natural extracts on mosquito larvae of *Aedes aegypti* (Diptera: Culicidae). *International Journal of Mosquito Research*, *3*(6), 26–30.

Vokou, D., Douvli, P., Blionis, G. J., & Halley, J. M. (2003). Effects of monoterpenoids, acting alone or in pairs, on seed germination and subsequent seedling growth. *Journal of Chemical Ecology*, *29*(10), 2281-2301. https://doi.org/10.1023/a:1026274430898

Wang, X.-G., Wei, X.-Y., Huang, X.-Y., Shen, L.-T., Tian, Y.-Q., & Xu, H.-H. (2011). Insecticidal constructure and bioactivities of compounds from *Ficus sarmentosa* var. *henryi*. *Agricultural Sciences in China*, *10*(9), 1402–1409. https://doi.org/10.1016/S1671-2927(11)60133-8

Yasmin, M. S., Bachchu, M. A. A., & Hossain, M. A. (2017). Toxic and repellent effects of three botanical oils against adult *Aphis craccivora* Koch. (Homoptera: Aphididae) under laboratory conditions. *University Journal of Zoology Rajshahi University*, *36*, 39–48.

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Microbiological, Phytochemical Constituents, and Antioxidant Properties of Fermented Green Robusta Coffee Beans

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ABSTRACT

Robusta coffee is one of Malaysia's most planted species due to its ability to adapt to the local climate. Nonetheless, the coffee species was perceived as having lower quality and economic value due to bitterness and astringency. It is widely believed that higher caffeine and chlorogenic acid contents in Robusta coffee beans contributed to the unfavourable bitter and astringent flavour. Hence, the present study intends to evaluate the effect of spontaneous wet fermentation (SWF) of locally grown Robusta (*Coffea canephora* L.) coffee towards the microbiological properties, phytochemical constituents, in particular caffeine and chlorogenic acids (CGA), total phenolic content (TPC), and antioxidant properties. The SWF of green Robusta coffee beans from University Agricultural Park (UAP), Universiti Putra Malaysia, Serdang, Selangor, took place at ambient temperatures between 25 to 28°C, and the pH decreased from 5.2 to 3.64 over five days of fermentation. The total plate count, lactic acid bacteria (LAB) and yeasts were significantly increased to approximately 7 Log_{10} CFU/g. The SWF has reduced caffeine content by 35%, while the CGA has decreased by roughly 20%. The SWF also led to an increase in TPC of approximately 31.5% and an increase in antioxidant activity of approximately 60%.

Keywords: Antioxidant activities, caffeine, chlorogenic acid, Robusta coffee, spontaneous wet fermentation

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INTRODUCTION

The diversity in chemical compositions, such as chlorogenic acids (CGA) in the green Robusta coffee beans, notably influences the coffee brew's sensory characteristics, ultimately affecting its economic value in the trading market (Fioresi et al., 2021). Based on studies, it has been observed that green Robusta coffee beans contain higher concentrations of caffeine and CGA, both of which exhibit bitterness and astringency taste in the coffee brew and are regarded as lower quality compared with green Arabica coffee beans (Bicho et al., 2011; de Melo Pereira, de Carvalho Neto, Júnior, et al., 2019; Seninde & Chambers IV, 2020). Nonetheless, the phytochemical constituents of green Robusta coffee beans can be significantly influenced by various factors, including the agronomic environment and the methods used during post-harvest processing (Maxiselly et al., 2023). For instance, caffeine and CGA concentrations in green coffee beans negatively correlated with altitude (Girma et al., 2020). Likewise, the environment of coffee cultivation has a significant impact on the microbial communities. The bacteria communities interaction was reported to predominate at higher altitudes due to larger levels of soil organic matter and nutrients, whereas fungi-bacteria communities interaction predominates at lower altitudes (Veloso et al., 2020).

Post-harvest spontaneous wet fermentation (SWF) involves immersing the pulped coffee cherries in water at ambient temperature to degrade the inner mucilage of Robusta coffee. The SWF process was greatly affected by the variation of naturally occurring microbial communities (Velmourougane, 2013). For example, the presence of yeast in the SWF might improve the levels of isoamyl alcohol, acetaldehyde, and acetate, consequently resulting in improved roasted coffee brew sensory attributes (Elhalis, Cox, Frank, et al., 2020; Hadj Salem et al., 2020; Pereira et al., 2020). While the presence of bacteria capable of producing pectin-related enzymes is favourable in the SWF (Silva et al., 2013). Steer fermentation is undisputedly appropriate because viable microbial strains such as *Pseudomonas* sp. and *Aspergillus* sp. can be inoculated to achieve the desired result to target the degradation of caffeine and CGA in green Robusta coffee beans (Gokulakrishnan et al., 2005; Tai et al., 2014). In stark contrast, the results of SWF may not be as favourable as those of steer fermentation since microbial profiles are highly dependent on the coffee-growing environment. Also, to our knowledge, there has been no report of the efficiency of spontaneous wet fermentation in reducing caffeine and CGA in locally planted Robusta coffee. Therefore, this research aims to evaluate the SWF microbiological properties as well as the impacts of SWF on the phytochemical constituents of green Robusta coffee beans, specifically caffeine and CGA, along with TPC and antioxidant properties.

METHODS

Preliminary Screening of Washed Green Robusta Coffee Beans Samples Collected from Various Locations

Washed green Robusta bean samples were acquired from four different regions of Malaysia: (1) Sik, Kedah in the north, (2) University Agricultural Park (UAP), Universiti Putra Malaysia, Serdang, Selangor in the centre, (3) Kluang, Johor in the south, and (4) Tenom, Sabah in the east. The washed green Robusta beans were ground to a powder that passed through 600–710 microns (Liu et al., 2018).

Extraction of Washed Green Robusta Coffee Beans Samples Collected from Various Locations. The coffee extraction was conducted according to Rukayadi et al. (2008). Approximately 4 g of fermented green Robusta coffee powder was mixed with 100 ml ethanol (Merck, Germany) and shaken (170 rpm) for 1 hr at ambient room temperature (27°C) by orbital shaker-incubator (Biosan, Latvia). The mixture was placed in the dark for 48 hr at room temperature. The mixture was then sonicated using an ultrasonicator (Fisherbrand, USA) at 70% amplitude for 10 min in an ice bath before filtering using a Buchner funnel and Whatman No. 1 filter paper (Whatman, USA). Then, ethanol was removed using a rotary evaporator (Eyela, Japan) at 60°C under a vacuum. The coffee extracts were used for subsequent phytochemical constituent quantification, TPC, and antioxidant assays.

Quantifying Phytochemical Constituents in Green Robusta Coffee Beans using High-performance Liquid Chromatography (HPLC). The quantification of phytochemical constituents was performed in accordance with Abrahão et al. (2019). HPLC (Shimadzu, Japan) was used to quantify seven phytochemical compounds: (1) caffeine, (2) CGA, (3) caffeic acids, (4) 3,4 dihydrobenzoic acids, (5) 4 dihydroxybenzoic acid, (6) *p*-coumaric, (7) quercetin, and (8) kaempferol. The reverse phase column C-18 was used with a flow rate of 1 ml/min, mobile phase methanol, water, and acetic acid (Merck, Germany) (70:28:2 v/v/v), and a wavelength of 280 nm for caffeine with mobile phase methanol (Merck, Germany) and 0.1% formic acid (Merck, Germany), a wavelength of 320 nm for CGA, caffeic acids, and *p*-coumaric, a wavelength of 280 nm for 3,4 dihydrobenzoic acids and 4 dihydroxybenzoic acids, and a wavelength of 320 nm for quercetin and kaempferol. A five-point standard curve (R^2 = 0.99) was developed for each compound for phytochemical quantification in the sample. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the standard error (SE) of the intercept and the standard deviation (SD) of the intercept from the standard curve. The sample with the highest level of caffeine and CGA was chosen to proceed with the subsequent SWF experiments because a sample with a low level of caffeine and CGA may not be able to be identified and quantified using HPLC after SWF and roasting.

TPC Comparison between Washed Green Robusta Coffee Beans from Various Locations. The TPC analysis was performed in accordance with Acidri et al. (2020), with minor modifications to the serial dilutions and three replications. The coffee extracts were diluted in five serial dilutions (0.2–1 mg/ml), and 1 ml was transferred to a universal container. The samples/gallic acid standard (0.02–0.1 mg/ml) were incubated with 5 ml of 10% Follin-Ciocalteu's reagent (Merck, Germany) for 1 min and then reacted with 4 ml of 20% (w/v) sodium carbonate (Friendemann Schmidt, Australia) solution in a dark ambient condition for 30 min. The absorbance was read at 765 nm using a spectrophotometer (Biomate 3). A five-point standard calibration curve (y = 3.47x + 0.1446) with R^2 = 0.9934 was used to calculate the TPC content.

Antioxidant Properties Comparison between Washed Green Robusta Coffee Beans from Various Locations. The Trolox equivalent antioxidant capacity (TEAC) was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay in accordance with Acidri et al. (2020), with minor modifications to the serial dilutions and three replications. The green coffee extracts were diluted with methanol in 10-fold serial dilutions (10–0.001 mg/ml), with 1 ml transferred into a new universal bottle. The green coffee extracts and freshly prepared Trolox (6–hydroxy–2,5,7,8–tetramethyl–chroman–2–carboxylic acid) standards (Aldrich, USA) were incubated with 9 ml of freshly prepared 0.1 mmol/l DPPH solution (Sigma-Aldich, Germany) for 10 min in a dark ambient environment. The absorbance was measured at 519 nm using a spectrophotometer (Biomate 3) with methanol (Merck, Germany) solvent as blank and DPPH (Sigma-Aldich, Germany) working solution as a control. The percentage inhibition of the DPPH radical was calculated through % inhibition = $(Abs_{control} - Abs_{sample}) / Abs_{control} - Abs_{blank}) \times 100\%$. The concentrations that caused a 50% decrease in the initial concentrations of the DPPH radical (half maximal inhibitory concentration [IC_{50}]) and antioxidant capacity of the samples, i.e., TEAC, were determined from the absorbance curve (y = 9.4141x + 0.0233) with R^2 = 0.9910.

The antioxidant-reducing power was performed using a ferric-reducing antioxidant power (FRAP) assay performed in accordance with Tran et al. (2020) with three replications. The FRAP working solution was freshly prepared by mixing 300 mM acetate buffer (Merck, Germany, pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (Tokyo Chemical Industry, Japan), 40 mM hydrochloric acid (HCl, Fisher Scientific, Malaysia) and 20 mM iron(III) chloride ($FeCl_3$, R and M Chemicals, Malaysia) in the ratio 10:1:1. The mixture was incubated for 30 min at 37°C. The extracts were diluted in five serial dilutions (0.1–0.5 mg/ml), from which 0.5 ml was transferred into the new universal bottle. The samples/freshly prepared Trolox (Aldrich, USA) were incubated with 9.5 ml of FRAP working solution and allowed to stand for 30 min in a dark ambient environment. The

absorbance was measured at 593 nm using a spectrophotometer (Biomate 3, USA) with FRAP working solution as blank. The antioxidant capacity of the samples was calculated.

Physical Characteristics Comparison between Green Robusta Coffee Beans from Various Locations. The dimension of washed green Robusta coffee beans was performed in accordance with Bote and Vos (2017) with minor modifications of the sample size and three replications. Ten washed green Robusta coffee beans were randomly selected to measure their dimension using a calliper. The surface with the longest length was measured as length, the surface with the greatest width was measured as width, and the thickness was measured with the flat surface facing downwards.

The hardness of washed green Robusta coffee beans was measured in accordance with Pittia et al. (2007), with minor modifications of beans used and working temperature, and with three replications. The hardness of washed green Robusta coffee beans (10 beans) was measured using a TA.XTplusC texture analyser (Stable Micro Systems, England) equipped with a 1,000 N load cell at a rate of 0.83 cm/s and the working temperature was 25°C. The coffee beans were placed on the analyser plate with the flat side up.

The colour analysis was performed in accordance with Wongsa et al. (2019) with three replications. The colour of washed green Robusta coffee beans (10 g) was measured using a colourimeter (Konika Minolta CR 400 Chroma Meter, Japan) to determine the colour parameter values, i.e., L^* ($L^* = 100$ means white, $L^* = 0$ means black), a^* [redness (+) and greenness (−)], and b^* [yellowness (+) and blueness (−)].

Spontaneous Wet Fermentation (SWF) of Robusta Coffee from UAP

After a preliminary screening, Robusta coffee from UAP, UPM, was chosen for SWF. The ripe Robusta coffee cherries were harvested by hand using a sterile glove and container [(sprayed with 70% alcohol (Merck, Germany)]. The cherries' fruit skin (exocarp) and part of the mucilage (mesocarp) were manually pulped using a sterile [(spray with 70% alcohol (Merck, Germany)] mortar and pestle upon arrival at the UPM laboratory. The pulped cherries were spontaneously fermented using ultrapure water (Arium 611 UV, Germany) at ambient temperature for 0 to 5 days. Zero-hour fermentation (day 0) was used as a negative control. Fermentation temperature and pH (Delta 320 pH meter, USA) readings were taken for 0 (day 0), 24 hr (day 1), 48 hr (day 2), 72 hr (day 3), 96 hr (day 4), and 120 hr of fermentation (day 5).

Temperature and pH Evaluation during Fermentation. The internal fermentation temperature and pH were measured daily throughout the SWF periods, i.e., from day 0 (0 hr) to day 5 (120 hr), using a glass-electrode pH meter (Delta 320 pH meter, USA), with three replications. Prior to the measurement, the pH meter was calibrated with pH 4.1 and pH 7.0 buffer solutions.

Microbiological Analysis. The United States Food and Drug Administration (FDA) (n.d.) performed the microbe enumeration and direct plating with minor solution volume adjustments. The analysis was performed with three replications. Fermented pulped cherries were transferred into a stomacher bag containing 100 ml of 1% peptone water (Oxoid CM 0509, United Kingdom) to determine the microbial population throughout 5 days of SWF. The bags were gently shaken for 5 min. Up to 10^{-8} serial dilutions in 1% nutrient broth (Oxoid CM0001, United Kingdom) were performed. For the total plate count enumeration, an aliquot (100 µl) of the three most diluted solutions was pipetted in duplicate onto plate count agar (Oxoid CM0325, United Kingdom) and incubated at 28°C for 48 hr. While De Man-Rogosa-Sharpe (MRS) agar (Oxoid CM0361, United Kingdom) was used for LAB enumeration at 37°C for 48 hr, potato dextrose agar (BD Difco, USA) was used for yeast and mould enumeration at 28°C for 48 hr. The visible colonies formed in the agar were calculated in logarithmic numbers of colony-forming units per gram ($\log_{10}$ CFU/g).

Extraction, Quantification of Phytochemical Constituents, TPC, and Antioxidant Activity Analysis of SWF Green Robusta Coffee Beans. The green Robusta coffee beans were dried to 10–12% moisture, and the parchments were manually removed. Following this, the extraction was conducted according to the methods outlined in the preceding extraction procedures. The phytochemical constituent quantification, TPC analysis, DPPH radical assay, and FRAP assay were conducted according to the methods outlined in the preceding analysis procedures.

Hardness SWF Green Robusta Coffee Beans. The hardness of SWF green Robusta coffee beans was conducted according to the methods outlined in the preceding hardness testing procedures.

Statistical Analysis

All results were reported as mean ± standard deviation (SD), and statistical analyses were performed using one-way analysis of variance (ANOVA). Tukey's multiple range tests were used with a probability of $p < 0.05$ to identify significant differences between the results. General linear model (GLM) regression analysis was used to explain the variance of the dependent variables, i.e., (1) pH, (2) LAB, (3) caffeine, (4) CGA, (5) caffeic acid, (6) TPC, and (7) antioxidant capacity influenced by the independent variables, i.e., fermentation periods through adjusted R^2. The direction and strength of correlation between the variables were evaluated using Pearson correlation. The software for statistical analysis was Minitab V.19 (Minitab Inc., USA).

RESULTS AND DISCUSSION

Phytochemical Constituents of Washed Green Robusta Coffee Beans from Various Locations

In the current study, seven phytochemicals were attempted to be measured using

HPLC; nonetheless, only four were detected (> LOD) statistically, as shown in Table 1. Because 4 dihydroxybenzoic acids, *p*-coumaric acid, quercetin, and kaempferol were either not detected or lower than LOQ throughout the analysis, the results were reported as undetected (ND). Among the four phytochemicals, caffeine had the highest concentration in all instances, ranging from 1 to 6%, followed by CGA, ranging from 0.6 to 2.1% per gram of dry weight (DW). Washed green Robusta coffee beans from UAP have the highest concentration of all locations. This observation could be attributable to the fact that the site of the plantation has the highest average temperature and lowest average yearly precipitation compared to other locations (Table 2). Such agroclimatic conditions enhance the propensity for smaller bean size, significantly impacting phytochemical constituents (Kath et al., 2021).

Table 1
Quantification of washed green coffee Robusta beans phytochemical constituents using high-performance liquid chromatography

Phytochemicals	Sik	UAP	Kluang	Tenom	LOD	LOQ
Chlorogenic acid (mg/g DW)	16.40 ± 1.10 ^{b}	21.05 ± 1.44 ^{a}	15.00 ± 0.76 ^{b}	6.69 ± 0.40 ^{c}	2.25 ± 0.00	6.92 ± 0.00
Caffeine (mg/g DW)	24.24 ± 0.08 ^{b}	60.26 ± 0.30 ^{a}	22.44 ± 0.23 ^{b}	9.19 ± 0.08 ^{c}	5.80 ± 0.00	17.55 ± 0.00
Caffeic acid (mg/g DW)	2.29 ± 0.11 ^{b}	3.53 ± 0.19 ^{a}	2.48 ± 0.00 ^{b}	1.52 ± 0.01 ^{c}	1.13 ± 0.00	3.54 ± 0.00
3,4-dihydroxybenzoic acid (mg/g DW)	6.45 ± 0.04 ^{a}	4.66 ± 0.08 ^{b}	4.43 ± 0.02 ^{b}	3.50 ± 0.08 ^{c}	1.29 ± 0.00	4.03 ± 0.00
4 dihydroxybenzoic acid	ND	ND	ND	ND	1.29 ± 0.00	4.03 ± 0.00
p-coumaric acid	ND	ND	ND	ND	5.15 ± 0.00	15.62 ± 0.00
Quercetin	ND	ND	ND	ND	1.45 ± 0.00	4.67 ± 0.00
Kaempferol	ND	ND	ND	ND	1.43 ± 0.00	4.34 ± 0.00

Note. DW = Dry wieght; UAP = University Agricultural Park; LOD = Limit of detection; LOQ = Limit of quantification; ND = Not detected; $^{a\text{-}c}$ Mean values ± standard deviations within the same row without a common superscript are significantly different ($p < 0.05$)

Table 2
Climate conditions in different sampling areas

	Sik	UAP	Kluang	Tenom
Average temperature (°C)	24.99 – 33.21	25.57 – 34.56	25.56 – 31.7	25.6 – 28.56
Average annual precipitation (mm)	84.3	26.77	54.14	92.73
Average rainy days per year	191.2	57.13	142.09	207.34
Average days without rain per year	173.8	307.87	222.91	157.66
Elevation (m)	3.33	44.67	18.79	281.51

Note. Data were obtained from https://weatherandclimate.com/; UAP = University Agricultural Park

In contrast, the lowest caffeine and CGA concentrations were observed in the washed green beans from Tenom, where the plantation takes place at a higher altitude. This observation is consistent with that of Girma et al. (2020), who made a similar observation; nevertheless, the study did not clarify the causes behind this occurrence. Other researchers believe that one of the major causes was the influence of altitude's environmental temperature (both air and soil temperature) during coffee cherries' development on a number of metabolic pathways that directly affect the CGA, lipids, and soluble sugar levels (Joët et al., 2010). Caffeic acids, one of the CGA precursors that exhibit a striking similarity trend in Table 1, could support the claim that altitude and environmental temperature influence CGA metabolism. Additionally, the higher the altitude and cooler the temperature at which coffee beans are harvested, the higher their sensory score (Barbosa et al., 2012). Such a discovery has raised the alarm about global warming, which could substantially impact coffee's sensory quality.

Caffeine, CGA, and caffeic acid were not significantly different between Sik and Kluang washed green Robusta coffee beans, most probably due to the comparable growing conditions at both plantations, including low altitude and average temperature. Irrigation, on the other hand, was a crucial component for the survival and development of coffee plants, but it also had no major effect on the phytochemical composition of green coffee beans (da Silva et al., 2005).

The soil composition influenced by the application of fertilisers exerts a significant influence on the phytochemicals of green beans; however, the present study did not report on the type and schedule of fertiliser and pesticide application because farmers were unwilling to disclose commercially sensitive information. Nevertheless, it has been reported that the availability of soil nutrients such as nitrogen, magnesium, potassium, calcium, and phosphate has a substantial positive correlation with caffeine and CGA (Getachew et al., 2022).

TPC and Antioxidant Activity of Washed Green Robusta Coffee Beans from Various Locations

The total phenolic contents of washed green Robusta coffee beans from various locations were illustrated in Figure 1, and it showed that the TPC of washed green Robusta coffee beans varies on their geographical origins. Likewise, green coffee beans from UAP had the highest TPC concentration compared to other regions, but it was insignificant compared to green coffee beans from Sik. Again, green coffee beans from Tenom had the lowest TPC by almost 50% compared to green coffee beans from UAP and Sik. Although CGA is a phenolic compound, it would be too facile to conclude that lower CGA levels in coffee beans from Tenom, as outlined in Table 1, result in low TPC because not all TPC is CGA. A trace amount of 3,4-dihydroxybenzoic acid detected in all cases can also be classified as phenolic compounds. The present TPC result is consistent with the findings of Martinez et

al. (2021), who demonstrated that plantation altitude may not significantly affect TPC in green coffee beans and that other factors, such as maturity phases, may influence it.

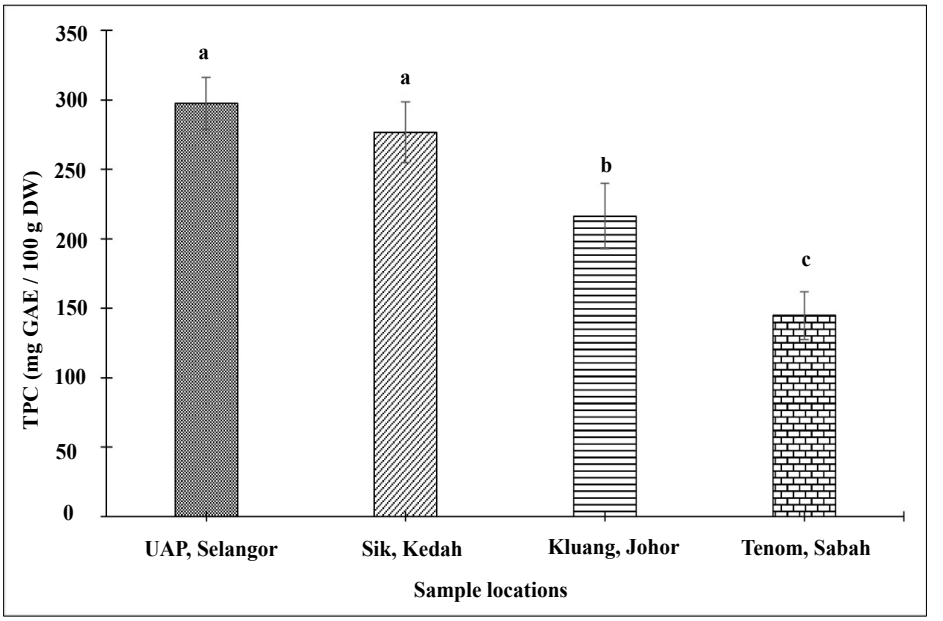

Figure 1. TPC of washed green Robusta coffee beans from various locations

Note. UAP = University Agricultural Park; [a-c] Mean values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

Concerning antioxidant activity, Figure 2 illustrates the antioxidant-reducing power and TEAC of washed green Robusta coffee beans in various regions from the FRAP and DPPH assays, along with the IC_{50}. The antioxidant-reducing power of washed green Robusta coffee beans from UAP was approximately 2.5 to 5 times greater than that of other regions, as measured by the FRAP assay. It may suggest that phytochemical compounds that stimulate antioxidant activity are particularly prone to the single-electron transfer (SET) mechanism. UAP still contained significantly higher TEAC than other regions for the TEAC, but there was not as substantial a difference as in the reducing power. The TEAC of washed green beans from UAP was roughly 10% to 50% higher compared with other regions. In line with the antioxidant-reducing power and TEAC from FRAP and DPPH assays, UAP beans had the lowest antioxidant concentration required to scavenge 50% of the initial DPPH radicals (IC_{50}). It suggests that the antioxidant activity of UAP beans is more potent than beans from other regions. The reducing power and TEAC result indicated that Tenom's beans have the lowest antioxidant activity.

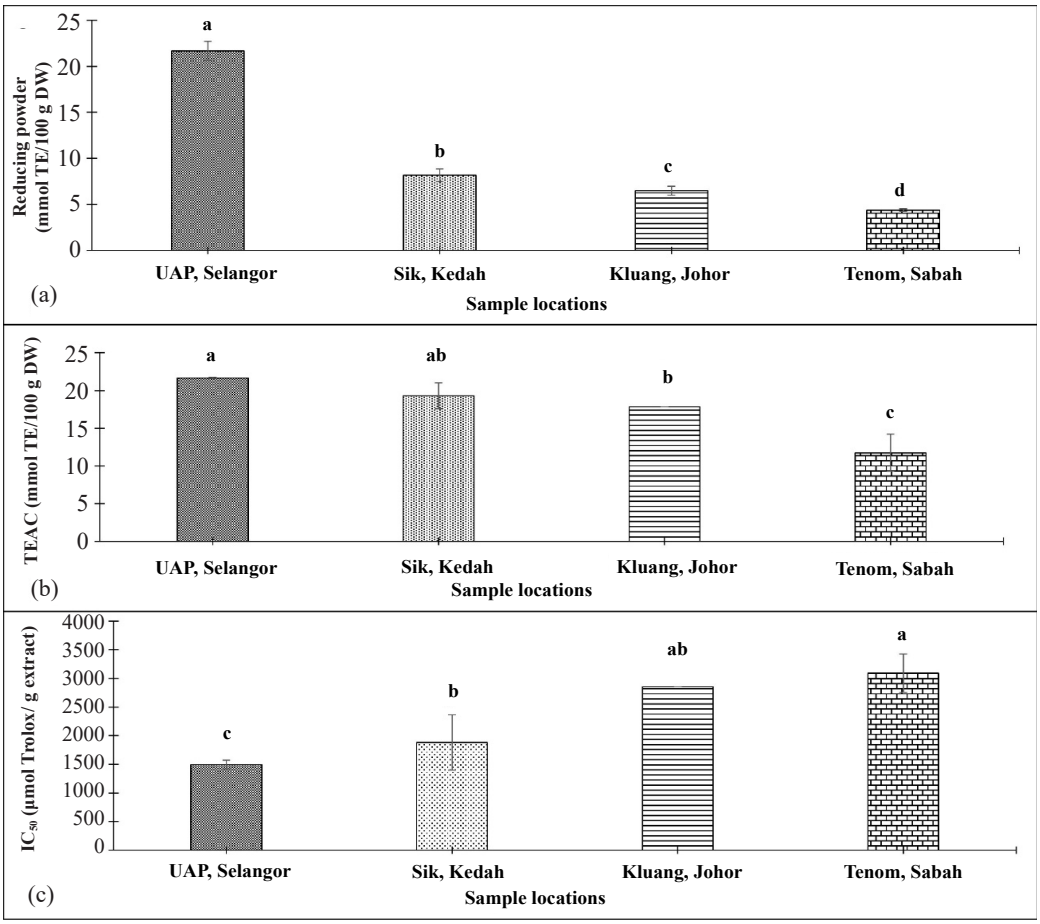

Figure 2. Antioxidant properties of washed green Robusta coffee beans from various locations. (a) Reducing power, (b) Trolox equivalent antioxidant capacity (TEAC), and (c) Half maximal inhibitory concentration (IC_{50})

Note. UAP = University Agricultural Park; [a-c] Mean values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

Physical Properties of Washed Green Robusta Coffee Beans from Various Locations

As washed coffee Robusta beans from Tenom were compared to other locations, the physical dimensions of length, width, and thickness were significantly larger, as illustrated in Figure 3, possibly due to higher altitude (Bote & Vos, 2017). Bean size has often been one of the primary physical attributes used to determine the worth of coffee in commerce; larger beans tend to have a higher economic value. Also, larger beans (from higher altitudes) are perceived to give superior cup quality, possibly due to lower caffeine and CGA concentrations, as shown in Table 1. Green beans from UAP were considerably smaller in length, width, and thickness compared to the other samples, yet they contained the most caffeine, CGA, TPC, and antioxidant properties (Table 1, Figures 1 and 2). The

preliminary screening results suggested that larger beans with greater economic worth may not always possess superior bioactive characteristics. Similar reports showed that coffee beans rated as having the best quality do not always exhibit the highest antioxidant activity and that Robusta coffee, which is perceived to be of poorer quality, has higher antioxidant activity than Arabica coffee beans (Jeszka-Skowron et al., 2016; Ramalakshmi et al., 2008). The "lower quality" label ascribed to Robusta coffee may result from a poor sensory experience from greater caffeine and CGA concentrations. The measurement and identification of sensory attributes were not conducted in this research. This observation has been noted and will be considered for future studies.

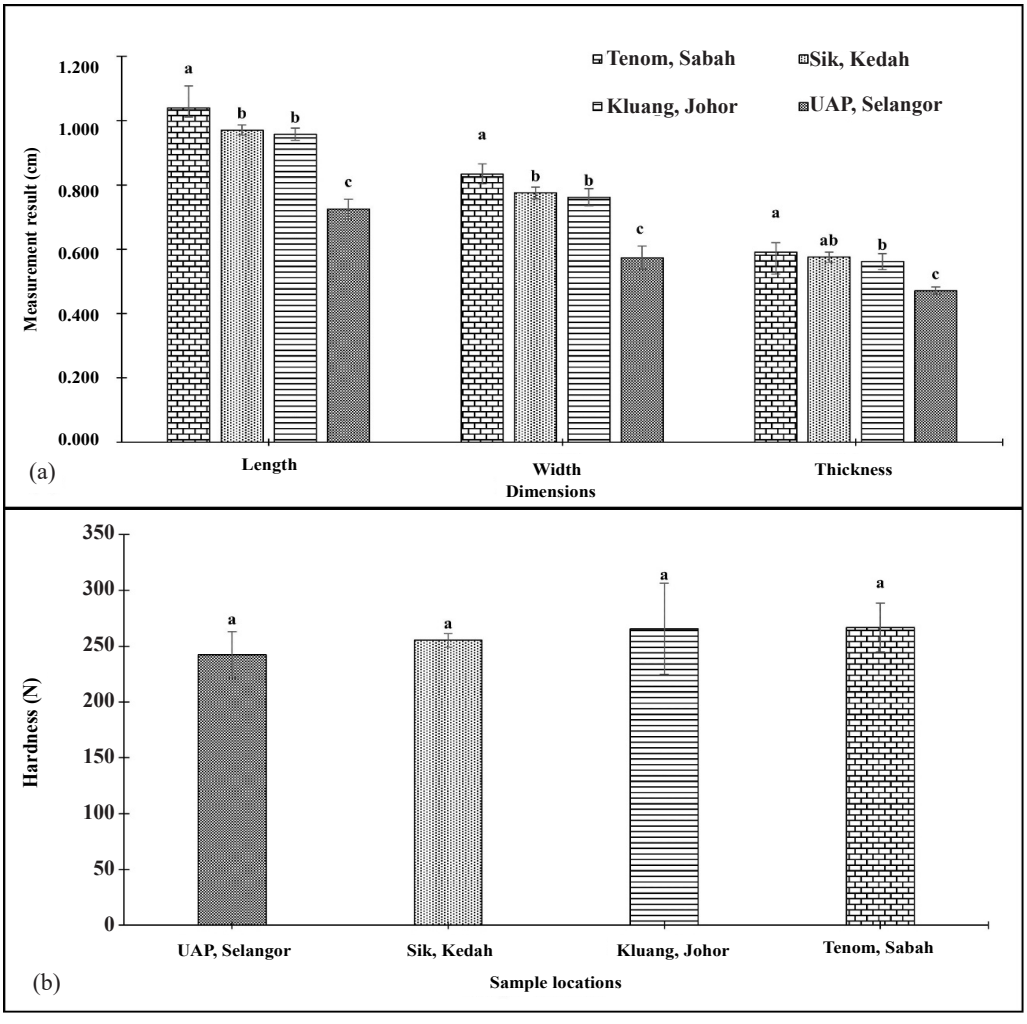

Figure 3. (a) Physical dimension and (b) hardness of washed green coffee beans from various locations

Note. UAP = University Agricultural Park; [a-c] Mean values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

Regarding the hardness of green Robusta coffee beans, there were no statistically significant differences among sampling locations, as illustrated in Figure 3. It is likely because all beans were subjected to a similar post-harvest wash process. It was believed that linear galactomannans were the primary component of the cell wall responsible for the hardness of green Robusta coffee beans (Li et al., 2021). Nonetheless, the hardness of fermented Robusta coffee beans will be compared in the subsequent SWF sections.

Table 3 depicts the colour of washed green Robusta coffee beans from various locations; all beans exhibited a greenish-brown hue. There was no significant variation in lightness (L^*) in all situations. At the same time, UAP coffee beans exhibited a darker brown pigmentation with a lower red/green value (a^*) and blue/yellow value (b^*).

Table 3
Colour properties of washed green Robusta coffee beans from various locations

Colour	Sik	UAP	Kluang	Tenom
L^*	50.68 ± 2.45 ^{a}	52.07 ± 2.60 ^{a}	54.17 ± 0.54 ^{a}	55.56 ± 0.11 ^{a}
a^*	4.20 ± 0.05 ^{a}	3.68 ± 0.03 ^{c}	4.83 ± 0.11 ^{b}	3.57 ± 0.11 ^{c}
b^*	19.08 ± 1.67 ^{a}	15.46 ± 1.10 ^{b}	21.56 ± 0.39 ^{a}	20.96 ± 0.11 ^{a}

Note. UAP = University Agricultural Park; $^{a-c}$ Means values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

SWF Temperature and pH

Robusta coffee from UAP was chosen for the SWF study because it contains the highest levels of caffeine and CGA. Such a large quantity will provide assurance that both compounds can still be detected and measured by HPLC even if their concentrations fall over SWF time. Over the SWF periods, the fermentation temperature (Figure 4) varied between 26 and 28°C, with the lowest temperature being on day five at 26.38°C. Although some food fermentations, such as cocoa and vinegar, resulted in exothermic reactions that could reach as high as 50°C, the temperature fluctuation in the current study was mostly influenced by the open environment because SWF was not conducted in the bioreactor model. Its purpose is to imitate real-world SWFs that lack particular environmental control. So, at the end of the study, it can be suggested that SWF of green Robusta coffee beans is still possible without a complicated fermentation model that is economically advantageous and practical for small businesses and farmers.

Regarding fermentation pH, the initial pH at day 0 was 5.20, decreasing significantly throughout the five days of SWF. As depicted in Figure 4, the pH reached its lowest value between 3.64 and 3.70 on days 3 and 4, but it slightly rebounded to 4.05 on day 5. This pH rise may be attributed to a decreased LAB load, which declined

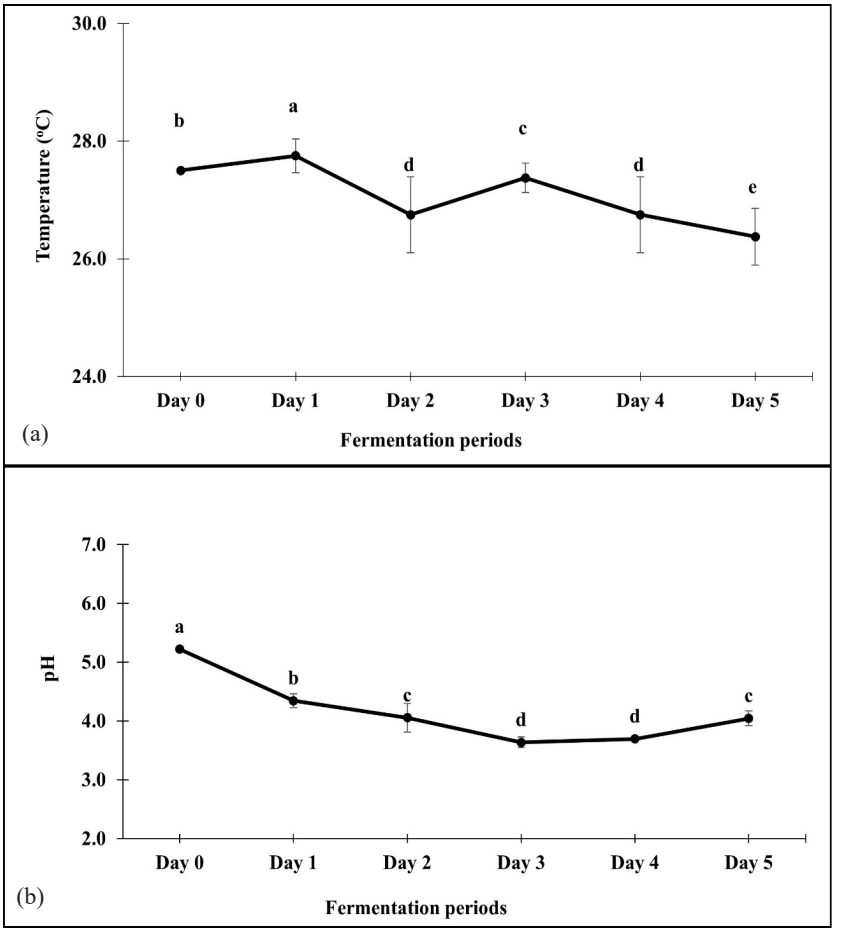

Figure 4. (a) Spontaneous wet fermentation temperature and (b) pH along the fermentation periods from day 0 to day 5

Note. [a-c] Mean values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

from 7.32 $\log_{10}$ CFU/g on day 4 to 7.12 $\log_{10}$ CFU/g on day 5. Furthermore, it is noticeable that the total plate count exhibited a similar reduction trend from 7.65 to 7.44 $\log_{10}$ CFU/g. This observation suggests that bacteria growth may have entered the death phase during an extended wet fermentation. The following microbiological properties discussion comprehensively analysed the correlation between LAB loads and pH. The presence of lactic acids from LAB could cause the pH to drop because the low oxygen SWF environment (facultative anaerobe) provides a favourable environment for their growth (the following discussion in the microbiological properties section elucidates the correlation between pH and LAB).

Also, an acidic environment is necessary for the dissociation of green coffee Robusta beans mucilage and the formation of aromatic compounds in roasted coffee

beans such as (1) aldehydes, (2) long chain alcohols, (3) carboxylic acids, and (4) ketones (de Melo Pereira, de Carvalho Neto, de O. Junqueira, et al., 2019; Schwan & Fleet, 2014; Velmourougane, 2013). Hence, there exists a mutually advantageous interaction in which the dissociation of mucilage provides vital nutrients to the microflora population. On the other hand, pulped coffee beans exposed to a low oxygen and pH environment could experience abiotic stress, resulting in the synthesis of γ-aminobutyric acid (GABA), which is renowned for its calming and relaxing effects. Additionally, the germination of coffee beans by isocitrate lyase in the initial two days of SWF could significantly increase GABA levels as well as alter several phytochemical concentrations, including caffeine (Kim Kim et al., 2018; Zhang et al., 2019).

SWF Microbiological Properties

Present SWF postulates the participation of complicated naturally occurring microorganisms such as yeasts and bacteria (Figure 5). From day 0 to day 5, the total plate count increased significantly from 5.82 $\log_{10}$ CFU/g to 7.44 $\log_{10}$ CFU/g. However, the bacteria loads did not significantly differ after reaching 7.3 $\log_{10}$ CFU/g. According to Pearson correlation, the total plate count was highly positively correlated ($p = 0.000$, $r = 0.950$) with LAB loads as well as the pH ($p = 0.000$, $r = -0.899$). The subsequent LAB discussion further discussed the correlation and effects between total plate count, LAB and pH variable. This increment of total plate count after 24 hr of soaking pulped beans to between 7 to 8 $\log_{10}$ CFU/g was comparable with another study indicating fermentation (Nasanit & Satayawut, 2015). It indicates that alteration in phytochemical contents,

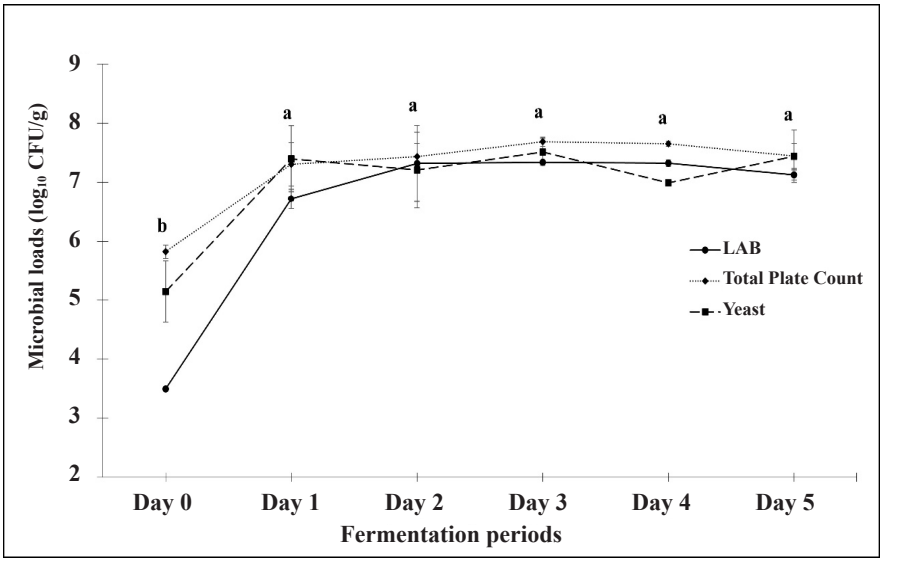

Figure 5. Spontaneous wet fermentation temperature microbial load from day 0 to day 5

Note. [a-b] Mean values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

antioxidant properties, and physicochemical properties could be observed, and the details will be discussed in subsequent phytochemical, antioxidants, and physical properties sections.

With regard to bacteria proliferation trends throughout SWF periods, different authors reported different proliferation trends. Nasanit and Satayawut (2015) reported that the initial total plate count loads were between 6 to 7 $\log_{10}$ CFU/g, and the trend decreased gradually after 24 hr to the end of 48 hr of fermentation. However, Elhalis, Cox, and Zhao (2020) and Evangelista et al. (2015), in line with the present study, reported that the initial loads of total plate count were between 3.8 to 5 $\log_{10}$ CFU/g, then increased significantly to between 7 and 8 $\log_{10}$ CFU/g and remained steady until the end of 48 hr fermentation periods. The initial rapid rise in the number of bacteria could be attributable to the rapid multiplication of microbes following adaptation to the fermentation medium. In contrast, the bacteria load remains constant (stationary phase) after that, which may be due to nutrient depletion and ribosome hibernation to conserve energy. The variability of microbial trends between studies is a peculiarity of SWF because of the diversity of microbial profiles intimately linked to the plantation environment; therefore, SWF remains the greatest barrier to the consistency of the final product when compared to steer fermentation (Bertranda, 2019).

Lactic acid fermentation is considered the most important trait of SWF, and its function has been described in the preceding pH section. In the present study, convincing evidence of LAB fermentation was observed, with a roughly 3-fold increase in LAB in merely 24 hr of SWF but a constant range of 7 $\log_{10}$ CFU/g throughout the fermentation periods. When the total plate count and LAB loads were compared, it was discovered that approximately 60% of the bacteria from the total plate count could be LAB at the initial SWF. Similarly, a study reported that LAB loads were up to 60% prevalent in the microflora community of SWF (de Oliveira Junqueira et al., 2019). After 24 hr of SWF, bacteria began to be displaced by LAB, plausible due to intolerance for the hostile acidic environment, and from 48 hr of SWF onwards, more than 90% of the bacteria in the total plate count could be LAB. Researchers have effectively isolated *Lactobacillus* sp., such as *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus casei*, and *Lactobacillus mesenteroides* from SWF and recognised its significant role in the dissociation of mucilage, altering phytochemical constituents such as caffeine and enhancement of the coffee brew's sensory profile (de Carvalho Neto et al., 2018; Djossou et al., 2011; Nasanit & Satayawut, 2015; Purwoko et al., 2022; Ribeiro et al., 2020).

Since lactic acid is the chief metabolite produced by LAB, the drop in pH in the current study was strongly believed to be attributable to lactic acid from LAB (S.-J. Lee et al., 2021). The GLM and correlation analysis presented in Tables 4 and 5 validated the significance and strength

of the association between fermentation times, pH, and LAB loads. The *p*-values for all the analysed dependent variables in GLM were less than 0.05, confirming a statistically significant relationship with the independent variables. The coefficient in the regression equation showed the distance between the factor levels and the overall mean. If the coefficient exhibits a positive value, an increase in the independent variable corresponds to an increase in the mean value of the dependent variable. Conversely, the negative coefficient exhibits an opposite relationship. For instance, the overall mean for the pH regression equation was 4.152. On day 0 of SWF, the pH increased by 1.068 units from the overall mean. Likewise, the first day of SWF had a positive coefficient with a slight rise of 0.203 units from the total pH mean, yet the pH was still lower than day 0. Continuing SWF from day 2 to day 5 resulted in lower pH values than the overall pH means. The comparison of the positive coefficient on day 1 and the negative coefficient on day 2 suggests that it is preferable to conduct SWF for a minimum of two consecutive days since the low pH of SWF appeared to be more prevalent from the second day of SWF forward. The adjusted R^2 of the fermentation period and pH equal 93.33%, indicating that if there is a variation in pH, 93.33% is due to the change in fermentation periods, and only 6.67% is due to error or unexplained factors. All following GLMs were interpreted in the same way. In terms of the intensity and direction of correlation, fermentation periods strongly correlated with LAB loads

Table 4
GLM of fermentation periods with pH, lactic acid bacteria (LAB), caffeine, chlorogenic acid (CGA), caffeic acid, total phenolic content (TPC), and antioxidant properties of fermented green Robusta coffee beans

Independent variable	Dependent variables	Model *p*-value	Regression equation
Fermentation periods	pH	0.000	pH = 4.152 + 1.068 fermentation day 0 + 0.203 fermentation day 1 - 0.087 fermentation day 2 - 0.517 fermentation day 3 - 0.462 fermentation day 4 - 0.207 fermentation day 5 Adjusted R^2 = 93.33%
	LAB	0.000	LAB = 6.677 - 3.183 fermentation day 0 + 0.037 fermentation day 1 + 0.638 fermentation day 2 + 0.858 fermentation day 3 + 0.958 fermentation day 4 + 0.692 fermentation day 5 Adjusted R^2 = 93.92%
	Caffeine	0.000	Caffeine = 63.007 + 15.265 fermentation day 0 - 2.751 fermentation day 3 - 12.514 fermentation day 5 Adjusted R^2 = 99.85%

Table 4 *(Continue)*

Independent variable	Dependent variables	Model *p*-value	Regression equation
	CGA	0.000	CGA = 22.071 + 2.675 fermentation day 0 - 1.020 fermentation day 3 - 1.655 fermentation day 5 Adjusted R^2 = 83.53%
	Caffeic acid	0.001	Caffeic acid = 3.765 - 1.041 fermentation day 0 - 0.237 fermentation day 3 + 1.279 fermentation day 5 Adjusted R^2 = 98.57%
	TPC	0.000	TPC = 279.490 - 76.800 fermentation day 0 + 24.000 fermentation day 1 + 18.000 fermentation day 2 + 20.400 fermentation day 3 - 0.100 fermentation day 4 + 14.400 fermentation day 5 Adjusted R^2 = 62.47%
	TEAC	0.000	TEAC = 20.184 - 5.375 fermentation day 0 + 1.410 fermentation day 1 + 1.458 fermentation day 2 + 0.801 fermentation day 3 + 0.824 fermentation day 4 + 0.883 fermentation day 5 Adjusted R^2 = 96.89%
	Reducing power	0.000	Reducing power = 20.367 - 6.498 fermentation day 0 + 1.237 fermentation day 1 + 1.286 fermentation day 2 + 0.273 fermentation day 3 + 1.734 fermentation day 4 + 1.969 fermentation day 5 Adjusted R^2 = 88.05%

Table 5
Pearson correlations of fermentation period with pH, lactic acid bacteria (LAB), caffeine, chlorogenic acid (CGA), caffeic acid, total phenolic content (TPC), and antioxidant properties of fermented green Robusta coffee beans

	Fermentation periods	LAB loads	Caffeine	CGA	TPC	Reducing power
Microbiological properties						
LAB loads	0.738					
pH	-0.789	-0.905				
Phytochemical constituents						
Caffeine	-0.998					
CGA	-0.914		0.925			
Caffeic acid	0.954		-0.938	-0.781		

Table 5 *(Continue)*

	TPC and antioxidant properties			
TPC	0.451			
Reducing power	0.683		0.776	
TEAC	0.573		0.882	0.927

Note. All correlations were significant ($p < 0.05$)

while strongly correlated negatively with pH. The LAB loads demonstrated a very significant negative correlation with pH; as LAB loads increased, SWF's pH decreased.

Yeast, likewise, was a participatory microbe in the SWF, with the load significantly increasing from 5.15 to 7.44 $\log_{10}$ CFU/g from day 0 to day 5. The load did not differ significantly after reaching 7.4 $\log_{10}$ CFU/g on the first day of SWF. Several yeast strains, including *Meyerozyma caribbica*, *Hanseniaspora uvarum*, *Torulaspora delbrueckii*, *Pichia kudriavzevii*, *Pichia fermentans*, *Pichia kluyveri*, and *Saccharomyces cerevisiae*, have been isolated from SWF and show remarkable results in modulating phytochemical contents and improving sensory quality (Elhalis, Cox, Frank, et al., 2020; Evangelista et al., 2014; Evangelista et al., 2015). Yeast-fermented green coffee beans contained significantly more isoamyl alcohol, ethanol, acetaldehyde, and ethyl acetate than beans fermented without yeast growth (Elhalis, Cox, Frank, et al., 2020). On the contrary, yeast was found to lower by up to 50% of (1) N-alkanoyl-5-hydroxytryptamides (C-5HTs), (2) cafestol, and (3) kahweol, which are associated with gastric irritation and blood cholesterol increase (Tinoco et al., 2019).

Comparing wet and dry post-harvest processes (SSF), wet fermentation of Robusta beans may be advantageous to SSF, especially in terms of food safety and timing efficiency. Certain foodborne pathogens can be inhibited from growing or reproducing in the acidic environment of wet fermentation (current study pH 3.6–4.95) (Kim, Wilkins, et al., 2018). The absence of mould in the present study reduced the possibility of mycotoxin synthesis, such as ochratoxin A, which typically exists in SSF (Schwan & Fleet, 2014). In addition, the SSF process is incredibly time-consuming, requiring up to 30 days of direct sun-drying and being highly weather and environment-dependent. Also, elevated carbon dioxide levels (350–400 vs 650–1,200 ppm), temperature increases (2–5°C) and drought stress hasten coffee bean fungi deterioration (Medina et al., 2017). Regarding water-soluble polysaccharides, wet-processed coffee beans yield more than dry-process beans (Tarzia et al., 2010).

Phytochemical Constituents of Green Robusta Coffee Beans after SWF

After SWF, green coffee Robusta beans from days 0, 3, and 5 were chosen to examine the influence of phytochemical constituents, particularly caffeine and CGA, as shown

in Figure 6 and chromatogram, as shown in Figure 7. All examined phytochemicals exhibited significant differences at each fermentation stage, with caffeine and CGA showing decremental trends, whereas caffeic acids showed incremental trends, and 3,4-dihydroxybenzoic acid showed no significant changes. The caffeine concentration decreased by around 35%, whereas CGA decreased by approximately 20% at the end of SWF. The increment in caffeic acids is partly thought to be produced by CGA catabolism during SWF. It should be noted that SWF does not necessarily cause the alteration of caffeine and CGA concentrations in green coffee beans, as Joët et al. (2010) reported. However, the results indicate that SWF has successfully decreased the quantities of caffeine and CGA in green Robusta coffee beans. Further validation of the regression and correlation between SWF, caffeine and CGA was conducted using GLM (Table 4) and Pearson statical analysis (Table 5). Based on the GLM, fermentation periods were highly associated with the variation of caffeine and CGA (adjusted $R^2 > 80\%$), and both compounds showed a very strong negative correlation with fermentation periods; the longer the Robusta coffee beans fermented, the lower the CGA and caffeine contents.

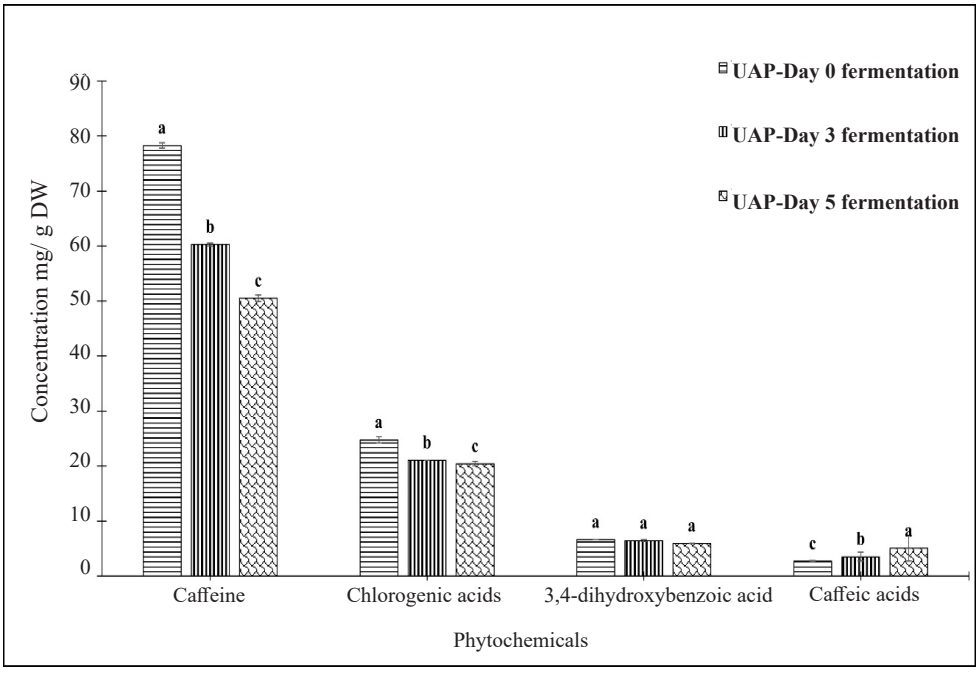

Figure 6. Comparison of phytochemical constituents of green Robusta coffee beans at certain SWF periods

Note. UAP = University Agricultural Park; [a-c] Mean values ± standard deviations without a common superscript within the group are significantly different ($p < 0.05$)

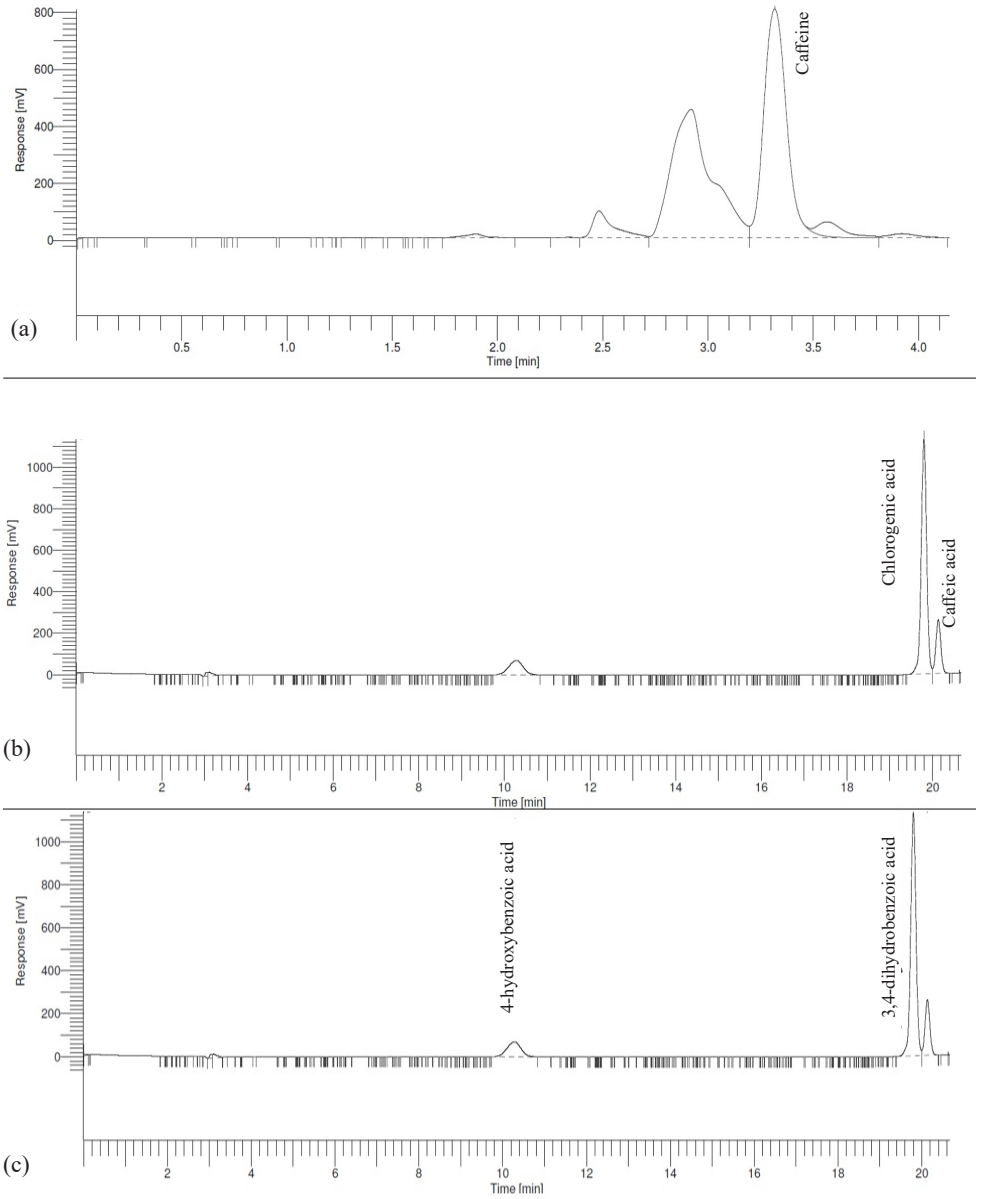

Figure 7. High-performance liquid chromatography chromatogram for fermented green Robusta coffee beans after 5 days of spontaneous wet fermentation. (a) caffeine, (b) chlorogenic acid and caffeic acid, and (c) 4-hydroxybenzoic acid and 3,4-dihydrobenzoic acid

It is deduced that *Pseudomonas* sp., *Lactobacillus* sp., and *Bacillus* sp. were present in the SWF, given that these bacteria were capable of degrading caffeine and CGA in green coffee beans and were prevalent in the soil of Peninsular and East Malaysia (Gokulakrishnan et al., 2005; Mun & Ling, 2022; Purwoko et al., 2022; Tripathi et al.,

2012; Vega et al., 2021). It also implies that comparable SWF results may be observed in other locations in Malaysia.

Without alternative nitrogen sources, such as urea and ammonium, caffeine will serve as the sole nitrogen supply for microbial survival (Hakil et al., 1999). Fungi (e.g., *Aspergillus* sp., *Penicillium* sp., and *Rhizopus* sp.) can degrade caffeine into theophylline (1,3-dimethylxanthine), while bacteria (e.g., *Pseudomonas* sp. and *Serratia* sp.) can degrade caffeine into theobromine (3,7-dimethlxanthine). These molecules were then metabolised to generate xanthine, which was then transformed into carbon dioxide and ammonia by purine catabolism (Gummadi et al., 2012; Gokulakrishnan et al., 2005). While degradation of CGA by *Lactobacillus* sp. results in the synthesis of hydroxycinnamic acids such as ferulic acid, quinic acid, and caffeic acid, additional decarboxylation and reduction could lead to the production of 4-vinylcathecol and dihydrocaffiec acid (Filannino et al., 2015; Rogozinska et al., 2021).

TPC and Antioxidant Properties of Green Robusta Coffee Beans after SWF

The result of TPC across 5-day fermentation periods is presented in Table 6. The TPC of non-fermented green Robusta coffee beans (day 0) was significantly lower compared with all fermented green coffee beans. Likewise, the antioxidant properties of unfermented green Robusta coffee beans (day 0) are significantly lower than those of fermented green Robusta coffee beans. The GLM in Table 4 confirmed that the independent variable of fermentation periods can be used to explain the variation among the dependent variables of TPC and antioxidant properties. During the SWF periods, microbial metabolism resulted in the degradation of organic compounds and cell wall structure, such as (1) cellulose, (2) hemicellulose, and (3) lignin, by microorganisms, thereby increasing the solubility of the interior cell components of coffee beans, which could contribute to an increase in TPC (Kurniawati et al., 2016). The evidence of softer fermented Robusta coffee beans as a result of cell wall degradation will be addressed in the subsequent section on coffee beans' hardness. Also, microbial metabolism can alter the profile of phenolic compounds, for example, by converting them to aglycone flavonoid form, which contributes to an increase in antioxidant activity, and also by metabolising CGA to hydroxycinnamic acids, which have been observed to be substantially connected with antioxidant power (Huynh et al., 2014; Kurniawati et al., 2016; Mullen et al., 2013). The Pearson correlations in Table 5 reveal a high positive correlation between TPC and the antioxidant properties of fermented green Robusta coffee beans.

Even though GLM and Pearson correlations supported the effects of fermentation durations on TPC and antioxidant properties, the connection was only somewhat moderately positive. Perhaps the TPC and antioxidant properties trends were associated more with microbial loads than fermentation durations, given that

Table 6
TPC and antioxidant properties of non-fermented and fermented green Robusta coffee beans

Fermentation day	Extract yield (%)	Total phenolic content (mg GAE/100 g DW)	IC_{50} (µg/ml)	TEAC (mmol TE/ 100 g DW)	Reducing power (mmol TE/ 100 g DW)
0	15.00 ± 0.00	202.70 ± 20.61 [a]	217.50 ± 3.54 [a]	14.81 ± 0.14 [a]	13.87 ± 0.60
1	19.01 ± 1.40	303.52 ± 18.76 [b]	175.00 ± 1.41 [ab]	21.60 ± 0.09 [b]	21.60 ± 1.25
2	14.28 ± 2.51	297. 53 ± 18.74 [b]	112.50 ± 37.48 [b]	21.64 ± 0.08 [b]	21.65 ± 1.05
3	18.03 ± 2.79	299.94 ± 29.23 [b]	136.00 ± 22.63 [ab]	20.99 ± 0.26 [b]	20.64 ± 0.86
4	16.27 ± 0.56	279.35 ± 43.41 [b]	145.50 ± 14.85 [ab]	21.01 ± 0.34 [b]	22.10 ± 0.99
5	14.56 ± 1.91	293.93 ± 14.47 [b]	152.50 ± 19.09 [ab]	21.07 ± 1.14 [b]	22.24 ± 0.54

Table 7
Pearson correlations of microbial loads and total phenolic content (TPC), antioxidant properties, caffeine, and chlorogenic acid (CGA) of fermented green Robusta coffee beans

Variables	LAB	Total plate count	Yeast	TPC	Reducing power
Microbiological properties					
Total plate count	0.950				
Yeast	0.877	0.914			
TPC and antioxidant properties					
TPC	0.774	0.697	0.857		
Reducing power	0.942	0.898	0.840	0.716	
TEAC	0.900	0.914	0.901	0.786	0.924
Phytochemical constituents					
Caffeine	-0.920	-0.879	-0.895		
CGA	-0.948	-0.894	-0.951		

Note. All correlations were significant ($p < 0.05$); LAB = Lactic acid bacteria; TEAC = Trolox equivalent antioxidant capacity

the microbial loads in Figure 5 exhibited an identical trend pattern. This notion was further corroborated by Table 7 Pearson correlations, which revealed a stronger positive correlation between microbial loads and TPC and antioxidant capabilities. It suggests that in order to boost TPC and antioxidant levels even further, the microbial load should be increased, which may be accomplished using a stirred-tank bioreactor that can regulate and optimise fermentation conditions such as (1) selection and combination of inoculated strains (2) appropriate culture supplementation, (3) temperature (e.g., 30°C), (4) aeration (e.g., 1 L/min), and (5) agitation (200 rpm) (de Carvalho Neto et al., 2018; de Jesus Cassimiro et al., 2022; Haile & Kang, 2019; L. W. Lee et al., 2017; Martinez et al., 2019; Wang et al., 2020). This observation differed slightly with caffeine and CGA, as both phytochemicals strongly correlated with fermentation periods and microbial loads (Tables 5 and 7).

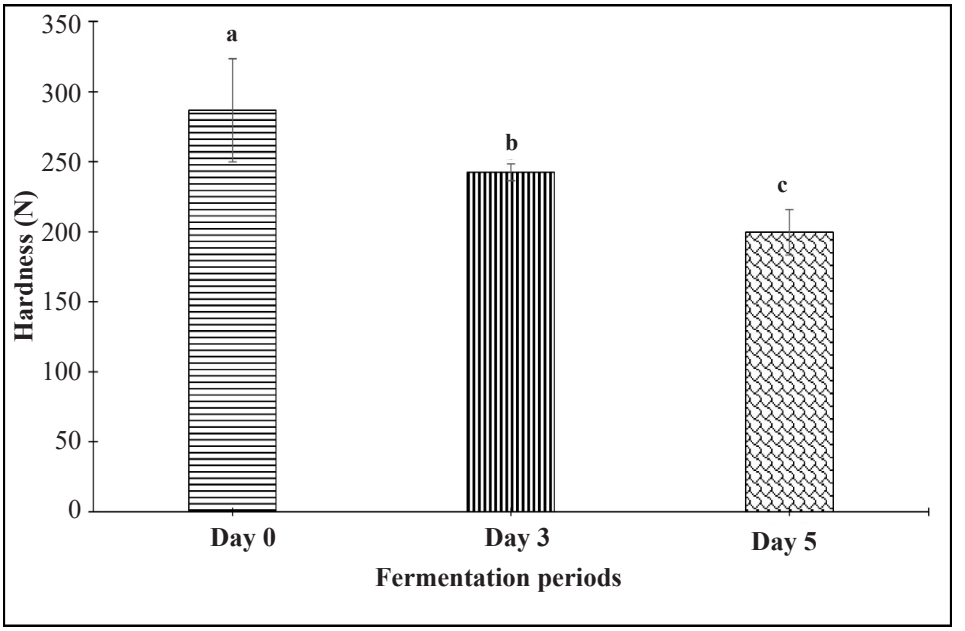

Figure 8. The hardness of fermented green Robusta coffee beans from University Agricultural Park after spontaneous wet fermentation

Note. [a-c] Mean values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

The Hardness of Green Robusta Coffee Beans after SWF

The hardness of green Robusta coffee beans after SWF was compared and presented in Figure 8. Compared to non-fermented beans, fermented Robusta coffee beans were significantly softer, with the beans that underwent 5 days of SWF being the softest by 30%. As previously discussed, this could be evidence of microbial pectinase, amylase,

cellulase, and protease activities from SWF that break down cell wall components (Elhalis et al., 2021). Germination during SWF may also contribute to the softer, fermented green Robusta coffee beans. During germination, α-galactosidase combines with (1→4)-*β*-mannan (endo-*β*-mannanase) and *β*-mannosidases to breakdown galactomannans, which are the principal component of the cell wall responsible for the hardness of green Robusta coffee beans (L. W. Lee et al., 2015).

CONCLUSION

This study compared physical and chemical characteristics, including phytochemicals and antioxidations, during the preliminary screening of green Robusta coffee beans from four locations in Malaysia, i.e., the north, centre, south, and east. The coffee plantation's agroclimatic conditions exert a substantial effect. Washed green Robusta coffee beans at a higher altitude showed greater size but contained less caffeine, CGA, TPC, and antioxidant properties. In comparison, the washed green Robusta coffee beans from UAP were the smallest in size but contained the highest levels of caffeine and CGA. Such characteristics typically have disadvantages in trading, as they are judged to be of low quality and to have a poor sensory profile. Due to its high caffeine and CGA concentration, Robusta coffee from UAP was chosen for further SWF study to determine whether its caffeine and CGA content could be reduced while retaining or improving its antioxidant properties. The five-day study of SWF revealed that SWF happened satisfactorily at ambient temperature without additional specified controls like fermentation in a bioreactor. LAB and yeast dominated the SWF throughout the five-day SWF period, reaching up to 7 $\log_{10}$ CFU/g. The pH dropped as low as 3.6, providing an essential medium for mucilage breakdown. The fermented beans were softer than the unfermented beans. The current SWF has decreased the caffeine concentration by 35% and the CGA content by 20% while increasing the TPC and antioxidant qualities by approximately 50% in fermented green Robusta coffee beans. The statistical analysis additionally revealed that fermentation periods may not be as impactful as microbial loads in enhancing TPC and antioxidant properties, whilst fermentation periods and microbial loads are equally important in reducing caffeine and CGA levels.

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REFERENCES

Abrahão, F. R., Rocha, L. C. R., Santos, T. A., do Carmo, E. L., Pereira, L. A. S., Borges, S. V., Pereira, R. G. F. A., & Botrel, D. A. (2019). Microencapsulation of bioactive compounds from espresso spent coffee by spray drying. *LWT*, *103*, 116–124. https://doi.org/10.1016/j.lwt.2018.12.061

Acidri, R., Sawai, Y., Sugimoto, Y., Handa, T., Sasagawa, D., Masunaga, T., Yamamoto, S., & Nishihara, E. (2020). Phytochemical profile and antioxidant capacity of coffee plant organs compared to green and roasted coffee beans. *Antioxidants*, *9*(2), 93. https://doi.org/10.3390/antiox9020093

Barbosa, J. N., Borém, F. M., Cirillo, M. A., Malta, M. R., Alvarenga, A. A., & Alves, H. M. R. (2012). Coffee quality and its interactions with environmental factors in Minas Gerais, Brazil. *Journal of Agricultural Science*, *4*(5), 181–190. https://doi.org/10.5539/jas.v4n5p181

Bertranda, R. L. (2019). Lag phase is a dynamic, organized, adaptive, and evolvable period that prepares bacteria for cell division. *Journal of Bacteriology*, *201*(7), e00697-18. https://doi.org/10.1128/JB.00697-18

Bicho, N. C., Leitão, A. E., Ramalho, J. C., & Lidon, F. C. (2011). Identification of chemical clusters discriminators of the roast degree in Arabica and Robusta coffee beans. *European Food Research and Technology*, *233*, 303–311. https://doi.org/10.1007/s00217-011-1518-5

Bote, A. D., & Vos, J. (2017). Tree management and environmental conditions affect coffee (*Coffea arabica* L.) bean quality. *NJAS: Wageningen Journal of Life Sciences*, *83*(1), 39–46. https://doi.org/10.1016/j.njas.2017.09.002

da Silva, E. A., Mazzafera, P., Brunini, O., Sakai, E., Arruda, F. B., Mattosso, L. H. C., Carvalho, C. R. L., & Pires, R. C. (2005). The influence of water management and environmental conditions on the chemical composition and beverage quality of coffee beans. *Brazilian Journal of Plant Physiology*, *17*(2), 229–238.

de Carvalho Neto, D. P., Kim, G. V., Finco, A. M. O., Letti, L. A. J., da Silva, B. J. G., Vandenberghe, L. P. S., & Soccol, C. R. (2018). Efficient coffee beans mucilage layer removal using lactic acid fermentation in a stirred-tank bioreactor: Kinetic, metabolic and sensorial studies. *Food Bioscience*, *26*, 80–87. https://doi.org/10.1016/j.fbio.2018.10.005

de Jesus Cassimiro, D. M., Batista, N. N., Fonseca, H. C., Naves, J. A. O., Dias, D. R., & Schwan, R. F. (2022). Coinoculation of lactic acid bacteria and yeasts increases the quality of wet fermented Arabica coffee. *International Journal of Food Microbiology*, *369*, 109627. https://doi.org/10.1016/j.ijfoodmicro.2022.109627

de Melo Pereira, G. V., de Carvalho Neto, D. P., de O. Junqueira, A. C., Karp, S. G., Letti, L. A. J., Júnior, A. I. M. I., & Soccol, C. R. (2019). A review of selection criteria for starter culture development in the food fermentation industry. *Food Reviews International*, *36*(2), 135–167. https://doi.org/10.1080/87559129.2019.1630636

de Melo Pereira, G. V., de Carvalho Neto, D. P., Júnior, A. I. M., Vásquez, Z. S., Medeiros, A. B. P., Vandenberghe, L. P. S., & Soccol, C. R. (2019). Exploring the impacts of postharvest processing on the aroma formation of coffee beans – A review. *Food Chemistry*, *272*, 441–452. https://doi.org/10.1016/j.foodchem.2018.08.061

de Oliveira Junqueira, A. C., de Melo Pereira, G. V., Medina, J. D. C., Alvear, M. C. R., Rosero, R., de Carvalho Neto, D. P., Enríquez, H. G., & Soccol, C. R. (2019). First description of bacterial and fungal communities in Colombian coffee beans fermentation analysed using Illumina-based amplicon sequencing. *Scientific Reports*, *9*, 8794. https://doi.org/10.1038/s41598-019-45002-8

Djossou, O., Perraud-Gaime, I., Mirleau, F. L., Rodriguez-Serrano, G., Karou, G., Niamke, S., Ouzari, I., Boudabous, A., & Roussos, S. (2011). Robusta coffee beans post-harvest microflora: *Lactobacillus plantarum* sp. as potential antagonist of *Aspergillus carbonarius*. *Anaerobe*, *17*(6), 267–272. https://doi.org/10.1016/j.anaerobe.2011.03.006

Elhalis, H., Cox, J., & Zhao, J. (2020). Ecological diversity, evolution and metabolism of microbial communities in the wet fermentation of

Australian coffee beans. *International Journal of Food Microbiology*, *321*, 108544. https://doi.org/10.1016/j.ijfoodmicro.2020.108544

Elhalis, H., Cox, J., Frank, D., & Zhao, J. (2020). The crucial role of yeasts in the wet fermentation of coffee beans and quality. *International Journal of Food Microbiology*, *333*, 108796. https://doi.org/10.1016/j.ijfoodmicro.2020.108796

Elhalis, H., Cox, J., Frank, D., & Zhao, J. (2021). Microbiological and biochemical performances of six yeast species as potential starter cultures for wet fermentation of coffee beans. *LWT*, *137*, 110430. https://doi.org/10.1016/j.lwt.2020.110430

Evangelista, S. R., da Cruz Pedroso Miguel, M. G., Silva, C. F., Pinheiro, A. C. M., & Schwan, R. F. (2015). Microbiological diversity associated with the spontaneous wet method of coffee fermentation. *International Journal of Food Microbiology*, *210*, 102–112. https://doi.org/10.1016/j.ijfoodmicro.2015.06.008

Evangelista, S. R., Silva, C. F., da Cruz Pedroso Miguel, M. G., de Souza Cordeiro, C., Pinheiro, A. C. M., Duarte, W. F., & Schwan, R. F. (2014). Improvement of coffee beverage quality by using selected yeasts strains during the fermentation in dry process. *Food Research International*, *61*, 183–195. https://doi.org/10.1016/j.foodres.2013.11.033

Filannino, P., Bai, Y., Di Cagno, R., Gobbetti, M., & Gänzle, M. G. (2015). Metabolism of phenolic compounds by *Lactobacillus* spp. during fermentation of cherry juice and broccoli puree. *Food Microbiology*, *46*, 272–279. https://doi.org/10.1016/j.fm.2014.08.018

Fioresi, D. B., Pereira, L. L., da Silva Oliveira, E. C., Moreira, T. R., & Ramos, A. C. (2021). Mid infrared spectroscopy for comparative analysis of fermented arabica and robusta coffee. *Food Control*, *121*, 107625. https://doi.org/10.1016/j.foodcont.2020.107625

Getachew, M., Tolassa, K., De Frenne, P., Verheyen, K., Tack, A. J. M., Hylander, K., Ayalew, B., & Boeckx, P. (2022). The relationship between elevation, soil temperatures, soil chemical characteristics, and green coffee bean quality and biochemistry in southwest Ethiopia. *Agronomy for Sustainable Development*, *42*, 61. https://doi.org/10.1007/s13593-022-00801-8

Girma, B., Gure, A., & Wedajo, F. (2020). Influence of altitude on caffeine, 5-caffeoylquinic acid, and nicotinic acid contents of Arabica coffee varieties. *Journal of Chemistry*, *2020*, 3904761. https://doi.org/10.1155/2020/3904761

Gokulakrishnan, S., Chandraraj, K., & Gummadi, S. N. (2005). Microbial and enzymatic methods for the removal of caffeine. *Enzyme and Microbial Technology*, *37*(2), 225–232. https://doi.org/10.1016/j.enzmictec.2005.03.004

Gummadi, S. N., Bhavya, B., & Ashok, N. (2012). Physiology, biochemistry and possible applications of microbial caffeine degradation. *Applied Microbiology and Biotechnology*, *93*, 545–554. https://doi.org/10.1007/s00253-011-3737-x

Hadj Salem, F., Lebrun, M., Mestres, C., Sieczkowski, N., Boulanger, R., & Collignan, A. (2020). Transfer kinetics of labeled aroma compounds from liquid media into coffee beans during simulated wet processing conditions. *Food Chemistry*, *322*, 126779. https://doi.org/10.1016/j.foodchem.2020.126779

Haile, M., & Kang, W. H. (2019). Isolation, identification, and characterization of pectinolytic yeasts for starter culture in coffee fermentation. *Microorganisms*, *7*(10), 401. https://doi.org/10.3390/microorganisms7100401

Hakil, M., Voisinet, F., Viniegra-González, G., & Augur, C. (1999). Caffeine degradation in solid state fermentation by *Aspergillus tamarii*: Effects of additional nitrogen sources. *Process Biochemistry*, *35*(1–2), 103–109. https://doi.org/10.1016/S0032-9592(99)00039-4

Huynh, N. T., Camp, J. V., Smagghe, G., & Raes, K. (2014). Improved release and metabolism of flavonoids by steered fermentation processes : A review. *International Journal of Molecular Sciences*, *15*(11), 19369–19388. https://doi.org/10.3390/ijms151119369

Jeszka-Skowron, M., Stanisz, E., & De Peña, M. P. (2016). Relationship between antioxidant capacity, chlorogenic acids and elemental composition of green coffee. *LWT*, *73*, 243–250. https://doi.org/10.1016/j.lwt.2016.06.018

Joët, T., Laffargue, A., Descroix, F., Doulbeau, S., Bertrand, B., de Kochko, A., & Dussert, S. (2010). Influence of environmental factors, wet processing and their interactions on the biochemical composition of green Arabica coffee beans. *Food Chemistry*, *118*(3), 693–701. https://doi.org/10.1016/j.foodchem.2009.05.048

Kath, J., Byrareddy, V. M., Mushtaq, S., Craparo, A., & Porcel, M. (2021). Temperature and rainfall impacts on robusta coffee bean characteristics. *Climate Risk Management*, *32*, 100281. https://doi.org/10.1016/j.crm.2021.100281

Kim, C., Wilkins, K., Bowers, M., Wynn, C., & Ndegwa, E. (2018). Influence of pH and temperature on growth characteristics of leading foodborne pathogens in a laboratory medium and select food beverages. *Austin Food Sciences*, *3*(1), 1031.

Kim, Y., Kim, Y., & Jhon, D.-Y. (2018). Changes of the chlorogenic acid, caffeine, γ-aminobutyric acid (GABA) and antioxdant activities during germination of coffee bean (coffea Arabica). *Emirates Journal of Food and Agriculture*, *30*(8), 675–680. https://doi.org/10.9755/ejfa.2018.v30.i8.1763

Kurniawati, N., Meryandini, A., & Sunarti, T. C. (2016). Introduction of actinomycetes starter on coffee fruits fermentation to enhance quality of coffee pulp. *Emirates Journal of Food and Agriculture*, *28*(3), 188–195. https://doi.org/10.9755/ejfa.2015-05-192

Lee, L. W., Cheong, M. W., Curran, P., Yu, B., & Liu, S. Q. (2015). Coffee fermentation and flavor - An intricate and delicate relationship. *Food Chemistry*, *185*, 182–191. https://doi.org/10.1016/j.foodchem.2015.03.124

Lee, L. W., Tay, G. Y., Cheong, M. W., Curran, P., Yu, B., & Liu, S. Q. (2017). Modulation of the volatile and non-volatile profiles of coffee fermented with *Yarrowia lipolytica*: II. Roasted coffee. *LWT*, *80*, 32–42. https://doi.org/10.1016/j.lwt.2017.01.070

Lee, S.-J., Jeon, H.-S., Yoo, J.-Y., & Kim, J.-H. (2021). Some important metabolites produced by lactic acid bacteria originated from kimchi. *Foods*, *10*(9), 2148. https://doi.org/10.3390/foods10092148

Li, Z., Zhang, C., Zhang, Y., Zeng, W., & Cesarino, I. (2021). Coffee cell walls - Composition, influence on cup quality and opportunities for coffee improvements. *Food Quality and Safety*, *5*, fyab012. https://doi.org/10.1093/fqsafe/fyab012

Liu, C., Yang, N., Yang, Q., Ayed, C., Linforth, R., & Fisk, I. D. (2018). Enhancing Robusta coffee aroma by modifying flavour precursors in the green coffee bean. *Food Chemistry*, *281*, 8–17. https://doi.org/10.1016/j.foodchem.2018.12.080

Martinez, S. J., Bressani, A. P. P., Dias, D. R., Simão, J. B. P., & Schwan, R. F. (2019). Effect of bacterial and yeast starters on the formation of volatile and organic acid compounds in coffee beans and selection of flavors markers precursors during wet fermentation. *Frontiers in Microbiology*, *10*, 1287. https://doi.org/10.3389/fmicb.2019.01287

Martinez, S. J., Simão, J. B. P., Pylro, V. S., & Schwan, R. F. (2021). The altitude of coffee cultivation causes shifts in the microbial community assembly and biochemical compounds in natural

induced anaerobic fermentations. *Frontiers in Microbiology*, *12*, 671395. https://doi.org/10.3389/fmicb.2021.671395

Maxiselly, Y., Humaira, D. S., Sari, D. N., & Suherman, C. (2023). Morpho-physiological traits and phytochemical compositions of *Coffea canephora* beans from Lampung for various harvesting stages and soaking durations. *International Journal of Plant Biology*, *14*(3), 746–754. https://doi.org/10.3390/ijpb14030055

Medina, A., Akbar, A., Baazeem, A., Rodriguez, A., & Magan, N. (2017). Climate change, food security and mycotoxins: Do we know enough? *Fungal Biology Reviews*, *31*(3), 143–154. https://doi.org/10.1016/j.fbr.2017.04.002

Mullen, W., Nemzer, B., Stalmach, A., Ali, S., & Combet, E. (2013). Polyphenolic and hydroxycinnamate contents of whole coffee fruits from China, India, and Mexico. *Journal of Agricultural and Food Chemistry*, *61*(22), 5298–5309. https://doi.org/10.1021/jf4003126

Mun, C. L., & Ling, C. M. W. V. (2022). Tropical soil bacterial diversity in Sabah, Malaysia. *Sains Malaysiana*, *51*(2), 451–460. https://doi.org/10.17576/jsm-2022-5102-10

Nasanit, R., & Satayawut, K. (2015). Microbiological study during coffee fermentation of *Coffea arabica* var. chiangmai 80 in Thailand. *Kasetsart Journal - Natural Science*, *49*(1), 32–41.

Pereira, L. L., Guarçoni, R. C., Pinheiro, P. F., Osório, V. M., Pinheiro, C. A., Moreira, T. R., & ten Caten, C. S. (2020). New propositions about coffee wet processing: Chemical and sensory perspectives. *Food Chemistry*, *310*, 125943. https://doi.org/10.1016/j.foodchem.2019.125943

Pittia, P., Nicoli, M. C., & Sacchetti, G. (2007). Effect of moisture and water activity on textural properties of raw and roasted coffee beans. *Journal of Texture Studies*, *38*(1), 116-134. https://doi.org/10.1111/j.1745-4603.2007.00111.x

Purwoko, T., Suranto., Setyaningsih, R., & Marliyana, S. D. (2022). Chlorogenic acid and caffeine content of fermented robusta bean. *Biodiversitas*, *23*(2), 902–906. https://doi.org/10.13057/biodiv/d230231

Ramalakshmi, K., Rahath Kubra, I., & Jagan Mohan Rao, L. (2008). Antioxidant potential of low-grade coffee beans. *Food Research International*, *41*(1), 96–103. https://doi.org/10.1016/j.foodres.2007.10.003

Ribeiro, L. S., da Cruz Pedrozo Miguel, M. G., Martinez, S. J., Bressani, A. P. P., Evangelista, S. R., Silva e Batista, C. F., & Schwan, R. F. (2020). The use of mesophilic and lactic acid bacteria strains as starter cultures for improvement of coffee beans wet fermentation. *World Journal of Microbiology and Biotechnology*, *36*, 186. https://doi.org/10.1007/s11274-020-02963-7

Rogozinska, M., Korsak, D., Mroczek, J., & Biesaga, M. (2021). Catabolism of hydroxycinnamic acids in contact with probiotic *Lactobacillus*. *Journal of Applied Microbiology*, *131*(3), 1464–1473. https://doi.org/10.1111/jam.15009

Rukayadi, Y., Shim, J.-S., & Hwang, J.-K. (2008). Screening of Thai medicinal plants for anticandidal activity. *Mycoses*, *51*(4), 308–312. https://doi.org/10.1111/j.1439-0507.2008.01497.x

Schwan, R. F., & Fleet, G. H. (Eds.) (2014). Cocoa and coffee fermentations (1st ed.). CRC Press. https://doi.org/10.1201/b17536

Seninde, D. R., & Chambers IV, E. (2020). Coffee flavor : A review. *Beverages*, *6*(3), 44. https://doi.org/10.3390/beverages6030044

Silva, C. F., Vilela, D. M., de Souza Cordeiro, C., Duarte, W. F., Dias, D. R., & Schwan, R. F. (2013). Evaluation of a potential starter culture for enhance quality of coffee fermentation. *World Journal of Microbiology and Biotechnology*, *29*, 235–247. https://doi.org/10.1007/s11274-012-1175-2

Tai, E.-S., Hsieh, P.-C., & Sheu, S.-C. (2014). Effect of polygalacturonase and feruloyl esterase from *Aspergillus tubingensis* on demucilage and quality of coffee beans. *Process Biochemistry*, *49*(8), 1274–1280. https://doi.org/10.1016/j.procbio.2014.05.001

Tarzia, A., dos Santos Scholz, M. B., & de Oliveira Petkowicz, C. L. (2010). Influence of the postharvest processing method on polysaccharides and coffee beverages. *International Journal of Food Science and Technology*, *45*(10), 2167–2175. https://doi.org/10.1111/j.1365-2621.2010.02388.x

Tinoco, N. A. B., Pacheco, S., Godoy, R. L. O., Bizzo, H. R., de Aguiar, P. F., Leite, S. G. F., & Rezende, C. M. (2019). Reduction of $^{\beta}$N-alkanoyl-5-hydroxytryptamides and diterpenes by yeast supplementation to green coffee during wet processing. *Food Research International*, *115*, 487–492. https://doi.org/10.1016/j.foodres.2018.10.007

Tran, T. M. K., Kirkman, T., Nguyen, M., & Vuong, Q. V. (2020). Effects of drying on physical properties , phenolic compounds and antioxidant capacity of Robusta wet coffee pulp (*Coffea canephora*). *Heliyon*, *6*(7), e04498. https://doi.org/10.1016/j.heliyon.2020.e04498

Tripathi, B. M., Kim, M., Singh, D., Lee-Cruz, L., Lai-Hoe, A., Ainuddin, A. N., Go, R., Rahim, R. A., Husni, M. H. A., Chun, J., & Adams, J. M. (2012). Tropical soil bacterial communities in Malaysia: pH dominates in the equatorial tropics too. *Microbial Ecology*, *64*, 474–484. https://doi.org/10.1007/s00248-012-0028-8

United State Food and Drug Administration. (n.d.). *Bacteriological analytical manual*. FDA. https://www.fda.gov/food/laboratory-methods-food/bacteriological-analytical-manual-bam

Vega, F. E., Emche, S., Shao, J., Simpkins, A., Summers, R. M., Mock, M. B., Ebert, D., Infante, F., Aoki, S., & Maul, J. E. (2021). Cultivation and genome sequencing of bacteria isolated from the coffee berry borer (*Hypothenemus hampei*), with emphasis on the role of caffeine degradation. *Frontiers in Microbiology*, *12*, 6447688. https://doi.org/10.3389/fmicb.2021.644768

Velmourougane, K. (2013). Impact of natural fermentation on physicochemical, micarobiological and cup quality characteristics of arabica and robusta coffee. *Proceedings of the National Academy of Sciences, India, Section B: Biological Sciences*, *83*, 233–239. https://doi.org/10.1007/s40011-012-0130-1

Veloso, T. G. R., da Silva, M. D. C. S., Cardoso, W. S., Guarçoni, R. C., Kasuya, M. C. M., & Pereira, L. L. (2020). Effects of environmental factors on microbiota of fruits and soil of *Coffea arabica* in Brazil. *Scientific Reports*, *10*, 14692. https://doi.org/10.1038/s41598-020-71309-y

Wang, C., Sun, J., Lassabliere, B., Yu, B., & Liu, S. Q. (2020). Coffee flavour modification through controlled fermentation of green coffee beans by *Lactococcus lactis* subsp. *cremoris*. *LWT*, *120*, 108930. https://doi.org/10.1016/j.lwt.2019.108930

Wongsa, P., Khampa, N., Horadee, S., Chaiwarith, J., & Rattanapanone, N. (2019). Quality and bioactive compounds of blends of Arabica and Robusta spray-dried coffee. *Food Chemistry*, *283*, 579–587. https://doi.org/10.1016/j.foodchem.2019.01.088

Zhang, S. J., de Bruyn, F., Pothakos, V., Torres, J., Falconi, C., Moccand, C., Weckx, S., & de Vuyst, L. (2019). Following coffee production from cherries to cup: Microbiological and metabolomic analysis of wet processing of *Coffea arabica*. *Applied and Environmental Microbiology*, *85*(6), e02635-18. https://doi.org/10.1128/AEM.02635-18

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Screening of Atrazine Tolerant Aquatic Plant and Roles of Plant Growth Regulators on Plant Growth and Atrazine Tolerance

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ABSTRACT

The extensive use of atrazine to control weeds in agricultural areas has contaminated atrazine in surface water and groundwater. Atrazine contamination in water resources causes human health concerns. Thus, this study investigated the possible use of aquatic plants for removing atrazine from contaminated water. The experiment was performed under plant nursery conditions and divided into two parts: (1) the atrazine-tolerant plants were screened, and (2) the most atrazine-tolerant plant was used for atrazine phytoremediation stimulated by plant growth regulators. The results showed that atrazine was toxic to all aquatic plants, as the dry weight of the plants was significantly decreased when exposed to 20 mg/L of atrazine (P<0.05). Based on five aquatic plants grown under 2.5–20 mg/L atrazine-contaminated water, *Azolla microphylla* Kaulf. was the most tolerant aquatic plant and was more suitable for use in atrazine phytoremediation than the other aquatic plants (*Ceratophyllum demersum* L., *Eichhornia crassipes* (Mart.) Solms, *Hydrilla verticillata* (L. f.) Royle, and *Salvinia cucullata* Roxb. ex Bory). The total chlorophyll, carotenoid, and proline contents in the biomass of *A. microphylla* cultured in 2.5–20 mg/L of atrazine did not significantly differ between the atrazine concentrations (P>0.05). Meanwhile, the proline contents in the other four aquatic plants increased with increasing atrazine concentrations, and the chlorophyll content significantly decreased with an increase in the atrazine concentration. However, *A. microphylla* could not remove atrazine from contaminated water, and the application of plant growth regulators (6-benzyladenine, gibberellic acid, indole-3-butyric acid, and salicylic acid) did not improve the atrazine removal from water. Atrazine in the water was around 21–26 mg/L on

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day five of *A. microphylla* cultivation compared to the initial concentration (25 mg/L). Using a plant growth regulator was ineffective for stimulating growth and atrazine removal by *A. microphylla*. Future research should explore other potential mechanisms for enhancing atrazine removal by *A. microphylla*.

Keywords: Atrazine, *Azolla*, herbicide, phytoremediation, plant growth regulator

INTRODUCTION

Atrazine is a widely used herbicide to control broadleaf weeds and annual grasses in field crops, such as corn and sugarcane (Steffens et al., 2022). Global use of atrazine is around 70,000–90,000 tons annually (H. He et al., 2019). In Thailand, atrazine is one of the top five imported herbicides (Aungudornpukdee, 2019) because it is inexpensive and efficiently controls weeds (H. He et al., 2019). The amount of atrazine used in sugarcane fields was 480–640 g/m^2 in Thailand (Ratchawang et al., 2022). The extensive use of atrazine for a long time and its chemical structure's stability makes it a ubiquitous contaminant in the environment (H. He et al., 2019; Ratchawang et al., 2022). Contamination by atrazine has been reported in surface water, sediment, and soil in many countries, including Thailand (Phewnil et al., 2012), China (Sun et al., 2017), and Iran (Almasi et al., 2020). For example, the average concentrations of atrazine in the topsoil and subsoil in the Huay Kapo Watershed, Nam Nao District, Phetchabun Province, Thailand, were 133.59 and 183.23 µg/kg, respectively (Phewnil et al., 2010). The contaminations of atrazine in the water and sediment in the agricultural catchment at Nong Bua reservoir, Wiang Sa District, Nan Province, Thailand, were 0.00016 µg/L and 0.00023 µg/kg, respectively (Thitiphuree et al., 2013). The mean concentration of atrazine in agricultural soils around the Yangtze River Delta, China, was 5.7 µg/kg (Sun et al., 2017). The concentration of atrazine in the water of the Shadegan wetland, Iran, ranged between 0 and 2,175.8 µg/L (Almasi et al., 2020). Atrazine applied to the soil leaches into water reservoirs (Rostami et al., 2021), and contamination by atrazine in water resources increases the risk of atrazine in drinking water. According to the United States Environmental Protection Agency and the European Community guidelines, the maximum concentration of atrazine in drinking water should not surpass 3.0 and 0.1 µg/L, respectively (H. He et al., 2019; Marecik et al., 2012). Using atrazine-contaminated water as a source of human drinking water is a public health concern because atrazine is an endocrine disruptor (Rostami et al., 2021; Steffens et al., 2022), and long-term human exposure to atrazine causes damage to the endocrine system (H. He et al., 2019). Moreover, preterm birth was reported in people who have consumed atrazine-contaminated water (Almberg et al., 2018).

Phytoremediation uses plants to decontaminate organic and inorganic pollutants from contaminated sites (Rostami et al., 2021). Phytoremediation of atrazine

by aquatic plants is interesting as a means to remove atrazine from contaminated water (Marecik et al., 2012). The possible mechanisms for the plant to decontaminate atrazine from the polluted water are phytodegradation: the organic contaminant degradation in plant tissue by plant enzymes, rhizodegradation: the exudation of root exudates from plant roots to stimulate the organic contaminant degradation around the root zone, and phytoaccumulation: the accumulation of organic contaminants into plant biomass (Ansari et al., 2020; Kooh et al., 2018; Q. Wang et al., 2012). Several plant species have been reported to remove atrazine from contaminated water, including sweet flag (*Acorus talamus* L.) (Marecik et al., 2012), *Iris pseudacorus* L., *Lythrum salicaria* L., and *Acorus calamus* L. (Q. Wang et al., 2012). Suitable characteristics for plants used in phytoremediation are high biomass, rapid growth under several environmental conditions, and tolerance to toxic contaminants (Sood et al., 2012). However, the sensitivity of aquatic plants to atrazine contamination is a limiting factor in the success of phytoremediation. The toxicity of atrazine has been reported in several aquatic plants, including broadleaf cattail (*Typha latifolia* L.) and narrow-leaf cattail (*Typha angustifolia* L.) (Marecik et al., 2012). Atrazine may inhibit photosynthesis and chlorosis and reduce plant biomass as a response of the plants to the toxicity of atrazine (Rostami et al., 2021; Sánchez et al., 2017).

Using an exogenous plant growth regulator is one way to reduce the toxic effects of contaminants on plants, and they can promote the growth of plants under abiotic stress conditions (Rahman et al., 2023; Y. He et al., 2022). Many plant growth regulators, including indole butyric acid, gibberellin, salicylic acid, and 6-benzyladenine, have been used to mitigate the toxic effects of abiotic stress on plants, including heavy metal stress (Rostami et al., 2021), drought stress (Li et al., 2018), and waterlogging stress (J. Wang et al., 2021). Probable mechanisms for plant growth regulators to alleviate toxic effects in plants grown under abiotic stress involve mediating the antioxidant defense systems and eliminating reactive oxygen species (Emamverdian et al., 2020; J. Wang et al., 2021; Li et al., 2018). For example, indole-3-butyric acid promotes the growth of adventitious roots by controlling antioxidant defense systems in mung bean (*Vigna radiata* (L.) Wilczek) for seedlings grown under cadmium and drought stresses (Li et al., 2018). Gibberellin relieved the toxicity of arsenic in rice (*Oryza sativa* L.) seedlings by reducing the arsenic accumulation in the root (Y. He et al., 2022). Salicylic acid also increases the tolerance of plants grown under heavy metal stress by stimulating antioxidant enzyme synthesis (Emamverdian et al., 2020). Reducing ethylene formation by salicylic acid application has been reported in rice grown under arsenic contamination (Khan et al., 2013, 2021). Exogenous 6-benzyladenine helped improve *Zea mays* L. tolerance to water logging by mitigating the reactive oxygen species produced under

waterlogging stress (J. Wang et al., 2021). However, no reports have investigated the effects of plant growth regulators on aquatic plant growth and the removal of atrazine under atrazine stress. Even though plant growth regulators may improve the growth of plants under atrazine stress to a similar trend as abiotic stress, described above, this study was performed to select the atrazine tolerant plants from five aquatic plant species (*A. microphylla*, *C. demersum*, *E. crassipes*, *H. verticillata*, and *S. cucullata*) because it is the first step of phytoremediation process. Then, the effect of exogenous plant growth regulators (indole butyric acid, gibberellic acid, salicylic acid, and 6-benzyladenine) on plant growth and atrazine remediation by the most tolerant aquatic plants was also determined.

MATERIALS AND METHODS

Preparation of Atrazine-contaminated Water

The atrazine (6-chloro-N2-ethyl-N4-isopropyl-1,3,5-triazine-2,4-diamine 80% w/w) was purchased from an agrochemical shop under the trade name Weethong (V. C. S. Agro Chem Company Limited, Thailand). The atrazine-contaminated water used in the phytotoxicity testing experiment was prepared by dissolving atrazine powder in tap water to give final concentrations of 0, 2.5, 5, 10, and 20 mg/L concentrations. The concentration of atrazine in the phytoremediation experiment was prepared as described previously to give a final 25 mg/L concentration.

Plant Preparation

The aquatic plants, *C. demersum*, *E. crassipes*, *H. verticillate*, and *S. cucullate* were purchased from a plant shop in Maha Sarakham Province, Thailand, and *A. microphylla* was purchased from a plant shop in Khonkaen Province, Thailand. All aquatic plants were gently rinsed with tap water and kept in a plant nursery before use. The environmental conditions in the plant nursery were natural sunlight and actual air temperature. The *A. microphylla* used in the phytoremediation experiment was purchased from the plant shop simultaneously. The plant sample was gently rinsed and mixed in a plastic basin before the experiment. The plant sample was weighed and divided into treatments.

Atrazine Phytotoxicity Testing

The experiment was performed under plant nursery conditions with natural sunlight and actual air temperature in November 2022 in Thailand. The atrazine phytotoxicity testing on the five aquatic plants was performed under a completely randomized design with one factor: atrazine concentration (0, 2.5, 5, 10, and 20 mg/L). The fresh weights of *A. microphylla*, *C. demersum*, *E. crassipes*, *H. verticillata*, and *S. cucullata* at the beginning of the experiment were 7, 30, 20, 30, and 40 g, respectively. Then, each aquatic plant was cultured in a plastic cup containing 500 ml of water contaminated with each concentration of atrazine for five days. The experiment was performed with five replicates. The growth of each plant was observed at the end

of the experiment, including fresh weight, dry weight, chlorophyll *a*, chlorophyll *b*, total chlorophyll, carotenoid, and proline contents. The relative growth rate (RGR) was calculated on a fresh weight basis, as described in Equation 1 (Riaz et al., 2017). The most tolerant aquatic plant was chosen for the atrazine phytoremediation in the next experiment.

$$\text{Relative growth rate (RGR)} = [\ln (W_2) - \ln (W_1)]/t_2 - t_1 \quad [1]$$

where, W_1 = plant weight at the beginning of the experiment; W_2 = plant weight at the last day of the experiment; t_1 = time at the beginning of the experiment; t_2 = time at the last day of the experiment.

Atrazine Phytoremediation Experiment

The experiment was performed in Thailand under plant nursery conditions with natural sunlight and actual air temperature in January 2023. The atrazine phytoremediation experiment was performed under a factorial completely random design (CRD) with 2 x 3 factors. The first factor was atrazine concentration (0 and 25 mg/L), and the second factor was the application of different plant growth regulators, indole butyric acid, gibberellic acid, salicylic acid, and 6-benzyladenine, at concentrations of 0, 1, and 10 mg/L. Atrazine-contaminated water was prepared by dissolving atrazine powder in tap water to give the final concentration of atrazine of 25 mg/L, and non-contaminated water served as a control. Indole butyric acid (Fluka, China), gibberellic acid (Sigma-Aldrich, USA), salicylic acid (Sigma-Aldrich, USA), and 6-benzyladenine (HiMedia Laboratories Pvt Ltd, India) were added separately to atrazine-contaminated water and non-contaminated water to give final concentrations for each plant growth regulator of 0, 1, and 10 mg/L. Then, 7 g of *A. microphylla* was cultured in atrazine-contaminated and non-contaminated water for five days. The experiment was performed with six replicates. The growth of *A. microphylla* and atrazine remaining in the water was observed. The growth of *A. microphylla* was observed by fresh weight, dry weight, chlorophyll *a*, chlorophyll *b*, total chlorophyll, carotenoid, proline, phenolic compound, and flavonoid compound contents. The RGR was also calculated on a fresh weight basis, as in Equation 1.

Atrazine Extraction and Analysis

The atrazine remaining in the water from each treatment was determined by sending it for analysis at the Central Laboratory (Thailand) Co. Ltd. (Khon Kaen branch), and each treatment was analyzed in triplicate. Atrazine was extracted and analyzed using the EPA508 method (Munch, 1995). Briefly, 500 ml of the water sample was mixed with 100 ml of dichloromethane (RCI Labscan Ltd., Thailand). The mixture was shaken in a separation funnel for 2 min and left for 10 min to separate into two layers. The dichloromethane phase was filtered through sodium sulfate (J. T. Baker, USA), and the water phase was further extracted two more times with dichloromethane using

the previously described method. Then, the dichloromethane phase of each extraction was combined, and the dichloromethane extract was dried with a rotary evaporator (EYELA, model EVC2000, Japan). The extract was redissolved with sodium acetate solution (RCI Labscan Ltd., Thailand) and mixed for 15 s with a vortex mixer. The extract of 1 ml was transferred into the vial, and the atrazine in the extract was analyzed.

The atrazine concentrations in the extracts and standards were measured using a gas chromatography-mass spectrometric detector (Model 6890 Network GC System, Agilent Technologies, China), and the separation was achieved using an HP-5MS column (0.25 nm x 250 µm x 30 m, Agilent J&W, China). The sample volume injected into the column was 2 µl under splitless conditions. The oven temperature was 80°C, followed by a linear increase of 10°C per min to 200°C and held for 2 min. The temperature was increased from 200 to 230ºC at 10°C per minute and held for 5 min. The internal quality control for atrazine analysis was reported as the percentage of atrazine recovery. It was performed by spiking 10 µg/L of atrazine into the clean water and extracting it with the same procedure as for the atrazine extraction of the samples. The percentage of atrazine recovery was 100%. The relative percent difference RPD was calculated from duplicates of the sample, with each being 10% of the sample, and the percentage of RPD was less than 20%. The calibration curve was generated from 3–5 points of known atrazine concentration. The reagent blank was analyzed for atrazine, and atrazine was not detected. The limit of detection (LOD) and the limit of quantitation (LOQ) were also included. The detection limit was 10 µg/L, and the quantification limit was 100 µg/L.

Proline Content

The proline content in the plant biomass was determined according to the methods described by Ábrahám et al. (2010) and Bates et al. (1973). For this, 300 mg of fresh plant material was grounded in 5 ml of 3% (w/v) sulphosalicylic acid (Loba Chemie Pvt Ltd, India) in liquid nitrogen, and the sample was centrifuged at 5,120 x *g* for 15 min. Next, 2 ml of the supernatant was transferred and mixed with 2 ml of glacial acetic acid (QRëc, New Zealand) and 2 ml of acid ninhydrin (Kemaus, Australia). Then, the mixture was incubated at 100ºC for 1 hr. The reaction in an ice bath was terminated for 10 min. Afterwards, 4 ml of toluene (Fisher Chemical, United Kingdom) was added and mixed with a vortex mixer for 20 min. The solution was left to separate into two layers before the toluene phase with a red color was transferred to determine the absorbance with a spectrophotometer (EMCLAB, Germany) at a wavelength of 520 nm. The amount of proline was calculated using a reference standard curve of L-proline (Sigma-Aldrich, USA) solution.

Chlorophyll and Carotenoid Contents

Chlorophyll and carotenoid contents were determined according to the methods described in Lichtenthaler (1987) as well

as Sardoei and Rahbarian (2014). For this, 200 mg of fresh plant material was ground in acetone (AnaPure, New Zealand). Then, the mixture was centrifuged at 1,280 x *g* for 5 min. The supernatant was then transferred into a new tube, and the volume with acetone was adjusted to 15 ml. The absorbance was determined with a spectrophotometer (EMCLAB, Germany) at 662, 647, and 470 nm wavelengths. Then, the chlorophyll *a* [2], chlorophyll *b* [3], total chlorophyll [4], and carotenoid [5] contents were calculated using these equations:

Chlorophyll *a* = (12.25 x A662) - (2.79 x A647) [2]

Chlorophyll *b* = (21.50 x A647) - (5.10 x A662) [3]

Total chlorophyll = Chlorophyll *a* + Chlorophyll *b* [4]

Carotenoids = (1000 x A470) - (1.82 x Chlorophyll *a*) - (85.02 x Chlorophyll *b*)/198 [5]

Phenolic and Flavonoid Contents

The crude extract was prepared using methods adapted from Kumari and Pandey-Rai (2018). For this, 100 mg of the dry plant material was grounded to a fine powder and then extracted with 30 ml of 90% ethanol (QRëc, New Zealand) and shaken at 150 rpm for 24 hr. The plant's fine powder was macerated for six days and shaken occasionally daily. The fine powder was filtered through filter paper (Whatman No. 1) to give the crude extract, and the fine powder was repeatedly extracted according to the method described previously one further time. Then, the crude extracts were combined, and the volume was reduced with a rotary evaporator (Buchi Syncore, Switzerland).

The total phenolic content was investigated using the Folin-Ciocalteu method, as described in Lertcanawanichakul et al. (2019). For this, 50 µl of crude extract was mixed with 25 µl of 10% (v/v) Folin-Ciocalteu reagent (Merck, USA), 50 µl of 7.5% sodium carbonate (Ajax FineChem Pyt Ltd, New Zealand), and 50 µl of reverse osmosis water, mixed thoroughly and was allowed to react at 45°C for 45 min. The absorbance was determined with a microplate reader (BMG LABTECH, SPECTROstar®Nano, Germany) at the wavelength of 765 nm. The total phenolic compounds in the sample were calculated using a gallic acid (Sigma-Aldrich, China) standard curve.

The total flavonoid content was investigated using the aluminum chloride colorimetric assay described in Phonprapai and Oontawee (2019). For this, 80 µl of the crude extract was mixed with 50 µl of 2% (w/v) aluminum chloride (Ajax FineChem Pyt Ltd, New Zealand) and 100 µl of 10% (v/v) ethanol (QRëc, New Zealand) and allowed to react under dark conditions for 30 min. The absorbance was determined with a microplate reader (BMG LABTECH, SPECTROstar®Nano, Germany) at a wavelength of 425 nm. The total flavonoid compound in the sample was calculated using a quercetin (Sigma-Aldrich, China) standard curve.

Statistical Analysis

One-way and two-way analysis of variance (ANOVA) were used to analyze the phytotoxicity and plant growth regulator experiments via Microsoft Excel 2019, respectively. The least square difference (LSD) was used for pairwise comparison.

RESULTS AND DISCUSSION

Growth of Aquatic Plants under Various Concentrations of Atrazine

Aquatic plants are a suitable choice to be used as atrazine phytoremediators in aquatic environments because aquatic plants naturally grow in the water, have contact with the contaminant directly, and can adapt to aquatic environmental conditions (Sood et al., 2012). The results in this study revealed that atrazine concentrations ranging from 2.5–20 mg/L exerted toxicity to all aquatic plants used in this study when considering the dry weight of the aquatic plants. The dry weights of *A. microphylla*, *C. demersum*, *E. crassipes*, *H. verticillate*, and *S. cucullata* decreased to 56.9–90.5% of the plants in non-contaminated water when atrazine concentration increased to 20 mg/L, the most toxic concentration (Table 1). In addition, the relative growth rate of all plants decreased significantly with increasing concentrations of atrazine ($P<0.05$) (Table 1). The reduction in the dry weight of the five aquatic plants was related to the decrease in the total chlorophyll content in the biomass of the aquatic plants (Table 1). The chlorophyll contents of *A. microphylla*, *C. demersum*, *E. crassipes*, *H. verticillata*, and *S. cucullata* grown in non-contaminated water were higher than atrazine-contaminated water (Table 1). The chlorophyll contents were decreased in *A. microphylla*, *H. verticillate*, and *S. cucullata* when the atrazine concentration was over 2.5 mg/L and decreases in the chlorophyll contents were observed in *C. demersum* and *E. crassipes* when the atrazine concentration was over 5 mg/L (Table 1). In general, the total chlorophyll content usually decreased in proportion to the increase in the atrazine concentration (Phewnil et al., 2012), which was also observed in all aquatic plants used in this study. The chlorophyll content in *A. microphylla* did not decrease further when the atrazine concentration increased from 5 to 20 mg/L. The total chlorophyll content in *A. microphylla* grown under different concentrations of atrazine did not significantly differ from each other ($P>0.05$). The reductions in the chlorophyll content are a sign of atrazine toxicity because the toxic effect of atrazine was to inhibit photosystem II in plants (Salem & El-Sobki, 2021; Yang & Zhang, 2020). If protein and photosynthetic pigment in the plant photosystem are destroyed, the ability to fix carbon and plant growth will decrease (Yang & Zhang, 2020). Thus, the loss of chlorophyll content from atrazine toxicity results in photosynthesis inhibition, which can decrease plant biomass (Phewnil et al., 2012; Yang & Zhang, 2020). Phytotoxic effects from atrazine have been previously reported; for example, 2.5 mg/L of atrazine inhibited growth, decreased the fresh weight and dry weight, and decreased the chlorophyll content in *Lemna perpusilla* Torr. after seven days of cultivation (Phewnil

et al., 2012). Gao et al. (2011) also reported that 10 µg/L of atrazine decreased the fresh weight and chlorophyll content in *Zostera marina* L., and 86.67% of plants died after exposure to 100 µg/L of atrazine. Decreasing weight and transpiration rate of *T. latifolia* were observed when plants were exposed to 20 µg/L of atrazine (Pérez et al., 2022). Exposure to atrazine at 2 nmol/L for two days decreased the chlorophyll content of *Phaeodactylum tricornutum* Pt-1 to only 37.5% compared to the control without atrazine exposure (Yang & Zhang, 2020). Yang et al. (2019) also reported that the genes encoding for proteins in photosystem II (*PsbO*, *PsbP*, *PsbU*, *PsbQ*, and *Psb27*) and genes encoding for electron transport in *Phaeodactylum tricornutum* Pt-1 were repressed under atrazine exposure (Yang et al., 2019). Another impact of atrazine on plants is tissue necrosis (Phewnil et al., 2012), but it was not observed in *A. microphylla*, *C. demersum*, *E. crassipes*, *H. verticillata*, and *S. cucullata* in this work. None of the aquatic plants showed any phytotoxic symptoms, and the plants looked green when observed by the naked eye.

The carotenoid contents in plant biomass of *A. microphylla* and *S. cucullata* grown in atrazine-contaminated water did not significantly differ from that grown in non-contaminated water (P>0.05) (Table 1). It indicated that atrazine was not toxic to both plants. Meanwhile, a decrease in the carotenoid content was detected in *C. demersum* and *E. crassipes* after atrazine exposure (Table 1). The fluctuation in carotenoid content is a sign of plant response to abiotic stress because carotenoids can act as an antioxidant molecule to neutralize the free radicals produced from photosynthetic reactions in plants (Kopsell et al., 2009). Thus, increasing the carotenoid content is a plant response mechanism to abiotic stress found in *H. verticillata* (Table 1). The decrease in the carotenoid content in *C. demersum* L. and *E. crassipes* may be because these plants used carotenoid molecules to neutralize the toxic effect of the free radicals produced during plant growth under atrazine contamination. Oxidative stress is a sign of toxicity in plants grown under atrazine exposure (Singh et al., 2018). Plants usually overcome oxidative stress by enzymatic and non-enzymatic mechanisms (Singh et al., 2018), and the production of carotenoids is a non-enzymatic mechanism plants use to detoxify the free radicals (Kumari & Pandey-Rai, 2018; Pérez-Gálve et al., 2020). The atrazine tolerance in *A. microphylla* and the proline content in aquatic plants was confirmed again because the proline content in *A. microphylla* was constant between plants grown under atrazine contamination and non-contamination conditions. However, the proline content in *C. demersum*, *E. crassipes*, *H. verticillata*, and *S. cucullata* increased when the atrazine concentration was increased. Increasing the proline content in the plant is another plant response mechanism to oxidative stress (Bibi et al., 2019) because proline can also act as an antioxidant molecule in plants (Din et al., 2020). Increased proline content has been reported in maize seedlings exposed to atrazine at 500 and 1,000 mg/L (Bibi et al., 2019).

Table 1
Relative growth rate (RGR), dry weight, chlorophyll content, carotenoid content, and proline content in five aquatic plants grown in atrazine-contaminated water for five days (data shown as mean ± SE)

Atrazine concentration (mg/L)	RGR (mg/g/day)	Dry weight (% of control)	Chlorophyll *a* (µg/g FW)	Chlorophyll *b* (µg/g FW)	Total Chlorophyll (µg/g FW)	Carotenoid (µg/g FW)	Proline (µg/g FW)
Salvinia cucullata							
0	0.084 ± 0.004 a	100.00 ± 0.44 a	319.28 ± 5.25 a	307.91 ± 7.09 a	627.19 ± 1.84 a	50.83 ± 3.41 a	6.97 ± 0.37 d
2.5	0.075 ± 0.002 b	94.16 ± 0.60 b	283.76 ± 1.79 b	289.14 ± 6.21 ab	572.90 ± 4.53 b	57.58 ± 2.24 a	9.26 ± 0.17 c
5	0.047 ± 0.004 c	92.31 ± 0.49 c	244.36 ± 2.81 c	273.51 ± 3.88 b	517.87 ± 5.97 c	59.22 ± 2.52 a	9.35 ± 0.09 c
10	0.024 ± 0.003 d	91.39 ± 0.54 cd	234.48 ± 5.10 c	282.71 ± 5.89 b	517.19 ± 8.45 c	54.73 ± 3.06 a	11.47 ± 0.25 b
20	0.014 ± 0.002 e	90.24 ± 0.25 d	233.88 ± 4.13 c	278.38 ± 6.84 b	512.26 ± 2.89 c	54.45 ± 2.45 a	13.68 ± 0.31 a
Eichhornia crassipes							
0	0.034 ± 0.003 a	100.00 ± 4.16 a	446.03 ± 23.64 a	212.94 ± 21.01 a	658.97 ± 3.94 a	161.66 ± 20.55 a	6.12 ± 0.29 c
2.5	0.033 ± 0.002 a	95.67 ± 5.15 a	324.23 ± 29.70 b	280.94 ± 18.82 a	605.16 ± 17.60 a	111.36 ± 15.81 ab	8.83 ± 0.31 b
5	0.028 ± 0.004 ab	73.55 ± 3.82 b	285.42 ± 2.90 b	218.28 ± 52.96 a	503.70 ± 50.61 b	79.03 ± 31.07 b	8.67 ± 0.29 b
10	0.019 ± 0.004 bc	76.90 ± 4.01 b	129.85 ± 5.32 c	88.82 ± 17.63 b	218.67 ± 18.48 c	81.12 ± 14.55 b	11.22 ± 0.29 a
20	0.017 ± 0.004 c	67.43 ± 5.39 b	104.65 ± 7.55 c	75.41 ± 19.03 b	180.06 ± 19.22 c	58.32 ± 16.42 b	10.71 ± 0.15 a
Ceratophyllum demersum							
0	0.018 ± 0.003 a	100.00 ± 0.62 a	427.68 ± 7.38 a	216.01 ± 6.37 a	643.68 ± 4.89 a	168.34 ± 11.55 a	8.24 ± 0.17 c
2.5	-0.039 ± 0.007 b	96.00 ± 0.94 b	399.84 ± 3.95 b	218.44 ± 10.93 a	618.28 ± 7.12 a	172.98 ± 16.13 a	7.90 ± 0.15 c
5	-0.068 ± 0.007 c	90.86 ± 1.20 c	148.68 ± 2.86 c	108.95 ± 8.87 b	257.62 ± 9.29 b	40.62 ± 1.67 b	9.26 ± 0.45 b
10	-0.070 ± 0.004 c	90.51 ± 1.41 c	82.09 ± 1.84 d	92.94 ± 17.98 b	175.03 ± 18.87 c	59.59 ± 11.06 b	9.69 ± 0.29 b
20	-0.086 ± 0.008 c	90.51 ± 1.81 c	63.45 ± 1.59 e	80.36 ± 17.08 b	143.81 ± 18.38 c	28.55 ± 9.62 b	11.05 ± 0.31 a
Hydrilla verticillata							
0	0.041 ± 0.004 a	100.00 ± 0.82 a	593.18 ± 26.31 a	791.32 ± 6.15 a	1384.50 ± 20.74 a	-57.61 ± 9.26 d	8.16 ± 0.29 c

Table 1 *(Continue)*

Atrazine concentration (mg/L)	RGR (mg/g/day)	Dry weight (% of control)	Chlorophyll *a* (µg/g FW)	Chlorophyll *b* (µg/g FW)	Total Chlorophyll (µg/g FW)	Carotenoid (µg/g FW)	Proline (µg/g FW)
2.5	0.030 ± 0.004 b	87.17 ± 1.65 b	459.16 ± 24.51 b	649.84 ± 3.96 b	1109.00 ± 21.40 b	13.21 ± 10.44 c	7.82 ± 0.22 d
5	-0.021 ± 0.002 c	59.47 ± 0.59 c	298.88 ± 9.35 c	474.00 ± 6.85 c	772.91 ± 6.46 c	65.68 ± 9.07 b	9.35 ± 0.09 b
10	-0.045 ± 0.004 c	55.53 ± 1.04 d	293.71 ± 7.80 c	444.66 ± 2.75 d	738.37 ± 10.55 c	72.78 ± 14.78 b	9.52 ± 0.09 b
20	-0.053 ± 0.004 c	56.93 ± 1.43 cd	147.19 ± 26.34 d	278.88 ± 4.42 e	426.07 ± 22.66 d	138.11 ± 8.49 a	10.62 ± 0.09 a
Azolla microphylla							
0	0.021 ± 0.004 a	100.00 ± 2.84 a	164.63 ± 4.63 a	93.15 ± 1.81 a	257.78 ± 5.67 a	73.03 ± 3.83 a	9.52 ± 0.52 a
2.5	-0.036 ± 0.004 b	90.07 ± 1.81 b	147.65 ± 4.90 b	61.07 ± 11.62 b	208.72 ± 9.29 b	60.44 ± 3.07 a	9.35 ± 0.68 a
5	-0.046 ± 0.007 bc	89.36 ± 2.07 b	136.94 ± 3.82 b	60.95 ± 5.76 b	197.89 ± 3.23 b	65.77 ± 6.35 a	10.62 ± 1.11 a
10	-0.046 ± 0.009 bc	83.69 ± 1.42 b	138.38 ± 2.81 b	58.83 ± 9.40 b	197.20 ± 7.00 b	59.72 ± 6.24 a	10.79 ± 0.22 a
20	-0.059 ± 0.002 c	75.89 ± 3.98 c	143.11 ± 2.31 b	55.84 ± 2.65 b	198.95 ± 1.17 b	58.06 ± 0.61 a	10.96 ± 0.29 a

Note. Different lowercase letters show significant differences ($P<0.05$) between atrazine concentrations within the same aquatic plant

Growth of *A. microphylla* under Plant Growth Regulator Application

Based on the dry weight, total chlorophyll, carotenoid, and proline contents of the tested aquatic plants described above, *A. microphylla*, the model aquatic plant, was selected for atrazine remediation in the next experiment because it is the most atrazine-tolerant plant of those tested. When observed by the naked eye, *A. microphylla* showed no sign of phytotoxicity at 20 mg/L of atrazine (Figure 1). Another suitable characteristic of *Azolla* for phytoremediation is its rapid growth. It is a free-floating plant that would be easy to manage after the phytoremediation process. Aquatic ferns have been reported to be used in the phytoremediation of various pollutants, namely heavy metals and pesticides (Sood et al., 2012). The atrazine remediation experiment was performed by growing *A. microphylla* in 25 mg/L of atrazine and using a plant growth regulator (indole butyric acid, gibberellin, salicylic acid, or 6-benzyladenine) to promote the growth of *A. microphylla* under atrazine contamination. There was a significant interaction between atrazine concentration and different types of plant growth regulators for all plant traits (Table 2). In atrazine-contaminated water, only 10 mg/L gibberellic acid, 1 mg/L indole butyric acid, and 1 mg/L 6-benzyladenine could increase the dry weight of *A. microphylla* significantly compared with plants exposed to atrazine without any plant growth regulator application. All plant growth regulators could significantly increase the relative growth rate in non-contaminated water. However, only 10 mg/L 6-benzyladenine, 1 mg/L indole butyric acid, 1–10 mg/L salicylic acid, and 10 mg/L gibberellic acids could increase the relative growth rate of *A. microphylla* in atrazine-contaminated water significantly (Table 2). Without a plant growth regulator, the results revealed that 25 mg/L atrazine decreased the total chlorophyll and carotenoid content and increased the proline content of *A. microphylla* (Table 2). The proline content of *A. microphylla* grown under atrazine contamination was higher than that grown under the non-contaminated condition. It was evident in the response of *A. microphylla* grown under 25 mg/L of atrazine, whereas proline acts as an antioxidant molecule that plants synthase in response to atrazine stress (Bibi et al., 2019). In addition, the phenolic and flavonoid contents in *A. microphylla* grown under atrazine-contaminated water without the plant growth regulator application did not significantly differ (P>0.05) from that with the plant growth regulator (6-benzyladenine, gibberellic acid, indole butyric acid, and salicylic acid) application (Table 2). Likewise, phenolic and flavonoid compounds are

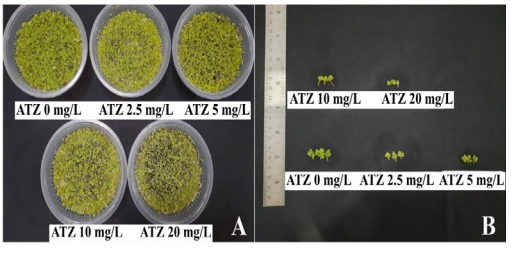

Figure 1. Growth of *Azolla microphylla* grown under atrazine-contaminated water in a concentration range from 0–20 mg/L for five days
Note. ATZ = Atrazine

secondary metabolites that protect plants from oxidative stress in plants (Kiani et al., 2021). Plant homeostasis between reactive oxygen species and phenolic or flavonoid compounds was a general mechanism of plant adaptation to abiotic stress (Kiani et al., 2021). The unchanged amount of phenolic and flavonoid compounds in *A. microphylla* may be due to the tolerant nature of the plant to atrazine. Thus, increasing the synthesis of phenolic and flavonoid compounds in response to atrazine stress was unnecessary for *A. microphylla* in this study. The exogenous plant growth regulator used did not affect the amount of both compounds; it may be due to the plant growth regulator not inducing the synthesis of phenolic and flavonoid compounds. Meanwhile, the previous research by Kumari and Pandey-Rai (2018) reported that exogenous plant growth regulators could induce the synthesis of phenolic and flavonoid compounds in plants grown under abiotic stress.

The plant growth regulators (6-benzyleadenine, indole butyric acid, salicylic acid, and gibberellic acid) used in this study did not improve *A. microphylla* growth under atrazine contamination. However, 10 mg/L of 6-benzyladenine and 10 mg/L of gibberellic acid tended to increase the dry weight, total chlorophyll content, and carotenoid content in *A. microphylla* grown under atrazine contamination to a greater extent than the other plant growth regulators (Table 2). Both plant growth regulators were used to stimulate growth, conserve chlorophyll content, and alleviate the toxic effects of abiotic stress on plants by various mechanisms. For example, gibberellic acid at 100 ppm has increased the weight of wheat grown under heat stress. However, it did not affect the level of antioxidant enzymes, lipid peroxidation, and membrane stability (Nagar et al., 2021). Pre-treatment of wheat seeds with 0.01–1.0 µM gibberellic acid alleviated Ni toxicity by increasing the chlorophyll content and decreasing the percentage of electrolyte leakage (Siddiqui et al., 2011). The 6-benzyladenine at 10 µM alleviated the abiotic stress from salt in *Solanum melongena* Mill. by increasing the chlorophyll content, decreasing the superoxide anion production, decreasing the malondialdehyde content, and increasing the antioxidant enzymes and proline content (Wu et al., 2014). Moreover, 0.5 mM of 6-benzyleadenine also promoted the shoot and root growth, reduced the superoxide anion and hydrogen peroxide accumulation, reduced the malondialdehyde content, and increased the antioxidant enzymes in a maize waterlogging sensitive strain (SY-XT1) grown under waterlogging conditions (J. Wang et al., 2021). However, promoting the growth of *A. microphylla* by salicylic acid and indole butyric acid was not observed in this study. However, both plant growth regulators have been used to stimulate the growth and tolerance of plants under heavy metal stress with a similar mechanism as other plant growth regulators (Kumari & Pandey-Rai, 2018; Šípošová et al., 2021). The concentration of the plant growth regulator also influenced the response of plants (Šípošová et al., 2021). This study found that only ten mg/L of 6-benzyladenine

Table 2

Relative growth rate (RGR), dry weight, chlorophyll, carotenoid, proline, phenolic, and flavonoid compounds in Azolla microphylla *grown under atrazine-contaminated water with various plant growth regulators used for five days (data shown as mean ± SE)*

Plant Growth Regulator	RGR (mg/g/day)	Dry weight (% of control)	Chlorophyll *a* (µg/g FW)	Chlorophyll *b* (µg/g FW)	Total chlorophyll (µg/g FW)	carotenoid (µg/g FW)	Proline (µg/g FW)	Phenolic (µg gallic acid/ mg DW)	Flavonoid (µg quercetin/ mg DW)
Non-contamination									
Indole butyric acid (1 mg/L)	0.039 ± 0.001 Aa	103.88 ± 1.32 Aab	87.21 ± 1.60 Abc	39.58 ± 2.07 Ad	126.79 ± 1.54 Ad	24.82 ± 1.19 Aab	83.65 ± 1.22 Bb	N.D.	N.D.
Indole butyric acid (10 mg/L)	0.025 ± 0.005 Aa	99.46 ± 2.09 Ab	88.59 ± 0.89 Abc	43.96 ± 2.90 Ad	132.56 ± 2.71 Ad	22.17 ± 1.77 Ab	79.18 ± 1.04 Bbc	N.D.	N.D.
Gibberellic acid (1 mg/L)	0.039 ± 0.003 Aa	104.20 ± 1.75 Aab	91.87 ± 1.32 Abc	55.56 ± 1.96 Bc	147.43 ± 3.02 Ac	23.02 ± 0.05 Aab	81.74 ± 1.81 Bbc	N.D.	N.D.
Gibberellic acid (10 mg/L)	0.039 ± 0.002 Aa	106.14 ± 1.45 Aa	113.79 ± 1.77 Aa	70.85 ± 2.28 Ab	184.63 ± 0.77 Aa	25.07 ± 1.40 Aab	69.60 ± 2.63 Bc	N.D.	N.D.
Salicylic acid (1 mg/L)	0.033 ± 0.005 Aa	101.40 ± 1.58 Ab	97.27 ± 1.48 Ab	62.47 ± 1.77 Abc	159.74 ± 1.03 Ab	27.68 ± 0.42 Aa	73.44 ± 2.18 Bc	N.D.	N.D.
Salicylic acid (10 mg/L)	0.030 ± 0.004 Aa	99.78 ± 1.84 Ab	63.86 ± 1.94 Acd	47.01 ± 2.49 Acd	110.87 ± 2.44 Ae	19.47 ± 1.57 Ab	92.59 ± 1.22 Ba	N.D.	N.D.
6-benzyladenine (1 mg/L)	0.033 ± 0.004 Aa	105.06 ± 2.43 Aab	77.77 ± 22.46 Acd	88.89 ± 10.66 Aa	166.66 ± 11.87 Ab	18.50 ± 4.36 Ab	92.59 ± 1.22 Ba	N.D.	N.D.
6-benzyladenine (10 mg/L)	0.037 ± 0.002 Aa	103.66 ± 1.73 Aab	100.67 ± 0.63 Aab	61.43 ± 0.97 Abc	162.10 ± 1.43 Ab	26.10 ± 0.74 Aab	75.35 ± 1.65 Bc	N.D.	N.D.
No plant growth regulator	0.011 ± 0.002 Ab	100.00 ± 1.75 Ab	83.75 ± 0.60 Abc	52.84 ± 1.40 Acd	136.59 ± 1.88 Ad	23.51 ± 0.49 Aab	91.95 ± 4.30 Ba	N.D.	N.D.
Atrazine-contamination									
Indole butyric acid (1 mg/L)	0.013 ± 0.003 Bb	101.40 ± 0.77 Aab	61.04 ± 1.70 Bb	38.66 ± 5.07 Ac	99.69 ± 4.43 Bc	24.89 ± 2.76 Aa	107.28 ± 1.81 Ac	11.40 ± 0.93 a	109.40 ± 13.09 a
Indole butyric acid (10 mg/L)	-0.038 ± 0.006 Bd	76.94 ± 0.94 Be	37.99 ± 1.23 Bc	33.76 ± 2.32 Ac	71.75 ± 3.53 Be	12.05 ± 0.25 Bc	130.91 ± 4.22 Aa	12.43 ± 1.38 a	121.28 ± 11.88 a

Table 2 *(Continue)*

Plant Growth Regulator	RGR (mg/g/day)	Dry weight (% of control)	Chlorophyll *a* (µg/g FW)	Chlorophyll *b* (µg/g FW)	Total chlorophyll (µg/g FW)	carotenoid (µg/g FW)	Proline (µg/g FW)	Phenolic (µg gallic acid/ mg DW)	Flavonoid (µg quercetin/ mg DW)
Gibberellic acid (1 mg/L)	-0.023 ± 0.008 Bc	75.75 ± 1.47 Be	53.41 ± 1.95 Bbc	72.34 ± 3.42 Aa	125.75 ± 1.88 Bb	9.17 ± 1.74 Bcd	104.09 ± 1.22 Ac	13.86 ± 2.40 a	173.78 ± 9.32 a
Gibberellic acid (10 mg/L)	0.024 ± 0.014 Aa	103.45 ± 1.48 Aa	93.57 ± 2.70 Ba	57.53 ± 4.64 Bb	151.09 ± 2.65 Ba	23.11 ± 1.97 Aa	88.12 ± 2.66 Ae	11.09 ± 2.55 a	116.45 ± 21.02 a
Salicylic acid (1 mg/L)	0.001 ± 0.007 Bb	84.16 ± 1.22 Bcd	44.40 ± 0.79 Bc	37.96 ± 2.33 Bc	82.35 ± 2.94 Bd	5.83 ± 0.79 Bd	114.94 ± 2.76 Ab	17.18 ± 2.97 a	110.01 ± 16.87 a
Salicylic acid (10 mg/L)	0.010 ± 0.006 Bb	81.90 ± 0.91 Bd	43.75 ± 1.52 Bc	41.28 ± 2.20 Ac	85.03 ± 2.92 Bd	3.09 ± 0.44 Bd	116.22 ± 1.65 Ab	13.64 ± 1.58 a	137.43 ± 16.91 a
6-benzyladenine (1 mg/L)	-0.012 ± 0.001 Bc	86.32 ± 1.29 Bc	63.79 ± 1.31 Ab	54.29 ± 1.51 Bb	118.08 ± 0.78 Bbc	17.58 ± 1.02 Ab	106.64 ± 1.61 Ac	13.97 ± 1.09 a	108.72 ± 21.14 a
6-benzyladenine (10 mg/L)	0.029 ± 0.004 Aa	98.92 ± 1.46 Bb	92.33 ± 1.42 Aa	63.76 ± 1.78 Aab	156.09 ± 1.80 Aa	22.53 ± 0.88 Aa	95.15 ± 2.63 Ad	11.91 ± 1.14 a	116.11 ± 15.92 a
No plant growth regulator	-0.024 ± 0.002 Bc	82.00 ± 0.97 Bd	61.49 ± 1.24 Bb	46.85 ± 2.38 Abc	108.35 ± 1.33 Bc	14.64 ± 1.97 Bbc	118.14 ± 1.22 Ab	14.76 ± 2.11 a	160.41 ± 13.04 a
Atrazine	**	**	**	**	**	**	**	-	-
Plant growth regulator	**	**	**	**	**	**	**	-	-
Atrazine x PGR	**	**	**	**	**	**	**	-	-

Note. Different lowercase letters show significant differences (P<0.05) between plant growth regulators within the same atrazine concentration; different capital letters show significant differences (P<0.05) between with and without atrazine at the same plant growth regulator (PGR); N.D. = Not determined; ** = Statistical significance (P<0.05)

and gibberellic acid stimulated the growth of *A. microphylla*. However, 1 mg/L of both plant growth regulators did not increase the dry weight, total chlorophyll content, and carotenoid content in *A. microphylla*. The findings in this study correspond with the study of Šípošová et al. (2021), who revealed that indole butyric acid at 10^{-9} M promoted the growth of maize under soil contaminated with 50 mM of cadmium nitrate, while 10^{-7} M of indole butyric acid inhibited the growth of maize under cadmium nitrate contamination (Šípošová et al., 2021).

Removal of Atrazine by *A. microphylla*

Cultivation of *A. microphylla* was unable to remove atrazine from contaminated water because the amount of atrazine remaining in the water after five days of *A. microphylla* cultivation was 22.67 mg/L, which was not significantly different from the amount of atrazine remaining in the unplanted control (25.33 mg/L). The initial concentration of tested atrazine was 25 mg/L (Table 3). The application of the plant growth regulators (6-benzyleadenine, indole butyric acid, salicylic acid, and gibberellic acid) did not improve the ability of *A. microphylla* to remove atrazine from the contaminated water (Table 3). The atrazine remaining in the water was around 21–26 mg/L when the plant growth regulator was applied, and the amount of atrazine remaining was not significantly different from the treatment without the plant growth regulator application ($P>0.05$). However, using 10 mg/L of 6-benzyladenine and 1 mg/L of indole butyric acid stimulates the removal of atrazine by *A. microphylla* compared to using both concentrations of salicylic acid. However, the amount of atrazine remaining in the water when using each type of plant growth regulator did not significantly differ from that without the plant growth regulator application ($P>0.05$). Based on our results,

Table 3
Atrazine remaining in water after cultivation of Azolla microphylla *with various plant growth regulators for five days (data shown as mean ± SE)*

Treatment	Atrazine (mg/L)
Indole butyric acid (1 mg/L)	21.50 ± 1.04 b
Indole butyric acid (10 mg/L)	26.00 ± 1.32 a
Gibberellic acid (1 mg/L)	23.92 ± 1.92 ab
Gibberellic acid (10 mg/L)	22.83 ± 0.17 ab
Salicylic acid (1 mg/L)	25.50 ± 1.04 a
Salicylic acid (10 mg/L)	25.42 ± 0.79 a
6-benzyladenine (1 mg/L)	22.17 ± 0.73 ab
6-benzyladenine (10 mg/L)	21.00 ± 0.29 b
No plant growth regulator	22.67 ± 0.17 ab
Non-cultivation of *A. microphylla*	25.33 ± 2.13 a

Note. Different lowercase letters show significant differences ($P<0.05$) between plant growth regulators within the same atrazine concentration

it was difficult to indicate the possible mechanisms of atrazine removal by *A. microphylla* because the amount of atrazine in contaminated water did not decrease significantly (P>0.05) between planting and not planting in atrazine-contaminated water. It suggests that *A. microphylla* could tolerate atrazine only. However, it could not remove atrazine from contaminated water. The findings in this study contradicted the previous studies by other researchers. For example, aquatic macrophytes, namely *I. pseudacorus*, *L. salicaria*, and *A. calamus*, could degrade atrazine by 75.6, 65.5, and 61.8%, respectively, when cultivated under hydroponic conditions for 20 days with an initial atrazine concentration of 4 mg/L (Q. Wang et al., 2012). The main mechanism of atrazine removal was the activity of plants to degrade atrazine and other mechanisms to remove atrazine, such as microbial degradation and abiotic degradation (Q. Wang et al., 2012). The reason why *A. microphylla* could not improve the atrazine removal from water in this study, possibly due to limited atrazine-degrading microorganisms found in the water used. Tap water was used to prepare the atrazine-contaminated water in this study, despite heterotrophic bacteria being a common microorganism found in tap water (Harnroongroj et al., 2012). Atrazine degradation activity was generally poor under sterilized conditions (Q. Wang et al., 2012). Moreover, the period of *A. microphylla* cultivation in this study was short, at only five days for this experiment. *Azolla microphylla* and related microorganisms might not have adapted to degrade atrazine during the experiment. The study by Q. Wang et al. (2012) reported that *I. pseudacorus*, *L. salicaria*, and *A. calamus* took 20 days for atrazine removal. However, the period for atrazine removal varied depending on the plant species. Marecik et al. (2021) reported that cultivation of *A. calamus* under hydroponic conditions for seven days could reduce atrazine by 57% (the initial concentration of atrazine was 3.5 g/L) and 97% of atrazine was removed after extending the time for *A. calamus* to 21 days. Meanwhile, *T. latifolia* took 50 days for 90% atrazine removal under hydroponic conditions (Marecik et al., 2021). Based on the cultivation of aquatic plants in other studies, it can be suggested that the cultivation period of *A. microphylla* was extended for more than five days; the atrazine may be removed.

Despite there being no atrazine removal by *A. microphylla* in this study, however, the mechanism for pollutant removal in genus *Azolla* in water is often by accumulation or phytoextraction, such as for cadmium (Rai, 2008) and methyl violet 2B dye (Kooh et al., 2018). Applying plant growth regulators in this study did not increase the plant capacity to remove atrazine. It may be due to the concentration of the plant growth regulator being suitable only to stimulate plant growth but not for increased atrazine removal. In addition, some plant growth regulators, such as cytokinin and salicylic acid, have been reported to decrease pollutant accumulation in some plants and algae. For example, cytokinin decreased Pb accumulation in

the algae cells of *Acutodesmus obliquus* (Piotrowska-Niczyporuk et al., 2018). Salicylic acid application to hemp grown in cadmium-contaminated sand decreased the Cd uptake into plants (Shi et al., 2009). If the atrazine-degrading microorganisms were insufficient, it is possible that some plant growth regulators that supported plant-microbe interactions were not working well. Thus, the use of *A. microphylla* in atrazine phytoremediation in the future should be done with the augmentation of atrazine-degrading microorganisms combined with the cultivation of *A. microphylla* in contaminated water.

CONCLUSION

Azolla microphylla was an atrazine-tolerant plant, but it could not remove atrazine when cultivated in contaminated water alone. About 22.67 mg/L of atrazine was remained in water at the end of experiment. Applying salicylic and indole butyric acid did not promote the growth and phytoremediation by *A. microphylla* grown in atrazine-contaminated water. Gibberellic acid and 6-benzyladenine were suitable for stimulating the growth of *A. microphylla* under atrazine contamination. Percentage of dry weight and total chlorophyll content of *A. microphylla* were 103 and 156.09 ug/g fresh weight when recieving 10 mg/l of giberellic acid and 6-benzyladenine, respectively. However, the suitable concentration should be determined when using both plant growth regulators. The application of *A. microphylla* and atrazine-degrading microorganisms may be interesting in aiding atrazine degradation. This assumption should be investigated in future work.

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REFERENCES

Ábrahám, E., Hourton-Cabassa, C., Erdei, L., & Szabados, L. (2010). Methods for determination of proline in plants. In R. Sunkar (Ed.), *Plant stress tolerance: Methods in molecular biology* (Vol. 639, pp. 317-331). Humana Press. https://doi.org/10.1007/978-1-60761-702-0_20

Almasi, H., Takdastan, A., Jaafarzadeh, N., Babaei, A. A., Birgani, Y. T., Cheraghian, B., Saki, A., & Jorf, S. (2020). Spatial distribution, ecological and health risk assessment and source identification of atrazine in Shadegan international wetland, Iran. *Marine Pollution Bulletin*, *160*, 111569. https://doi.org/10.1016/j.marpolbul.2020.111569

Almberg, K. S., Turyk, M. E., Jones, R. M., Rankin, K., Freels, S., & Stayner, L. T. (2018). Atrazine contamination of drinking water and adverse birth outcomes in community water systems with elevated atrazine in Ohio, 2006-2008. *International Journal of Environmental Research and Public Health*, *15*(9), 1889. https://doi.org/10.3390/ijerph15091889

Ansari, A. A., Naeem, N., Gill, S. S., & AlZuaibr, F. M. (2020). Phytoremediation of contaminated waters: An eco-friendly technology based on aquatic macrophytes application. *Egyptian Journal of Aquatic Research*, *46*(4), 371-376. https://doi.org/10.1016/j.ejar.2020.03.002

Aungudornpukdee, P. (2019). Pesticide use and environmental contamination a study in Khao Koh District, Phetchabun Province, Thailand. *Journal of Health Research*, *33*(2), 173-182. https://doi.org/10.1108/JHR-12-2018-0174

Bates, L. S., Waldren R. P., & Teare I. D. (1973). Rapid determination of free proline for water stress studies. *Plant and Soil*, *39*, 205-207. https://doi.org/10.1007/BF00018060

Bibi, S., Khan, S., Taimur, N., Daud, M. K., & Azizullah, A. (2019). Responses of morphological, physiological, and biochemical characteristics of maize (*Zea mays* L.) seedlings to atrazine stress. *Environmental Monitoring and Assessment*, *191*, 717. https://doi.org/10.1007/s10661-019-7867-4

Din, B. U., Amna., Rafiquee, M., Javed, M. T., Kamran, M. A., Mehmood, S., Khan, M., Sultan, T., Munis, M. F. H., & Chaudhary, H. J. (2020). Assisted phytoremediation of chromium spiked soils by *Sesbania sesban* in association with *Bacillus xiamenensis* PM14: A biochemical analysis. *Plant Physiology and Biochemistry*, *146*, 249-258. https://doi.org/10.1016/j.plaphy.2019.11.010

Emamverdian, A., Ding, Y., & Mokhberdoran, F. (2020). The role of salicylic acid and gibberellin signaling in plant responses to abiotic stress with an emphasis on heavy metals. *Plants Signaling and Behavior*, *15*(7), 1777372. https://doi.org/10.1080/15592324.2020.1777372

Gao, Y., Fang, J., Zhang, J., Ren, L., Mao, Y., Li, B., Zhang, M., Liu, D., & Du, M. (2011). The impact of the herbicide atrazine on growth and photosynthesis of seagrass, *Zostera marina* (L.), seedlings. *Marine Pollution Bulletin*, *62*(8), 1628-1631. https://doi.org/10.1016/j.marpolbul.2011.06.014

Harnroongroj, T., Leelaporn, A., Limsrivanichayakorn, S., Kaewdaeng, S., & Harnroongroj, T. (2012). Comparison of bacterial count in tap water between first burst and running tap water. *Journal of the Medical Association of Thailand*, *95*(5), 712-715.

He, H., Liu, Y., You, S., Liu, J., Xiao, H., & Tu, Z. (2019). A review on recent treatment technology for herbicide atrazine in contaminated environment. *International Journal of Research and Public Health*, *16*(24), 5129. https://doi.org/10.3390/ijerph16245129

He, Y., Zhang, T., Sun, Y., Wang, X., Cao, Q., Fang, Z., Chang, M., Cai, Q., & Luo, Q. (2022). Exogenous IAA alleviates arsenic toxicity to rice and reduces arsenic accumulation in rice grains. *Journal of Plant Growth Regulation*, *41*, 734-741. https://doi.org/10.1007/s00344-021-10336-z

Khan, M. I. R., Iqbal, N., Masood, A., Per, T. S. & Khan, N. A. (2013) Salicylic acid alleviates adverse effects of heat stress on photosynthesis through changes in proline production and ethylene formation. *Plant Signaling and Behavior*, *8*(11), e26374. https://doi.org/10.4161/psb.26374

Khan, M. I. R., Jahan, B., AlAjmi, M. F., Rehman, M. T., Iqbal, N., Irfan, M., Sehar, Z., & Khan, N. A. (2021). Crosstalk of plant growth regulators protects photosynthetic performance from arsenic damage by modulating defense systems in rice. *Ecotoxicology and Environmental Safety*, *222*, 112535. https://doi.org/10.1016/j.ecoenv.2021.112535

Kiani, R., Arzani, A., & Mirmohammady Maibody, S. A. M. (2021). Polyphenols, flavonoids, and antioxidant activity involved in salt tolerance in wheat, *Aegilops rica* and their amphidiploids. *Frontiers in Plant Science*, *12*, 646221. https://doi.org/10.3389/fpls.2021.646221

Kooh, M. R. R., Lim, L. B. L., Lim, L.-H., & Malik, O. A. (2018). Phytoextraction potential of water fern (*Azolla pinnata*) in the removal of a hazardous dye, methyl violet 2B: Artificial

neural network modeling. *International Journal of Phytoremediation*, *20*(5), 424-431. https://doi.org/10.1080/15226514.2017.1365337

Kopsell, D. A., Armel, G. R., Mueller, T. C., Sams, C. E., Deyton, D. E., Mcelroy, J. S., & Kopsell, D. E. (2009). Increase in nutritionally important sweet corn kernel carotenoids following mesotrione and atrazine applications. *Journal of Agricultural and Food Chemistry*, *57*(14), 6362-6368. https://doi.org/10.1021/jf9013313

Kumari, A., & Pandey-Rai, S. (2018). Enhanced arsenic tolerance and secondary metabolism by modulation of gene expression and proteome profile in *Artemisia annua* L. after application of exogenous salicylic acid. *Plant Physiology and Biochemistry*, *132*, 590-602. https://doi.org/10.1016/j.plaphy.2018.10.010

Lertcanawanichakul, M., Chawawisit, K., & Hiransai, P. (2019). Biological activities of extracts from some local plants in Pakpanang, Nakhon Si Thammarat Province: Antioxidant and antibacterial activity. *Rajamangala University of Technology Srivijaya Research Journal*, *11*(2), 279-289.

Li, S.-W., Zeng, X.-Y., Leng, Y., Feng, L., & Kang, X.-H. (2018). Indole-3-butyric acid mediates antioxidative defense systems to promote adventitious rooting in mung bean seedlings under cadmium and drought stresses. *Ecotoxicology and Environmental Safety*, *161*, 332-341. https://doi.org/10.1016/j.ecoenv.2018.06.003

Lichtenthaler, H. K. (1987). Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. In R. Douce & L. Packer (Eds.), *Methods in enzymology* (Vol. 148, pp. 350-382). Academic Press. https://doi.org/10.1016/0076-6879(87)48036-1

Marecik, R., Białas, W., Cyplik, P., Ławniczak, Ł., & Chrzanowski, Ł. (2012). Phytoremediation potential of three wetlands plant species toward atrazine in environmentally relevant concentrations. *Polish Journal of Environmental Studies*, *21*(3), 697-702.

Munch, J. W. (Ed.) (1995). *Determination of chlorinated pesticides in water by gas chromatography with an electron capture detector*. Environmental Protection Agency.

Nagar, S., Singh, V. P., Arora, A., Dhakar, R., Singh, N., Singh, G. P., Meena, S., Kumar, S., & Shiv Ramakrishnan, R. (2021). Understanding the role of gibberellic acid and paclobutrazol in terminal heat stress tolerance in wheat. *Frontiers in Plant Science*, *12*, 692252. https://doi.org/10.3389/fpls.2021.692252

Pérez, D. J., Doucette, W. J., & Moore, M. T. (2022). Atrazine uptake, translocation, bioaccumulation and biodegradation in cattail (*Typha latifolia*) as a function of exposure time. *Chemosphere*, *287*(Part 1), 132104. https://doi.org/10.1016/j.chemosphere.2021.132104

Phewnil, O., Panichsakpatana, S., Tungkananuruk, N., & Pitiyont, B. (2010). Atrazine transport from the maize (*Zea mays* L.) cultivated upland soil in Huay Kapo Watershed, Nam Nao District, Phetchabun Province, Thailand. *Thai Journal of Agricultural Science*, *43*(3), 119-127.

Phewnil, O.-A., Tungkananurak, N., Panichsakpatana, S., Pitiyont, B., Siripat, N., & Watanabe, H. (2012). The residues of atrazine herbicide in stream water and stream sediment in Huay Kapo Watershed, Phetchabun Province, Thailand. *Environment and Natural Resources Journal*, *10*(1), 42-52.

Phonprapai, C., & Oontawee, S. (2019). Development of extraction process for preparing high antioxidant extracts from Thai herbs. *Thai Journal of Science and Technology*, *8*(5), 479-492. https://doi.org/10.14456/tjst.2019.54

Piotrowska-Niczyporuk, A., Bajguza, A., Zambrzycka-Szelewab, E., & Bralska, M. (2018). Exogenously applied auxins and cytokinins ameliorate lead

toxicity by inducing antioxidant defense system in green alga *Acutodesmus obliquus*. *Plant Physiology and Biochemistry*, *132*, 535-546. https://doi.org/10.1016/j.plaphy.2018.09.038

Rahman, S. U., Li, Y., Hussain, S., Hussain, B., Khan, W.-u.-D., Riaz, L., Ashraf, M. N., Khaliq, M. A., Du, Z., & Cheng, H. (2023). Role of phytohormones in heavy metal tolerance in plants: A review. *Ecological Indicators*, *146*, 109844. https://doi.org/10.1016/j.ecolind.2022.109844

Rai, P. K. (2008). Technical note: Phytoremediation of Hg and Cd from industrial effluents using an aquatic free floating macrophyte *Azolla pinnata*. *International Journal of Phytoremediation*, *10*(5), 430-439. https://doi.org/10.1080/15226510802100606

Ratchawang, S., Chotpantarat, S., & Charoenrojying, P. (2022). Assessment of atrazine migration in soil and groundwater using nitrate as an indicator in an intensively cultivated sugarcane field, Suphan Buri Province, Thailand. *Frontiers in Earth Science*, *10*, 855599. https://doi.org/10.3389/feart.2022.855599

Riaz, G., Tabinda, A. B., Iqbal, S., Yasar, A., Abbas, M., Khan, A. M., Mahfooz, Y., & Baqar, M. (2017). Phytoremediation of organochlorine and pyrethroid pesticides by aquatic macrophytes and algae in freshwater systems. *International Journal of Phytoremediation*, *19*(10), 894-898. https://doi.org/10.1080/15226514.2017.1303808

Rostami, S., Jafari, S., Moeini, Z., Jaskulak, M., Keshtgar, L., Badeenezhad, A., Azhdarpoor, A., Rostami, M., Zorena, K., & Dehghani, M. (2021). Current methods and technologies for degradation of atrazine in contaminated soil and water: A review. *Environmental Technology and Innovation*, *24*, 102019. https://doi.org/10.1016/j.eti.2021.102019

Salem, R. E. M. E., & El-Sobki, A. E. A. (2021). Physiological and biochemical parameters as an index for herbicides damage in wheat plants. *Egyptian Academic Journal of Biological Sciences*, *13*(2), 25-35. https://doi.org/10.21608/eajbsf.2021.182445

Sánchez, V., López-Bellido, F. J., Cañizares, P., & Rodríguez, L. (2017). Assessing the phytoremediation potential of crop and grass plants for atrazine-spiked soils. *Chemosphere*, *185*, 119-126. https://doi.org/10.1016/j.chemosphere.2017.07.013

Sardoei, A. S., & Rahbarian, P. (2014). Effect of different media on chlorophyll and carotenoids of ornamental plants under system mist. *European Journal of Experimental Biology*, *4*(2), 366-369.

Shi, G. R., Cai, Q. S., Liu, Q. Q., & Wu, L. (2009). Salicylic acid-mediated alleviation of cadmium toxicity in hemp plants in relation to cadmium uptake, photosynthesis, and antioxidant enzymes. *Acta Physiologia Plantarum*, *31*, 969-977. https://doi.org/10.1007/s11738-009-0312-5

Siddiqui, M. H., Al-Whaibi, M. H., & Basalah, M. O. (2011). Interactive effect of calcium and gibberellin on nickel tolerance in relation to antioxidant systems in *Triticum aestivum* L. *Protoplasma*, *248*, 503-511. https://doi.org/10.1007/s00709-010-0197-6

Singh, S., Kumar, V., Chauhan, A., Datta, S., Wani, A. B., Singh, N., & Singh, J. (2018). Toxicity, degradation and analysis of the herbicide atrazine. *Environmental Chemistry Letters*, *16*, 211-237. https://doi.org/10.1007/s10311-017-0665-8

Šípošová, K., Labancová, E., Kuˇcerová, D., Kollárová, K., & Vivodová, Z. (2021). Effects of exogenous application of indole-3-butyric acid on maize plants cultivated in the presence or absence of cadmium. *Plants*, *10*(11), 2503. https://doi.org/10.3390/plants10112503

Sood, A., Uniyal, P. L., Prasanna, R., & Ahluwalia, A. S. (2012). Phytoremediation potential of aquatic macrophyte, *Azolla*. *Ambio*, *41*, 122-137. https://doi.org/10.1007%2Fs13280-011-0159-z

Steffens, C., Ballen, S. C., Scapin, E., da Silva, D. M., Steffens, J., & Jacques, R. A. (2022). Advances of nanobiosensors and its application in atrazine detection in water: A review. *Sensors and Actuators Reports*, *4*, 100096. https://doi.org/10.1016/j.snr.2022.100096

Sun, J. T., Pan, L. L., Zhan, Y., Tsang, D. C. W., Zhu, L. Z., & Li, X. D. (2017). Atrazine contamination in agricultural soils from the Yangtze River Delta of China and associated health risks. *Environmental Geochemistry and Health*, *39*, 369-378. https://doi.org/10.1007/s10653-016-9853-x

Thitiphuree, T., Kitana, J., Varanusupakul, P., & Kitana, N. (2013). Atrazine contamination and potential health effects on freshwater mussel *Uniandra contradens* living in agricultural catchment at Nan Province, Thailand. *EnvironmentAsia*, *6*(1), 13-18. https://doi.org/10.14456/ea.2013.3

Wang, J., Wang, D., Zhu, M., & Li, F. (2021). Exogenous 6-benzyladenine improves waterlogging tolerance in maize seedlings by mitigating oxidative stress and upregulating the ascorbate-glutathione cycle. *Frontiers in Plant Science*, *12*, 680376. https://doi.org/10.3389/fpls.2021.680376

Wang, Q., Zhang, W., Li, C., & Xiao, B. (2012). Phytoremediation of atrazine by three Emergent hydrophytes in a hydroponic system. *Water Science & Technology*, *66*(6), 1282-1288. https://doi.org/10.2166/wst.2012.320

Wu, X., He, J., Chen, J., Yang, S., & Zha, D. (2014). Alleviation of exogenous 6-benzyladenine on two genotypes of eggplant (*Solanum melongena* Mill.) growth under salt stress. *Protoplasma*, *251*, 169-176. https://doi.org/10.1007/s00709-013-0535-6

Yang, L., & Zhang, Y. (2020). Effects of atrazine and its two major derivatives on the photosynthetic physiology and carbon sequestration potential of a marine diatom. *Ecotoxicology and Environmental Safety*, *205*, 111359. https://doi.org/10.1016/j.ecoenv.2020.111359

Yang, L., Li, H., Zhang, Y., & Jiao, N. (2019). Environmental risk assessment of triazine herbicides in the Bohai Sea and the Yellow Sea and their toxicity to phytoplankton at environmental concentrations. *Environment International*, *133*(Part A), 105175. https://doi.org/10.1016/j.envint.2019.105175

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Short Communication

Spore Germination of *Diplazium simplicivenium* Holtt. (Athyriaceae) in Peninsular Malaysia

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ABSTRACT

Diplazium simplicivenium Holtt. a species of fern in Peninsular Malaysia. This study reports an efficient method *for D. simplicivenium* spore sterilisation and the effect of plant growth regulators (PGRs) via green globular bodies (GGB). Sterilisation with 0.1% mercuric chloride effectively allowed spore germination. The result showed that the culture media supplemented with 0.5 mg/L gibberellin positively affects the weight of GGB and the number of shoots with significant differences (ANOVA, $p < 0.05$). The soaking technique established in this study for spore sterilisation is an efficient approach, and the optimal plant and concentration of plant hormones were identified. This procedure can be applied to other indigenous ferns and closely similar species.

Keywords: *Diplazium*, fern, *in vitro*, micropropagation, spore

INTRODUCTION

Fern is a vascular plant widely distributed worldwide with approximately 12,000 species (Sharpe et al., 2010). Malaysia has 1,165 fern taxa, 647 of which are found in Peninsular Malaysia (Maideen et al., 2019, 2020). However, the diversity of the plant decreases due to many factors, such as habitat loss and climate change. *Diplazium simplicivenium* is a fern species known as "pokok paku" among Malaysians. The species belongs to the Athyriaceae and can potentially be used as an ornamental plant due to the uniqueness of its frond and daily life as a rope for tying goods and vegetables (Abrori et al., 2022). This species had been found in

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shady mountain valleys and exposed areas near roadside drains. In the last few decades, anthropogenic disturbances and climate change have resulted in the loss of habitat for plant species such as ferns in the Cameron Highlands, making it more challenging for fern species to develop. In Asia, particularly in Peninsular Malaysia, more studies are needed on *in vitro* spore propagation of ferns. For instance, two studies *in vitro* of fern *Platycerium coronarium* by Taha et al. (2011) and *Cyathea latebrosa* by Nadhirah et al. (2022). The current research deals with the *in vitro* spore propagation of *D. simplicivenium*, which will provide insight into conservation strategies.

MATERIAL AND METHODS

Culture Media

Spores had been used to germinate in a Murashige and Skoog (1962) (Duchefa Biochemie BV, Netherlands) medium with macronutrients at half strength. Each treatment's medium is pH-adjusted to 5.8 and contains 3% (w/v) sucrose (Duchefa Biochemie BV, Netherlands) and 0.4% (w/v) gelrite (Duchefa Biochemie BV, Netherlands) before being autoclaved at 121°C for 15 min.

Spore Collection

Mature pinnae bearing *D. simplicivenium* spores were freshly collected from Parit Fall, Cameron Highland, Pahang, Malaysia (Figures 1a and b). The mature pinnae were then rinsed in a conical flask for 10 min with running tap water.

Sterilisation of Spores

The sterilising procedure was essential before beginning the germination procedure. Mature pinnae were treated with a fungicidal solution consisting of 1 g of benomyl (Benex 500 WP, Imaspro Corporation Berhad, Malaysia) with 3–5 drops of Tween-20 (Sigma-Aldrich, Germany) and 200 ml of distilled water before being shaken for an hour on an orbital shaker. The pinnae were then rinsed with sterile distilled water three times. Then, the soak method (SM) for treatment is used.

Soak Method

The pinnae were soaked in mercuric (II) chloride ($HgCl_2$) (Sigma-Aldrich, Germany) for 10 min in each treatment (0.05, 0.1, 0.5, and 1.0%). The pinnae were then air-dried on filter paper after being rinsed with three distilled waters. After drying, the spores were scraped off the pinnae and cultured on Murashige and Skoog (1962) medium with ½ strength of macronutrients (Figure 1c). The spores were incubated in the growth chamber with a photoperiod of 16 hr at 25°C and white fluorescent light at a light level of 3,000 lux. All experiments were done in aseptic conditions.

GGB Induction and Multiplication

After four weeks in culture, spores began to germinate, and after three months, several gametophytes appeared. Each GGB induced throughout all treatments was fragmented into four segments, each measuring 1.0 cm in diameter and 1.0 g in weight. In Figure 1 (e, f, g, h, i, j, k, l, m, n, o, p, q, and r) order

to study the effect of cytokinin on GGB induction, GGB was cultivated with 0.5, 1.0, 3.0, and 5.0 mg/L on various culture medium such 2,4-dichlorophenoxyacetic acid (2,4-D) (Duchefa Biochemie BV, Netherlands), gibberellin (GA3) (Duchefa Biochemie BV, Netherlands) and a combination of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6 benzylaminopurine (BAP) (0.5 mg/L 2,4-D + 2.0 mg/L BAP; 1.0 mg/L 2,4-D + 4.0 mg/L BAP; 3.0 mg/L 2,4-D + 12.0 mg/L BAP; 5.0 mg/L 2,4-D + 20.0 mg/L BAP). Half strength of Murashige and Skoog (MS) culture media without cytokinin was used as a control. After 12 weeks of culture, the final fresh weight and the quantity of GGB shoots were recorded.

Data Analysis

Using IBM SPSS Statistics software (version 27), data on the final fresh weight and the number of shoots from GGB were analysed using analysis of variance (ANOVA), and the differences between means were analysed by pairwise comparisons test with a 5% probability. Accordingly, the study's findings were provided.

RESULTS AND DISCUSSION

Ferns are commonly cultivated *in vitro* via spores. The serious issue of spore loss and contamination remains, although several techniques have been discovered for spore surface sterilisation and sowing (Wu et al., 2009). A simple and highly efficient approach has been developed for the first study's *in vitro* micropropagation of *D. simplicivenium*.

The *in vitro* spore production technique includes several life cycle stages, from spores through gametophytes and sporophytes. The growth of gametophytes begins with the outgrowth of the protonemal cell, or both, and develops through several morphological appearances, such as filamentous, spatulate, and heart-shaped. Surface sterilisation is the crucial first phase in the aseptic culture of the spore culture (Dyer, 1979).

The soak method, used in a recent study by Nadhirah et al. (2022), proved to be the most effective way to propagate *C. latebrosa* because it can minimise fungal contamination and spore loss when using 0.1% $HgCl_2$ and 30% NaOCl, while achieving high germination rates of 90 and 80%, respectively. Both studies utilised the same type of explant (pinnae); however, compared with ours, which mainly used $HgCl_2$, their study used two types of disinfectant, $HgCl_2$ and NaOCl. However, the findings showed that 0.1% $HgCl_2$ is an efficient disinfectant in both studies.

Golamaully et al. (2015) demonstrated that spore surface sterilisation of *D. proliferium* was efficient at lower concentrations of $HgCl_2$ (0.05%) without using fungicide. However, this study's higher concentrations of $HgCl_2$ (0.1%) using fungicide were efficient. Since the spore surface sterilisation in this study used the entire pinnae, whereas Goullamaully et al. (2015) only used the scarpered spore that dropped during air drying, the area of the sterilisation surface may influence the concentration needed for surface sterilisation. The highest percentage of

spore germination (87.5±2.20) was observed in the 0.1% $HgCl_2$ treatment.

Our studies show that 0.1% of $HgCl_2$ plus fungicide was efficient in spore sterilisation, resulting in a high germination rate (87%). However, the study by Golamaully et al. (2015) in *D. proliferium* showed that 0.05% of $HgCl_2$ without fungicide was efficient in spore sterilisation. Spores provide all the nutrients required for early growth and are a common beginning material for fern development. Regarding the species, it might take a few days to a few months for fern spores to germinate (Chou et al., 2007; Fernández & Revilla, 2003). Thus, during the early stages of germination, the low-nutrient medium of the Murashige and Skoog (1962) medium is commonly used and can be modified. However, this study supports the use of half MS for spore germination and the development of a tiny filament known as protonema after 30-40 days of cultivation (Figure 1a).

Tables 1 and 2 represent the mean percentage of succession toward weight and the number of shoots in GGB for three months. The medium GA_3 supplement (0.5 mg/L) generated the best results in terms of GGB weight (3.46±0.08) and shoot number (20±2.38). This treatment resulted in the growth of greenish GGB with rhizomes (Figure 1f). The treatment was subsequently added with GA_3 (1.0 mg/L), which had similar effects in terms of weight (2.55±0.10) and the number of shoots (11.25±0.97). However, this treatment resulted in brownish GGB and the formation of rhizomes (Figure 1g).

In this study, GGB cultivated on GA_3-containing media resulted in varied success in shoot initiation and weight, which was influenced by the concentration (Table 2). Similar results were also observed on *P. coronarium* (Taha et al., 2011) *and Pteris tripatita* (Ravi et al., 2014). *Lygodium japonicum* (Takeno & Furuya, 1977), *Blechnum spicant* (Fernández et al., 1997), *Anemia phyllitidis* (Kaźmierczak, 1998, 2003), and *Osmundastrum cinnamomeum* (Babenko et al., 2018).

In the previous study, the plant growth regulator had proven varied effects on gametophyte growth. For instance, adding GA_3 to the media at concentrations of 0.5, 5.0, and 50 M dramatically slowed the formation of gametophytes in *B. spicant* at all phases of ontogenesis. They inhibited sporophytes from growing (Fernández et al., 1997). Then, in the *A. phyllitidis* thallium, GA_3 affected cell division, which resulted in a decline in cell division and the appearance of smaller thalli. Prothallium enlarged because of the expansion of individual cells (Kaźmierczak, 1998, 2003). Similar outcomes were shown in *O. cinnamomeum* (L.) C. Presl, where reduced hormone concentration significantly increased the size of the prothallium, while a slight rise in concentration significantly decreased the size of the thallus (Hollingsworth et al., 2012). In MS media supplemented with 1.0 and 1.5 mg/L GA_3, Taha et al. (2011) reported that *P. coronarium* successfully regenerated sporophytes from gametophyte explants. The results reported by Ravi et al. (2014) in their study on *P. tripatita* at

lower concentrations of this hormone also influence the overall morphological growth of gametophytes.

Regenerated plantlets were placed on a medium containing 0.5 mg/L GA_3 to sustain this species. Through a series of acclimatisation stages, the *in vitro*-grown full plantlets (Figure 1r) were transplanted outdoors. Plantlets were transplanted into pots made of a 3:1 mixture of peat moss and vermiculite (Figure 1s). In the glasshouse, they were kept at a relative humidity of around 70 with 75% shade. After six weeks, a 95% survival rate resulted (Figure 1s, 1t, and 1u).

Table 1
Percentage of germination and contamination from sterilisation method with different concentrations of mercuric (II) chloride ($HgCl_2$)

Disinfectant	Concentration (%)	Contamination	Germination	Remarks
$HgCl_2$	0.05	94.8 ± 2.00	0.00 ± 0.00	Fungus growth
	0.10	45.0 ± 3.37	87.5 ± 2.20	Germinate
	0.50	14.0 ± 1.60	24.5 ± 5.90	Germinate
	1.00	3.80 ± 1.10	0.00 ± 0.00	Over sterile

Note. Results represent mean±standard error mean (SEM)

Table 2
Effect of 2,4-dichlorophenoxyacetic acid (2,4-D), gibberellin (GA_3), and combination of 2,4-D and 6-benzylaminopurine (BAP) on weight and shoot from green globular bodies (GGB) Diplazium simplicivenium after 12 weeks of culture

Type of media	Weight of GGB	Number of shoots
Half Murashige and Skoog (MS)	0.73 ± 0.03 ^{b}	6.25 ± 0.32 ^{d}
0.5 mg/L 2,4-D	1.39 ± 0.05 ^{cd}	5.00 ± 0.49 ^{cd}
1.0 mg/L 2,4-D	0.83 ± 0.04 ^{b}	1.58 ± 0.25 ^{ab}
3.0 mg/L 2,4-D	1.27 ± 0.04 ^{cd}	0.00 ± 0.00 ^{a}
5.0 mg/L 2,4-D	1.28 ± 0.42 ^{cd}	0.00 ± 0.00 ^{a}
0.5 mg/L GA_3	3.46 ± 0.08 ^{f}	20.0 ± 2.38 ^{f}
1.0 mg/LGA_3	2.55 ± 0.10 ^{e}	11.25 ± 0.97 ^{e}
3.0 mg/L GA_3	1.44 ± 0.08 ^{d}	1.75 ± 0.37 ^{ab}
5.0 mg/L GA_3	2.38 ± 0.08 ^{e}	3.33 ± 0.432 ^{bc}
0.5 mg/L 2,4-D + 2.0 mg/L BAP	1.20 ± 0.05 ^{c}	1.75 ± 0.32 ^{ab}
1.0 mg/L 2,4-D + 4.0 mg/L BAP	1.34 ± 0.04 ^{cd}	1.50 ± 0.31 ^{ab}
3.0 mg/L 2,4-D + 12 mg/L BAP	0.73 ± 0.03 ^{b}	0.00 ± 0.00 ^{a}
5.0 mg/L 2,4-D + 20 mg/L BAP	0.41 ± 0.02 ^{a}	0.00 ± 0.00 ^{a}


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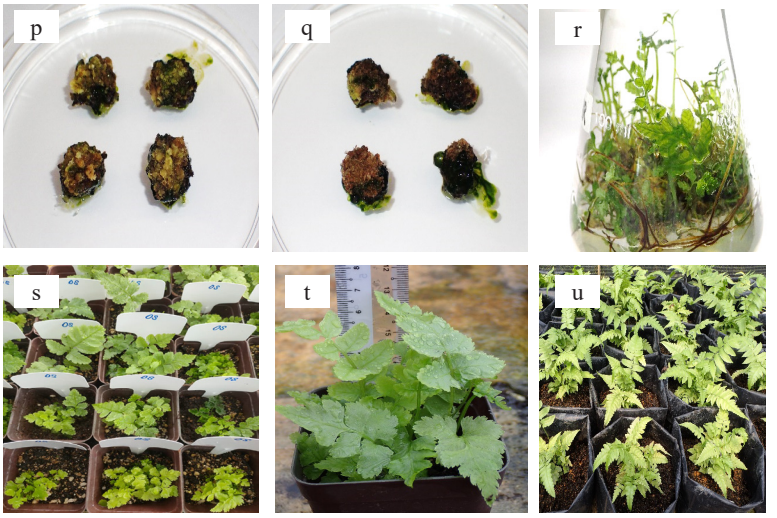

Figure 1. *In vitro* regeneration of *Diplazium simplicivenium*. (a) Mature pinnae of *D. simplicivenium*, (b) sporangium, (c) spore germination on ½ Murashige and Skoog (MS) for 4 weeks of sowing, (d) spore germination on ½ MS for 8 weeks of sowing, (e) green globular bodies (GGB) multiplication on ½ MS (control) for three months, (f) GGB growth on 0.5 mg/L gibberellin (GA_3), (g) GGB growth on 1.0 mg/L GA_3, (h) GGB growth on 3.0 mg/L GA_3, (i) GGB growth on 5.0 mg/L GA_3, (j) GGB growth on 0.5 mg/L 2,4-D, (k) GGB growth on 1.0 mg/L 2,4-D, (l) GGB growth on 3.0 mg/L 2,4-D, (m) GGB growth on 5.0 mg/L 2,4-D, (n) GGB growth on 0.5 mg/L 2,4-D + 2.0 mg/L BAP, (o) GGB growth on 1.0 mg/L 2,4-D + 4.0 mg/L BAP, (p) GGB growth on 3.0 mg/L 2,4-D + 12.0 mg/L BAP, (q) GGB growth on 5.0 mg/L 2,4-D + 20.0 mg/L BAP, (r) plantlets growth on 0.5 mg/L GA_3 for 3 months, (s) Plantlets planted in 72-hole plug for 1 month, (t) plantlets planted in 72-hole plug for 2 months, and (u) Acclimatised plant cultivated in the glasshouse for 4 months

CONCLUSION

This article reports on *in vitro* spore culture for *D. simplicivenium* via GGB. Based on the result, the mean germination was significantly higher in 0.1 and 0.5% $HgCl_2$, respectively. It has been observed that 0.5 mg/L GA_3 culture medium followed by 1.0 mg/L GA_3 medium is best for GGB of *D. simplicivenium* toward efficient weight and quantity of shoots. An efficient spore culture approach would allow for large-scale production of *D. simplicivenium*, which might contribute to conserving species on the verge of extinction and be applied to propagate other fern species.

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REFERENCES

Abrori, F. M., Saraswati., Wijarini, F., & Fatmawati. (2022). Introducing the ferns through comics: Visualisation of ethnopteridology study of Dayak Lundayeh tribe. In *IOP Conference Series: Earth and Environmental Science* (Vol. 1083, No. 1, p. 012013). IOP Publishing. https://doi.org/10.1088/1755-1315/1083/1/012013

Babenko, L. M., Romanenko, K. O., Shcherbatiuk, M. M., Vasheka, O. V., Romanenko, P. O., Negretsky, V. A., & Kosakivska, I. V. (2018).

Effects of exogenous phytohormones on spore germination and morphogenesis of *Polystichum aculeatum* (L.) Roth gametophyte *in vitro* culture. *Cytology and Genetics*, *52*, 117–126. https://doi.org/10.3103/S0095452718020032

Chou, H.-M., Huang, Y.-M., Wong, S.-L., Hsieh, T.-H., Hsu, S.-Y., & Chiou, W.-L. (2007). Observations of gametophytes and juvenile sporophytes of *Archangiopteris somaí* Hayata (Marattiaceae), an endangered fern in Taiwan. *Botanical Studies*, *48*, 205-213.

Dyer, A. F. (1979). The experimental biology of ferns. *Transactions of the Botanical Society of Edinburgh*, *43*(2), 75-90. https://doi.org/10.1080/03746607908685341

Fernández, H., & Revilla, M. A. (2003). *In vitro* culture of ornamental ferns. *Plant Cell, Tissue and Organ Culture*, *73*, 1-13. https://doi.org/10.1023/A:1022650701341

Fernández, H., Bertrand, A. M., Feito, I., & Sanchez-Tames, R. (1997). Gametophyte culture *in vitro* and antheridiogen activity in *Blechnum spicant*. *Plant Cell, Tissue and Organ Culture*, *50*, 71–74. https://doi.org/10.1023/A:1005962726905

Golamaully, Z. M., Bhoyroo, V., Nazurally, N., & Gopal, V. (2015). *In vitro* cultures as an aid to conservation of indigenous ferns: *Diplazium proliferum*. *International Journal of Plant Biology*, *6*(1), 6020. https://doi.org/10.4081/pb.2015.6020

Hollingsworth, S. N., Andres, E. A., & Greer, G. K. (2012). Pheromonal interactions among gametophytes of *Osmundastrum cinnamomeum* and the origins of antheridiogen systems in leptosporangiate ferns. *International Journal of Plant Sciences*, *173*(4), 382–390. https://doi.org/10.1086/664717

Kaźmierczak, A. (1998). Studies on morphology and metabolism of prothalli during GA_3-induced formation of antheridia in *Anemia phyllitidis*. *Acta Physiologiae Plantarum*, *20*, 277–283. https://doi.org/10.1007/s11738-998-0059-4

Kaźmierczak, A. (2003). Induction of cell division and cell expansion at the beginning of gibberellin A_3-induced precocious antheridia formation in *Anemia phyllitidis* gametophytes. *Plant Science*, *165*(5), 933–939. https://doi.org/10.1016/s0168-9452(03)00217-6

Maideen, H. M. K, Salleh, N. I. M., & Khaduwi, N. A. M. (2020). *Paku pohon Cyatheaceae* [Cyatheaceae tree spikes]. Penerbit Universiti Kebangsaan Malaysia.

Maideen, H., Farhana, N., Nadirah, N., Khaduwi, N. A., & Norhazrina, N. (2019). Ferns and lycophytes of Pulau Pangkor and its vicinity. *The Malaysian Forester*, *82*(1), 77–86.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, *15*(3), 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nadhirah, N., Maideen, H., Rahman, Z. A., & Othman, A. N. (2022). Optimizing *in vitro* surface sterilization of *Cyathea latebrosa* spore. *Malaysian Applied Biology*, *51*(5), 159–163. https://doi.org/10.55230/mabjournal.v51i5.2422

Ravi, B. X., Robert, J., & Gabriel, M. (2014). *In vitro* spore germination and gametophytic growth development of a critically endangered fern *Pteris tripartite* Sw. *African Journal of Biotechnology*, *13*(23), 2350-2358. https://doi.org/10.5897/ajb2013.13419

Sharpe, J. M, Mehltreter, K., & Walker, L. R. (2010). Ecological importance of ferns. In K. Mehltreter & L. R. Walker (Eds.), *Fern ecology* (pp. 1-21). Cambridge University Press. https://doi.org/10.1017/CBO9780511844898.002

Taha, R. M., Haron, N. W., & Wafa, S. N. (2011). Morphological and tissue culture studies of *Platycerium coronarium*, a rare ornamental fern species from Malaysia. *American Fern Journal*, *101*(4), 241–251. https://doi.org/10.1640/0002-8444-101.4.241

Takeno, K., & Furuya, M. (1977). Inhibitory effect of gibberellins on archegonial differentiation in *Lygodium japonicum*. *Physiologia Plantarum*, *39*(2), 135–138. https://doi.org/10.1111/j.1399-3054.1977.tb04024.x

Wu, H., Chen, P.-T., Yuan, L.-P., & Chen, L.-Q. (2009). An efficient method for surface sterilization and sowing fern spores *in vitro*. *American Fern Journal*, *99*(3), 226–230. https://doi.org/10.1640/0002-8444-99.3.226

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Correlation Among Physical Properties of Parboiled Milled Rice During Hydrothermal Pretreatment Processing

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ABSTRACT

The rice cultivar MR297 has a high harvesting yield but a low milling and head rice yield. Parboiling is one of the methods to increase the head yield of rice varieties. The colour of parboiled rice is intense due to husk colour absorption during soaking. This study researched the influence of hydrothermal pretreatment (soaking time and temperature) on the physical properties such as dimension, thousand kernel weight (TKW), density, volume expansion (V_e), colour, and hardness of parboiled milled rice. MR297 raw rice cultivars were soaked at 1:1 (w/w). Paddy was soaked at 50, 60, and 70°C for 1, 2, 3, 4, and 5 hr and steamed at 100°C for 20 min prior to drying for 24 hr at 38°C. It was discovered that the effects of soaking time and temperature varied with the physical properties of rice. Soaking time and temperatures were found to be significant (p<0.05) in terms of dimensional properties (length [L], width [W], and thickness [T]), TKW, density, V_e, colour (L*, a*, b*) and hardness of parboiled rice. The L, W, T, V_e, TKW, a*, and b* increase proportionally as time and temperature increased, while the density and L* were negatively correlated. The hardness was negatively correlated with the L and colour b* (yellowness) rice measurements. The principal component analysis (PCA) results revealed that the

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soaking time was associated with L, W, T, density, V_e, and colour properties based on the loading analysis in the PC1. In contrast, the temperature, TKW and hardness were associated with PC2.

Keywords: Correlation, hydrothermal, paddy MR297, parboiling, physical properties, soaking condition

INTRODUCTION

A new rice cultivar created by the Malaysian Agricultural Research and Development Institute (MARDI) is called MR297. The parents of MRQ76 and P446 were used to begin breeding MR297 in 2006/2007. The cultivars were then made public in the off-season of 2014. MARDI SIRAJ 297 is another name for MR297 (Ramli & Kamaruzaman, 2020). The cultivars MR253, MR263, and MR269 are well known for yielding crops of greater quality. Esa et al. (2020) found that MR297 types had higher disease resistance than MR263, which was advantageous for farmers. However, MR263 has a 65.5% milling yield and an 88% head rice yield, while the milling yield of this crop is only 61.4% and the head rice yield is only 67.8%. Because of this, the variety may have a lesser economic value to the milling factories. By using the parboiling technique, broken rice varieties might be fixed instead. Parboiling gelatinises the whole grain's starch to maximise head rice's recovery and reduce breakage, which has been a considerable loss during processing (Buggenhout et al., 2014).

Rice starch undergoes structural and physical modifications during parboiling (Dutta & Mahanta, 2012; Iqbal et al., 2021; Muchlisyiyah et al., 2023; Roy et al., 2019). Before milling, the parboiling method calls for soaking, steaming, and drying the rice. This process aims to enhance rice's physical, functional, and nutritional properties. Hydrothermal treatment is a crucial phase in the parboiling process that significantly impacts the overall quality of the parboiled rice. Hydrothermal treatments can enhance the physical properties of rice grain during parboiling, such as its size, hardness, and colour (Azuka et al., 2021; Saleh & Meullenet, 2015; Sivakamasundari et al., 2020). The treatment also gives the rice a yellowish tone, changing its colour (Saleh et al., 2018). Rice's dimensional characteristics, such as length and the length-to-breadth ratio, are positively correlated. On the other hand, the colour of rice grains has a detrimental effect on the price of rice at the point of marketing price (Rachmat et al., 2016). The primary reason for consumers' preference for local rice is mostly attributed to its physical attributes and aroma, which are comparable to those of locally produced fragrant rice (Rahim et al., 2023).

Previous studies have been researched on the influence of single soaking treatment on the physical properties of rice and paddy (Bello et al., 2004, 2007; Ji-u & Inprasit, 2019; Rattanamechaiskul et al., 2023). Some of the previous scientific papers reported on the physical and chemical changes to the rice kernel during soaking (Behera & Sutar, 2018; Hu et al., 2021; Liu et al., 2021; Saleh et al., 2018; Zhu et al., 2019). Mir and Bosco

(2013) examined how soaking affected the physical and practical characteristics of rice farmed in temperate parts of India. The parameters were tested on the soaked paddy, which will undergo further changes in the other steps of parboiling. Other studies also examined the effect of soaking, along with the other parboiling step parboiling to the physicochemical properties of parboiled rice (Ebuehi & Oyewole, 2008; Jayaraman et al., 2019; Panda et al., 2021; Roy et al., 2019; Y. Tian et al., 2014; Z. Wu et al., 2021). To the best of our knowledge, there are still limited studies regarding the effect of soaking treatment on the physical characteristics of dried parboiled rice. Because there is no information on the soaking behaviour of parboiled milled Malaysian rice varieties, the current study sought to evaluate the influence of time (1, 2, 3, 4, and 5 hr) and temperature (50, 60, and 70°C) as the effect of hydrothermal pretreatment processing of MR297 parboiled rice on the physical characteristics changes, and to determine the best correlations can display the soaking behaviour of rice under the analysed condition.

METHODOLOGY

Sample Preparation

Fresh paddy (MR297) was sown in June 2022 and harvested in November 2022 by a regional farmer in Tanjung Karang, Selangor, Malaysia. Fresh paddy grain was delivered to the laboratory and kept at 10°C in an airtight container until further analysis. A moisture analyser was used to examine the initial moisture content of the paddy (MX50, A&D, USA). The initial moisture content was 19.5±0.6%. During the cleaning process, sticks, stones, leaves, and other plant fragments were physically removed from the foreign materials (Reddy & Chakraverty, 2004).

Parboiling Procedure

The parboiling procedure consisted of soaking, steaming, and dehydrating. Fresh paddy from MR297 was parboiled in the laboratory using a heat immersion procedure. In the beginning, a water bath (WNB22, Memmert, Germany) was utilised to heat 100 ml of water in a beaker glass size 250 ml to the desired temperature (50, 60, and 70°C). The beaker was sealed with aluminium foil. When the water in the beaker glass attained the desired temperature (50, 60, or 70°C), 100 g of paddy was added (1:1 paddy to water ratio) to prevent excessive evaporation (Jannasch & Wang, 2020). Gentle mixing was performed. The paddy was soaked for one, two, three, four, and five hours in a separate beaker glass for each temperature to ensure that the paddy was thoroughly saturated. The paddy was steamed for 20 min at 100°C using a domestic steamer (VC1401, Tefal, France). The steamed paddy was then cooled at ambient temperature for one hour (Bootkote et al., 2016). Then, the paddy was dehydrated in an oven at 38°C for 24 hr (DO6836, Memmert, Germany) (J. Tian et al., 2018; Roy et al., 2019; Villanova et al., 2017).

Using a dehusker (THU-35, Satake Corporation, Japan), dried parboiled rice

was dehulled and milled for 150 s (TM05-C, Satake, Japan) (Liang et al., 2008). The head rice grains were sorted from broken rice using a laboratory-grade instrument (TWL05C-T, Satake Corporation, Japan). The term "head rice yield" (HRY) refers to the ratio of dried parboiled rough rice mass to head parboiled rice mass. The physical characteristics of the milled rice were examined next, including dimension, TKW, density, volume expansion, colour, and hardness.

Determination of Physical Properties

Dimensions. Using a vernier calliper (Mitutoyo Corporation, Japan), the average length (L), width (W), and thickness (T) of 100 paddy grains were calculated (±0.01 mm) (Zainal & Shamsudin, 2021). Figure 1 depicts the axis's major (L), medium (W), and minor (T) dimensions.

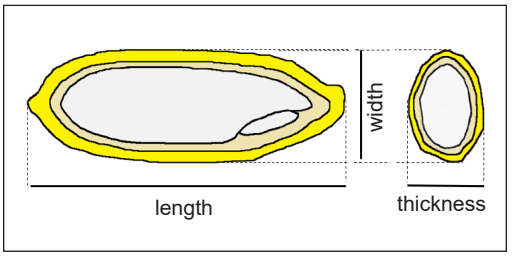

Figure 1. Dimensional characteristics of MR297 paddy grain

Thousand Kernel Weight (TKW). TKW measures paddy yield and milling quality (W. Wu et al., 2018). TKW is the weight of one thousand seeds in g. TKW was determined by manually counting and weighing one hundred grains of milled head rice on an electronic scale (ER-120A, AND, Japan) (±0.0001 g). The results were then multiplied by ten to calculate the mass of one thousand grains.

Volume Expansion. Rice grain volume represents the quantity of space occupied by the entire grain. The volume of the paddy was assumed to be constant and calculated using the relationship that follows between its dimensions (Bhat & Riar, 2016; Mir & Bosco, 2013; Nádvorníková et al., 2018):

$$V_c = \frac{\frac{\pi}{6} L\,(WT)^2}{4}$$

[Equation 1]

where, Vc = Volume calculated (mm^3); L = Major axis (length) (mm); W = Medium axis (width) (mm); and T = Minor axis (thickness) (mm)

The volume expansion was a dimensionless number calculated by the ratio of parboiled paddy volume divided by the raw paddy volume:

$$\textit{Volume expansion} = \frac{\textit{Parboiled paddy volume}}{\textit{Raw paddy volume}}$$

[Equation 2]

Density. Paddy's density was determined by calculating the mass divided by its volume.

$$\rho_T = \frac{\textit{Mass of grain}}{\textit{True volume}}$$

[Equation 3]

Colour. Using a colourimeter (FRU WR10, China), 50 g of each variety of milled rice was randomly selected and placed in a Petri dish. The colour reader was calibrated using a whiteboard with black-and-white writing. L (lightness), a* (redness), and b* (yellowness) scales were measured by placing the colourimeter next to the Petri dish containing the rice samples and photographing it with a 40 mm lens against a plain white paper background (Shamsudin et al., 2021; Shen et al., 2009).

Hardness. The greatest force applied during the initial compression was used to determine the hardness. The hardness of samples was analysed by using a texture analyser (TA-XT2, Stable Micro Systems, United Kingdom). Individual rice grains were compressed using a single compression force-versus-time program at a test speed of 2 mm/s and a post speed of 10 mm/s using a stainless-steel probe with a 5 mm diameter (P5) (Kumar & Prasad, 2018).

Statistical Analysis

Each experiment was carried out three times. The effects of soaking time and temperature on dimensions, physical, colour and hardness were assessed using analysis of variance (two-way-ANOVA). Tukey's honestly significant difference (HSD) tests (p<0.05) were performed to investigate the significant impact across polishing time levels. Pearson correlation analysis was used to analyse the connections between the physical characteristics of the parboiled rice MR297. To illustrate the change in parboiled paddy and rice properties at various soaking times and temperatures, the PCA was carried out using the Minitab Software (version 19).

RESULTS AND DISCUSSION

Effect of Soaking Time and Temperature on the Dimensional Properties of Parboiled Paddy

The results showed a significant effect of the soaking time on the L, W, and T of MR297 long-grain paddy (p<0.05). The soaking temperature significantly affected the W and T of the parboiled paddy. The interaction effect between soaking time and soaking temperature is not statistically significant, indicating that the influence of soaking time remained constant across all levels of soaking temperature and vice versa. Table 1 displays the various dimensional properties of parboiled MR297 long-grain paddy with varying soaking times and temperatures. It was found that there is an increase of the W (2.30–2.36 mm) and T (2.03–2.06 mm) of parboiled paddy with increasing soaking time and temperature (p<0.05). However, the L of the parboiled paddy slightly increases with the increase of soaking time (p<0.05), and there is no significant increase of the L of the paddy by the increasing soaking temperature (p>0.05).

Due to the physical and chemical interactions that occur during hydrothermal treatment, the paddy endures distinct transformations. The L, W, and T of the rice grew by 12 to 15% from its unprocessed state. According to previous research, parboiling decreases unpolished and brown

Table 1
Effect of soaking time and temperature of parboiled rice on dimensional properties

	L (mm)	W (mm)	T (mm)
Soaking time (hr)			
1	10.16±0.07^{b}	2.30±0.02^{c}	2.03±0.02^{c}
2	10.30±0.02^{a}	2.31±0.01^{c}	2.04±0.02^{c}
3	10.32±0.01^{a}	2.32±0.01^{b}	2.05±0.01^{bc}
4	10.37±0.04^{a}	2.34±0.01^{b}	2.06±0.02^{b}
5	10.37±0.01^{a}	2.36±0.02^{a}	2.09±0.02^{a}
Soaking temperature (^{o}C)			
50	10.29±0.11^{a}	2.31±0.02^{b}	2.03±0.02^{c}
60	10.31±0.08^{a}	2.33±0.02^{a}	2.06±0.02^{b}
70	10.32±0.08^{a}	2.34±0.02^{a}	2.07±0.02^{a}

Note. Data are expressed mean±SD; Different letters indicate statistically significant differences exist $p<0.05$ for each column. Means do not share a letter is significantly different. Tukey's test was applied with 95% simultaneous confidence intervals. L = Length; W = Width; T = Thickness

rice's length and dorsiventral diameter (Rocha-Villarreal et al., 2018). Due to the irreversible swell of starch granules caused by water diffusion and heat treatment, parboiled rice kernels are bulkier and shorter than non-parboiled rice kernels (Patindol et al., 2008). This change was observed during parboiling under the surplus water system but not under water restrictions like dry heat and pressure (Sittipod & Shi, 2016). During the soaking process, the volume of milled rice changed as water molecules moved from the external medium to the interior kernel (Hu et al., 2021). Table 1 shows that as time and temperature increase, the growth in dimension will mostly affect the medium and minor axes rather than the major axis (L). It indicates that the husk is swollen. Due to water penetrating amorphous starch granules and forming hydrate through hydrogen bonding, paddy's dimensions increased (Ji-u & Inprasit, 2019). After the first hour of soaking, the growth of the paddy's size slows down (Hanucharoenkul et al., 2021; Ji-u & Inprasit, 2019; Yadav & Jindal, 2007).

Effect of Soaking Time and Temperature on the TKW of Parboiled Paddy

The soaking time and temperature affected the TKW of parboiled paddy (Table 2). The TKW increased substantially from 27.70 to 28.04 g as the soaking time increased from 1 to 4 hr ($p<0.05$). However, 5 hr of immersion demonstrated that the TKW decreased with time. At a given level of parboiling, the TKW mass will increase. Our research revealed that the TKW of parboiled paddy increased to a certain point in time and temperature, which decreased as the soaking time increased. The soluble amylose will significantly decrease at a high parboiling temperature, so the parboiled rice weight will appear quite lower (Dutta & Mahanta, 2012; Tao et al., 2019). The levels

Table 2
Effect of soaking time and temperature of parboiled rice on thousand kernel weight (TKW), density, and volume expansion

	TKW (g)	Density (g/mL)	Volume expansion
Soaking time (hr)			
1	27.70±0.54^{b}	0.95±0.02^{a}	1.69±0.07^{d}
2	27.93±0.64^{ab}	0.94±0.01^{ab}	1.72±0.05^{d}
3	28.12±0.62^{ab}	0.92±0.02^{bc}	1.77±0.04^{c}
4	28.36±0.27^{a}	0.90±0.01^{c}	1.82±0.03^{b}
5	28.04±0.20^{ab}	0.85±0.03^{d}	1.91±0.07^{a}
Soaking temperature (^{o}C)			
50	27.68±0.47^{b}	0.93±0.03^{a}	1.72±0.08^{c}
60	27.95±0.43^{b}	0.90±0.04^{b}	1.80±0.08^{b}
70	28.46±0.25^{a}	0.90±0.04^{b}	1.83±0.08^{a}

Note. Data are expressed mean±SD; Different letters indicate statistically significant differences exist $p<0.05$ for each column. Means do not share a letter is significantly different. Tukey's test was applied with 95% simultaneous confidence intervals

of soluble amylose influence the weight of parboiled rice per thousand kernels.

Effect of Soaking Time and Temperature on the Volume Expansion

Table 2 demonstrates the effect of various soaking times and temperatures on the volume expansion of parboiled rice. In this study, the volume expansion increased from 1.69 to 1.91 with increasing duration and temperature of the soaking process ($p<0.05$). The increase of dimensions (L, W, and T) in the "Effect of Soaking Time and Temperature on the Dimensional Properties of Parboiled Paddy" affected the volume expansion of the parboiled paddy. The volume of the paddy was determined using a mathematical method that incorporates the measurements of the paddy's dimensions (Bhat & Riar, 2016; Mir & Bosco, 2013; Nádvorníková et al., 2018). Consequently, the dimension enhancement resulting from hydrothermal treatment would result in a corresponding rise in volume expansion. The greater the severity of the parboiling procedure, the greater the increase in volume expansion (Wahengbam & Hazarika, 2019).

Effect of Soaking Time and Temperature on the Density

Table 2 also shows the effect of different soaking times and temperatures in the parboiled conditions on the density. In this study, the density was decreased with increasing time and temperature of the soaking process ($p<0.05$). Parboiled paddy has a density that ranges from 0.85 to 0.95 g/ml. In this study, the density decreased (0.95 to 0.85 g/ml) as the soaking process' time and temperature increased ($p<0.05$). The decrease in density might be due to the increase in volume expansion in the "Effect of Soaking Time and Temperature on the

Volume Expansion." The ratio between mass and volume of paddy calculated the density of paddy. The increase in mass was found to be smaller than the increase in the dimensions. The mass was more influenced by the change of mass of the rice inside the paddy, whereas the husk was more influenced by the dimensional change. The density of paddy is lower than that of dehusked paddy due to eliminating the bran component during the milling procedure. The bran has a relatively low density, and its removal leads to an increased dehusked paddy density. Rice is traded by weight on international markets; grains with a higher density fetch a higher price. According to Mir et al. (2013), some rice varieties grown in temperate India were 1.22–1.40 g/ml. Nigerian native rice has a density ranging from 1.03–2.07 g/ml (Azuka et al., 2021). The density of the parboiled rice will differ due to the range in size and TKW, as discussed previously.

Effect of Soaking Time and Temperature on the Colour

The lightness of milled rice reduced when it was parboiled. According to the results of the two-way ANOVA, there is no interaction impact between soaking time and soaking temperature. The average value of lightness, yellowness, and redness of milled parboiled rice as impacted by the soaking process conditions is shown in Table 3. The lightness dropped significantly (67.89–63.95) when the soaking time and temperature increased (p<0.05). The degree of consumer acceptance regarding rice exports is contingent upon the observable yellowness exhibited by parboiled milled rice. Table 3 illustrates the effect of parboiled rice soaking time and temperature on yellowness (b*) attributes. The yellowness increased significantly (30.12–33.49) with increasing temperature time and temperature (Table 3). Also, the redness of parboiled milled rice has an impact on consumer acceptability of rice

Table 3
Effect of soaking time and temperature of parboiled rice on colour properties

	L*	a*	b*
Soaking time (hr)			
1	67.89±1.48^{a}	7.34±0.30^{c}	30.12±1.03^{c}
2	67.12±1.28^{a}	7.48±0.20^{c}	31.45±1.47^{bc}
3	65.43±0.96^{b}	8.20±0.19^{b}	31.87±1.60^{b}
4	65.19±0.90^{b}	8.24±0.28^{b}	32.14±1.32^{ab}
5	63.95±0.58^{c}	8.92±0.29^{a}	33.49±2.53^{a}
Soaking temperature (°C)			
50	67.05±1.87^{a}	7.83±0.60^{b}	30.13±0.49^{c}
60	65.60±1.66^{b}	8.07±0.61^{a}	32.07±1.59^{b}
70	65.11±1.18^{b}	8.20±0.68^{a}	33.24±1.86^{a}

Note. Data are expressed mean±SD; Different letters indicate statistically significant differences exist p< 0.05 for each column. Means do not share a letter is significantly different. Tukey's test was applied with 95% simultaneous confidence intervals. L* = Lightness; a* = Redness; b* = Yellowness

markets. Table 3 illustrates the effect of parboiled rice soaking time and temperature on redness (a*) attributes. The redness (a*) grew significantly (p<0.05) as the soaking time and temperature increased (Table 3).

The soaking temperature intensified the colour (Mir & Bosco, 2013). The endosperm absorbs the husk colour during the process, causing the rice to turn amber when parboiled. The elevated levels of reducing sugar, free a-amino nitrogen, and glucose-to-fructose isomerisation in parboiled rice indicate a non-enzymatic Maillard colour change (Balbinoti et al., 2018). Increasing the soak water temperature, steaming time, and steaming pressure for poorly hydrated grains improves the colour of parboiled rice (Hu et al., 2019). Higher soaking temperatures cause the husk to absorb more colour, and coloured materials like folic acid absorb into the soaking water, colouring the rice kernel. Lamberts et al. (2008) found that the transformation from white to amber rice after parboiling was caused by the diffusion of bark and bran pigments, as well as non-enzymatic Maillard and enzymatic browning, to a lesser extent. Maillard reactions (increased furosine levels) following gelatinisation led to the darkening of rice. Pigment taken from husks altered the colour (Sareepuang et al., 2008).

Effect of Soaking Time and Temperature on the Hardness

It was found that the hardness was significantly affected by the different soaking times and temperatures of the parboiling process. The hardness increased (3,077.26–3,268.67 g) with the increasing temperature of the soaking process. However, the hardness was significantly (p<0.05) decreased (3,545.83–3,087.82 g) with the increase of time of the soaking process. The hardness of the kernel is influenced by soaking time and temperature. According to Graham-Acquaah et al. (2015), the stiffness of rice increases with steaming time and decreases with soaking water temperature. After parboiling, the rice kernel's ultimate tensile strength and elastic modulus are raised four to five times. The steaming duration and starch gelatinisation directly influence Hardness levels.

Table 4 illustrates how soaking time and temperature treatment affected the parboiled rice's hardness. An increase in soaking time would result in a reduction in hardness (p<0.05). However, a slight

Table 4
Effect of soaking time and temperature of parboiled rice on hardness

Time	Hardness (g)
Soaking time (hr)	
1	3545.43±125.50^{a}
2	3244.05±203.21^{b}
3	3025.02±54.97^{bc}
4	3008.21±171.96^{bc}
5	3087.82±78.26^{c}
Temperature (°C)	
50	3077.26±217.29^{a}
60	3200.40±206.82^{ab}
70	3268.67±274.59^{b}

Note. Data are expressed mean±SD; Different letters indicate statistically significant differences exist p<0.05 for each column. Means do not share a letter is significantly different. Tukey's test was applied with 95% simultaneous confidence intervals

increase in hardness was observed after 5 hr of soaking time. At the beginning of the soaking period, the hardness was decreased upon amylose leaching; the increase in soaking time would severe the gelatinisation of rice starch. Upon heating, the process of leaching caused the migration of short-chain amylose, which subsequently penetrated the indentations on the surface of the cooked rice. During this period, the amylose with elongated chains underwent cross-linking, resulting in the eventual formation of three-dimensional network architectures by hydrogen bonding (Yang et al., 2016). Rising the soaking temperature would result in an elevation in rice hardness (p<0.05). The observed phenomenon can be attributed to the heightened gelatinisation of starch, which is directly influenced by the duration of soaking (Miah et al., 2002).

Parboiled rice has a distinct texture when cooked and less breaking when milled because of its increased strength. Gelatinisation, thermal starch degradation, and recrystallisation of starch with lipid-amylose inclusion complexes are the three processes that contribute to the altered hardness of rice that has been parboiled (Dutta & Mahanta, 2012). The types of starch polymorphs generated in rice during the parboiling process determine the texture of the rice after it has been cooked.

The Correlation Between Dimensional, Physical, Colour, and Texture Properties

Pearson's correlation coefficients among the physical characteristics of parboiled rice with the different soaking times and temperatures are shown in Table 5. There is a significant correlation across the different properties of parboiled rice MR297, including hardness, L, W, T, V_e, TKW, density, and colour L, a, and b (p<0.05). The hardness was highly negatively correlated with the major axis (r=-0.770, p<0.01) and a* (r=-0.0557, p<0.005). The correlation coefficients between L and the W, T, V_e, TKW, $a*$, and $b*$ were highly positive with values of 0.758, 0.634, 0.766, 0.556, 0.761, and 0.612, respectively. Meanwhile, correlation coefficients between the major axis and the L* and density were highly negative -0.734 and -0.0798, respectively (p<0.05).

The medium axis was highly positively correlated with the minor axis (r=0.931, p<0.01), volume expansion (r=0.985, p<0.01), TKW (r=0.565, p<0.05), a$*$ (r=0938, p<0.01), and $b*$ (r=0.825, p<0.01). The correlation coefficients between the minor axis with volume expansion, TKW, a*, and $b*$ were highly positive with values of 0.973, 0.645, 0.831, and 0.867, respectively (p<0.01). On the other hand, correlation coefficients between the medium axis and the minor axis with density and L* were highly negative -0.968, -0.927, -0.924, and -0.895, respectively (p<0.01). These results indicate that the increase of dimensions decreased proportionally with the increase in density and L*. The same results were found by Megat-Ahmad-Azman et al. (2020), in which the dimensions were negatively correlated with the density.

As indicated in Table 5, volume expansion increases proportionally with

Table 5
Pearson correlation coefficients for physical properties of parboiled paddy and milled rice MR297

	Hardness	L	W	T	V	TKW	density	L*	a*	b*
Hardness	1									
L	-.770**	1								
W	-0.426	.758**	1							
T	-0.158	.634*	.931**	1						
V	-0.362	.766**	.985**	.973**	1					
TKW	-0.158	.556*	.565*	.645**	.624*	1				
Density	0.392	-.734**	-.968**	-.924**	-.966**	-0.407	1			
L*	0.456	-.798**	-.927**	-.895**	-.937**	-.670**	.889**	1		
a*	-.557*	.761**	.938**	.831**	.911**	0.432	-.928**	-.927**	1	
b*	-0.214	.612*	.825**	.867**	.863**	.652**	-.781**	-.808**	.745**	1

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

TKW (r=0.624, p<0.05), a* (r=0.911, p<0.01), and b* (r=0.863, p<0.01). The volume expansion was negatively correlated with the density (−0.966) and L* (−0.934). TKW was highly positively correlated with b* (r=0.652, p<0.01). TKW was conversely highly negative with L* (r=-0.670, p<0.01). These results indicate volume expansions and TKW decreased proportionally with the decrease in b*. However, it also decreased proportionally with the increase in L*. The correlation coefficient between density and L* was highly positive, with a value of 0.889. Volume had a highly negative correlation with a* (−0.928) and b (-0.781) (p<0.01). L* was highly negatively correlated with the a∗ (r=-0.927, p<0.01) and b∗ (r=-0.808, p<0.01). Meanwhile, a∗ was highly positively correlated with the b∗ (0.745). The results showed that the increase in volume expansion, TKW, a* and b* decreased proportionally with density and L*.

Figure 2 displays the score plot of the physical and dimensional characteristics of MR297 parboiled paddy grain and the hardness and colour of milled parboiled rice as the effect of temperature and time of soaking. This plot is based on the principal component analysis. The PCA score plot shows the variations in the characteristics of MR297 across a range of different treatments. The variance was divided into two halves. The first (PC1) and second (PC2) principal components (PC) accounted for 71.5% and 17.4%, which together accounted for 88.9% of the variation (Table 6). The PC1 was by far the most important component for providing data that was plotted in the PCA. According to Table 7, PC1 considers all physical properties except hardness, TKW, and temperature of soaking to be equally important. However, the important qualities in PC2 were temperature, hardness, and TKW, which were less important in PC1. Additionally, Figure 2 noted that the sample load positively towards the PC1, the higher the soaking temperature. Also, the increase in soaking time was loading

Table 6
Explained variance (Eigenanalysis) of the correlation matrix

Eigenvalue	8.585	2.089	0.789	0.227	0.143	0.082	0.034	0.030	0.013	0.008	0.000	0.000
Proportion	0.715	0.174	0.066	0.019	0.012	0.007	0.003	0.002	0.001	0.001	0.000	0.000
Cumulative	0.715	0.889	0.955	0.974	0.986	0.993	0.996	0.998	0.999	1.000	1.000	1.000

Table 7
Correlation loadings for paddy in different temperatures and times of soaking and 12 physical characteristics

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12
Temperature	0.158	0.592	0.174	-0.232	0.068	-0.275	-0.487	0.025	0.042	-0.469	-0.023	0.011
Time	0.298	-0.318	-0.044	0.110	0.037	0.173	-0.088	0.703	-0.352	-0.370	0.026	0.030
Hardness	-0.165	0.531	-0.422	0.298	-0.182	-0.183	0.034	0.459	-0.067	0.374	0.025	-0.003
L	0.286	-0.235	0.375	-0.048	-0.598	-0.459	-0.079	0.156	0.176	0.256	-0.034	0.152
W	0.333	-0.008	-0.171	0.093	0.016	0.313	-0.567	-0.253	-0.234	0.377	-0.039	0.411
T	0.318	0.186	-0.215	0.199	-0.204	0.114	0.453	-0.150	0.246	-0.373	-0.057	0.542
V expansion	0.336	0.055	-0.138	0.108	-0.185	0.155	-0.005	-0.096	0.068	-0.013	-0.695	-0.547
TKW	0.224	0.313	0.629	0.420	0.107	0.360	0.126	0.064	0.075	0.164	0.245	-0.160
Density	-0.321	0.053	0.348	-0.043	0.261	0.048	0.060	0.237	0.002	0.112	-0.663	0.434
Lightness	-0.330	-0.015	-0.039	-0.178	-0.389	0.555	-0.285	0.211	0.508	-0.090	0.072	-0.002
a*	0.320	-0.140	-0.188	-0.019	0.544	-0.151	-0.078	0.219	0.658	0.188	0.038	-0.010
b*	0.296	0.230	-0.004	-0.757	-0.006	0.227	0.338	0.148	-0.151	0.269	0.060	-0.008

Note. PC 1-12 = Principal components 1-12, respectively; L = Length; W = Width; T = Thickness; V expansion = Volume expansion; TKW = Thousand kernel weight; a* = Redness ; b* = Yellowness

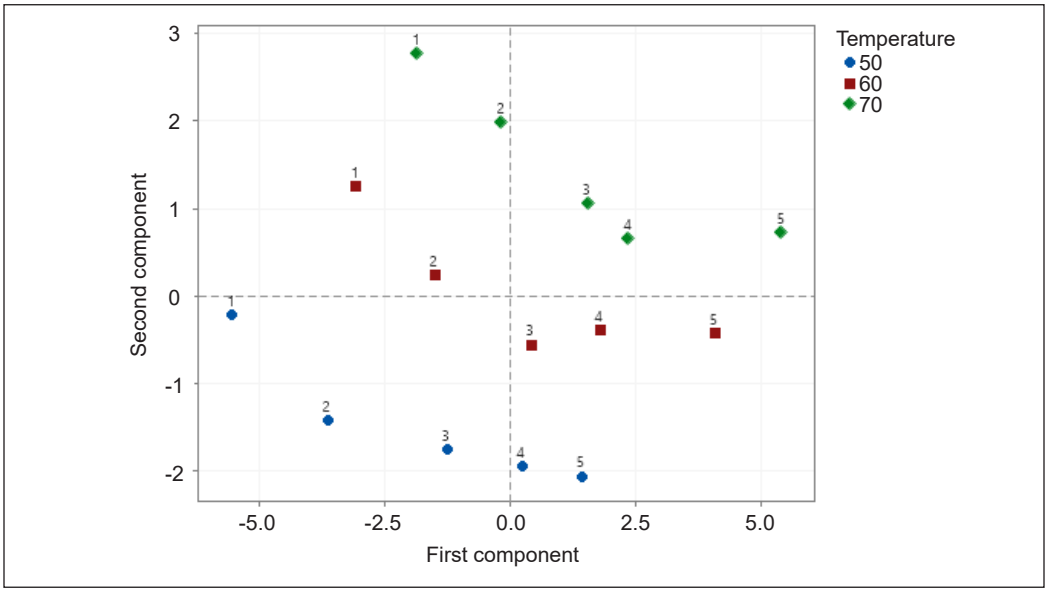

Figure 2. Score plot for principal components 1 and 2

positively towards the increase of the soaking temperature. It was further agreed by the study's earlier portion, where the time and temperature of the soaking treatment during parboiling affects proportionally with no interaction for the dimensional, physical, hardness, and colour properties of parboiled paddy and milled rice.

CONCLUSION

Soaking time and temperature were all important in parboiled paddy and rice properties. However, there was no interaction between time and temperature due to all the changes in physical properties. The hydrothermal treatment has expanded the dimensions of parboiled rice compared to raw rice; therefore, it has increased the volume expansion. Hydrothermal also modified the TKW, density, and colour properties of parboiled rice. The colour turns into a yellowish colour due to the Maillard reaction and the imbibition of the colour of the husk. Increasing the soaking duration decreases the hardness, while raising the soaking temperature increases the hardness. Several significant correlations between the properties of parboiled rice were discovered. The dimensions, volume expansion, TKW, a* and b* were negatively correlated with the density and L*. After parboiling, the hardness was negatively correlated with L and b*. The study will be very important for parboiled rice producers regarding the effect of soaking time and temperature on the physical characteristics of parboiled paddy and rice.

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REFERENCES

Azuka, C. E., Nkama, I., & Asoiro, F. U. (2021). Physical properties of parboiled milled local rice varieties marketed in South-East Nigeria. *Journal of Food Science and Technology*, *58*, 1788–1796. https://doi.org/10.1007/s13197-020-04690-1

Balbinoti, T. C. V., Nicolin, D. J., de Matos Jorge, L. M., & Jorge, R. M. M. (2018). Parboiled rice and parboiling process. *Food Engineering Reviews*, *10*, 165–185. https://doi.org/10.1007/s12393-018-9177-y

Behera, G., & Sutar, P. P. (2018). A comprehensive review of mathematical modeling of paddy parboiling and drying: Effects of modern techniques on process kinetics and rice quality. *Trends in Food Science and Technology*, *75*, 206–230. https://doi.org/10.1016/j.tifs.2018.03.015

Bello, M. O., Tolaba, M. P., & Suarez, C. (2007). Water absorption and starch gelatinization in whole rice grain during soaking. *LWT - Food Science and Technology*, *40*(2), 313–318. https://doi.org/10.1016/j.lwt.2005.09.017

Bello, M., Tolaba, M. P., & Suarez, C. (2004). Factors affecting water uptake of rice grain during soaking. *LWT - Food Science and Technology*, *37*(8), 811–816. https://doi.org/10.1016/j.lwt.2004.02.014

Bhat, F. M., & Riar, C. S. (2016). Cultivars effect on the physical characteristics of rice (rough and milled) (*Oryza sativa* L.) of temperate region of Kashmir (India). *Journal of Food Science and Technology*, *53*, 4258–4269. https://doi.org/10.1007/s13197-016-2420-8

Bootkote, P., Soponronnarit, S., & Prachayawarakorn, S. (2016). Process of producing parboiled rice with different colors by fluidized bed drying technique including tempering. *Food and Bioprocess Technology*, *9*, 1574–1586. https://doi.org/10.1007/s11947-016-1737-7

Buggenhout, J., Brijs, K., Van Oevelen, J., & Delcour, J. A. (2014). Milling breakage susceptibility and mechanical properties of parboiled brown rice kernels. *LWT - Food Science and Technology*, *59*(1), 369–375. https://doi.org/10.1016/j.lwt.2014.05.001

Dutta, H., & Mahanta, C. L. (2012). Effect of hydrothermal treatment varying in time and pressure on the properties of parboiled rices with different amylose content. *Food Research International*, *49*(2), 655–663. https://doi.org/10.1016/j.foodres.2012.09.014

Ebuehi, O. A. T., & Oyewole, A. C. (2008). Effect of cooking and soaking on physical, nutrient composition and sensory evaluation of indigenous and foreign rice varieties in Nigeria. *Nutrition and Food Science*, *38*(1), 15-21. https://doi.org/10.1108/00346650810847972

Esa, N., Puteh, A., Mat, M., Ismail, R., & Yusop, M. R. (2020). Increasing yield of susceptible and resistant rice blast cultivars using silicon fertilization. *Indonesian Journal of Agricultural Science*, *21*(2), 49. https://doi.org/10.21082/ijas.v21n2.2020.p49-58

Graham-Acquaah, S., Manful, J. T., Ndindeng, S. A., & Tchatcha, D. A. (2015). Effects of soaking and steaming regimes on the quality of artisanal parboiled rice. *Journal of Food Processing and Preservation*, *39*(6), 2286–2296. https://doi.org/10.1111/jfpp.12474

Hanucharoenkul, P., Theerathanan, C., & Pongsawatmanit, R. (2021). Influence of soaking temperature and time on the kinetics of water

absorption and pasting properties of glutinous rice. *Agriculture and Natural Resources*, *55*, 193–200. https://doi.org/10.34044/j.anres.2021.55.2.05

Hu, Z., Shao, Y., Lu, L., Fang, C., Hu, X., & Zhu, Z. (2019). Effect of germination and parboiling treatment on distribution of water molecular, physicochemical profiles and microstructure of rice. *Journal of Food Measurement and Characterization*, *13*, 1898–1906. https://doi.org/10.1007/s11694-019-00108-5

Hu, Z., Yang, Y., Lu, L., Chen, Y., Zhu, Z., & Huang, J. (2021). Kinetics of water absorption expansion of rice during soaking at different temperatures and correlation analysis upon the influential factors. *Food Chemistry*, *346*, 128912. https://doi.org/10.1016/j.foodchem.2020.128912

Iqbal, S., Zhang, P., Wu, P., Ge, A., Ge, F., Deng, R., & Chen, X. D. (2021). Evolutions of rheology, microstructure and digestibility of parboiled rice during simulated semi-dynamic gastrointestinal digestion. *LWT, 148*, 111700. https://doi.org/10.1016/j.lwt.2021.111700

Jannasch, A., & Wang, Y.-J. (2020). Development of a limited-water soaking method on the fortification of rice with calcium and iron by parboiling. *Journal of Cereal Science*, *94*, 103014. https://doi.org/10.1016/j.jcs.2020.103014

Jayaraman, R., Uluvar, H., Khanum, F., & Singh, V. (2019). Influence of parboiling of red paddy varieties by simple hot soaking on physical, nutrient, phytochemical, antioxidant properties of their dehusked rice and their mineral, starch, and antioxidant's bioaccessibility studies. *Journal of Food Biochemistry*, *43*(7), e12839. https://doi.org/10.1111/jfbc.12839

Ji-u, P., & Inprasit, C. (2019). Effect of soaking conditions on properties of Khao Dawk Mali 105. *Agriculture and Natural Resources*, *53*, 378–385. https://doi.org/10.34044/j.anres.2019.53.4.08

Kumar, S., & Prasad, K. (2018). Effect of parboiling and puffing processes on the physicochemical, functional, optical, pasting, thermal, textural and structural properties of selected Indica rice. *Journal of Food Measurement and Characterization*, *12*, 1707–1722. https://doi.org/10.1007/s11694-018-9786-4

Lamberts, L., Rombouts, I., Brijs, K., Gebruers, K., & Delcour, J. A. (2008). Impact of parboiling conditions on Maillard precursors and indicators in long-grain rice cultivars. *Food Chemistry*, *110*(4), 916–922. https://doi.org/10.1016/j.foodchem.2008.02.080

Liang, J., Li, Z., Tsuji, K., Nakano, K., Robert Nout, M. J., & Hamer, R. J. (2008). Milling characteristics and distribution of phytic acid and zinc in long-, medium- and short-grain rice. *Journal of Cereal Science*, *48*(1), 83–91. https://doi.org/10.1016/j.jcs.2007.08.003

Liu, J., Liu, Y., Wang, A., Dai, Z., Wang, R., Sun, H., Strappe, P., & Zhou, Z. (2021). Characteristics of moisture migration and volatile compounds of rice stored under various storage conditions. *Journal of Cereal Science*, *102*, 103323. https://doi.org/10.1016/J.JCS.2021.103323

Megat-Ahmad-Azman, P. N., Shamsudin, R., Che-Man, H., & Ya'acob, M. E. (2020). Kinetics of quality changes in soaking water during the retting process of pepper berries (*Piper nigrum* L.). *Processes*, *8*(10), 1255. https://doi.org/10.3390/pr8101255

Miah, M. A. K., Haque, A., Douglass, M. P., & Clarke, B. (2002). Parboiling of rice. Part II: Effect of hot soaking time on the degree of starch gelatinization. *International Journal of Food Science and Technology*, *37*(5), 539–545. https://doi.org/10.1046/j.1365-2621.2002.00611.x

Mir, S. A., & Bosco, S. J. D. (2013). Effect of soaking temperature on physical and functional properties of parboiled rice cultivars grown in temperate region of India. *Food and Nutrition*

Sciences, *4*(3), 282–288. https://doi.org/10.4236/fns.2013.43038

Mir, S. A., Bosco, S. J. D., & Sunooj, K. V. (2013). Evaluation of physical properties of rice cultivars grown in the temperate region of India. *International Food Research Journal*, *20*(4), 1521-1527.

Muchlisyiyah, J., Shamsudin, R., Basha, R. K., Shukri, R., How, S., Niranjan, K., & Onwude, D. (2023). Parboiled rice processing method, rice quality, health benefits, environment, and future perspectives: A review. *Agriculture*, *13*(7), 1390. https://doi.org/10.3390/agriculture13071390

Nádvorníková, M., Banout, J., Herák, D., & Verner, V. (2018). Evaluation of physical properties of rice used in traditional Kyrgyz Cuisine. *Food Science and Nutrition*, *6*(6), 1778–1787. https://doi.org/10.1002/fsn3.746

Panda, B. K., Panigrahi, S. S., Mishra, G., & Shrivastava, S. L. (2021). Microwave-assisted hydration of freshly harvested paddy (*Oryza sativa* L.): Process development based on soaking characterization and energy utilization. *Food and Bioprocess Technology*, *14*, 1844–1856. https://doi.org/10.1007/s11947-021-02682-3

Patindol, J., Newton, J., & Wang, Y.-J. (2008). Functional properties as affected by laboratory-scale parboiling of rough rice and brown rice. *Journal of Food Science*, *73*(8), E370–E377. https://doi.org/10.1111/j.1750-3841.2008.00926.x

Rachmat, R., Thahir, R., & Gummert, M. (2016). The empirical relationship between price and quality of rice at market level in West Java. *Indonesian Journal of Agricultural Science*, *7*(1), 27-33. https://doi.org/10.21082/ijas.v7n1.2006.p27-33

Rahim, H., Ariff, E. E. E., & Hosni, H. (2023). Bridging the gap between consumer preferences and the development of fragrant rice varieties in Malaysia. *Agricultural Research*, *12*, 462-472. https://doi.org/10.1007/s40003-023-00666-7

Ramli, A., & Kamaruzaman, R. (2020). Breeding strategies for rice in Malaysia. In P. Lestari, K. Mulya, D. W. U. D. Satyawan, Supriadi., & Mastur. (Eds.), *Strategies and technologies for the utilization and improvement* (pp. 25-36). IAARD Press.

Rattanamechaiskul, C., Junka, N., & Prakotmak, P. (2023). Modeling and optimization of moisture diffusion of paddy and brown rice during thermal soaking. *Journal of Food Process Engineering*, *46*(4), e14902. https://doi.org/10.1111/jfpe.14302

Reddy, B. S., & Chakraverty, A. (2004). Physical properties of raw and parboiled paddy. *Biosystems Engineering*, *88*(4), 461–466. https://doi.org/10.1016/j.biosystemseng.2004.05.002

Rocha-Villarreal, V., Serna-Saldivar, S. O., & García-Lara, S. (2018). Effects of parboiling and other hydrothermal treatments on the physical, functional, and nutritional properties of rice and other cereals. *Cereal Chemistry*, *95*(1), 79–91. https://doi.org/10.1002/cche.10010

Roy, M., Dutta, H., Jaganmohan, R., Choudhury, M., Kumar, N., & Kumar, A. (2019). Effect of steam parboiling and hot soaking treatments on milling yield, physical, physicochemical, bioactive and digestibility properties of buckwheat (*Fagopyrum esculentum* L.). *Journal of Food Science and Technology*, *56*, 3524–3533. https://doi.org/10.1007/s13197-019-03849-9

Saleh, M., & Meullenet, J.-F. (2015). Cooked rice texture and rice flour pasting properties; Impacted by rice temperature during milling. *Journal of Food Science and Technology*, *52*, 1602–1609. https://doi.org/10.1007/s13197-013-1180-y

Saleh, M., Akash, M., & Ondier, G. (2018). Effects of temperature and soaking durations on the hydration kinetics of hybrid and pureline parboiled brown rice cultivars. *Journal of Food Measurement and Characterization*, *12*, 1369–1377. https://doi.org/10.1007/s11694-018-9751-2

Sareepuang, K., Siriamornpun, S., Wiset, L., & Meeso, N. (2008). Effect of soaking temperature on physical, chemical and cooking properties of parboiled fragrant rice research unit of drying technology for agricultural products. *World Journal of Agricultural Sciences*, *4*(4), 409–415.

Shamsudin, R., Ariffin, S. H., Zainol@Abdullah, W. N. Z., Azmi, N. S., & Abdul-Halim, A. A. (2021). Modelling the kinetics of color and texture changes of Dabai (*Canarium odontophyllum* Miq.) during blanching. *Agronomy*, *11*(11), 2185. https://doi.org/10.3390/agronomy11112185

Shen, Y., Jin, L., Xiao, P., Lu, Y., & Bao, J. (2009). Total phenolics, flavonoids, antioxidant capacity in rice grain and their relations to grain color, size and weight. *Journal of Cereal Science*, *49*(1), 106–111. https://doi.org/10.1016/j.jcs.2008.07.010

Sittipod, S., & Shi, Y.-C. (2016). Changes of starch during parboiling of rice kernels. *Journal of Cereal Science*, *69*, 238–244. https://doi.org/10.1016/j.jcs.2016.03.015

Sivakamasundari, S. K., Moses, J. A., & Anandharamakrishnan, C. (2020). Effect of parboiling methods on the physicochemical characteristics and glycemic index of rice varieties. *Journal of Food Measurement and Characterization*, *14*, 3122–3137. https://doi.org/10.1007/s11694-020-00551-9

Tao, K., Li, C., Yu, W., Gilbert, R. G., & Li, E. (2019). How amylose molecular fine structure of rice starch affects functional properties. *Carbohydrate Polymers*, *204*, 24–31. https://doi.org/10.1016/j.carbpol.2018.09.078

Tian, J., Cai, Y., Qin, W., Matsushita, Y., Ye, X., & Ogawa, Y. (2018). Parboiling reduced the crystallinity and *in vitro* digestibility of non-waxy short grain rice. *Food Chemistry*, *257*, 23–28. https://doi.org/10.1016/j.foodchem.2018.03.005

Tian, Y., Zhao, J., Xie, Z., Wang, J., Xu, X., & Jin, Z. (2014). Effect of different pressure-soaking treatments on color, texture, morphology and retrogradation properties of cooked rice. *LWT - Food Science and Technology*, *55*(1), 368–373. https://doi.org/10.1016/J.LWT.2013.09.020

Villanova, F. A., Vanier, N. L., de Avila Madruga, N., Pesek, J., Matyska-Pesek, M., Elias, M. C., & de Oliveira, M. (2017). Improvement of the quality of parboiled rice by using anti-browning agents during parboiling process. *Food Chemistry*, *235*, 51–57. https://doi.org/10.1016/j.foodchem.2017.05.053

Wahengbam, E. D., & Hazarika, M. K. (2019). Quality of ready-to-eat *komal chawal* produced by brown rice parboiling method. *Journal of Food Science and Technology*, *56*, 187–199. https://doi.org/10.1007/s13197-018-3472-8

Wu, W., Zhou, L., Chen, J., Qiu, Z., & He, Y. (2018). GainTKW: A measurement system of thousand kernel weight based on the android platform. *Agronomy*, *8*(9), 178. https://doi.org/10.3390/agronomy8090178

Wu, Z., He, Y., Yan, W., Zhang, W., Liu, X., Hui, A., Wang, H., & Li, H. (2021). Effect of high-pressure pre-soaking on texture and retrogradation properties of parboiled rice. *Journal of the Science of Food and Agriculture*, *101*(10), 4201–4206. https://doi.org/10.1002/jsfa.11058

Yadav, B. K., & Jindal, V. K. (2007). Modeling changes in milled rice (*Oryza sativa* L.) kernel dimensions during soaking by image analysis. *Journal of Food Engineering*, *80*(1), 359–369. https://doi.org/10.1016/J.JFOODENG.2006.06.005

Yang, L., Sun, Y.-H., Liu, Y., Mao, Q., You, L.-X., Hou, J.-M., & Ashraf, M. A. (2016). Effects of leached amylose and amylopectin in rice cooking liquid on texture and structure of cooked rice. *Brazilian Archives of Biology and Technology*, *59*, e16160504. https://doi.org/10.1590/1678-4324-2016160504

Zainal, N., & Shamsudin, R. (2021). Physical properties of different cultivar local glutinous rice (susu and siding) and commercial Thai cultivar. *Advances in Agricultural and Food Research Journal*, 2(1), a0000178. https://doi.org/10.36877/aafrj.a0000178

Zhu, L., Cheng, L., Zhang, H., Wang, L., Qian, H., Qi, X., & Wu, G. (2019). Research on migration path and structuring role of water in rice grain during soaking. *Food Hydrocolloids*, *92*, 41–50. https://doi.org/10.1016/j.foodhyd.2019.01.051

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Effect of Preharvest Treatment Using Jasmonic Acid and Methyl Jasmonate on the Physicochemical Properties and Antioxidant Activities of Red-fleshed Dragon Fruit (*Hylocereus polyrhizus* L.)

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ABSTRACT

This study investigated the effect of exogenous plant growth regulators (PGR), namely jasmonic acid (JA) and methyl jasmonate (MeJA), on the physicochemical properties of flesh and peels of red-fleshed dragon fruit (*Hylocereus polyrhizus*). The fruit was sprayed with 100 and 1,000 ppm of JA and MeJA at 15 and 22 days of anthesis and harvested after 35 days. Then, the flesh and peels were analyzed for total soluble solids (TSS), total betacyanins, betanin, total phenolics (TP), total flavonoids (TF), and color characteristics. The fruit peels contained significantly higher (p<0.05) TP and antioxidant activities compared to flesh. No significant difference was detected between the variables in the peels, except for significantly higher (p<0.05) of total betacyanins (~295.6 and ~299.9 mg/100 g) and TP (~614.1 and 566.1 mg GAE/100 g) were recorded in control and 100 ppm MeJA, respectively. In the flesh, 1,000 ppm MeJA-treated fruit possessed the highest total betacyanins (~139.2 mg/100 g), betanin (~356.0 mg/g), TP (~244.9 mg GAE/100 g), TF (~329.0 mg CE/100 g), Trolox equivalent antioxidant capacity (TEAC) (63.2 μmol TE/g) and reducing power (~21.5 μmol TE/g). Overall, 1,000 ppm MeJA was more effective in enhancing the accumulation of bioactive compounds and antioxidant activities in the flesh of red-fleshed dragon fruit compared to other PGR treatments.

Keywords: Betacyanins, fruit preservation, pitaya, plant growth regulator, plant hormone, shelf life extension, sustainable agriculture

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INTRODUCTION

Preference and consumption of fruit depend on several aspects, including the fruit's visual cues and taste and nutritional benefits. The red-fleshed dragon fruit (*Hylocereus polyrhizus*) has been recognized for its attractiveness, unique shape, intense red colors, juiciness, sweetness, and pleasant taste. It is a native of Southern Mexico and Central America (Abirami et al., 2021). Dragon fruit has become popular in Asia, and cultivation is expanding in countries such as Vietnam, Thailand, Taiwan, the Philippines, and Malaysia (Hossain et al., 2021). Three main components in dragon fruit include the pulp (47.40%–73.76%), peel (36.70%–37.60%), and seed (2.70%–14.67%) with an average weight of approximately 300–350 g. It can be consumed as a dessert or salad or used to produce various products such as juices, jams, candies, and ice creams. Other than flesh, the fruit peels are enriched with beneficial polyphenols, especially betacyanins, that could be further processed into valuable products such as natural food colorants (Jalgaonkar et al., 2022).

Notably, the red-fleshed's nutrient composition, minerals, and vitamins vary by cultivar, agricultural region and climate, plant feeding and nutrition, ripening stage, harvesting duration, and storage conditions (Attar et al., 2022). The bioactive components, including vitamin C, phenolic acids, betalains, and flavonoids, are ubiquitously found in the fruit to serve a variety of protective functions such as antioxidant, antimicrobial, anticancer, and antidiabetic activities (Nishikito et al., 2023). These molecules have become increasingly important in the human diet and remarkably increased the market of red-fleshed dragon fruit domestically and internationally (Jalgaonkar et al., 2022).

Specific metabolic pathways have created natural pigments and can be classified into several large groups, such as carotenoids, betalains, chlorophyll, and anthocyanins. Betalains are recognized as a group of water-soluble nitrogen-containing pigments. They are also known as chromo-alkaloids due to the presence of nitrogen in the basic structural components (Ren et al., 2017). Betalains can be further classified into two primary structural subgroups, betacyanins and betaxanthins, which are responsible for the reddish-violet and yellowish-orange colors, respectively (Miguel, 2018).

Betacyanins abundant in red-fleshed dragon fruit, identified as betanin, isobetanin, philocactin, and hilocerenin (Cheok et al., 2022). These compounds are important in providing an intense red color in the fruit peels and flesh. Notably, betacyanin stability is affected by various factors, including temperature, pH, water activity, light intensity, oxygen, and antioxidants (Calva-Estrada et al., 2022). These factors might lead to the deformation or degradation of betacyanin compounds. Given the brilliant red color of the fruit, it can fulfill other functions, such as attracting pollinators for pollination, seed dispersal, photosynthesis, and protection against biotic and abiotic stress (Sadowska-Bartosz & Bartosz, 2021).

Recently, the study of betacyanins in the red-fleshed dragon fruit has attracted much attention due to current evidence about the positive influences on the human body, as it also acts as an antioxidant (Paśko, Galanty, Zagrodzki, Ku, et al., 2021).

Therefore, the increased demand for tropical fruit, including dragon fruit, results in the agroindustry continuing to expand the production of high-quality fruit. Furthermore, new approaches using pre-treatment to improve the quality of fruit crops are continuously being developed. One of the pre-treatments developed involves gene regulation modification by applying plant growth regulators (PGR) on fruit crops (Ordoñez Trejo et al., 2023). The application of PGR is widely used in modern agriculture to enhance fruit crops' physicochemical properties and desirable bioactive compounds, improving the market's desirable characteristics. PGR are chemical compounds that are synthesized artificially to mimic and perform the same function as naturally occurring plant hormones, including auxins, cytokines, gibberellins, acetic acid, ethylene, salicylic acid, and the jasmonates group (Mukherjee et al., 2022).

PGR could effectively improve the characteristics and accumulation of bioactive compounds in fruit crops (Ordoñez Trejo et al., 2023). However, the effectiveness of PGR performance may also be affected by factors such as application method, time of application, concentration, crop stage, plant species, and the environmental conditions in which plants are cultivated (Khan et al., 2020). Moreover, PGR can be applied to plants through a variety of methods. The most common include foliar application, soaking, pre-plant seeding, and injection.

Jasmonic acid (JA) and its derivatives were called 'jasmonates.' Naturally, jasmonates are lipid-derived compounds synthesized via the octadecanoid pathway (Figure 1). Wasternack and Strnad (2018) reported that the initial step of JA synthesis occurs in the membrane chloroplast, where α-linolenic acid from membrane lipids is released. Moreover, the synthesis of JA in the chloroplast is regulated by enzymes such as lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). These enzymes are important in various ways; LOX contributes to ripening, aroma, and flavor development, AOS can act as antioxidants and defense mechanisms against pathogens and insects, and AOC is linked to environmental stress responses, fruit ripening and senescence. Also, these enzymes are responsible for forming oxophytodienoic acid (OPDA). Hence, the ultimate form of JA could be produced when OPDA is transported into peroxisomes and oxidized by the process of β-oxidation. In summary, after the formation of OPDA, the jasmonic acid biosynthetic pathway continues through a series of enzymatic conversions to produce active jasmonic acid. This molecule then plays a crucial role in regulating various processes contributing to fruit quality, including ripening, aroma, flavor, defense responses, and stress tolerance. In addition,

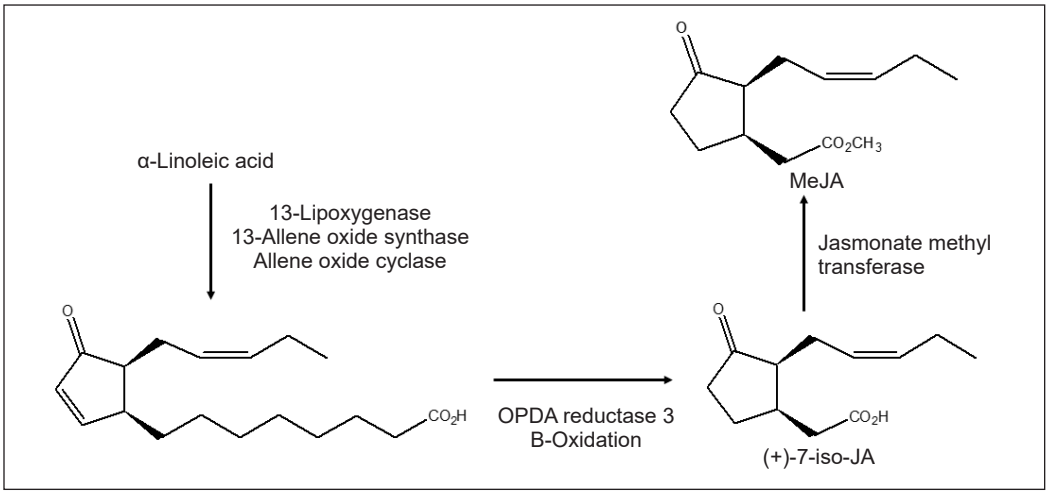

Figure 1. Structure and biosynthesis of jasmonates
Note. OPDA = 12-oxophytodienoic acid; JA = Jasmonic acid; MeJA = Methyl jasmonate (Source: Taki-Nakano et al., 2014)

methyl jasmonate (MeJA) could be formed if the JA produced is further altered in the cytoplasm by jasmonate methyl transferase (Ruan et al., 2019).

The information about exogenous JA and MeJA regulating the bioactive compounds in red-fleshed dragon fruit is limited. The research problem in this study revolves around the limited knowledge regarding the impact of exogenous JA and MeJA on the regulation of bioactive compounds in red-fleshed dragon fruit, the unexplored effects of preharvest treatment with varying concentrations of JA and MeJA on the physicochemical properties and antioxidant activity, as well as inconsistency in the quality of both the flesh and peels of this fruit. Therefore, this study aims to investigate the effect of preharvest treatment using 100 and 1,000 ppm of JA and MeJA on the physicochemical properties, bioactive compounds, antioxidant activity, and color improvement of the flesh and peels of red-fleshed dragon fruit. In addition, the Pearson correlation, general linear model (GLM), and principal component analysis (PCA) were also performed to correlate between the variables studied in the fruit flesh and peels. Overall, preharvest treatment with a higher concentration of JA and MeJA at 1,000 ppm was hypothesized to significantly influence the variables tested on the flesh and peels of red-fleshed dragon fruit compared to 100 ppm.

MATERIALS AND METHODS

Chemicals

The solvent and reagent used were of analytical and high-performance liquid chromatography (HPLC) grade. Folin–Ciocalteu's phenol reagent and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were obtained from Merck (Germany). Betanin, 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and 6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (USA). Ethanol, trifluoroacetic acid (TFA) 98%, and acetonitrile were purchased from Fisher Scientific (United Kingdom).

Agronomic Practices of the Plant

The agronomic practices of the red-dragon fruit plant were carried out at the Multi Rich Pitaya farm located in Sepang, Selangor, Malaysia (GPS: 2.805723, 101.7407). The planting distance was 3 m × 3 m using a single post system. The age of the plant was six years. Five kg of organic manure was applied to each plant once a year. In contrast, chemical fertilizer containing nitrogen (N), phosphorus (P), and potassium (K) was applied at the ratio of 2:10:10 (v:v:v) for every month. Irrigation and pest or disease management are interconnected aspects of modern agriculture. Hence, proper irrigation practices, including water management, can help prevent the development and spread of pests and diseases. Also, integrating irrigation management into a holistic pest and disease management strategy is crucial for maintaining crop health and maximizing yields while minimizing the use of chemical controls.

Phytohormone Preharvest Treatments

The application of PGR was carried out according to the method by H. Wang et al. (2019) with slight modifications. The fruit was sprayed before 9 a.m. (28°C, 63% relative humidity [RH]) with 100 and 1,000 ppm of JA and MeJA after 15 and 22 d of anthesis. The spraying volume was around 8-10 ml, covering the entire fruit. The sprayer was designed to deliver smaller droplets (<150 μm) to control the drifting of treatments. At least 10 flowers were treated in each treatment, where control samples were the red-fleshed dragon fruit flowers without any treatment. The fruit was then harvested after 35 d of anthesis. The criteria for harvesting the fruit involve a vibrant red skin color and a uniform shape, with each fruit weighing around 300 g. The harvested fruit were packed in the polystyrene box (55 length [L] × 42 width [W] × 30 height [H] cm; ±70 L) and then transported in air-conditioned transportation (25°C, 27% RH) to the laboratory and kept at 4°C until further analysis.

Preparation of Red-fleshed Dragon Fruit Extract

Flesh. Preliminary experiments were conducted to determine the conditions favoring better betacyanins extraction. The extraction methods were performed according to Naderi et al. (2012) with slight modifications, whereas the extraction of betacyanins from flesh used a 1:1 ratio of flesh/ethanol (w/v). A 100 g of the mashed flesh fruit was typically macerated with 100 ml of 50% aqueous ethanol for 15 min under cooling conditions (4°C).

Peel. Fruit peels were separated from the flesh and dried for 48 hr. Freeze-dried peels were ground and passed through 20 mesh sieves. Then, 4 g of peel powder was added to 100 ml of 50% ethanol to be macerated for 15 min under cooling conditions (4°C).

The homogenized sample was centrifuged at 769 × *g* and 4°C for 15 min, followed by vacuum filtration using Whatman No.1 filter paper (Sigma-Aldrich, USA). The extraction for both flesh and peels was conducted in triplicate. Then, the supernatants were kept at -20°C for further analysis.

Determination of Total Soluble Solids (TSS)

TSS of red-fleshed dragon fruit flesh was determined using an ATAGO digital handheld pocket refractometer PAL-08S (Japan). The values in °Brix were taken in triplicate measurements.

Determination of Total Betacyanins

Total betacyanins were quantified according to the Naderi et al. (2012) method with slight modifications, whereby the solvent used was 50% ethanol instead of deionized water. The extracted samples were diluted 20-fold with ethanol and kept in the dark for 20 min for equilibrium. The following equation describes the quantification of total betacyanins:

$$\text{Total betacyanins (mg/L)} = \frac{A \times DF \times MW \times 1{,}000}{\varepsilon \times L} \quad (1)$$

where, A = absorption at 540 nm, F = dilution factor, MW = 550 g/mol (molecular weight of betanin), ε = 60,000 L/mol·cm (molar extinction coefficient of betanin in ethanol), and L = 1 cm (pathlength of the cuvette). Total betacyanins were calculated and expressed in mg per gram (mg/g).

$$\text{Total betacyanins (mg/g)} = \frac{\text{Total betacyanins } (\frac{\text{mg}}{\text{L}}) \times (L)}{\text{Sample (g)}} \quad (2)$$

Quantification of Betanin Using HPLC

Betanin was determined based on the method of Naderi et al. (2012) with slight modifications. The mobile phase contained a mixture of 90% solvent A (0.5% aqueous TFA) and 10% solvent B (acetonitrile). Briefly, a Purospher STAR RP18 end-capped column (250 mm × 4.6 mm i.d., particle size of 5 µm, Merck, Germany) in liquid chromatographic apparatus (Waters Corp., USA), equipped with a Waters 2478 two-channel UV detector was used to separate and quantify the betanin at 540 nm. The flow rate was 1 ml/min, and the injection volume was 10 µl for 15 min of analysis. The calibration curve for betanin was plotted in the range of 10.0–2.0 mg/ml.

Determination of Total Phenolics (TP)

TP was determined according to the Folin Ciocalteu's reagent as described by Ramli et al. (2014) with slight modifications. The TP was quantified using the standard curve of gallic acid (0.25–0.05 mg/ml in water), which was expressed in mg gallic acid equivalent (GAE) per 100 g (mg GAE/100 g).

Determination of Total Flavonoids (TF)

The TF in red-fleshed dragon fruit was analyzed using the method by Senevirathna

et al. (2021). It was calculated using the standard curve of catechin (300–50 mg/L), which was expressed in mg of catechin equivalent per 100 g (mg CE/100 g).

Determination of Trolox Equivalent Antioxidant Capacity (TEAC)

The antioxidant capacity of flesh and peels was carried out according to Nawawi et al. (2023) with slight modifications in the solvent used, concentration of DPPH solution and the ratio of sample to DPPH solution. An amount of 0–2,000 µM of Trolox solution was measured using a spectrophotometer at 517 nm. The antioxidant capacity was expressed as µmol of Trolox equivalents (TE) per gram of the samples (µmol TE/g).

Determination of Ferric-reducing Antioxidant Power (FRAP)

The ferric-reducing antioxidant power (FRAP) was analyzed according to Azman et al. (2022). The concentration of Trolox solution was 0 – 1,500 µM, and reducing power was expressed as µmol of TE per gram (µmol TE/g).

Determination of Color

The color of the flesh and peels of red-fleshed dragon fruit were measured by using a colorimeter (CR-410, Minolta, Japan) based on three coordinates units: L^* (lightness/darkness), a^* (redness/greenness), and b^* (yellowness/blueness), which white tiles are used to calibrate the instrument.

Chroma (C) is the quantitative attribute that describes the intensity of a color, while hue (h°) provides a qualitative attribute of color characterized as reddish, greenish, yellowish, and bluish, respectively. The chroma and hue angle were calculated using the equations below (Wrolstad & Smith, 2017):

$$(C) = [(a^*)^2 + (b^*)^2]^{1/2} \quad (3)$$

$$(h°) = \text{Arctan}\ (b^*/a^*) \quad (4)$$

Statistical Analysis

One-way analysis of variance (ANOVA) was performed in all statistical analyses. In this study, a significant level of 95% (p<0.05) was applied in Tukey's multiple range tests. The correlations between tested parameters were also evaluated using the linear Pearson correlation, GLM and PCA. The statistical analysis was performed using the Minitab V.19 software (Minitab Inc., USA).

RESULTS AND DISCUSSION

According to the preliminary test, freeze-drying is not an effective option to dry the flesh of red-fleshed dragon fruit due to the fruit flesh being high in sugar such as glucose, fructose, and some oligosaccharide (Huang et al., 2021), which produces a sticky powder when freeze-dried. Therefore, in this study, the fresh weight (FW) results were converted into dry weight (DW) based on the moisture content of the flesh (84.0 ± 0.2%). Also, in the preliminary test, the extraction efficiency using methanol or ethanol has been compared. Ethanol was more efficient in extracting bioactive compounds in red-fleshed dragon fruit flesh

and peels due to the polarity of betacyanins. Halimfanezi and Asra (2020) state that betacyanins are more hydrophilic and easier to dissolve in ethanol.

Total Soluble Solids (TSS)

TSS was assessed as an indicator of the sweetness of the fruit. The effects of MeJA and JA foliar treatment on the TSS of fruit flesh are shown in Table 1. After the application of PGR at different concentrations, 100 ppm MeJA, 100 ppm JA, 1,000 ppm MeJA and 1,000 ppm JA, TSS values in the fruit flesh demonstrated a significant (p<0.05) increase (13.13–13.97 ºBrix) compared to the control sample (~12.40 ºBrix). Moreover, statistical analysis verified that only PGR types and their concentrations significantly influenced (p<0.05) the TSS values, while the interaction between these variables showed no significant differences (Table 2).

Similar results were reported in previous studies about the effect of preharvest treatments on the TSS of different fruit species. Due to external preharvest treatment, the TSS value in blackberry (Hull Thornless) increased to 35% after the treatment using 0.1 mM MeJA (S. Y. Wang et al., 2008). On the other hand, TSS in peaches increased by 8% after being treated with 0.1 mmol/L MeJA at 5°C during 14 d of storage (Meng et al., 2009). An increase in TSS value might be due to the conversion of polysaccharides into sugars in the presence of organic acids (Batista-Silva et al., 2018; Gupta et al., 2023).

Total Betacyanins

As reported previously, red-fleshed dragon fruit is a good source of betacyanins, as it is responsible for the red-violet color of the fruit. In this study, the MeJA and JA at different concentrations (100 and 1,000 ppm) were used to provoke the possible stimulation of betacyanins in the flesh and peels, as shown in Table . GLM analysis in Table 2 showed the significant influence of PGR concentrations and the interaction between the PGR types and concentrations (p<0.05). Higher concentrations of PGR efficiently elevated the production of

Table 1
Total soluble solids (TSS), total betacyanins, and betanin in the flesh and peels of untreated (control) and treated red-fleshed dragon fruit with plant growth regulators (PGR) at different concentrations

Treatments (ppm)	TSS (°Brix) (Flesh)	Total betacyanins (mg/100 g)		Betanin (mg/g)	
		Flesh	Peels	Flesh	Peels
Control	12.4 ± 0.1^{c}	107.2 ± 1.0^{Bd}	295.6 ± 1.9^{Aa}	244.6 ± 1.7^{Bd}	734.7 ± 40.8^{Aa}
100 MeJA	13.9 ± 0.1^{a}	109.5 ± 1.6^{Bcd}	299.9 ± 1.6^{Aa}	318.1 ± 1.1^{Bc}	779.87 ± 103.4^{Aa}
100 JA	14.0 ± 0.1^{a}	116.0 ± 2.4^{Bc}	163.4 ± 1.0^{Ad}	328.8 ± 15.7^{Abc}	363.8 ± 58.6^{Ab}
1000 MeJA	13.1 ± 0.1^{b}	139.2 ± 0.1^{Ba}	251.9 ± 6.8^{Ab}	440.4 ± 7.3^{Ba}	583.9 ± 27.9^{Aab}
1000 JA	13.9 ± 0.1^{a}	127.0 ± 3.1^{Bb}	236.5 ± 0.1^{Ac}	356.0 ± 2.2^{Bb}	722.7 ± 35.7^{Aa}

Note. JA = Jasmonic acid; MeJA = Methyl jasmonate. Values with the same letter $^{a, b,}$ and ^{c} in each row are not significantly different (p>0.05). Values with the same letter ^{A} and ^{B} in each column are not significantly different (p>0.05)

Table 2
Summary of the estimation results of the general linear model (GLM)

Analysis of variance	Parameters (*p*-values)		
	Types of PGR	Concentration of PGR	Types of PGR × Concentration of PGR
Flesh			
Total Soluble Solids	0.048	0.000	0.074
Total Betacyanins	0.125	0.000	0.001
Betanin	0.001	0.000	0.000
TP	0.339	0.001	0.014
TF	0.020	0.000	0.000
TEAC	0.000	0.000	0.002
Reducing Power	0.003	0.000	0.000
*L**	0.511	0.055	0.844
*a**	0.522	0.009	0.710
*b**	0.445	0.000	0.838
Peels			
Total Betacyanins	0.000	0.000	0.000
Betanin	0.031	0.019	0.001
TP	0.010	0.000	0.000
TF	0.000	0.132	0.000
TEAC	0.364	0.016	0.743
Reducing Power	0.674	0.303	0.890
*L**	0.001	0.966	0.046
*a**	0.010	0.453	0.001
*b**	0.990	0.156	0.106

Note. TP = Total phenolics; TF = Total flavonoids; TEAC = Trolox equivalent antioxidant capacity. Parameters with $p>0.05$ are not significantly different

betacyanins in the fruit flesh. Among the concentrations tested, the most effective ($p<0.05$) was 1,000 ppm MeJA with total betacyanins of ~139.2 mg/100 g. The result also showed that the application of MeJA is more effective than JA in accumulating betacyanins in the flesh. It might be related to the physical properties of the MeJA that are higher in volatility and hydrophobicity than JA, which make it significantly easier to pass through the plant stomata and reach the inside of the cytoplasm (Li et al., 2018).

A similar finding was reported by Mustafa et al. (2018). In contrast, applying 0.1 mM MeJA on red-fleshed dragon fruit during postharvest treatment increased the accumulation of betacyanins in the fruit, ranging from 40–50 g/kg at 6°C during 14 d of storage. The resulting accumulation of bioactive compounds such as betalain might be due to activating the phenylpropanoid pathway that MeJA and JA regulate. Betalain is a derivation of tyrosine. Thus, phenylpropanoid biosynthesis was acknowledged to be the upstream pathway

of the tyrosine pathway (Yadav et al., 2020). In peaches, a modulating initial effect of PGR was reported similarly, whereas MeJA stimulated the expression levels of anthocyanin-associated genes that resulted in anthocyanin accumulation in the peach fruit (Wei et al., 2017). Therefore, this study demonstrates that PGR could modify the pathway associated with bioactive compounds to increase their concentration in fruit.

In contrast to the flesh, the peels in fruit treated with 100 ppm MeJA and control samples exhibited the highest total betacyanins, ~299.9 and ~295.6 mg/100 g, respectively (Table 1). Statistical analysis also detected a significant influence of the types of PGR, concentrations used and the interaction between the types of PGR and concentrations on the total betacyanins in fruit peels (Table 2).

Overall, total betacyanins in peels (~163.4 – ~299.9 mg/100 g) were higher compared to the flesh (~107.2 – ~139.2 mg/100 g). This result was in agreement with the study by Khoo et al. (2022), where red-fleshed dragon fruit peel had higher betacyanins (~35.12 mg/g FW) than in the flesh (~0.15 mg/g FW). During the extraction process, the broken seeds can also contribute to the degradation of betacyanins in the flesh (Naderi et al., 2012).

Preharvest and postharvest treatments can have distinct physiological effects on fruit, leading to different outcomes in terms of quality and responses. Preharvest treatments

Table 3
Pearson correlation between analysis measured in the flesh and peels of red-fleshed dragon fruit

Analysis 1	Analysis 2	Correlation	*p*-value
Flesh			
Betanin	Total betacyanins	0.923	0.000
TP	Total betacyanins	0.899	0.000
Reducing power	Total betacyanins	0.953	0.000
TF	Total betacyanins	0.935	0.000
TP	Betanin	0.837	0.003
Scavenging activity	Betanin	0.733	0.016
Reducing power	Betanin	0.965	0.000
TF	Betanin	0.829	0.003
Reducing power	TP	0.917	0.000
TF	TP	0.916	0.000
Reducing power	Scavenging activity	0.657	0.039
TF	Reducing power	0.909	0.000
Peels			
Betanin	Total betacyanins	0.874	0.001
TF	Total betacyanins	0.910	0.000
TF	Betanin	0.748	0.013

Note. TP = Total phenolics; TF = Total flavonoids. Analysis with $p<0.05$ is significantly different

involve applying various substances (such as chemicals or growth regulators) to plants before harvesting. These treatments can affect the plant's physiological processes during its growth and development. They can influence nutrient uptake, hormone regulation, and stress responses. On the other hand, postharvest treatments occur after the fruit is harvested and aim to maintain or improve its quality during storage, transportation, and sale. These treatments can include control of ripening, prevent spoilage, and enhance shelf life. It is important to note that while preharvest and postharvest treatments can have varying effects, they are interconnected. Preharvest conditions can set the stage for how fruit responds to postharvest treatments. Additionally, understanding the interactions between these treatments and the JA pathway requires careful consideration of the specific plant species, environmental conditions, and physiological processes involved (Baek et al., 2021).

Betanin

In this study, betanin was the major betacyanin found in the flesh and peels of red-fleshed dragon fruit (Figure 2), which agrees with the data obtained by Naderi et al. (2012). According to Table 1, a significantly higher (p<0.05) concentration of betanin in the flesh (~440.4 mg/g) was recorded in the fruit treated with 1,000 ppm MeJA. However, unlike the peel, no significant difference was observed between all treatments, including the control sample. The Pearson correlation in Table 3 indicated a strong correlation (p<0.05) between the total betacyanins and betanin in the fruit flesh (r = 0.923) and peels (r = 0.874). Also, GLM analysis showed that the types of PGR, concentrations used, and interactions between the types of PGR and their concentrations significantly affected (p<0.05) the betanin in the flesh and peels (Table 2).

On the other hand, Naderi et al. (2012) also quantified the other types of betacyanins such as isobetanin, phyllocactin, isophyllocactin, and hylocerenin. However, only betanin was quantified in this study due to the limitation of the standard. In addition, other studies also reported the findings of anthocyanins in red-fleshed dragon fruit, including cyanidin 3-*O*-glucoside, delphinidin 3-*O*-glucoside, and pelargonidin 3-*O*-glucoside in the average concentration of 12.67, 0.82, and 1.76 mg/100 g DW, respectively (Saenjum et al., 2021). Notably, none of these anthocyanins were detected in this study samples during the preliminary test. It might be due to the extraction process using an acidified hydroalcoholic solution at pH 2 by Saenjum et al. (2021), compared to 50% ethanol in this study.

Total Phenolics (TP) and Total Flavonoids (TF)

Red dragon fruit is known for its phenolic and flavonoid compounds with antioxidant properties. Chen et al. (2021) found that 80 different phenolic compounds were tentatively characterized in dragon fruit, including 25 phenolic acids and 38 flavonoids. The total phenolics and flavonoids in red dragon fruit can vary

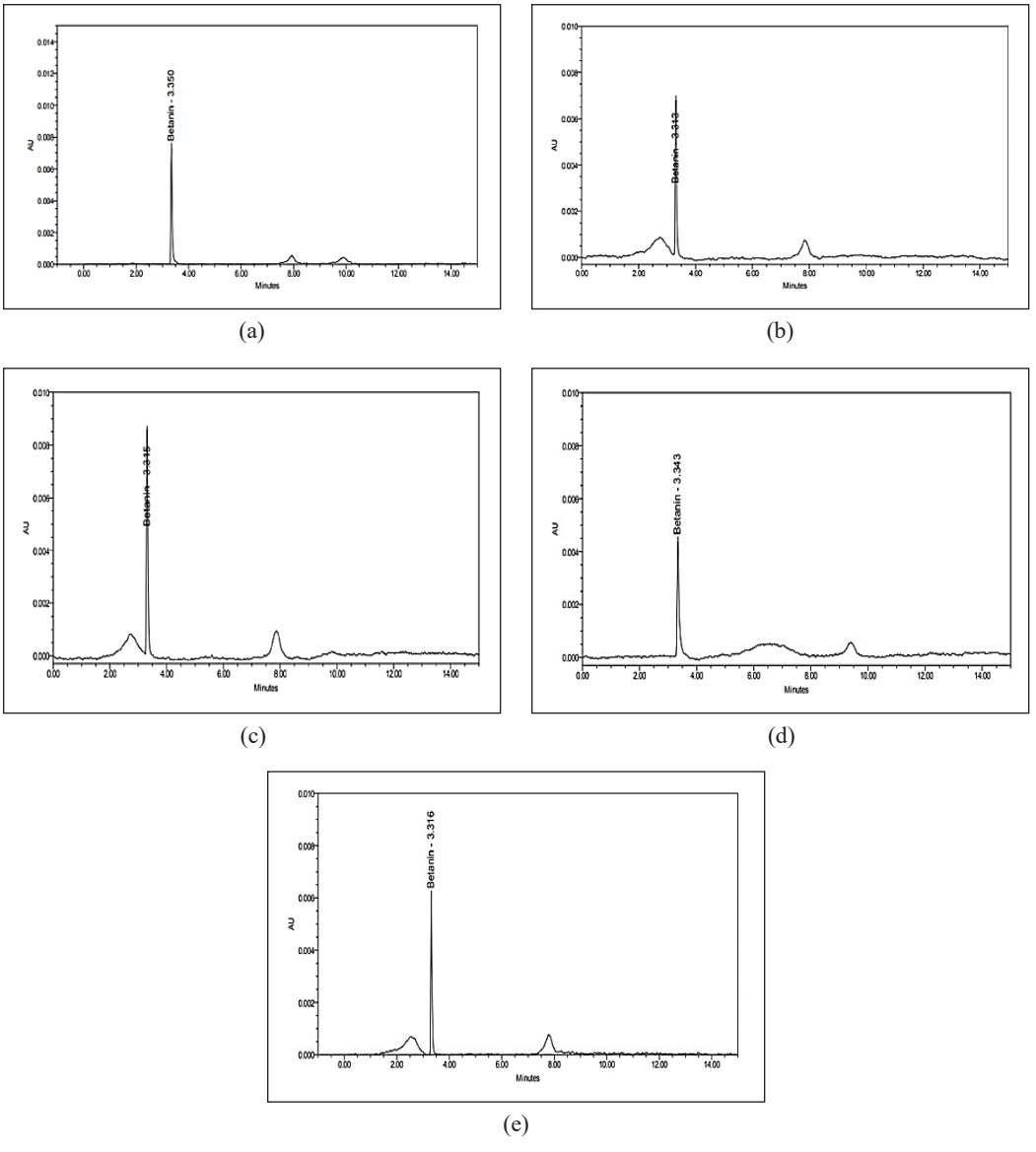

Figure 2. Typical high-performance liquid chromatography (HPLC) chromatogram of (a) standard betanin and betanin detected in red-fleshed dragon fruit flesh of (b) 1,000 ppm JA, (c) 1,000 ppm MeJA and fruit peels of (d) 1,000 ppm JA, and (e) 1,000 ppm MeJA at 540 nm
Note. JA = Jasmonic acid; MeJA = Methyl jasmonate

depending on geographical origin and extraction methods (Paśko, Galanty, Zagrodzki, Luksirikul, et al., 2021).

This study reported the effect of PGR preharvest treatment on the TP and TF of red-fleshed dragon fruit flesh and peels (Table 4). The fruit treated with 1,000 ppm MeJA possessed a significantly higher (p<0.05) TP (~244.9 mg GAE/100 g) and TF values (~329.0 mg GAE/100 g)

Table 4
Total phenolics (TP), total flavonoids (TF), Trolox equivalents antioxidant capacity (TEAC), and reducing power in the flesh and peels of untreated (control) and treated red-fleshed dragon fruit with plant growth regulators (PGR) at different concentrations

Treatments (ppm)	TP		TF		TEAC		Reducing power	
	Flesh	Peels	Flesh	Peels	Flesh	Peels	Flesh	Peels
	mg GAE/100 g		mg CE/100 g		µmol TE/g		µmol TE/g	
Control	208.3 ± 2.0^{Bb}	614.1 ± 5.7^{Aa}	225.5 ± 6.1^{Bbc}	774.7 ± 14.3^{Ab}	33.2 ± 0.2^{Bc}	90.3 ± 13.3^{Aa}	15.7 ± 0.2^{Bd}	44.4 ± 4.5^{Aa}
100 MeJA	202.1 ± 5.1^{Bb}	566.1 ± 4.3^{Aa}	201.8 ± 9.2^{Bc}	846.6 ± 37.5^{Aa}	60.4 ± 5.3^{Ba}	75.4 ± 4.9^{Aa}	16.9 ± 0.2^{Bc}	40.8 ± 11.9^{Aa}
100 JA	213.8 ± 20.3^{Bb}	458.1 ± 21.9^{Ab}	234.2 ± 0.1^{Bb}	640.3 ± 24.8^{Ac}	44.2 ± 1.0^{Bb}	66.4 ± 1.9^{Aa}	18.0 ± 0.5^{Bb}	36.4 ± 3.3^{Aa}
1000 MeJA	244.9 ± 3.3^{Ba}	300.1 ± 26.8^{Ac}	329.0 ± 6.1^{Ba}	787.2 ± 21.7^{Aab}	63.2 ± 1.4^{Ba}	70.7 ± 0.1^{Aa}	21.5 ± 0.1^{Ba}	36.9 ± 0.4^{Aa}
1000 JA	220.4 ± 6.5^{Bab}	486.5 ± 26.7^{Ab}	255.7 ± 12.2^{Bb}	724.7 ± 28.1^{Ab}	43.8 ± 0.12^{Bb}	66.0 ± 3.3^{Ab}	18.3 ± 0.0^{Bb}	36.1 ± 9.0^{Aa}

Note. JA = Jasmonic acid; MeJA = Methyl jasmonate. Values with the same letter $^{a, b,}$ and ^{c} in each row are not significantly different (p>0.05). Values with the same letter ^{A} and ^{B} in each column are not significantly different (p>0.05)

compared to other parameters, including control samples. According to the statistical analysis, the concentrations of PGR used and the interactions between the types of PGR and their concentrations significantly influenced (p<0.05) the TP values in the fruit flesh (Table 2). Also, in the flesh, there were strong correlations (p<0.05) between TP and total betacyanins (r = 0.899) and betanin (r = 0.837). Moreover, strong correlations (p<0.05) were detected between TF and total betacyanins (r = 0.935) and betanin (r = 0.829) (Table 3).

In the case of fruit peels, control and 100 ppm MeJA demonstrated a significantly higher TP (p<0.05), ~614.1 mg GAE/100 g, and ~566.1 mg GAE/100 g, respectively, compared to other samples. On the other hand, higher TF values were recorded in 100 ppm MeJA (~846.6 mg GAE/100 g) and 1,000 ppm MeJA (~787.2 mg GAE/100 g). Overall, TF in the flesh and TP in the peels were significantly influenced (p<0.05) by the types of PGR, their concentrations and the interactions between the PGR types and their concentrations. In the peels, TF was strongly correlated (p<0.05) to total betacyanins (r = 0.910) and betanin (r = 0.748).

The effect of PGR treatment on the increment of the TP is also reported in other cultivar fruits. In the study by Ghasemzadeh et al. (2016), a significant increase of 64% in TP and 234% in TF value was reported in sweet potato root after being subjected to the treatment using 100 µM MeJA. Hu et al. (2022) also claimed that applying JAs can activate a signal molecule that could trigger the plant defense systems and increase phenolic synthesis.

Antioxidant Activities (TEAC and Reducing Power)

Two or more assays are commonly used to determine the antioxidant capacity of the plant extract. Various assays are used to determine antioxidant activity because they could exert their effect in various mechanisms, such as scavenging radicals, sequestering transition metal ions and decomposing hydrogen peroxide (Chaves et al., 2020). Hence, the chosen best-suited method should consider the function to be evaluated. In this study, the TEAC and FRAP assays were used to evaluate the antioxidant capacities of the red-fleshed dragon fruit. In general, the TEAC assay was used to measure the antioxidant capacity, while the FRAP assay was used to determine the reducing power of samples (Chen et al., 2021).

As shown in Table 4, the TEAC values were highest in the fruit flesh after 100 and 1,000 ppm MeJA treatments, resulting in ~60.4 and ~63.2 µmol TE/g, respectively. The highest reducing power in the flesh was detected in the fruit treated with 1,000 ppm MeJA (~21.5 µmol TE/g). According to the GLM analysis, the types of PGR, concentrations used, and their interactions significantly influenced (p<0.05) the TEAC and reduced power in the fruit flesh.

The stronger antioxidant activity in red-fleshed dragon fruit is likely due to the

Table 5
Color characteristics in the flesh and peel of untreated (control) and treated red-fleshed dragon fruit with plant growth regulators (PGR) at different concentrations, as measured by colorimeter

Flesh						
Treatments (ppm)	*L**	*a**	*b**	C	h°	Visualization
Control	29.85 ± 0.69^{a}	36.92 ± 1.61^{a}	-2.10 ± 0.28^{c}	36.98 ± 1.61^{a}	-0.06 ± 0.01^{a}	
100 MeJA	30.09 ± 0.76^{a}	36.30 ± 1.19^{a}	-1.71 ± 0.54^{bc}	36.34 ± 1.19^{a}	-0.05 ± 0.01^{a}	
100 JA	30.22 ± 0.60^{a}	37.14 ± 0.83^{a}	-1.84 ± 0.56^{ab}	37.18 ± 0.82^{a}	-0.05 ± 0.02^{a}	
1,000 MeJA	29.34 ± 0.83^{a}	35.30 ± 1.83^{ab}	-0.43 ± 0.51^{a}	35.30 ± 1.82^{a}	-0.01 ± 0.01^{a}	
1,000 JA	29.64 ± 0.93^{a}	35.34 ± 1.93^{ab}	-0.63 ± 0.65^{a}	35.35 ± 1.94^{a}	-0.02 ± 0.02^{a}	
Peels						
Treatments (ppm)	*L**	*a**	*b**	C	h°	Visualization
Control	40.72 ± 3.07^{ab}	41.12 ± 3.67^{a}	8.41 ± 1.40^{a}	42.02 ± 3.35^{a}	0.21 ± 0.05^{a}	
100 MeJA	39.67 ± 1.51^{ab}	40.38 ± 2.23^{ab}	7.54 ± 0.73^{a}	41.09 ± 2.13^{ab}	0.19 ± 0.02^{a}	
100 JA	42.11 ± 2.64^{ab}	40.09 ± 2.39^{ab}	8.17 ± 1.47^{a}	40.95 ± 2.23^{ab}	0.20 ± 0.04^{a}	
1,000 MeJA	39.25 ± 2.59^{b}	37.76 ± 3.51^{b}	8.35 ± 0.71^{a}	38.68 ± 3.42^{b}	0.22 ± 0.03^{a}	
1,000 JA	42.22 ± 2.14^{a}	42.94 ± 1.50^{a}	7.73 ± 0.70^{a}	43.64 ± 1.49^{a}	0.18 ± 0.02^{a}	

Note. JA = Jasmonic acid; MeJA = Methyl jasmonate. Visualization = Conversion of *L**, *a**, and *b** values to RGB (red, green, blue) color model (http://www.easyrgb.com/en/). C = Chroma; h° = Hue angle. Values with the same letter ^{a} and ^{b} in each row are not significantly different (p>0.05)

abundance of pigments such as betalains and phenolic compounds (Zitha et al., 2022), which is supported by positive correlations ($p<0.05$) between reducing power and TP ($r = 0.917$), TF ($r = 0.909$), total betacyanins ($r = 0.953$), and betanin ($r = 0.965$) in the flesh. Also, TEAC was positively correlated to betanin ($r = 0.733$). Due to the natural red pigment of the fruit that possibly interferes with the antioxidant assay, the assay used is highly dependent on the reduction or formation of color in the antioxidant reaction (Lu et al., 2021). In addition, without pre-treatment with PGRs, no modification of genes can occur to promote the formation of the betalains and phenolic compounds in the control samples. Therefore, this leads to low antioxidant activity detected in control samples due to the lower amount of bioactive compounds present.

Even though significantly higher ($p<0.05$) antioxidant activities were detected in peels than in fruit flesh, no significant differences were detected in TEAC and reducing power in fruit peels between all treatments, including control samples. This finding is in agreement with the previous finding by Le (2022), who reported that the peels contained a higher amount of radical scavenging compounds than flesh. Attar et al. (2022) revealed that the antioxidant activity presumed for the whole fruit does not always correlate to the concentration of its most abundant betalains. However, it is the consequence of the interaction of all the antioxidant compounds it contains.

Color Characteristics

Color can be considered an important factor for consumer acceptability. The changes in the L*, a*, b*, chroma, and hue angle values were observed to evaluate the effect of different concentrations of PGR on red coloration in red-fleshed dragon fruit. Table 5 shows no significant difference in the *L**, *a**, *b**, chroma, and hue angle values between the control and treated samples with PGR in the fruit flesh and peels. Previously, Öztürk et al. (2013) reported that the MeJA at a concentration of 4,480 mg/L that was applied to 'Fuji' apples promoted the development of red hue, which is opposite to the results obtained in this study.

However, *L**, *a**, *b**, chroma, and hue angle values in peels were relatively higher compared to the flesh. Higher *L** values indicated lower darkness, while higher *a** values showed increased redness. The combination of these values led to higher Chroma and hue angle values in the peels. This result might be due to the abundance of tiny seeds in the flesh fruit that interfered with the color measurement. According to Wu et al. (2019), the seeds may partially contribute to the darkening of fruit flesh, which can be proved by lower *L** values (~29.34 – ~30.22) that indicate higher darkness in the flesh.

In addition, the bract on the fruit peels also contributed to the interference of the peel color measurement, resulting in no significant difference observed in the color changes. As stated by Nguyen et al. (2021), the bract of dragon fruit could

maintain its appearance as 0.1 mM MeJA during postharvest treatment, delaying the degradation of the bract color. This result could result in high L^* values measured in the peel color. Therefore, no correlation was detected between L^*, a^*, b^*, chroma, and hue angle values with betacyanins or other phenolic contents.

Principal Component Analysis (PCA)

The score plot generated from PCA showed a relationship between PGR treatment at different concentrations and the results obtained from the analysis of the dragon fruit flesh and peels, respectively (Figure 3). In the flesh (Figures 3a–3c), the distribution of data analysis is defined by the 75.2%

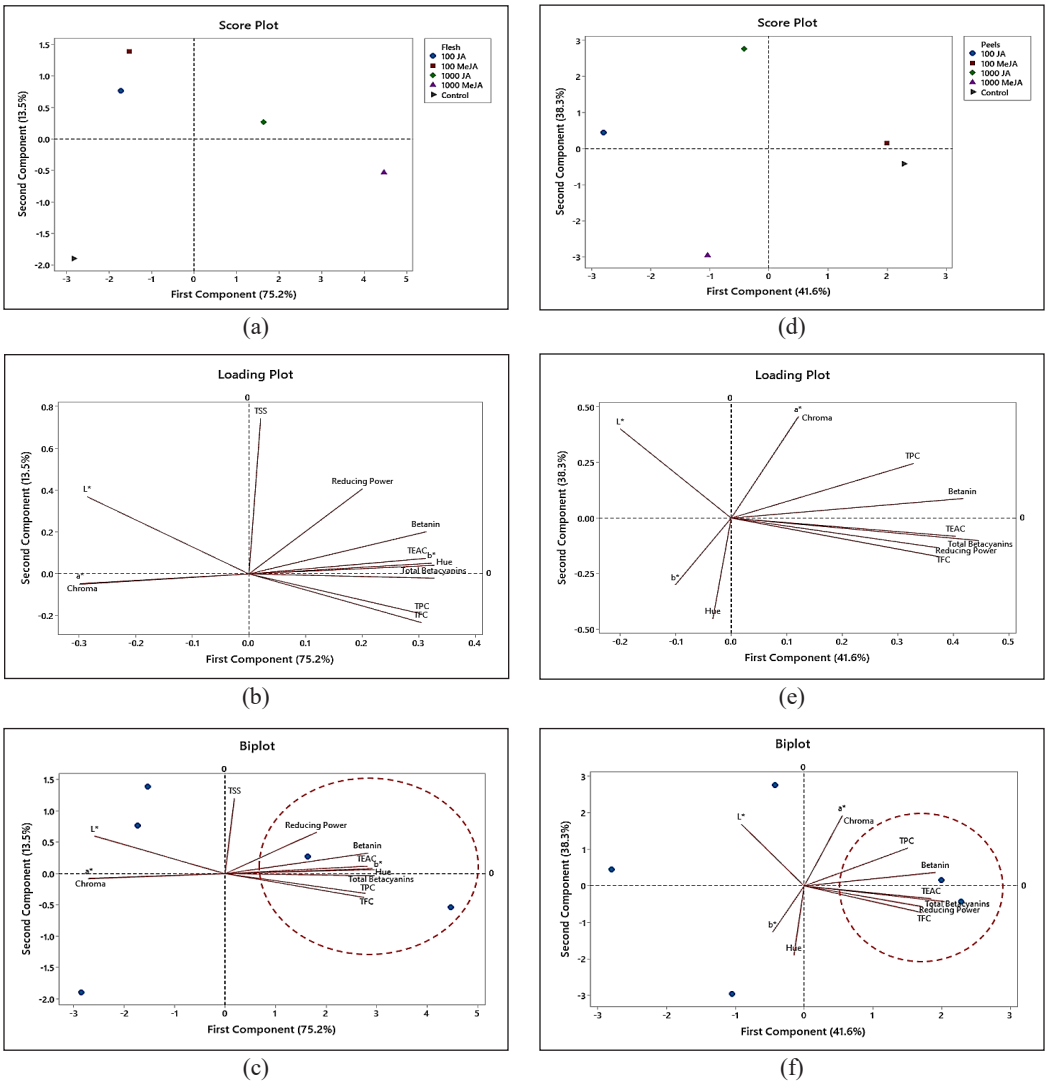

Figure 3. Score plot, loading plot, and bi-plot of principal component analysis based on the effect of two different concentrations of MeJA and JA on the flesh (a–c) and peels (d–f) of red-fleshed dragon fruit
Note. JA = Jasmonic acid; MeJA = Methyl jasmonate

PC1 and 13.5% PC2, where 1,000 ppm PGR, particularly MeJA, was positively correlated to betanin and total betacyanins compound, antioxidant activity, TF, and TP. PGR treatment using 1,000 MeJA efficiently increased the dragon fruit flesh's bioactive compounds and antioxidant activity.

On the other hand, Figures 3d–3f present the loading plot graph, PC1 and PC2 accounted for 41.6 and 38.3%, respectively. Overall, the biplot graph revealed that 100 MeJA and control samples were positively correlated with the betanin and total betacyanins compounds, antioxidant activity, TP, and TF. This finding contradicted the one obtained from the flesh, whereas peels with a lower concentration of MeJA (100 ppm) and untreated dragon fruit showed higher bioactive and antioxidant activity than 1,000 ppm.

CONCLUSION

Bioactive compounds in red-fleshed dragon fruit may vary depending on several factors, such as PGR types, concentrations used, and the interaction between types of PGR and their concentrations. In this study, the application of PGR influenced the accumulation of physicochemical and bioactive substances and antioxidant activities in red-fleshed dragon fruit, particularly the flesh part. PGR treatment using MeJA was more effective than JA. Treatment using 1,000 ppm MeJA effectively increased the total betacyanins, betanin, TSS, TP, TF, and antioxidant activity in the fruit flesh between the concentrations used. However, PGR treatments had little effect on fruit peel. The present correlation in the peels only exhibited between betacyanins, betanin, and TF. In summary, a comprehensive understanding of how JA and MeJA influence red-fleshed dragon fruit can provide valuable insights into plant biology, cultivation practices, and crop management strategies, leading to improved fruit quality, yield, and sustainability in agriculture.

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REFERENCES

Abirami, K., Swain, S., Baskaran, V., Venkatesan, K., Sakthivel, K., & Bommayasamy, N. (2021). Distinguishing three dragon fruit (*Hylocereus* spp.) species grown in Andaman and Nicobar Islands of India using morphological, biochemical and molecular traits. *Scientific Reports*, *11*, 2894. https://doi.org/10.1038/s41598-021-81682-x

Attar, Ş. H., Gündeşli, M. A., Urün, I., Kafkas, S., Kafkas, N. E., Ercisli, S., Ge, C., MIcek, J., & Adamkova, A. (2022). Nutritional analysis of red-purple and white-fleshed pitaya (*Hylocereus*) species. *Molecules*, *27*(3), 808. https://doi.org/10.3390/molecules27030808

Azman, E. M., Nor, N. D. M., Charalampopoulos, D., & Chatzifragkou, A. (2022). Effect of acidified water on phenolic profile and antioxidant activity of dried blackcurrant (*Ribes nigrum* L.) pomace extracts. *LWT*, *154*, 112733. https://doi.org/10.1016/j.lwt.2021.112733

Baek, M. W., Choi, H. R., Jae, L. Y., Kang, H.-M., Lee, O.-H., Jeong, C. S., & Tilahun, S. (2021).

Preharvest treatment of methyl jasmonate and salicylic acid increase the yield, antioxidant activity and GABA content of tomato. *Agronomy*, *11*(11), 2293. https://doi.org/10.3390/agronomy11112293

Batista-Silva, W., Nascimento, V. L., Medeiros, D. B., Nunes-Nesi, A., Ribeiro, D. M., Zsögön, A., & Araújo, W. L. (2018). Modifications in organic acid profiles during fruit development and ripening: Correlation or causation? *Frontiers in Plant Science*, *9*, 1689. https://doi.org/10.3389/fpls.2018.01689

Calva-Estrada, S. J., Jiménez-Fernández, M., & Lugo-Cervantes, E. (2022). Betalains and their applications in food: The current state of processing, stability and future opportunities in the industry. *Food Chemistry: Molecular Sciences*, *4*, 100089. https://doi.org/10.1016/j.fochms.2022.100089

Chaves, N., Santiago, A., & Alías, J. C. (2020). Quantification of the antioxidant activity of plant extracts: Analysis of sensitivity and hierarchization based on the method used. *Antioxidants*, *9*(1), 76. https://doi.org/10.3390/antiox9010076

Chen, Z., Zhong, B., Barrow, C. J., Dunshea, F. R., & Suleria, H. A. R. (2021). Identification of phenolic compounds in Australian grown dragon fruits by LC-ESI-QTOF-MS/MS and determination of their antioxidant potential. *Arabian Journal of Chemistry*, *14*(6), 103151. https://doi.org/10.1016/j.arabjc.2021.103151

Cheok, A., Xu, Y., Zhang, Z., Caton, P. W., & Rodriguez-Mateos, A. (2022). Betalain-rich dragon fruit (pitaya) consumption improves vascular function in men and women: A double-blind, randomized controlled crossover trial. *The American Journal of Clinical Nutrition*, *115*(5), 1418-1431. https://doi.org/10.1093/ajcn/nqab410

Ghasemzadeh, A., Talei, D., Jaafar, H. Z. E., Juraimi, A. S., Mohamed, M. T. M., Puteh, A., & Halim, M. R. A. (2016). Plant-growth regulators alter phytochemical constituents and pharmaceutical quality in sweet potato (*Ipomoea batatas* L.). *BMC Complementary and Alternative Medicine*, *16*, 152. https://doi.org/10.1186/s12906-016-1113-1

Gupta, V., Meena, N. K., Sharma, Y. K., & Choudhary, K. (2023). Comparative study of different polysaccharide-based edible coatings on physicochemical attributes and bioactive compounds of mango cv. Dashehari fruits. *eFood*, *4*(1), e55. https://doi.org/10.1002/efd2.55

Halimfanezi, L., & Asra, R. R. (2020). A review: Analysis of betacyanin levels in various natural products. *Asian Journal of Pharmaceutical Research and Development*, *8*(5), 88-95. https://doi.org/10.22270/ajprd.v8i5.846

Hossain, F. M., Numan, S. M. N., & Akhtar, S. (2021). Cultivation, nutritional value, and health benefits of dragon fruit (*Hylocereus* spp.): A review. *International Journal of Horticultural Science and Technology*, *8*(3), 259-269. https://doi.org/10.22059/ijhst.2021.311550.400

Hu, W., Sarengaowa., Guan, Y., & Feng, K. (2022). Biosynthesis of phenolic compounds and antioxidant activity in fresh-cut fruits and vegetables. *Frontiers in Microbiology*, *13*, 906069. https://doi.org/10.3389/fmicb.2022.906069

Huang, Y., Brennan, M. A., Kasapis, S., Richardson, S. J., & Brennan, C. S. (2021). Maturation process, nutritional profile, bioactivities and utilisation in food products of red pitaya fruits: A review. *Foods*, *10*(11), 2862. https://doi.org/10.3390/foods10112862

Jalgaonkar, K., Mahawar, M. K., Bibwe, B., & Kannaujia, P. (2022). Postharvest profile, processing and waste utilization of dragon fruit (*Hylocereus* spp.): A review. *Food Reviews International*, *38*(4), 733-759. https://doi.org/10.1080/87559129.2020.1742152

Khan, N., Bano, A. M. D., & Babar, A. (2020). Impacts of plant growth promoters and plant growth regulators on rainfed agriculture. *PLOS One*, *15*(4), e0231426. https://doi.org/10.1371/journal.pone.0231426

Khoo, H. E., He, X., Tang, Y., Li, Z., Li, C., Zeng, Y., Tang, J., & Sun, J. (2022). Betacyanins and anthocyanins in pulp and peel of red pitaya (*Hylocereus polyrhizus* cv. Jindu), inhibition of oxidative stress, lipid reducing, and cytotoxic effects. *Frontiers in Nutrition*, *9*, 894438. https://doi.org/10.3389%2Ffnut.2022.894438

Le, N. L. (2022). Functional compounds in dragon fruit peels and their potential health benefits: A review. *International Journal of Food Science and Technology*, *57*(5), 2571-2580. https://doi.org/10.1111/ijfs.15111

Li, X., Li, M., Wang, J., Wang, L., Han, C., Jin, P., & Zheng, Y. (2018). Methyl jasmonate enhances wound-induced phenolic accumulation in pitaya fruit by regulating sugar content and energy status. *Postharvest Biology and Technology*, *137*, 106-112. https://doi.org/10.1016/j.postharvbio.2017.11.016

Lu, W., Shi, Y., Wang, R., Su, D., Tang, M., Liu, Y., & Li, Z. (2021). Antioxidant activity and healthy benefits of natural pigments in fruits: A review. *International Journal of Molecular Sciences*, *22*(9), 4945. https://doi.org/10.3390/ijms22094945

Meng, X., Han, J., Wang, Q., & Tian, S. (2009). Changes in physiology and quality of peach fruits treated by methyl jasmonate under low temperature stress. *Food Chemistry*, *114*(3), 1028-1035. https://doi.org/10.1016/j.foodchem.2008.09.109

Miguel, M. G. (2018). Betalains in some species of the Amaranthaceae family: A review. *Antioxidants*, *7*(4), 53. https://doi.org/10.3390/antiox7040053

Mukherjee, A., Gaurav, A. K., Singh, S., Yadav, S., Bhowmick, S., Abeysinghe, S., & Verma, J. P. (2022). The bioactive potential of phytohormones: A review. *Biotechnology Reports*, *35*, e00748. https://doi.org/10.1016/j.btre.2022.e00748

Mustafa, M. A., Ali, A., Seymour, G., & Tucker, G. (2018). Treatment of dragonfruit (*Hylocereus polyrhizus*) with salicylic acid and methyl jasmonate improves postharvest physico-chemical properties and antioxidant activity during cold storage. *Scientia Horticulturae*, *231*, 89-96. https://doi.org/10.1016/j.scienta.2017.09.041

Naderi, N., Ghazali, H. M., Hussin, A. S. M., Amid, M., & Manap, M. Y. A. (2012). Characterization and quantification of dragon fruit (*Hylocereus polyrhizus*) betacyanin pigments extracted by two procedures. *Pertanika Journal of Tropical Agricultural Science*, *35*(1), 33-40.

Nawawi, N. I. M., Ijod, G., Abas, F., Ramli, N. S., Adzahan, N. M., & Azman, E. M. (2023). Influence of different drying methods on anthocyanins composition and antioxidant activities of mangosteen (*Garcinia mangostana* L.) pericarps and LC-MS analysis of the active extract. *Foods 12*(12), 2351. https://doi.org/10.3390/foods12122351

Nguyen, H. T., Boonyaritthongchai, P., Buanong, M., Supapvanich, S., & Wongs-Aree, C. (2021). Chitosan-and κ-carrageenan-based composite coating on dragon fruit (*Hylocereus undatus*) pretreated with plant growth regulators maintains bract chlorophyll and fruit edibility. *Scientia Horticulturae*, *281*, 109916. https://doi.org/10.1016/j.scienta.2021.109916

Nishikito, D. F., Borges, A. C. A., Laurindo, L. F., Otoboni, A. M. M. B., Direito, R., de Alvares Goulart, R., Nicolau, C. C., Fiorini, A. M. R., Sinatora, R. V., & Barbalho, S. M. (2023). Anti-inflammatory, antioxidant, and other health effects of dragon fruit and potential delivery systems for its bioactive compounds. *Pharmaceutics*, *15*(1), 159. https://doi.org/10.3390/pharmaceutics15010159

Ordoñez Trejo, E. J. O., Brizzolara, S., Cardillo, V., Ruperti, B., Bonghi, C., & Tonutti, P. (2023). The impact of PGRs applied in the field on the postharvest behavior of fruit crops. *Scientia Horticulturae*, *318*, 112103. https://doi.org/10.1016/j.scienta.2023.112103

Öztürk, B., Altuntas, E., Yildiz, K., Ozkan, Y., & Saracoglu, O. (2013). Effect of methyl jasmonate treatments on the bioactive compounds and physicochemical quality of' 'Fuji' apples. *Ciencia e Investigación Agraria*, *40*(1), 201-211. https://doi.org/10.4067/s0718-16202013000100018

Paśko, P., Galanty, A., Zagrodzki, P., Ku, Y. G., Luksirikul, P., Weisz, M., & Gorinstein, S. (2021). Bioactivity and cytotoxicity of different species of pitaya fruits – A comparative study with advanced chemometric analysis. *Food Bioscience*, *40*, 100888. https://doi.org/10.1016/j.fbio.2021.100888

Paśko, P., Galanty, A., Zagrodzki, P., Luksirikul, P., Barasch, D., Nemirovski, A., & Gorinstein, S. (2021). Dragon fruits as a reservoir of natural polyphenolics with chemopreventive properties. *Molecules*, *26*(8), 2158. https://doi.org/10.3390/molecules26082158

Ramli, N. S., Ismail, P., & Rahmat, A. (2014). Influence of conventional and ultrasonic-assisted extraction on phenolic contents, betacyanin contents, and antioxidant capacity of red dragon fruit (*Hylocereus polyrhizus*). *The Scientific World Journal*, *2014*, 964731. https://doi.org/10.1155/2014/964731

Ren, Y., Yang, J., Lu, B., Jiang, Y., Chen, H., Hong, Y., Wu, B., & Miao, Y. (2017). Structure of pigment metabolic pathways and their contributions to white tepal color formation of Chinese *Narcissus tazetta* var. *chinensis* cv Jinzhanyintai. *International Journal of Molecular Sciences*, *18*(9), 1923. https://doi.org/10.3390/ijms18091923

Ruan, J., Zhou, Y., Zhou, M., Yan, J., Khurshid, M., Weng, W., Cheng, J., & Zhang, K. (2019). Jasmonic acid signaling pathway in plants. *International Journal of Molecular Sciences*, *20*(10), 2479. https://doi.org/10.3390/ijms20102479

Sadowska-Bartosz, I., & Bartosz, G. (2021). Biological properties and applications of betalains. *Molecules*, *26*(9), 2520. https://doi.org/10.3390/molecules26092520

Saenjum, C., Pattananandecha, T., & Nakagawa, K. (2021). Antioxidative and anti-inflammatory phytochemicals and related stable paramagnetic species in different parts of dragon fruit. *Molecules*, *26*(12), 3565. https://doi.org/10.3390/molecules26123565

Senevirathna, S. S. J., Ramli, N. S., Azman, E. M., Juhari, N. H., & Karim, R. (2021). Optimization of the drum drying parameters and citric acid level to produce purple sweet potato (*Ipomoea batatas* L.) powder using response surface methodology. *Foods*, *10*(6), 1378. https://doi.org/10.3390/foods10061378

Taki-Nakano, N., Ohzeki, H., Kotera, J., & Ohta, H. (2014). Cytoprotective effects of 12-oxo phytodienoic acid, a plant-derived oxylipin jasmonate, on oxidative stress-induced toxicity in human neuroblastoma SH-SY5Y cells. *Biochimica et Biophysica Acta - General Subjects*, *1840*(12), 3413-3422. https://doi.org/10.1016/j.bbagen.2014.09.003

Wang, H., Wu, Y., Yu, R., Wu, C., Fan, G., & Li, T. (2019). Effects of postharvest application of methyl jasmonate on physicochemical characteristics and antioxidant system of the blueberry fruit. *Scientia Horticulturae*, *258*, 108785. https://doi.org/10.1016/j.scienta.2019.108785

Wang, S. Y., Bowman, L., & Ding, M. (2008). Methyl jasmonate enhances antioxidant activity and flavonoid content in blackberries (*Rubus* sp.) and

promotes antiproliferation of human cancer cells. *Food Chemistry, 107*(3), 1261-1269. https://doi.org/10.1016/j.foodchem.2007.09.065

Wasternack, C., & Strnad, M. (2018). Jasmonates: News on occurrence, biosynthesis, metabolism and action of an ancient group of signaling compounds. *International Journal of Molecular Sciences*, *19*(9), 2539. https://doi.org/10.3390/ijms19092539

Wei, J., Wen, X., & Tang, L. (2017). Effect of methyl jasmonic acid on peach fruit ripening progress. *Scientia Horticulturae*, *220*, 206-213. https://doi.org/10.1016/j.scienta.2017.03.004

Wrolstad, R. E., & Smith, D. E. (2017). Color analysis. In S. S. Nielsen (Ed.), *Food analysis* (pp. 545-555). Springer. https://doi.org/10.1007/978-3-319-45776-5_31

Wu, Y., Xu, J., He, Y., Shi, M., Han, X., Li, W., Zhang, X., & Wen, X. (2019). Metabolic profiling of pitaya (*Hylocereus polyrhizus*) during fruit development and maturation. *Molecules*, *24*(6), 1114. https://doi.org/10.3390/molecules24061114

Yadav, V., Wang, Z., Wei, C., Amo, A., Ahmed, B., Yang, X., & Zhang, X. (2020). Phenylpropanoid pathway engineering: An emerging approach towards plant defense. *Pathogens*, *9*(4), 312. https://doi.org/10.3390/pathogens9040312

Zitha, E. Z. M., Magalhães, D. S., do Lago, R. C., Carvalho, E. E. N., Pasqual, M., & Boas, E. V. D. B. V. (2022). Changes in the bioactive compounds and antioxidant activity in red-fleshed dragon fruit during its development. *Scientia Horticulturae*, *291*, 110611. https://doi.org/10.1016/j.scienta.2021.110611

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Physicochemical Properties, Proximate Composition, and Carotenoid Content of *Momordica cochinchinensis* L. Spreng (Gac) Fruit

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ABSTRACT

The study aims to determine the physical and chemical properties of *Momordica cochinchinensis* L. Spreng (gac) fruits. Fruit size varied, weighing 359.17 to 588.33g, with lengths of 11.10 to 13.92 cm and circumferences of 27.43 to 30.67 cm. Components included pulp (34.06 to 41.58%), seeds (23.11 to 29.70%), peel (16.65 to 20.60%), and aril (15.64 to 18.64%). Skin and aril colour parameters (L*, a*, b*) indicated maturity and carotenoid content. Aril had higher acidity (pH 5.54±0.02, titratable acidity [TA] 0.03 to 0.05g/L), total soluble solids (TSS, 11.57%±0.52 °Brix), and carbohydrates (55.6 g/100 g) than pulp (pH 5.65±0.02, TA 0.01 to 0.02g/L, TSS 4.90%±0.33 °Brix, carbohydrates 30.9 g/100 g). Peel contained most protein (6.2 g/100 g) and dietary fibre (56.9 to 58.1 g/100 g). Glucose and fructose were found in both pulp and aril. Potassium levels were highest in peel (817.59 mg/100 g), followed by pulp (658.20 mg/100 g) and aril (228.79 mg/100 g). Lycopene dominated carotenoids, especially in aril (31.7 to 103.7 mg/g). β-carotene, lutein, astaxanthin,

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and zeaxanthin were also present. β-carotene (2.9 to 9.6 mg/g) was second to lycopene, followed by astaxanthin (1.54 to 4.91 mg/g), lutein (0.16 to 1.35 mg/g), and zeaxanthin (0.35 to 1.49 mg/g), absent in pulp. These findings have implications for the food industry, offering insights into gac fruit's nutritional potential. Malaysian gac exhibited superior nutritional content, with pulp and aril as notable sources of carbohydrates and minerals for consumption and aril as a promising source of healthy oils.

Keywords: Carotenoids, minerals, *Momordica cochinchinensis* L. Spreng, physicochemical, proximate, sugar profiles

INTRODUCTION

Momordica cochinchinensis L. Spreng, commonly referred to as 'gac', belongs to the *Momordica* genus, which encompasses other species such as *Momordica charantia*, *Momordica balsamina*, *Momordica dioica*, and *Momordica tuberosa*. Gac falls under the melon family (Cucurbitaceae), sharing its botanical lineage with cucumbers, squash, luffa, and bitter melon (Chomicki et al., 2020; Dujardin & Kitthawee, 2013; Parks et al., 2013). This tropical plant is native to Southeast Asia and enjoys extensive cultivation in countries like Thailand, Vietnam, Laos, China, Japan, India, Cambodia, the Philippines, Malaysia, and Bangladesh (Kubola et al., 2013; Mai et al., 2013; Mukherjee et al., 2022). Gac goes by various names in different regions, including spiny gourd, spiny bitter gourd, cochinchin gourd, sweet gourd (in English), Fak kao (in Thailand), gac (in Vietnam), Mak kao (in Laos), Moc Niet Tu (in China), Bhat kerala (in India), Kushika and Mokubetsushi (in Japan), Hakur, Kakrol, and Kakor (in Hindustan), and Teruah (in Malaysia) (Huynh & Nguyen, 2020; Kubola et al., 2013).

Gac has gained popularity in Vietnam due to its health benefits and its traditional role in cooking. It is frequently used in Vietnam as a natural colouring agent for making glutinous rice, known as *xoi Gac*, a dish commonly served during festive occasions (Le et al., 2018; Phan-thi & Waché, 2014). In Thailand, gac is often enjoyed as a vegetable, particularly in its immature stage. The fruit pulp has a flavour resembling papaya and is either cooked or boiled, typically served with chilli paste or included in various curry dishes (Kubola et al., 2013).

Gac fruits exhibit significant variation in size and weight, and this variability can impact the concentrations of bioactive compounds, especially carotenoids. Factors such as the specific plant variety, local climate, harvest season, level of maturity, geographical location, and the use of fertilisers all contribute to the diversity in fruit size (Bhumsaidon & Chamchong, 2016). Various attributes related to fruit quality, including size, weight, skin colour, firmness, thickness of the aril (the fleshy part surrounding the seeds), and colour, play a pivotal role in determining consumer acceptance and pricing. Traditionally,

Vietnamese consumers prefer gac fruits weighing approximately 1.2–2 kg and possessing good firmness, red skin, and a thick, dark-red aril (Tran et al., 2016).

The gac fruit consists of two primary parts: the mesocarp and the endocarp. The mesocarp, which makes up nearly 50% of the fruit's weight, is thick, spongy, and has an orange colour. On the other hand, the endocarp is composed of red, soft, and sticky arils that are approximately 1–3 mm thick and envelop black seeds. These arils contain a high concentration of valuable carotenoids, notably β-carotene and lycopene (Kha et al., 2013). Previous studies have documented β-carotene concentrations ranging from 0.083–0.769 mg/g and lycopene concentrations ranging from 0.380-0.408 mg/g in Vietnamese gac varieties (Kubola et al., 2013). Compared to tomatoes, gac arils can contain approximately 3 mg/g of lycopene in terms of fresh weight, while tomatoes have around 40–50 µg/g in fresh weight (Mai et al., 2013). Gac fruits are also abundant in other carotenoids, earning them the status of a "super fruit" due to their substantial bioactive and nutritional potential, including essential fatty acids, vitamin E, and lutein.

Moreover, gac fruits boast high concentrations of nutrients like vitamin E and unsaturated and polyunsaturated fatty acids, which are known for their beneficial impacts on human health. Approximately 70% of the total fatty acids found in gac arils are unsaturated, with 50% being polyunsaturated. These arils contain significant amounts of oleic acid, palmitic acid, linoleic acid, vitamin E, and omega-3 fatty acids. The presence of fat in the gac aril aided the absorption of carotenoids, vitamin E, and other fat-soluble nutrients (Kha et al., 2013).

Despite gac fruit being widespread in Eastern Asia, its limited exposure in terms of food preparation and knowledge of its nutritional advantages has hindered its presence in the market. As a result, gac is viewed as an indigenous fruit in Malaysia. Furthermore, there is a noticeable absence of studies exploring the physicochemical traits, proximate composition, and nutritional advantages of gac fruits. Therefore, this study aims to investigate the physicochemical characteristics, proximate composition, and carotenoid content of the peel, pulp, and aril of *Momordica cochinchinensis* L. Spreng (gac) fruits cultivated in Malaysia.

MATERIALS AND METHODS

Sample Preparation

Seven mature gac fruits, distinguished by their uniform yellow-red skin as documented in the research conducted by Kha et al. (2013), were procured from the International Tropical Fruits Network (TFNet) located in Serdang, Selangor, Malaysia. Upon its arrival at the laboratory, the fruit underwent a thorough cleaning under tap water. Processing of the fruit was carried out on the same day. The peel, pulp (endocarp), red sticky arils (mesocarp), and seeds were manually separated from the fruit's cavity and then stored at -80°C. These components were then subjected to freeze-drying (lyophilisation) at a temperature

of -45°C over 3 days, employing a Christ Beta 2-8 LD plus freeze dryer from GmbH (Germany). The various fruit parts were reduced and transformed into powdered using a Waring® stainless steel commercial blender to prepare the samples for analysis (Waring® Commercial, USA). The resulting powdered samples were securely sealed in opaque containers and maintained at -18°C until they were ready for further analysis.

Chemical and Reagents

The chemicals and reagents employed for the analysis of the proximate composition, as well as the carotenoid standards (β-carotene, lycopene, lutein, astaxanthin, and zeaxanthin), were procured from Sigma Chemical Co. (USA) and Fisher Scientific (USA), ensuring the highest available purity. For the high-performance liquid chromatography (HPLC) analysis, methanol, dichloromethane, and acetonitrile, which served as mobile phases, were obtained from Merck (Germany). Other solvents such as *n*-hexane, acetone, and ethanol were also sourced from Fisher Scientific (USA).

Determination of Physicochemical Properties of Gac Fruit

Determination of Weight, Length, and Circumference. The physical measurements of gac fruit, including weight, length, and circumference, were obtained using an analytical weigh balance, a Mitutoyo stainless steel calliper (0–6", made in Japan), and a flexible measuring tape. Fruit length was defined as the distance from the apex to the stem along the polar axis, while fruit circumference was measured as the maximum width perpendicular to the polar axis.

Determination of Fruit Colour. The colour of various parts of the gac fruit, including the skin, pulp, and aril, was quantified using a HunterLab Ultrascan® PRO USP1092 spectrophotometer (USA). These measurements were conducted under the D65/10 illuminant, and the results were expressed in terms of darkness to lightness (L*), greenness to redness (a*), and blueness to yellowness (b*). The L* value ranges from 0 (representing black) to 100 (indicating white). Multiple measurements were taken at three distinct points on the fruit's skin, pulp, and aril. The aril's colour was determined using a glass cuvette following a standardised protocol.

Determination of Soluble Solids Concentration. The gac fruit pulp and aril's total soluble solids (TSS) content was assessed using an Atago N1 Hand Refractometer (Japan). After separating the flesh and aril from the skin and seeds, 10 g of each tissue was homogenised with 40 ml of distilled water. Subsequently, the resultant mixture was filtered, and a small drop of the filtrate was carefully placed on the prism glass of the refractometer. The measurement of soluble solids concentration was conducted at a temperature of 27°C.

Determination of TA and pH. The remaining juice from the TSS determination was utilised to measure TA. It was accomplished

by titrating it with a 0.1 mol/L sodium hydroxide (NaOH) solution, employing 1% phenolphthalein as an indicator. The outcomes were expressed as a percentage of citric acid, calculated using the formula [(ml NaOH × 0.1 mol/L/Weight of the titrated sample) × 0.064 × 100%], a method specified by Tran et al. (2016). Furthermore, the pH of the juice was gauged using a Crison Micro pH 2000 glass electrode pH meter. This pH meter was calibrated using pH 4.0 and 7.0 buffer solutions as references.

Determination of Proximate Composition

The assessment of the proximate composition of gac peel, pulp, and aril adhered to the Association of Official Analytical Chemists (AOAC) (2000) standard method, a well-established and widely acknowledged protocol within scientific research and food analysis. This method is commonly employed to determine nutritional components in food samples.

Moisture Content. The moisture content in the gac sample was ascertained by subjecting roughly 2 g of the sample to a drying process within pre-dried crucibles at 100°C for 24 hr. Following this, after allowing the crucibles to cool in a desiccator, their weights were re-measured. The weight of the dried sample was divided by the initial sample weight and then multiplied by 100% to compute the percentage of dry matter. Subsequently, the moisture content was determined by subtracting the percentage of dry matter from 100%.

Ash Content. The ash content analysis of gac fruits followed the AOAC (2000) standard using the dried ashing method. Initially, 5 g of the sample was weighed and placed into crucibles. These crucibles underwent high-temperature ashing at 550°C for 24 hr. After cooling in a desiccator, the crucibles were reweighed to calculate the ash content as a percentage of the original sample weight.

Crude Protein Content. The protein content in gac fruit was determined via the Kjeldahl method. Initially, 1 g of the sample was weighed and placed into a digesting tube, along with Kjeltabs Cu 3.5 Catalyst tablets and concentrated sulfuric acid. Digestion continued until a green solution formed. After cooling, the solution was combined with a receiving solution containing boric acid and bromocresol indicator. NaOH was added, and distillation occurred using a Kjeltec™ Distillation Unit (Denmark). The resulting distillate underwent titration with 0.1 N hydrochloric acid (HCl) until a noticeable colour change was observed. The protein percentage was calculated using the formula:

$$\text{Protein (\%)} = [(T - B) \times N \times 14.007 \times 100\%] / \text{Weight of the sample (in mg)} \quad \text{[Equation 1]}$$

Where, T = Volume of the sample titration; B = Volume of the blank titration; N = Normality of hydrochloric acid

Crude Fat Content. The fat content in gac fruit was determined using the Soxhlet

method. Initially, 2 g of the sample were accurately weighed and then transferred to thimbles. These thimbles were subsequently placed within an extraction cup containing 70 ml of petroleum ether. After extraction, the cup containing the extracted fat was dried in an oven at 103°C for 2 hr. Following this, it was allowed to cool within a desiccator. The weight of the extraction cup was then recorded. The percentage of fat was calculated using the formula:

$$\text{Percentage of fat (\%)} = [(W3 - W2) \times 100\%] / W1 \quad \text{[Equation 2]}$$

Where, $W1$ = Weight of the initial sample; $W2$ = Weight of the extraction cup; $W3$ = Weight of the extraction cup along with the extracted fat

Total Available Carbohydrate Content. The total available carbohydrate content of the gac fruits was assessed using the Clegg-anthrone method, following the procedure outlined by Chin et al. (2023) as well as Ram and Shankar (2023). Initially, 2.5 g of the sample were mixed with 10 ml of water and agitated to disperse the sample evenly. Subsequently, 13 ml of 52% perchloric acid was added to the mixture, which was then stirred for 20 min before being diluted to a final volume of 100 ml. For the carbohydrate analysis, 10 ml of the extracted sample was diluted with 100 ml of water. Next, 1 ml of the diluted sample extract or a glucose standard solution was carefully transferred into a test tube, followed by adding 5 ml of anthrone reagent. The test tube was then placed in a boiling water bath for 12 min and cooled to room temperature. The absorbance of the resulting reaction mixture was measured at 630 nm against a blank. The formula (25 × b) / (a × w) was then employed to compute the total available carbohydrate content, expressed as a percentage of glucose by weight, where 'a' represents the absorbance of the glucose standard solution, 'b' is the absorbance of the sample extract, and 'w' signifies the weight of the sample in grams.

Total Dietary Fibre Content. The total dietary fibre in gac fruit was analysed using the AOAC (2000) method. The sample (1 g) was mixed with 50 ml of pH 6.0 phosphate buffer, and α-amylase was added. After 15 min of boiling in a water bath with periodic agitation, the mixture was cooled, the pH was adjusted, and protease was introduced. Subsequently, it was incubated in a 60°C water bath. Amyloglucosidase was then added, followed by another incubation at 60°C. After cooling, ethanol was used to precipitate the fibre. The precipitate was filtered, washed, and transferred to crucibles with celite. These crucibles were washed, dried, and weighed. The residues were analysed using the dried ashing and Kjeldahl methods. Finally, the crucibles, including celite and ash, were weighed to determine the total dietary fibre content.

Sugar Profiles

Sugars in gac peel, pulp, and aril were extracted following the method outlined by Zheng et al. (2016). In summary, 2 g of

powdered sample was homogenised with 5.0 ml of cold ethanol (80%). After incubation and centrifugation, the supernatants were combined and adjusted to a total volume of 25 ml, and a portion of the extract was dried for sugar analysis. Sugar analysis involved a chromatographic system with acetonitrile-water (80:20, v/v) as the mobile phase and an Agilent Hi-Plex H-cation exchange column (Agilent Technologies, USA). Detection was performed using a refractive index detector, and data analysis was conducted with a chromatography data system. Sugar concentrations were expressed as µg/g fresh weight (FW).

Mineral Analysis

Mineral concentrations in gac fruit parts (peel, pulp, and aril) were determined using atomic absorption spectrophotometry (AAS) with a PerkinElmer A Analyst 800 model (USA). Gac samples were ashed at 550°C for 5 hr, and the resulting ash was dissolved in concentrated hydrochloric acid (HCl). The diluted solution was then analysed using AAS under optimised conditions and calibrated using standard solutions.

Carotenoid Extraction Method

For carotenoid extraction from gac samples (peel, pulp, and aril), a modified method based on Auisakchaiyoung and Rojanakorn (2015) as well as Tran et al. (2016) was used. Approximately 2 g of gac powder were blended with 100 ml of a solvent mixture (n-hexane, acetone, and ethanol in a 50:25:25 ratio) and stirred for 30 min. Next, 15 ml of water was added, and the upper layer was filtered through a 0.45 µm filter paper. A 10 ml aliquot of the extract was evaporated using a rotary evaporator, repeated four times until the extract became colourless, and then used for total carotenoid measurement via high-performance *liquid chromatography* with diode-array detection (DAD-HPLC) analysis.

Determination of Carotenoid Content by HPLC. The extracted solution was diluted to 4 ml and filtered through a 0.45 µm membrane syringe filter into HPLC glass amber vials. Following Kubola et al. (2013), HPLC analysis used a 20 µl injection volume. Calibration curves for carotenoids (lutein, zeaxanthin, β-carotene, lycopene, and astaxanthin) were constructed with standard solutions. The analysis employed an Agilent 1100 Series HPLC system with a diode array detector (DAD, USA) and either an Alltech C-18 column (USA) or a YMC Carotenoids C-30 column (Japan). Columns were preconditioned with the mobile phase. The mobile phase consisted of methanol, acetonitrile, and dichloromethane in varying ratios, with a 1.0 to 1.2 ml/min flow rate. Column temperature was set at 30°C, and absorbance was monitored at specific wavelengths for each carotenoid. The mobile phase was filtered and degassed before HPLC analysis.

Statistical Analysis

The experiment was conducted in triplicate, and the results are reported as mean values with standard deviations (mean ± SD). One-way analysis of variance (ANOVA) was

performed using SPSS software (version 17.0) to assess differences between means, with a significance level set at $p<0.05$.

RESULTS AND DISCUSSION

Physicochemical Properties of Gac Fruit

Weight, Length, and Circumference of Gac Fruit. Figure 1 illustrates the different shapes (oblong and round) of the gac fruit in (a) and (b), as well as various maturity levels of the fruit in (c), (d), and (e). The figure also provides a visual representation of the different parts of the fruit, including the opened cut before part separation in (f), the skin and pulp after removing seeds and aril in (g), the seeds coated with the sticky aril in (h) and (i), the aril after separation from the seeds in (j), the dried seeds with aril in (k), the dried peel in (l), and the size of the gac seed in (m) and (n). The pulp, also known as mesocarp, is a significant component of fruits and vegetables, but it is often underutilised, except for green gac, commonly used in Thai cuisine (Kubola et al., 2013). Given the limited available information on the physical properties of gac fruit, various measurements such as weight, length, circumference, pulp thickness, as well as the weight percentage of seeds, pulp, aril, and peel in relation to the fruit weight, have been compiled and are presented in Table 1.

The collected data on the size and characteristics of gac fruit are significant for the food industry as they provide insights into the potential commercial utilisation of different fruit parts with optimal nutritional content. It is worth noting that there was variability in fruit size, leading to larger standard deviations in Table 1. In line with Müller-Maatsch et al. (2017), the study identified two types of gac fruit: oblong and round. The oblong fruits weighed between 500 and 1,600 g, ranging from 6 to 10 cm and a maximum length of 13 cm. On the other hand, the round fruits measured approximately 4-6 cm in length. In this investigation, the oblong-shaped fruits exhibited larger dimensions compared to the round-shaped ones, with weights of 588.33 versus 359.17 g, lengths of 13.92 versus 11.10 cm, and circumferences of 30.67 versus 27.43 cm, respectively.

Tran et al. (2016) observed that the size, including length and diameter, as well as the weight of gac fruit, is influenced by its maturity stage. However, there was no significant difference in length for gac fruit cultivated in Malaysia between the two types. In Assam, India, Hamidon et al. (2020) reported that gac fruit typically ranges in weight from 1 to 3 kg. Similarly, Tran et al. (2016) found that gac fruit grown in Sydney, Australia, had weights ranging from 1.04 to 1.73 kg, lengths varying between 21.33 and 26.33 cm, and diameters ranging from 42.50 to 50.83 cm, depending on the maturity stage, which ranged from green and immature to fully ripened and mature.

Conversely, Bhumsaidon and Chamchong (2016) noted that the average weight of gac fruit in Vietnam is approximately 710 g, with the aril accounting for 125 g (18%). In contrast, in Thailand, the average weight is around 438

Figure 1. *Momordica cochinchinensis* L. Spreng (gac) fruit and its part as shown as follows: (a) oblong and round shape of gac fruit from freezer; (b) post-thawed; (c) fully ripe (red) gac fruit; (d) fully ripe (red); (e) medium ripe (yellow); (f) interior view; (g) skin and pulp after seed and aril removal; (h) seeds coated by sticky aril; (i) seeds coated by sticky aril; (j) aril after separation from the seeds; (k) dried seeds with arils; (l) dried gac skin; (m) gac seeds; and (n) size of seed

g, with a higher percentage of aril (21% or 110 g). These findings suggest that the gac fruit in Malaysia, as studied in this research, tended to be smaller in size compared to reports from other regions. As noted by Parks et al. (2013), fruit size can impact the thickness of the aril, which generally increases with larger fruit sizes.

Table 1
Physical properties of gac fruits and seeds

Sample	Fruits												Seeds
	Weight (g)	Length (cm)	Circumference (cm)	Pulp thickness (mm)	Seed (g)	Seed % (g)	Pulp (g)	Pulp % (g)	Aril (g)	Aril % (g)	Peel (g)	Peel % (g)	Length (mm)
Oblong	588.33 ± 92.92	13.92 ± 0.92	30.67 ± 2.04	23.00 ± 2.00	135.97 ± 18.17	23.11 ± 19.56	244.65 ± 48.87	41.58 ± 52.59	109.65 ± 25.53	18.64 ± 27.47	97.93 ± 14.79	16.65 ± 15.92	20.50 ± 1.05
Round	359.17 ± 69.13	11.10 ± 0.85	27.43 ± 1.94	16.75 ± 1.89	106.67 ± 12.48	29.70 ± 18.06	122.33 ± 30.28	34.06 ± 43.81	56.17 ± 14.61	15.64 ± 21.13	74.00 ± 15.38	20.60 ± 22.24	16.17 ± 1.72

Note. Results are expressed as means ± standard deviations (n = 6)

Table 1 presents the relative weights of the four primary components of the gac fruit: peel, pulp, aril, and seeds, expressed as percentages of the total fruit weight. The findings reveal that the pulp constitutes the largest proportion of the fruit (34.06%–41.58%), followed by the seeds (23.11%–29.70%), peel (16.65%–20.60%), and the aril, which is of particular interest, makes up the smallest proportion (15.64%–18.64%) of the total fruit weight. The peel percentage in relation to the fruit weight in this study ranged from 16.65% to 20.60%. This finding is higher than that reported by Parks et al. (2013), who stated that the gac skin accounts for about 17% of the fruit weight. However, it is lower than the range of 13.89% to 15.16% reported by Tran et al. (2016) for fully ripe gac fruit.

In terms of pulp thickness, the study found it to be in the range of 16.75 to 23.00 mm (approximately 0.66 to 0.91"). It differs from the report by Chintan and Vijayakumar (2020), who stated that gac fruit grown in Vietnam has a pulp thickness of approximately half an inch (approximately 38.1 mm). The variation in pulp thickness could be attributed to the different fruit sizes in the respective studies. The pulp of the gac fruit in the study accounted for 34.06% to 41.58% of the fruit weight. This observation is lower than the values reported by Tran et al. (2016) (43.86% and 49% of fruit weight) and Parks et al. (2013) (52% to 75% of total fruit weight). The lower percentage of pulp weight in this study suggests that the gac fruit used was at a semi-ripe maturity stage, aligning with the findings of Tran et al. (2016), who reported a pulp weight of 36.20% at a similar stage.

Conversely, the aril contributed 15.64% to 18.64% of the fresh weight, which is significantly lower compared to the findings of Tran et al. (2016) (29.17% to 30.00%), who reported that the fruit flesh, encompassing the red soft and sticky aril, accounted for 25% of the fruit weight. Parks et al. (2013) also reported a range of 6 to 31% for the aril component of gac fruit. Additionally, other studies by Tran and Parks (2022) have reported aril percentages of 10%, 18%, and 24.6% of fruit weight, respectively.

The seeds of the gac fruit in this study accounted for 23.11 to 29.70% of the fresh weight, which is significantly higher compared to the findings of Tran and Parks (2022) (9.33% to 10.20%). This difference suggests that the quality of gac fruit grown in Malaysia may be lower due to its smaller size (less weight), a lower percentage of aril, and a higher percentage of seeds compared to other studies such as Parks et al. (2013) and Tran et al. (2016).

The size of the seeds in this study ranged from 16.17 to 20.50 mm in length. Chintan and Vijayakumar (2020) reported that each gac fruit has an average of 15 to 20 round, compressed, sculptured seeds. Parks et al. (2013) also stated that approximately 30 to 40 seeds per fruit, accounting for 18% to 30% of the fruit weight. According to Tran et al. (2016), the development of the seeds within the gac fruit is influenced by the harvest maturity stage, with a significant difference in the number of immature white

seeds compared to fully mature black seeds at different stages of maturity.

The Colour of Gac Fruit. The skin colour of gac fruit is an important sensory attribute, as highlighted by Angkananon and Anantawat (2015), and it is influenced by factors such as maturity index, as discussed by Kubola et al. (2013). This study observed various skin colours of mature gac fruits (Figures 1a, b, c, d, and e). Figure 1c and d represent ripe (red) gac fruits, while Figure 1e depicts a mature (yellow) gac fruit. Kubola et al. (2013) categorised gac fruits into three maturity indices: immature (green), mature or medium ripe (yellow), and fully ripe (red). Immature gac fruits are typically green in colour with white pulp and very small transparent seeds, commonly consumed as a vegetable in Thailand. Medium-ripe fruits have yellow peels, light yellow pulp, and white seed coats and are aged between 120 to 130 days. Fully ripe fruits, aged between 140 and 150 days, have orange pulp, red arils (mesocarp), and black seed coats. According to Kha et al. (2013), mature gac fruits can be identified by their uniform yellow to red skin colour and size. Gac fruit is generally seasonal, maturing from August to February (Mai et al., 2013). Determining maturity standards is crucial for achieving higher yields of gac aril for carotenoid and gac oil extraction purposes.

The skin colour of gac fruit was analysed using the L*, a*, and b* values, representing lightness, redness/greenness, and yellowness/blueness, respectively, as presented in Table 2. The results indicate that the pulp exhibited a lighter colour compared to the arils, while the arils displayed a redder colour compared to the pulp. Skin colour provides insights into the maturity stages of gac fruit, with higher L* values suggesting lighter skin, positive a* values indicating more redness, and positive b* values indicating greater yellowness. In this study, the L*, a*, and b* values for skin colour were 40.69±0.82, 43.93±0.59, and 37.38±1.67, respectively.

Tran et al. (2016) reported that fully ripened gac fruit in their study, characterised by orange or red skin, yellow pulp, and red arils, had L*, a*, and b* values of 42.17±3.83, 40.11±3.60, and 51.55±11.17, respectively. These results align with the findings here in terms of skin lightness and redness. However, there is a discrepancy in yellowness, as this study indicated less yellowness in gac skin. This difference can be explained by the colour-opponent

Table 2
Physicochemical characteristics of gac fruit

Fruit part	Colour			pH	Titratable acidity (g/L)	Total soluble solids
	L*	a*	b*			
Peel	40.69 ± 0.82	43.93 ± 0.59	37.38 ± 1.67	NA	NA	NA
Pulp	42.20 ± 1.99	24.68 ± 1.49	43.76 ± 4.33	5.65 ± 0.02	0.02 ± 0.01	4.90 ± 0.33
Aril	26.62 ± 1.10	36.35 ± 1.24	27.62 ± 2.63	5.54 ± 0.05	0.03 ± 0.02	11.57 ± 0.52

Note. Results are expressed as means ± standard deviations (n = 3); NA = Not applicable

theory, which suggests that colours cannot simultaneously appear as red and green or yellow and blue.

The gac aril's dark-red colour indicates its high content of lycopene, β-carotene, and oils (Tran et al., 2016). In this study, the aril colour was characterised by L*, a*, and b* values of 26.62±1.10, 36.35±1.24, and 27.62±2.63, respectively. Comparing these values to the findings of Mai et al. (2013), their fresh gac aril had colour indices (L*, a*, b*) of 44.0±1.4, 20.7±3.6, and 9.0±1.6, respectively. The results suggest that the aril colour in this study was darker than that reported by Mai et al. (2013) but exhibited less redness and yellowness. This colour variation could indicate that the gac aril in this study may contain a lower concentration of carotenoids compared to the peel. The red colour of the aril is likely attributed to the presence of carotenoids, particularly β-carotene and lycopene, in the fruit composition.

pH, TA, and TSS of Gac Fruit. The pH values for the edible parts of the gac fruit (pulp and aril) were 5.65±0.02 and 5.54±0.02, respectively, indicating slightly acidic conditions. TA ranged from 0.01 to 0.02 g/L for the pulp and 0.03 to 0.05 g/L for the aril. The aril also had higher TSS compared to the pulp, with a value of 11.57%±0.52 °Brix for the aril and 4.90%±0.33 °Brix for the pulp. It indicates that the gac aril is more acidic than the pulp, with an inverse relationship between pH and TA. Additionally, an increase in TA and total soluble solids were observed.

In comparison to the findings of Tran et al. (2016), this study showed lower pH, TA, and TSS values for the gac aril. Tran et al. (2016) reported pH, TA, and TSS values of 6.73±0.13, 0.26±0.01, and 15.80±0.13 for gac aril juice. In this study, the gac aril had a lower acidity level and lower total soluble solids compared to the findings of Tran et al. (2016). According to Tran et al. (2016), there is a close relationship between harvest maturity and TSS of gac aril juice, with an increase in TSS as maturity increases, followed by a decline at the final maturity stage or when fully ripe. Therefore, the pH and TA remain stable as the TSS increases during maturation.

Proximate Composition of Gac Fruit

The proximate composition analysis of gac fruit revealed that the pulp had the highest moisture content (94.9 g/100 g), followed by the aril (90.7 g/100 g) and the peel (88.1 g/100 g) (Table 3). It indicates that gac fruit grown in Malaysia had significantly higher moisture content compared to gac fruit grown in Vietnam, as reported by Gunasekaran et al. (2014) (88.6% moisture). Mai et al. (2013) also reported a high water content of 76.8% in gac aril. The moisture content tends to increase with the maturity of the fruit. The high moisture content in gac pulp and aril makes them suitable for juice extraction. However, it poses challenges in terms of fruit preservation and finding cost-effective drying methods. The selection of a drying technique for processing and preserving gac fruit aril powder is crucial to maintain its quality and achieve a high

Table 3
Proximate composition of gac fruit

Fruit part	Proximate composition*					
	Moisture	Ash	Total available carbohydrate	Crude fat	Crude protein	Total dietary fibre
Peel	88.1±0.25^{bc}	14.0 ± 0.66^{ab}	19.3 ± 0.82^{c}	1.6 ± 0.07^{b}	6.2 ± 0.26^{a}	56.9 ± 1.15^{a}
Pulp	94.9±0.26^{a}	17.3 ± 0.71^{a}	30.9 ± 0.57^{b}	1.6 ± 0.13^{b}	4.6 ± 0.22^{b}	33.3 ± 3.76^{b}
Arils	90.7±0.12^{b}	6.3 ± 0.55^{c}	55.6 ± 0.91^{a}	22.3 ± 2.33^{a}	5.8 ± 0.32^{ab}	7.0 ± 4.37^{c}

Note. Results are expressed as means ± standard deviations (n = 3); * g/100g fresh sample; $^{a-c}$ = Values with different superscript alphabet (small letters) within a column are significantly different (p<0.05)

yield of lycopene and β-carotene, which are valuable natural compounds for the food and pharmaceutical industries (Angkananon & Anantawat, 2015). Drying temperature has been found to significantly affect the contents of lycopene, β-carotene, and total phenolics in dried gac aril (Auisakchaiyoung & Rojanakorn, 2015).

In this study, gac pulp was found to have a high ash content ranging from 17.3 to 23.5 g/100 g, compared to the peel (14.0 to 15.9 g/100 g) and aril (6.3 to 13.1 g/100 g). There are limited reports on gac fruit's ash and minerals content, but Angkananon and Anantawat (2015) reported ash content of 0.7 mg and 0.58% in gac arils, respectively. It suggests that gac fruit in this study had higher mineral content than Angkananon and Anantawat's reported values (2015).

The gac aril in this study was found to have significantly higher available carbohydrate content (55.6 g/100 g) compared to the pulp (30.9 g/100 g) and peel (19.3 g/100 g). Angkananon and Anantawat (2015), as well as Gunasekaran et al. (2014), reported average carbohydrate content in gac fruit and gac fruit aril powders with maltodextrin addition of 7.6 and 73.25%, respectively.

The peel contained the highest protein content (6.2 g/100 g), followed by the aril (5.8 g/100 g) and pulp (4.6 g/100 g). These values were significantly higher compared to the reported values by Angkananon and Anantawat (2015) (0.31%), Chintan and Vijayakumar (2020) (0.6 g for fruit and 2.1 g for aril) as well as Gunasekaran et al. (2014) (1.5%). However, the gac aril in this study contained a significant fat content (22.3 g/100 g), while the peel and pulp had lower percentages (1.6 g/100 g). These values were significantly higher compared to the reported values by Angkananon and Anantawat (2015) (5.93%) as well as Gunasekaran et al. (2014) (0.1%).

The peel had the highest amount of total dietary fibre (56.9 to 58.1 g/100 g), followed by the pulp (33.3 to 37.1 g/100 g) and arils (7.0 to 11.4/100 g). These values were significantly higher compared to the reported values by Angkananon and Anantawat (2015) (15.16%) as well as Gunasekaran et al. (2014) (1.1%). The total energy contribution of gac fruit peel, pulp, and arils in this study was 116.4, 156.4, and 446.3 kcal/100 g, respectively. These values were significantly higher compared to the reported values by Gunasekaran et al. (2014) (37 kcal/100 g).

Sugar Profile Analysis of Gac Fruit

In this study, glucose was the main type of sugar in gac pulp and aril, with significant amounts of 6.66 and 7.55 g/100 g, respectively. Fructose was also detected in gac pulp and aril, with 5.84 and 6.45 g/100 g, respectively. No sucrose, maltose, or lactose was detected in either part of the gac fruit. There are no previous reports on the sugar profile of gac fruit from other researchers. The presence of glucose and fructose, monosaccharides, in gac pulp and aril corresponds to the carbohydrate content found in the total available carbohydrate analysis (Table 3). This finding indicates that gac aril contains significantly higher amounts of glucose and fructose compared to the pulp.

Mineral Composition of Gac Fruit

Mineral elements are essential for various physiological processes in the body. They are classified into macrominerals, required in larger quantities, and microminerals or trace elements, required in smaller quantities. Table 4 presents the mineral composition of gac peel, pulp, and aril. The pulp exhibited the highest ash content and significantly higher levels of most studied mineral elements, except for potassium and sodium, which were significantly higher in the peel. These findings are consistent with a study by Niyi et al. (2019) on the mineral composition of cucumber fruits, which found that potassium was highest in the peel, followed by the pulp and seeds. In the gac fruit, potassium was the most abundant mineral in the peel (817.59 mg/100g), followed by the pulp (658.20 mg/100g) and aril (228.79 mg/100g). As far as available information indicates, there are no published reports on the mineral composition of gac fruit. Potassium is a vital intracellular cation, calcium is important for bone and teeth

Table 4
Mineral composition of gac fruit

Minerals[$]	Peel	Pulp	Aril
Macronutrients			
Potassium	817.59±3.77[a]	658.20±16.96[b]	228.79±6.00[c]
Phosphorus	38.37±1.68[b]	53.97±0.88[a]	20.89±2.84[c]
Sodium	34.52±0.18[a]	5.75±0.10[b]	1.89±0.06[c]
Sulphur	18.27±0.28[ab]	20.07±1.22[a]	11.47±1.22[c]
Calcium	15.37±0.38[b]	17.58±6.63[a]	15.96±2.76[b]
Magnesium	14.95±0.35[a]	15.41±0.23[a]	11.34±0.66[b]
Micronutrients			
Aluminium	9.57±0.33[a]	0.63±0.01[b]	0.11±0.06[b]
Iron/Ferum	1.98±0.05[a]	0.71±0.13[b]	1.72±0.12[ab]
Zinc	1.77±2.11[a]	0.26±0.02[bc]	0.43±0.15[b]
Boron	0.23±0.04[ab]	0.27±0.00[a]	0.12±0.01[c]
Copper	0.21±0.01[a]	0.14±0.00[b]	0.09±0.01[c]
Manganese	0.13±0.00[a]	0.05±0.00[b]	0.09±0.01[ab]

Note. Results are expressed as means ± standard deviations (n = 2); [$] mg/100g fresh sample; [a – c] = Values with different superscript alphabet (small letters) within a row are significantly different (p<0.05)

health, and sodium plays a crucial role in regulating osmotic pressure in the body. Therefore, consuming gac pulp can provide a good source of minerals to support daily nutritional requirements.

Gac pulp was found to have the highest content of phosphorus, sulphur, calcium, and magnesium compared to the gac peel. The values for these minerals in gac pulp were 53.97, 20.07, 17.58, and 15.41 mg/100 g, respectively. Although gac aril contains lower amounts of minerals than the peel and pulp, it still had significant potassium, phosphorus, calcium, and magnesium levels at 228.79, 20.89, 15.96, and 11.34 mg/100 g, respectively. Additionally, all parts of the gac fruit (peel, pulp, and aril) were found to be good sources of phosphorus, with values of 38.37, 53.97, and 20.89 mg/100 g, respectively.

Carotenoids Composition of Gac Fruit

The carotenoid content of gac fruit, including lycopene, β-carotene, lutein, astaxanthin, and zeaxanthin, was analysed and is presented in Table 5. Lycopene was the most abundant carotenoid in gac fruit, particularly in the aril, with an average range of 31.7 to 103.7 mg/g of dry sample. β-carotene was the second most abundant carotenoid, with levels ranging from 2.9 to 9.6 mg/g of sample. Astaxanthin ranged from 1.54 to 4.91 mg/g, lutein from 0.16 to 1.35 mg/g, and zeaxanthin from 0.35 to 1.49 mg/g. Interestingly, zeaxanthin was not detected in the pulp.

When analysing the carotenoid composition, it was observed that the gac aril had the highest levels of lycopene, ranging from 31.7 to 103.7 mg/g, followed by the gac peel with a range of 6.3 to 15.5 mg/g, and the gac pulp with a range of 3.6 to 13.3 mg/g. The gac aril also contained significant amounts of β-carotene, ranging from 2.9 to 6.5 mg/g, while the gac peel had a slightly higher β-carotene content of 3.6 to 9.6 mg/g. Lutein content was higher in the gac aril compared to the peel, with a range of 0.2 to 1.4 mg/g. Astaxanthin content ranged from 1.5 to 3.6 mg/g in the gac pulp and 1.7 to 3.8 mg/g in the gac peel. Zeaxanthin was detected in the gac aril at levels ranging from 0.5 to 1.5 mg/g but was not detected in the gac pulp.

Comparing these findings to the study by Kubola and Siriamornpun (2011), results

Table 5
Carotenoids composition of gac fruit

Fruit part	Carotenoids composition (mg/g, dry material)				
	β-carotene	Lycopene	Lutein	Astaxanthin	Zeaxanthin
Peel	3.58±0.05 – 9.64±0.46	6.25±0.05 – 15.5±0.21	0.26±0.07 – 0.67±0.00	1.72±0.01 – 3.80±0.05	0.35±0.02 – 0.38±0.04
Pulp	3.04±0.00 – 6.10± 0.04	3.58±0.01 – 13.3±0.60	0.16±0.12 – 0.38±0.00	1.54±0.15 – 3.59±0.02	n.d[$]
Aril	2.89±0.01 – 6.45±0.01	31.69±0.12 – 103.7±0.15	0.20±0.03 – 1.35±0.00	3.23±0.44 – 4.91±0.04	0.52±0.02 – 1.49±0.03

Note. Results are expressed as means ± standard deviations (n = 3); [$] n.d = Non detected

showed higher levels of β-carotene and lycopene in both the gac peel and pulp. For instance, 1 g of gac peel contained 3.6–9.6 mg of β-carotene and 6.3–15.5 mg of lycopene, while Kubola and Siriamornpun (2011) reported lower values of 1.6–5.9 mg of β-carotene and 1.6–3.4 mg of lycopene. Similarly, 1 g of gac pulp contained 3.0–6.1 mg of β-carotene and 3.6–13.3 mg of lycopene, whereas Kubola and Siriamornpun (2011) reported lower values of 3.0–5.4 mg of β-carotene and 1.8–6.2 mg of lycopene. However, findings for lutein content were within a similar range to that reported by Kubola and Siriamornpun (2011), with values ranging from 0.2 to 1.4 mg/g in the gac aril. Additionally, results showed higher levels of lycopene and β-carotene in the gac aril compared to the findings of Angkananon and Anantawat (2015).

It is important to note that carotenoid content in gac fruit can vary due to variety, maturity stage, growing conditions, and storage conditions. Harvesting gac fruit at full maturity has resulted in the highest concentration of carotenoids, particularly lycopene and β-carotene, in the aril. Geographical and climate differences, as well as other environmental factors, can also influence the nutritional content of gac fruit samples. In comparison to the findings of Tran et al. (2016), the gac aril in this study from Malaysia contained less β-carotene but higher lycopene content, which may be attributed to variations in growing conditions and genetic factors. These factors should be considered when assessing the carotenoid content of gac fruit.

CONCLUSION

The study successfully met its objectives by investigating Malaysian gac fruits' physicochemical characteristics, proximate composition, and carotenoid content. These fruits distinguish themselves as "super fruits" due to their notably high carotenoid content. Malaysian gac fruits exhibit distinctions in size and proximate composition compared to other varieties. They offer essential nutritional qualities, particularly in the pulp and aril, and are abundant in carbohydrates, minerals, and potentially edible oils. This research underscores the potential of gac fruit as a valuable dietary addition and highlights the necessity for further exploration of its applications within the food and pharmaceutical industries. The study unveiled the variability in gac fruit components influenced by factors like maturity, location, and growth conditions. These factors encompass the content of the peel, the thickness of the pulp, the size of the seeds, and the colour of the skin, all serving as indicators of maturity. Gac pulp is well-suited for juice extraction, the peel contains a high ash content, and the aril excels in carbohydrates and fat. Gac pulp could serve as a source of minerals, particularly potassium. Carotenoid analysis identifies lycopene as the primary carotenoid, followed by β-carotene, lutein, astaxanthin, and zeaxanthin. These findings emphasise the importance of considering these factors to optimise the utilisation of gac fruit, particularly its valuable carotenoid content. Further research into preservation and utilisation methods has the potential

to unlock the full capabilities of gac fruit within the food and pharmaceutical sectors, aligning with the study's goals.

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REFERENCES

Angkananon, W., & Anantawat, V. (2015). Effects of spray drying conditions on characteristics, nutritional value and antioxidant activity of gac fruit aril powder. *Review of Integrative Business and Economics Research*, *4*(NRRU), 1-11.

Association of Official Analytical Chemists. (2000). *Official methods of analysis* (17th ed.). AOAC.

Auisakchaiyoung, T., & Rojanakorn, T. (2015). Effect of foam-mat drying conditions on quality of dried gac fruit (*Momordica cochinchinensis*) aril. *International Food Research Journal*, *22*(5), 2025-2031.

Bhumsaidon, A., & Chamchong, M. (2016). Variation of lycopene and beta-carotene contents after harvesting of gac fruit and its prediction. *Agriculture and Natural Resources*, *50*(4), 257-263. https://doi.org/10.1016/j.anres.2016.04.003

Chin, Y. Y., Chang, K. A., Ng, W. M., Eng, Z. P., Chew, L. Y., Neo, Y. P., Yan, S. W., Wong, C. L., Kong, K. W., & Ismail, A. (2023). A comparative evaluation of nutritional composition and antioxidant properties of six Malaysian edible seaweeds. *Food Chemistry Advances*, *3*, 100426. https://doi.org/10.1016/j.focha.2023.100426

Chintan, K. N., & Vijayakumar, R. (2020). Gac fruit – A tropical bioresource with power packed treasure of antioxidants. *Agriculture and Food*, *2*(5), 104-107.

Chomicki, G., Schaefer, H., & Renner, S. S. (2020). Origin and domestication of Cucurbitaceae crops: Insights from phylogenies, genomics, and archaeology. *New Phytologist*, *226*(5), 1240-1255. https://doi.org/10.1111/nph.16015

Dujardin, J.-P., & Kitthawee, S. (2013). Phenetic structure of two *Bactrocera tau* cryptic species (Diptera: Tephritidae) infesting *Momordica cochinchinensis* (Cucurbitaceae) in Thailand and Laos. *Zoology*, *116*(2), 129–138. https://doi.org/10.1016/j.zool.2012.07.004

Gunasekaran, N., Arumugam, A., & Perumal, S. (2014). Food prospects and nutraceutical attributes of *Momordica* species: A potential tropical bioresources – A review. *Food Science and Human Wellness*, *3*(3-4), 117–126. https://doi.org/10.1016/j.fshw.2014.07.001

Hamidon, A., Shah, R. M., Razali, R. M., & Lob, S. (2020). Effect of different types and concentration of rooting hormones on *Momordica cochinensis* (gac fruit) root vine cuttings. *Malaysian Applied Biology*, *49*(4), 127-132. https://doi.org/10.55230/mabjournal.v49i4.1602

Huynh, T., & Nguyen, M. H. (2020). Bioactive compounds from gac (*Momordica cochinchinensis* Lour. Spreng). In H. N. Murthy & V. A. Bapat (Eds.), *Bioactive compounds in underutilized fruits and nuts* (pp. 591-604). Springer. https://doi.org/10.1007/978-3-030-30182-8_40

Kha, T. C., Nguyen, M. H., Roach, P. D. Parks, S. E., & Stathopoulos, C. (2013). The underutilized gac fruit: Nutrient, composition, health benefits and options for processing. *Food Reviews International*, *29*(1), 92-106. https://doi.org/10.1080/87559129.2012.692141

Kubola, J., & Siriamornpun, S. (2011). Phytochemicals and antioxidant activity of different fruit fractions (peel, pulp, aril, and seed) of Thai gac (*Momordica cochinchinensis* Spreng). *Food Chemistry*, *127*(3), 1138-1145. https://doi.org/10.1016/j.foodchem.2011.01.115

Kubola, J., Meeso, N., & Siriamornpun, S. (2013). Lycopene and beta carotene concentration in aril oil of gac (*Momordica cochinchinensis* Spreng) as influenced by aril-drying process and solvents extraction. *FRIN*, *50*(2), 664–669. https://doi.org/10.1016/j.foodres.2011.07.004

Le, Q.-U., Lay, H.-L., Wu, M.-C., & Nguyen, T. H.-H. (2018). Natural plant colorants widely used in Vietnam traditional food culture. *Journal of Food, Nutrition and Agriculture*, *1*(1), 40-46. https://doi.org/10.21839/jfna.2018.v1i1.220

Mai, H. C., Truong, V., Haut, B., & Debaste, F. (2013). Impact of limited drying on *Momordica cochinchinensis* Spreng. aril carotenoids content and antioxidant activity. *Journal of Food Engineering*, *118*(4), 358–364. https://doi.org/10.1016/j.jfoodeng.2013.04.004

Mukherjee, P. K., Singha, S., Kar, A., Chanda, J., Banerjee, S., Dasgupta, B., Haldar, P. K., & Sharma, N. (2022). Therapeutic importance of Cucurbitaceae: A medicinally important family. *Journal of Ethnopharmacology*, *282*, 114599. https://doi.org/10.1016/j.jep.2021.114599

Müller-Maatsch, J., Sprenger, J., Hempel, J., Kreiser, F., Carle, R., & Schweiggert, R. M. (2017). Carotenoids from gac fruit aril (*Momordica cochinchinensis* [Lour.] Spreng.) are more bioaccessible than those from carrot root and tomato fruit. *Food Research International*, *99*(Part 2), 928-935. https://doi.org/10.1016/j.foodres.2016.10.053

Niyi, O. H., Jonathan, A. A., & Ibukun, A. O. (2019). Comparative assessment of the proximate, mineral composition and mineral safety index of peel, pulp, and seeds of cucumber (*Cucumis sativus*). *Open Journal of Applied Sciences*, *9*, 691-701. https://doi.org/10.4236/ojapps.2019.99056

Parks, S. E., Nguyen, M. H., Gale, D., & Murray, C. (2013). *Assessing the potential for a gac (Cochinchin gourd) industry in Australia*. https://researchdirect.westernsydney.edu.au/islandora/object/uws:18655

Phan-Thi, H., & Waché, Y. (2014). Isomerization and increase in the antioxidant properties of lycopene from *Momordica cochinchinensis* (gac) by moderate heat treatment with UV-Vis spectra as a marker. *Food Chemistry*, *156*, 58-63. https://doi.org/10.1016/j.foodchem.2014.01.040

Ram, B. M. V. S., & Shankar, V. S. (2023). Investigate the tray dried method to analyse the carbohydrate content in pumpkin (*Cucurbita*) and compare with oven dry method. *Journal of Survey in Fisheries Sciences*, *10*(1S), 2356-2369. https://doi.org/10.17762/sfs.v10i1S.468

Tran, X. T., & Parks, S. E. (2022). Improving cultivation of gac fruit. In M. Nguyen & T. C. Kha (Eds.), *Gac fruit: Advances in cultivation, utilization, health benefits and processing technologies* (pp. 1-14). CABI. https://doi.org/10.1079/9781789247329.0001

Tran, X. T., Parks, S. E., Roach, P. D., Golding, J. B., & Nguyen, M. H. (2016). Effects of maturity on physicochemical properties of gac fruit (*Momordica cochinchinensis* Spreng.). *Food Science and Nutrition*, *4*(2), 305-314. https://doi.org/10.1002/fsn3.291

Zheng, H., Zhang, Q., Quan, J., Zheng, Q., & Xi, W. (2016). Determination of sugars, organic acids, aroma components, and carotenoids in grapefruit pulps. *Food Chemistry*, *205*, 112-121. https://doi.org/10.1016/j.foodchem.2016.03.007

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Vigna marina as a Potential Leguminous Cover Crop for High Salinity Soils

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ABSTRACT

The beach bean (*Vigna marina*) exhibits robust growth in habitats characterised by sandy substrates, limited nutrient availability, and elevated saline levels. The utilisation of *V. marina*, a potentially beneficial leguminous cover crop, allows for its cultivation in regions characterised by soil salinity, hence facilitating the alleviation of environmental stress and the promotion of nitrogen fixation within the soil. A study assessed the feasibility of *V. marina* as a leguminous cover crop, in which this legume was cultivated in both coastal and inland soils. *Pueraria javanica* and *Mucuna bracteata*, widely recognised as established leguminous cover crops, were used as the control in this experiment. The observations involved were total plant biomass, nitrogenase activity, and leaf chlorophyll content of the host plants. The experiment consisted of five replicates arranged in a randomised complete block design, respectively. The effects of commercialised rhizobial compost on the development of the leguminous plants planted in both plots were also investigated. The results indicated that *V. marina* flourished in coastal and inland soils with the highest leaf chlorophyll concentration throughout the eight weeks of growth. It showed that *V. marina* has the potential to outperform the other two established leguminous cover crops when planted in highly salinised soils. The results also showed evidence that *V. marina* was an excellent potential leguminous cover crop, especially for any agricultural plots of high salinity soils, compared to the other two well-established leguminous cover crops, *P. javanica* and *M. bracteata*.

Keywords: Coastal soil, leguminous cover crop, *Mucuna bracteata*, *Pueraria javanica*, saline soils, *Vigna marina*

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INTRODUCTION

Due to high salt concentrations in the soil, most cereal plants are subjected to salinity stress conditions, and the area of land affected by the increase in salinity is expanding daily. Accordingly, soil salinity is one of the most detrimental environmental factors affecting agricultural plant productivity and food security (Sahbeni et al., 2023; Shrivastava & Kumar, 2015). Consequently, it may be the most influential ecological factor in worldwide agricultural productivity and food security (Sahbeni et al., 2023; Schwabe et al., 2006). Additionally, salinity is a significant hindrance to the growth of crop plants (Apse & Blumwald, 2002; Flowers, 2004; Munns & Tester, 2008; Witcombe et al., 2008). It also negatively impacts soil structure, nutrient availability, and plant growth, resulting in decreased cereal yields and, in extreme cases, desertification (Sahbeni et al., 2023; Shahid et al., 2018). Approximately one-third of all agricultural fields are becoming increasingly salinised, representing over a hundred countries with a wide range of climates (Khasanov et al., 2023). Estimates indicate that nearly one billion hectares of land are salinised globally, with 77 million hectares attributable to human activity (Khasanov et al., 2023; Squires & Glenn, 2011). Each year, up to two million hectares of land become salinised, substantially reducing agricultural productivity (Abbas et al., 2013; Khasanov et al., 2023). Due to its diverse climatic zones and physical terrain, Asia experiences many climate change effects, including sea level rise, saline water intrusion and soil salinisation (Hasnat et al., 2018). In low-rainfall regions of Malaysia, saline soils are prevalent (Paramananthan, 2013). Approximately 50% of Peninsular Malaysia's land is deemed unsuitable for agriculture (Abubakar et al., 2023), referred to as problematic soils, of which five per cent are affected by salinity (Aminuddin et al., 2005; Nordin et al., 2015).

Coastal areas account for around 13% of Malaysia's overall landmass (total area of 4.43 million ha), whilst 57% of this land is employed for agricultural purposes (Shultana et al., 2021). Nevertheless, the primary limitation in coastal regions is salinity caused by seawater infiltration (Herman et al., 2015; Shultana et al., 2021). As such, extensive efforts are being actively implemented to utilise or remediate the high-salinity soils for agricultural activities. Among the measures is introducing plant varieties that can tolerate and establish under high salinity stress conditions. In addition, the application of microorganisms that are tolerant to increased soil salinity and subsequently involved in soil reclamation, improving the soil conditions, and enhancing plant growth was also involved. In addition, the cultivation of leguminous cover crops that are tolerant to high salinity conditions also has great potential for implementation in the plantation sector. The legumes may protect the soil from erosion and, at the same time, improve the soil conditions that are suitable for agricultural activity. Potential leguminous plants suited for this purpose are known as *V. marina*. This lesser-known legume grew on coastal soils

(voucher specimens [USM Herbarium 11782]) and was deposited in the Universiti Sains Malaysia (USM) herbarium. This leguminous plant is referred to as nanea, dune bean, notched cowpea, and beach cowpea (Singh et al., 2019). Moreover, according to Singh et al. (2019), *V. marina* is widely distributed in tropical regions, exhibiting a floral structure resembling the mung bean and urd bean. Singh et al. (2019) investigated its ability to withstand salinity stress and possess salt-tolerant genes. It is also recommended for beachfront properties as a ground cover to prevent coastal erosion (Singh et al., 2019). It may benefit the agricultural activity of economic crops planted in high-salinity soil conditions. Therefore, an experiment was conducted to observe and evaluate the growth performance of *V. marina* under salinity stress conditions compared with established leguminous cover crops, *P. javanica* and *M. bracteata*, under two different soil classifications, coastal and inland, respectively.

MATERIALS AND METHODS

The seeds of *V. marina*, *P. javanica*, and *M. bracteata* provided by Agricultural Crop Trust (ACT) were germinated in bed using river sand as a medium under plant house conditions. Simultaneously, the seeds (according to respective treatments) were treated with 25 ml Commercial Rhizobial Compost (CRC) that contained rhizobial inoculum, provided by HUMIBOX (M) Sdn. Bhd. (Malaysia). After seven days of germination, the seeds were transferred to new pot trays under plant house conditions for the seedling establishment of each legume for 30 days of growth. The seeds were treated accordingly with 25 ml of CRC on day 7. After 30 days of growth, the established seedlings were transferred to respective experimental plots: (1) coastal plot (5°25'33.4"N 100°19'37.4" E) (at beach site of Tanjung Bungah, Penang, Malaysia) with soil conductivity at 138.7 mS/cm, and (2) inland plot (5°21'29.4"N 100°17'38.7" E) (at experimental plots of USM, Minden, Penang Malaysia) with soil conductivity at 59.6 mS/cm. All plants of both plots were observed (growth and development) for 60 days of growth (D_{60}). The seedlings were treated accordingly with 50 ml of CRC at day 37. Both experimental plots were supplied with rock phosphate (30% ± 1.0 P_2O_5 [PK Fertilizers Sdn. Bhd., Malaysia]) at a rate of 20 g/plant at day planting (day 0) and day 49 (100 g/plant of rock phosphate). Additionally, at day 0, all seedlings were supplied with 10 g of black organic fertiliser (ANITA, Malaysia) with an NPK ratio of 8:8:8.

The experiment consisted of two treatments with ten replications for each tested leguminous plant. The treatments were as follows: (1) + CRC and (2) – CRC, and were arranged in a randomised complete block design (RCBD) for D_{60}. Before the detrimental harvest at D_{60} commenced, each seedling was examined to assess the chlorophyll content in their leaves. This assessment involved the utilisation of a soil plant analysis development (SPAD) meter known as the MINOLTA™ SPAD-502

(Japan), which quantifies leaf greenness (Neufeld et al., 2006). Determining leaf chlorophyll content was conducted by utilising a standard curve of chlorophyll meter values and the corresponding leaf chlorophyll content (Amir et al., 2001). A sample of leaves with different greenness (yellow, light green, and dark green) was selected for chlorophyll analysis (MINOLTA™ SPAD-502) (Japan) and total leaf chlorophyll content analyses (Amir et al., 2001; Coombs et al., 1985). As for the nitrogenase enzyme activity, the nodules of each tested legume and plots were sampled and incubated in an airtight vessel (7 ml) for acetylene reduction assay (ARA). Subsequently, a substitution of acetylene gas was performed, wherein 0.7 ml (equivalent to 10%) of the air present in the vessel was replaced (Reis et al., 2015). The quantification of ethylene was conducted after a 24-hr incubation period, during which 1 ml of gas was taken in triplicate for each sample. All samples were incubated at room temperature within a light-restricted environment. The assay was performed utilising the Shimadzu Gas Chromatography (GC-2014, Japan) instrument, which was outfitted with a Supelco Carboxen 1004 stainless steel micro packed column measuring 2 m in length and 0.76 mm in internal diameter, equipped with a flame ionisation detector (FID) in the setup. The flow rate of nitrogen, which is employed as a carrier gas, was 30 ml/min. In the experiment, the temperature of the column, injection, and FID were consistently held at 80°C (column temperature) and 180°C (injection and FID temperature), respectively. The actual ethylene concentration was calculated by utilising the established standard curve of ethylene gas (C_2H_4, Supelco®, German) and analysing the corresponding peak region. The nitrogenase enzyme activities (µmol C_2H_4/g) were defined based on ethylene concentration (µmol C_2H_4) and the weight (g) of the tested nodules.

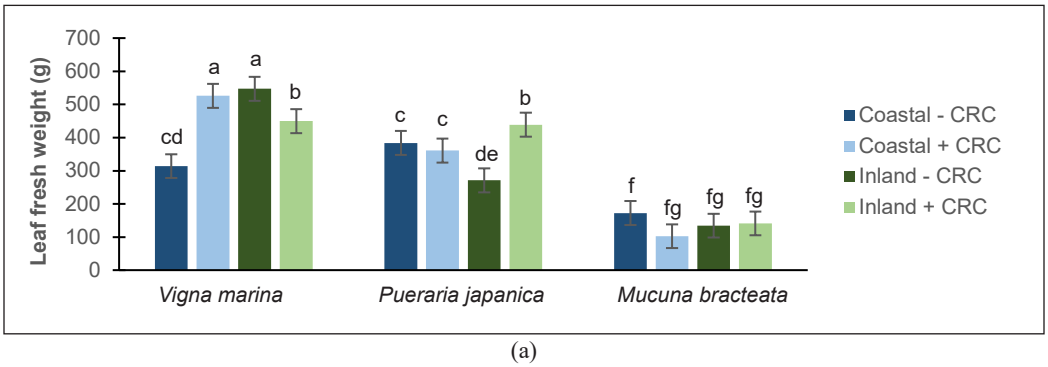
All treated seedlings were observed for plant biomass (fresh and dry weight) of leaf, above-ground, and root fresh and dry weight. The samples were oven-dried at 70°C to determine the dry weight (Ojo, 2001). Data collected were statistically analysed using IBM SPSS (version 26.0). Analysis of variance (ANOVA) was used to compute the *p*-value. At a significance level of $p < 0.05$, the Tukey honest significant difference (Tukey's HSD) test was utilised to determine the statistically significant difference between treatments. One-way ANOVA was performed to make comparisons between the means of the data.

RESULTS AND DISCUSSION

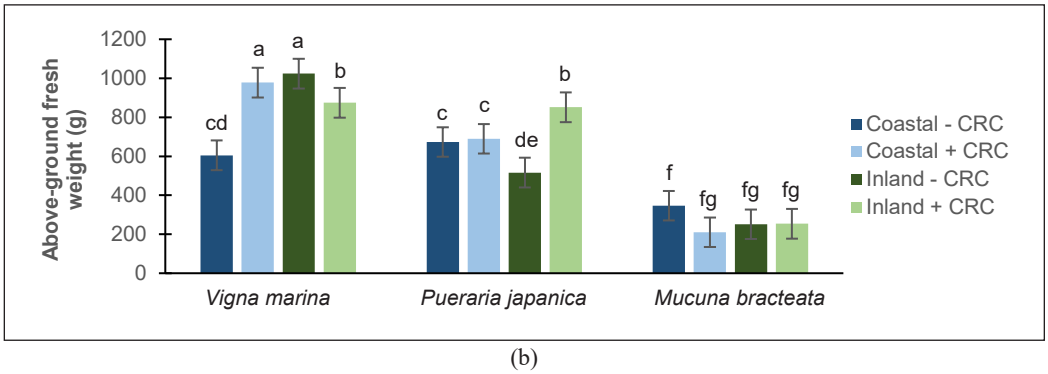
Plant Growth Observations

Treated leguminous cover crops were collected after D_{60} for coastal and inland soil plots. The plant samples were observed for leaves, roots, and above-ground plant biomass. Treated *V. marina* seedlings had the highest leaf fresh weight among the other plants tested (526.1–547.2 g), whereas *M. bracteata* had the lowest leaf fresh weight (102.73–134.49 g) (Figure 1a). On the other hand, the results differed

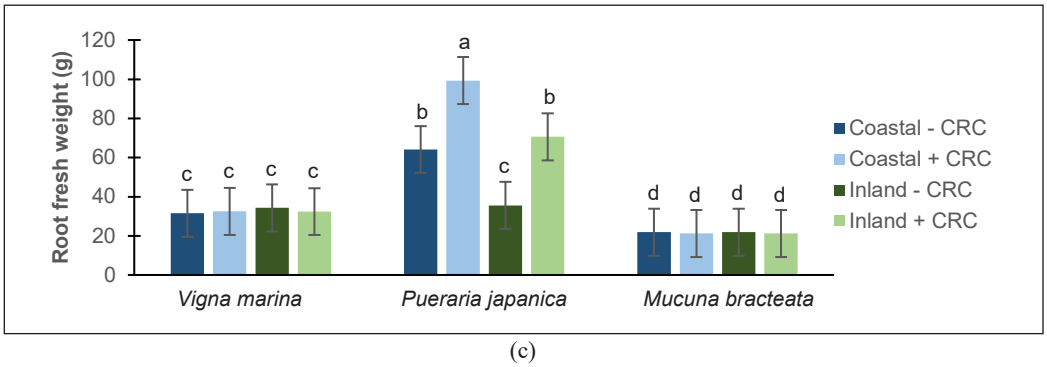
for root fresh weight, where *P. javanica* with CRC in coastal soil had the highest root fresh weight among the plants, which was 99.3 g compared to both *V. marina* and *M. bracteata* (Figure 1b). Meanwhile, the highest above-ground fresh weight among the plants tested was observed in *V. marina*, either treated with or without CRC for both inland and coastal soils (1,023.5 and 977.63 g, respectively). *M. bracteata* (with and without CRC in coastal and inland soils) had the lowest weight (Figure 1c).



(a)



(b)



(c)

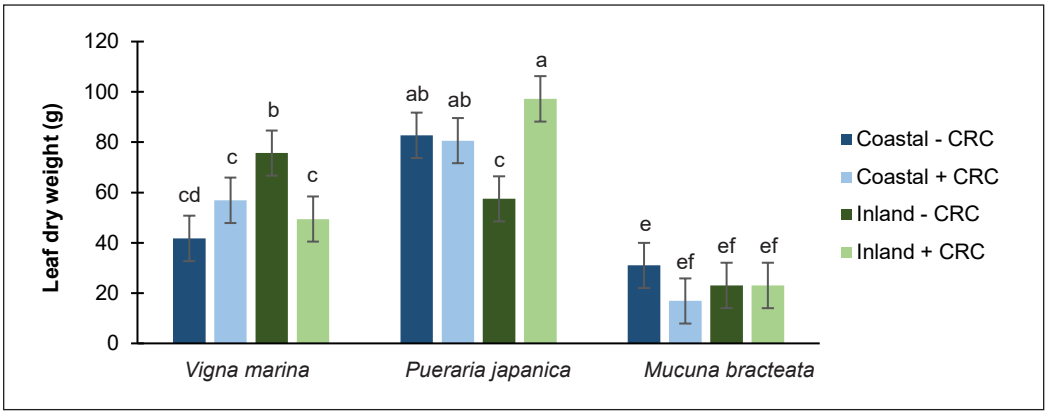
Figure 1. Means (± SE) fresh weight of leaf (a), above-ground (b), and root (c) of three leguminous cover crops (*Vigna marina*, *Pueraria javanica*, and *Mucuna bracteata*) with and without commercialised rhizobial compost (CRC) grown in two different soils, coastal and inland for 60 days

Note. Means with different letters are significantly different ($p < 0.05$) based on Tukey's honest significant difference

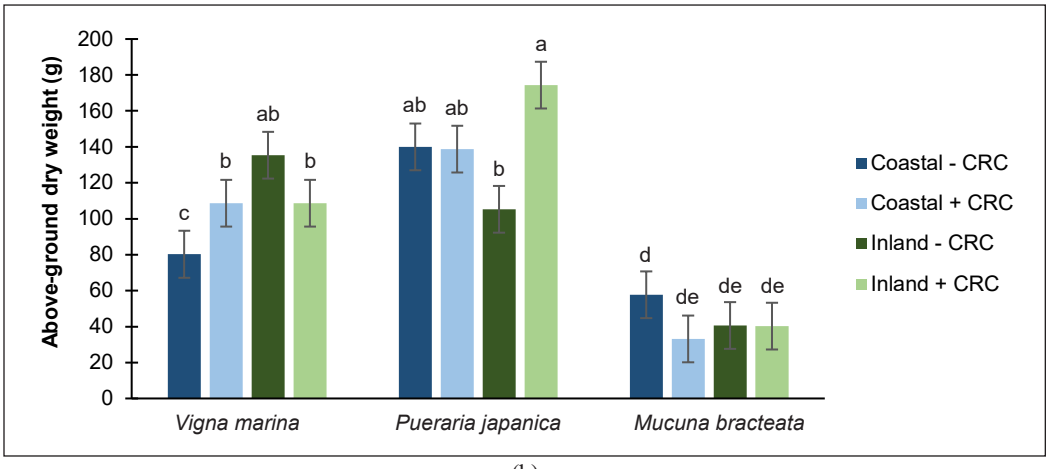
The results showed that the leaf dry weight of *P. javanica* (+ CRC) on inland soils was the highest, followed by treated *V. marina*. The leaf biomass was recorded in a range of 40.1 to 97.25 g. The lowest leaf dry weight was recorded for *M. bracteata* in a range of 16.9–23.07 g (Figure 2a). Other than that, the root dry weight of *P. javanica* with CRC in coastal soil still had the highest weight among the plants, which was 52.65 g, while the least were both *M. bracteata* with and without CRC in inland soil, which was 4.12 and 3.44 g, respectively (Figure 2b). Subsequently, the highest above-ground dry weight was recorded in *P. javanica* treated with CRC, followed by *V. marina* for coastal and inland soils tested (174.24–138 g) (Figure 2c).

The findings demonstrate that abiotic stress can inhibit plant growth and development, reduce yield, and, in extreme cases, cause plant death (Gechev & Petrov, 2020; Krasensky & Jonak, 2012). The global climate's continual deterioration and the rise in agrochemical utilisation and industrialisation contribute to the escalating severity of abiotic stressors on a global scale (Naing & Kim, 2021). Salinity is one of the most significant abiotic stresses that limits crop growth and productivity, as well as one of the world's earliest and most widespread environmental problems (Safdar et al., 2019). Due to high osmotic potential and specific ion toxicity, soil salinity can substantially inhibit seed germination and seedling growth (Rajabi et al., 2020). The presence of salinity has several effects on plants. One notable impact is reducing water availability to the roots due to the salt content in the soil solution (Safdar et al., 2019). Consequently, this hinders the plants' capacity to uptake water and essential minerals like potassium (K^+) and calcium (Ca^{2+}) (Yadav et al., 2020).

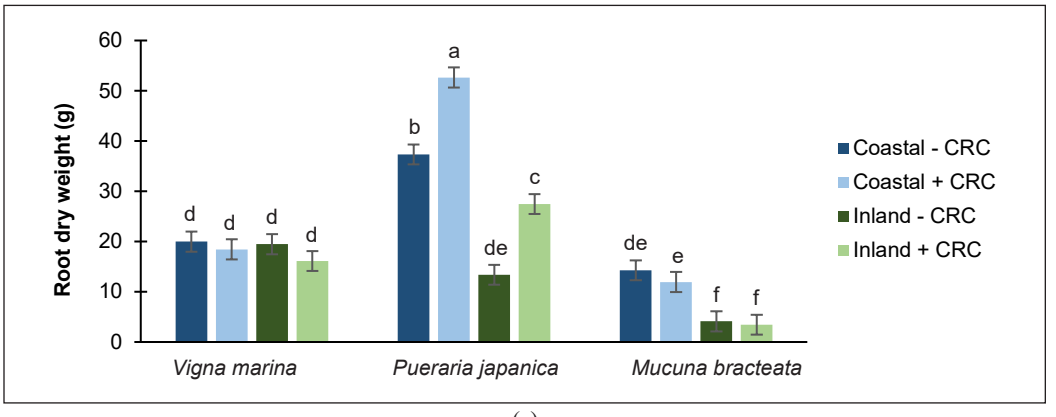
Additionally, according to Safdar et al. (2019), salt accumulation in different plant tissues can reach dangerous levels, hindering plant functionality and metabolic processes. The infiltration of sodium (Na^+) and chloride (Cl^-) ions into cells has been observed to have adverse effects on the integrity of the cell membrane and disrupt metabolic activities occurring within the cytosol (Yadav et al., 2020). Salinity inhibits seedling establishment by decreasing endogenous phytohormone levels (Egamberdieva & Kucharova, 2009; Ullah et al., 2021). Subsequent stages of shoot growth will be affected if seeded during the initial phase of salinity (El Sayed, 2011; Ullah et al., 2021; Wakeel et al., 2011). Although the seedlings are vigorous and generally grow during the second phase of salinity, the final yield will be affected (Ullah et al., 2021). Due to decreased stomatal conductivity, the plant's carbon fixation capacity is reduced, interfering with the catalytic activities of enzymes that fix carbon and destroy photosynthetic pigments (Omoto et al., 2012; Ullah et al., 2021). As a result, the shoot and root biomass of plants established in saline soil decreased significantly (Genc et al., 2019; Kalhoro et al., 2016; Ullah et al., 2021).



(a)



(b)



(c)

Figure 2. Means (± SE) dry weight of leaf (a), above-ground (b), and root (c) of three leguminous cover crops (*Vigna marina*, *Pueraria javanica*, and *Mucuna bracteata*) with and without commercialised rhizobial compost (CRC) grown in two different soils, coastal and inland for 60 days

Note. Means with different letters are significantly different ($p < 0.05$) based on Tukey's honest significant difference

Nitrogenase Activity

The results showed that treated *P. javanica* with CRC in inland soil had the highest nitrogenase enzyme activity among tested seedlings, which was 110.26 µmol C_2H_4/g.h compared to seedlings planted on coastal soil. In comparison, *V. marina* and *M. bracteata* recorded the lowest nitrogenase enzyme activity for both inland and coastal soils (Figure 3).

At the same time, similar findings were observed earlier by Abd-Alla et al. (2019), who stated that soil salinity negatively affects root colonisation, root filament infection by rhizobia, and the growth and viability of these bacteria. These abiotic elements have the potential to exert a detrimental impact on the advantageous characteristics and effectiveness of the inserted plant growth-promoting rhizobacteria (PGPR) inoculants (Egamberdieva et al., 2017). Eventually, this will hinder the ability of legumes to form and maintain nitrogen-fixing nodules in salt-affected soils as it inhibits the formation or development of nodules, thus reducing the performance of wholly developed nodules, resulting in a severe reduction in legume production (Rao et al., 2002; Wan et al., 2023). In addition, it has been observed that salt stress has a detrimental impact on the nutrient adsorption capacity of plants, specifically affecting the ability to adsorb nitrogen, leading to a decrease in plant development (Wan et al., 2023). Nitrogen may improve the plants' water utilisation and stem structure (Agami et al., 2018). The legumes relied on effective nodules where atmospheric nitrogen is converted to nitrate ion $(NO_3)^-$ and ammonium ion (NH_4^+) and absorbed by plants (López et al., 2018).

Leaf Chlorophyll Content

Treated plants had improved the leaf chlorophyll content over eight weeks of

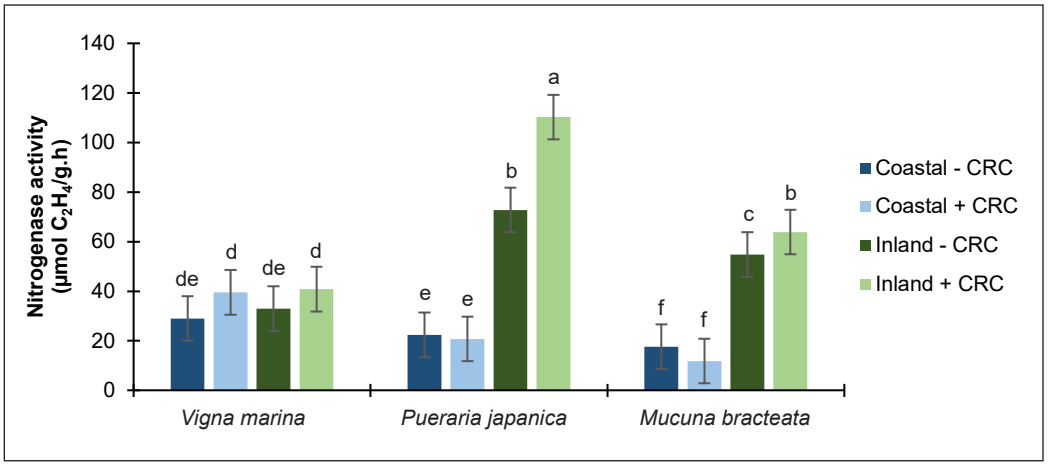


Figure 3. Means (± SE) nitrogenase activity of three leguminous cover crops (*Vigna marina*, *Pueraria javanica*, and *Mucuna bracteata*) with and without commercialised rhizobial compost (CRC) grown in two different soils, coastal and inland for 60 days

Note. Means with different letters are significantly different ($p < 0.05$) based on Tukey's honest significant difference

observation. The highest leaf chlorophyll content was observed on *V. marina* (- CRC) in coastal soil (0.95 mg chlorophyll/ mg leaf). In contrast, the lowest leaf chlorophyll content was shown on *M. bracteata* with CRC at 0.65 mg chlorophyll/ mg leaf (Figure 4). Similar observations were recorded for the seedlings grown on inland soils. The highest leaf chlorophyll content was observed on *V. marina* (+ and - CRC) in coastal soil (0.85 and 0.89 mg chlorophyll/mg leaf). At the same time, the lowest leaf chlorophyll content was depicted on *M. bracteata* at 0.65 mg chlorophyll/mg leaf (Figure 4).

The results have clearly shown that *V. marina* may overcome the high salinity conditions of the soil and maintain higher leaf chlorophyll content. The results also showed that the leaf colour of *M. bracteata* was considerably less vibrant than that of both *V. marina* and *P. javanica*. Increasing nitrogen accelerated the rate of photosynthesis, resulting in an increase in chlorophyll and a verdant leaf (Li et al., 2020; Mooney et al., 1995; Piccoli & Bottini, 2013; Wang et al., 2021). Since *M. bracteata* was less verdant than both *V. marina* and *P. javanica*, this explains why *M. bracteata* had the lowest leaf

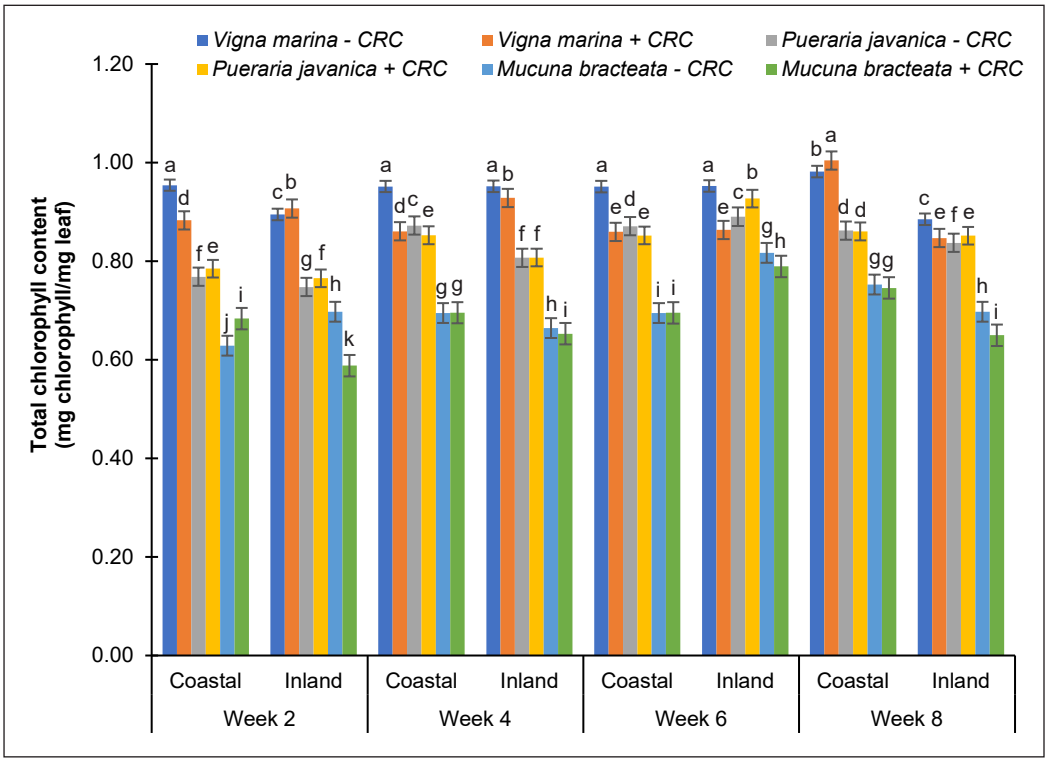


Figure 4. Means (± SE) leaf chlorophyll content of three leguminous cover crops (*Vigna marina*, *Pueraria javanica*, and *Mucuna bracteata*) with and without commercialised rhizobial compost (CRC) grown in two different soils, coastal (a) and inland (b) for 60 days

Note. Means with different letters are significantly different ($p < 0.05$) based on Tukey's honest significant difference

chlorophyll content. Earlier findings by Ali et al. (2004) and Muhamad Hassan et al. (2022) indicated that salinity decreased leaf chlorophyll content. Loss of chlorophyll due to salt stress may be associated with photoinhibition or reactive oxygen species (ROS) formation (Heidari, 2012; Kato & Shimizu, 1985; Kesawat et al., 2023).

Nounjan et al. (2020) observed that the impact of salt stress on thylakoid stacking results in a decrease in the chlorophyll *a*:*b* ratio. The loss of chlorophyll levels in response to saline stress is a widely seen occurrence attributable to multiple mechanisms. These factors include the inhibition of chlorophyll biosynthesis, produced by the activation of the chlorophyllase enzyme, as well as the destruction of cellular membranes due to chlorophyll breakdown mediated by salinity (Muhamad Hassan et al., 2022). The adverse effects of salt stress on plants are evident in their morphology, physiology, and biochemical properties: morphologically, plants experience stunted growth, chlorosis, and impaired seed germination; physiologically, salt stress inhibits photosynthesis and disrupts nutrient balance; biochemically, plants undergo oxidative stress, electrolyte leakage, and membrane disorganisation (Balasubramaniam et al., 2023). The decrease in photosynthesis under saline soils caused a reduction in chlorophyll content, as the chlorophyll index was shown to be reduced in sensitive genotypes under salt stress conditions, but it was observed to be raised in tolerant genotypes (Heidari, 2012; Masarmi et al., 2023).

CONCLUSION

The growth efficacy of *V. marina* was compared to that of two other well-established leguminous cover crops, *P. javanica* and *M. bracteata*. Although planted on coastal soils, *V. marina* performed exceptionally well as a potential leguminous cover crop with the highest leaf chlorophyll content and biomass compared to conventional leguminous cover crops, *P. javanica* and *M. bracteata*. Additionally, *V. marina* was also able to flourish in both coastal and inland soils. With the growing problem of saline soil in agricultural fields such as oil palm and rubber plantations, *V. marina* showed promising potential as the leguminous cover crop for selected agricultural fields and warranting further investigation.

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REFERENCES

Abbas, A., Khan, S., Hussain, N., Hanjra, M. A., & Akbar, S. (2013). Characterizing soil salinity in irrigated agriculture using a remote sensing approach. *Physics and Chemistry of the Earth*, *55-57*, 43-52. https://doi.org/10.1016/j.pce.2010.12.004

Abd-Alla, M. H., Nafady, N. A., Bashandy, S. R., & Hassan, A. A. (2019). Mitigation of effect of salt

stress on the nodulation, nitrogen fixation and growth of chickpea (*Cicer arietinum* L.) by triple microbial inoculation. *Rhizosphere*, *10*, 100148. https://doi.org/10.1016/j.rhisph.2019.100148

Abubakar, A., Ishak, M. Y., Bakar, A. A., Uddin, M. K., Ahmad, M. H., Seman, I. A., Ching, L. M., Ahmad, A., & Hashim, Z. (2023). Geospatial simulation and mapping of climate suitability for oil palm (*Elaeis guineensis*) production in Peninsular Malaysia using GIS/remote sensing techniques and analytic hierarchy process. *Modeling Earth Systems and Environment*, *9*, 73-96. https://doi.org/10.1007/s40808-022-01465-9

Agami, R. A., Alamri, S. A. M., Abd El-Mageed, T. A., Abousekken, M. S. M., & Hashem, M. (2018). Role of exogenous nitrogen supply in alleviating the deficit irrigation stress in wheat plants. *Agricultural Water Management*, *210*, 261-270. https://doi.org/10.1016/j.agwat.2018.08.034

Ali, Y., Aslam, Z., Ashraf, M. Y., & Tahir, G. R. (2004). Effect of salinity on chlorophyll concentration, leaf area, yield and yield components of rice genotypes grown under saline environment. *International Journal of Environmental Science and Technology*, *1*, 221-225. https://doi.org/10.1007/BF03325836

Aminuddin, B. Y., Ghulam, M. H., Wan Abdullah, W. Y., Zulkefli, M., & Salama, R. B. (2005). Sustainability of current agricultural practices in the Cameron Highlands, Malaysia. *Water, Air, and Soil Pollution: Focus*, *5*, 89-101. https://doi.org/10.1007/s11267-005-7405-y

Amir, H. G., Shamsuddin, Z. H., Halimi, M. S., Ramlan, M. F., & Marziah, M. (2001). Effects of *Azospirillum* inoculation on N_2 fixation and growth of oil palm plantlets at nursery stage. *Journal of Oil Palm Research*, *13*(1), 42-49.

Apse, M. P., & Blumwald, E. (2002). Engineering salt tolerance in plants. *Current Opinion in Biotechnology*, *13*(2), 146-150. https://doi.org/10.1016/S0958-1669(02)00298-7

Balasubramaniam, T., Shen, G., Esmaeili, N., & Zhang, H. (2023). Plants' response mechanisms to salinity stress. *Plants*, *12*(12), 2253. https://doi.org/10.3390/plants12122253

Coombs, J., Hind, G., Leegood, R. C., Tieszen, L. L., & Vonshak, A. (1985). Analytical techniques. In J. Coombs, D. O. Hall, S. P. Long, & J. M. O. Scurlock (Eds.), *Techniques in bioproductivity and photosynthesis* (2nd ed., pp. 219-228). Pergamon. https://doi.org/10.1016/B978-0-08-031999-5.50027-3

Egamberdieva, D., & Kucharova, Z. (2009). Selection for root colonising bacteria stimulating wheat growth in saline soils. *Biology and Fertility of Soils*, *45*, 563-571. https://doi.org/10.1007/s00374-009-0366-y

Egamberdieva, D., Davranov, K., Wirth, S., Hashem, A., & Abd_Allah, E. F. (2017). Impact of soil salinity on the plant-growth-promoting and biological control abilities of root associated bacteria. *Saudi Journal of Biological Sciences*, *24*(7), 1601-1608. https://doi.org/10.1016/j.sjbs.2017.07.004

El Sayed, H. E. S. A. (2011). Influence of salinity stress on growth parameters, photosynthetic activity, and cytological studies of *Zea mays* L. plant using hydrogel polymer. *Agriculture and Biology Journal of North America*, *2*(6), 907-920. https://doi.org/10.5251/abjna.2011.2.6.907.920

Flowers, T. J. (2004). Improving crop salt tolerance. *Journal of Experimental Botany*, *55*(396), 307-319. https://doi.org/10.1093/jxb/erh003

Gechev, T., & Petrov, V. (2020). Reactive oxygen species and abiotic stress in plants. *International Journal of Molecular Sciences*, *21*(20), 7433. https://doi.org/10.3390/ijms21207433

Genc, Y., Taylor, J., Lyons, G., Li, Y., Cheong, J., Appelbee, M., Oldach, K., & Sutton, T. (2019). Bread wheat with high salinity and sodicity tolerance. *Frontiers in Plant Science*, *10*, 1280. https://doi.org/10.3389/fpls.2019.01280

Hasnat, G. N. T., Kabir, M. A., & Hossain, M. A. (2018). Major environmental issues and problems of South Asia, particularly Bangladesh. In C. Hussain (Ed.), *Handbook of environmental materials management* (pp. 1-40). Springer. https://doi.org/10.1007/978-3-319-58538-3_7-1

Heidari, M. (2012). Effects of salinity stress on growth, chlorophyll content and osmotic components of two basil (*Ocimum basilicum* L.) genotypes. *African Journal of Biotechnology*, *11*(2), 379-384. https://doi.org/10.5897/AJB11.2572

Herman, T., Murchie, E. H., & Warsi, A. A. (2015). Rice production and climate change: A case study of malaysian rice. *Pertanika Journal of Tropical Agricultural Science*, *38*(3), 321-328.

Kalhoro, N. A., Rajpar, I., Kalhoro, S. A., Ali, A., Raza, S., Ahmed, M., Kalhoro, F. A., Ramzan, M., & Wahid, F. (2016). Effect of salt stress on the growth and yield of wheat (*Triticum aestivum* L.). *American Journal of Plant Sciences*, *7*, 2257-2271. https://doi.org/10.4236/ajps.2016.715199

Kato, M., & Shimizu, S. (1985). Chlorophyll metabolism in higher plants VI. Involvement of peroxidase in chlorophyll degradation. *Plant and Cell Physiology*, *26*(7), 1291-1301. https://doi.org/10.1093/oxfordjournals.pcp.a077029

Kesawat, M. S., Satheesh, N., Kherawat, B. S., Kumar, A., Kim, H.-U., Chung, S.-M., & Kumar, M. (2023). Regulation of reactive oxygen species during salt stress in plants and their crosstalk with other signaling molecules - Current perspectives and future directions. *Plants*, *12*(4), 864. https://doi.org/10.3390/plants12040864

Khasanov, S., Oymatov, R., & Kulmatov, R. (2023). Canopy temperature: As an indicator of soil salinity (a case study in Syrdarya province, Uzbekistan). In *IOP Conference Series: Earth and Environmental Science* (Vol. 1142, No. 1, p. 012109). IOP Publishing. https://doi.org/10.1088/1755-1315/1142/1/012109

Krasensky, J., & Jonak, C. (2012). Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *Journal of Experimental Botany*, *63*(4), 1593-1608. https://doi.org/10.1093/jxb/err460

Li, Y.-T., Li, Y., Li, Y.-N., Liang, Y., Sun, Q., Li, G., Liu, P., Zhang, Z.-S., & Gao, H.-Y. (2020). Dynamic light caused less photosynthetic suppression, rather than more, under nitrogen deficit conditions than under sufficient nitrogen supply conditions in soybean. *BMC Plant Biology*, *20*, 339. https://doi.org/10.1186/s12870-020-02516-y

López, S. M. Y., Sánchez, M. D. M., Pastorino, G. N., Franco, M. E. E., García, N. T., & Balatti, P. A. (2018). Nodulation and delayed nodule senescence: Strategies of two *Bradyrhizobium japonicum* isolates with high capacity to fix nitrogen. *Current Microbiology*, *75*, 997-1005. https://doi.org/10.1007/s00284-018-1478-0

Masarmi, A. G., Solouki, M., Fakheri, B., Kalaji, H. M., Mahgdingad, N., Golkari, S., Telesiński, A., Lamlom, S. F., Kociel, H., & Yousef, A. F. (2023). Comparing the salinity tolerance of twenty different wheat genotypes on the basis of their physiological and biochemical parameters under NaCl stress. *PLOS One*, *18*(3), e0282606. https://doi.org/10.1371/journal.pone.0282606

Mooney, H. A., Fichtner, K., & Schulze, E.-D. (1995). Growth, photosynthesis, and storage of carbohydrates and nitrogen in *Phaseolus lunatus* in relation to resource availability. *Oecologia*, *104*, 17-23. https://doi.org/10.1007/BF00365557

Muhamad Hassan, M. H., Awang, Y., Jaafar, J. N., Sayuti, Z., Othman Ghani, M. N., Mohamad Sabdin, Z. H., & Nazli, M. H. (2022). Effects of salinity sources on growth, physiological process, yield, and fruit quality of grafted rock melon (*Cucumis melo* L.). *Pertanika Journal of Tropical Agricultural Science*, *45*(4), 919-941. https://doi.org/10.47836/pjtas.45.4.05

Munns, R., & Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, *59*, 651-681. https://doi.org/10.1146/annurev.arplant.59.032607.092911

Naing, A. H., & Kim, C. K. (2021). Abiotic stress-induced anthocyanins in plants: Their role in tolerance to abiotic stresses. *Physiologia Plantarum*, *172*(3), 1711-1723. https://doi.org/10.1111/ppl.13373

Neufeld, H. S., Chappelka, A. H., Somers, G. L., Burkey, K. O., Davison, A. W., & Finkelstein, P. L. (2006). Visible foliar injury caused by ozone alters the relationship between SPAD meter readings and chlorophyll concentrations in cutleaf coneflower. *Photosynthesis Research*, *87*, 281-286. https://doi.org/10.1007/s11120-005-9008-x

Nordin, M. N., Lah, M. K., & Jahan, M. S. (2015). Effects of different salinity levels on rice production. *Australian Journal of Basic and Applied Sciences*, *9*, 524-530.

Nounjan, N., Mahakham, W., Siangliw, J. L., Toojinda, T., & Theerakulpisut, P. (2020). Chlorophyll retention and high photosynthetic performance contribute to salinity tolerance in rice carrying drought tolerance quantitative trait loci (QTLs). *Agriculture*, *10*(12), 620. https://doi.org/10.3390/agriculture10120620

Ojo, O. A. (2001). Assessment of nodulation of *Mucuna pruriens* by promiscuous indigenous rhizobia in the moist savanna zone of Nigeria. *World Journal of Microbiology and Biotechnology*, *17*, 429-432. https://doi.org/10.1023/A:1016769412363

Omoto, E., Taniguchi, M., & Miyake, H. (2012). Adaptation responses in C_4 photosynthesis of maize under salinity. *Journal of Plant Physiology*, *169*(5), 469-477. https://doi.org/10.1016/j.jplph.2011.11.009

Paramananthan, S. (2013). Managing marginal soils for sustainable growth of oil palms in the tropics. *Journal of Oil Palm, Environment and Health*, *4*, 1-16. https://doi.org/10.5366/jope.2013.1

Piccoli, P., & Bottini, R. (2013). Abiotic stress tolerance induced by endophytic PGPR. In R. Aroca (Ed.), *Symbiotic endophytes* (Vol. 37, pp. 151-163). Springer. https://doi.org/10.1007/978-3-642-39317-4_8

Rajabi Dehnavi, A., Zahedi, M., Ludwiczak, A., Cardenas Perez, S., & Piernik, A. (2020). Effect of salinity on seed germination and seedling development of sorghum (*Sorghum bicolor* (L.) Moench) genotypes. *Agronomy*, *10*(6), 859. https://doi.org/10.3390/agronomy10060859

Rao, D. L. N., Giller, K. E., Yeo, A. R., & Flowers, T. J. (2002). The effects of salinity and sodicity upon nodulation and nitrogen fixation in chickpea (*Cicer arietinum*). *Annals of Botany*, *89*(5), 563-570. https://doi.org/10.1093/aob/mcf097

Reis, V. M., Baldani, V. L. D., & Baldani, J. I. (2015). Isolation, identification, and biochemical characterization of *Azospirillum* spp. and other nitrogen-fixing bacteria. In F. Cassán, Y. Okon, & C. Creus (Eds.), *Handbook for* Azospirillum (pp. 3-26). Springer. https://doi.org/10.1007/978-3-319-06542-7_1

Safdar, H., Amin, A., Shafiq, Y., Ali, A., Yasin, R., Shoukat, A., Hussan, M. U., & Sarwar, M. I. (2019). A review: Impact of salinity on plant growth. *Nature and Science*, *17*(1), 34-40. https://doi.org/10.7537/marsnsj170119.06

Sahbeni, G., Ngabire, M., Musyimi, P. K., & Székely, B. (2023). Challenges and opportunities in remote sensing for soil salinization mapping and monitoring: A review. *Remote Sensing*, *15*(10), 2540. https://doi.org/10.3390/rs15102540

Schwabe, K. A., Kan, I., & Knapp, K. C. (2006). Drain water management for salinity mitigation in irrigated agriculture. *American Journal of Agricultural Economics*, *88*(1), 133-149. https://doi.org/10.1111/j.1467-8276.2006.00843.x

Shahid, S. A., Zaman, M., & Heng, L. (2018). Introduction to soil salinity, sodicity, and diagnostics techniques. In *Guideline for salinity*

assessment, mitigation and adaptation using nuclear and related techniques (pp. 1-42). Springer. https://doi.org/10.1007/978-3-319-96190-3_1

Shrivastava, P., & Kumar, R. (2015). Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi Journal of Biological Sciences*, *22*(2), 123-131. https://doi.org/10.1016/j.sjbs.2014.12.001

Shultana, R., Kee Zuan, A. T., Yusop, M. R., Saud, H. M., & El-Shehawi, A. M. (2021). *Bacillus tequilensis* strain 'UPMRB9'improves biochemical attributes and nutrient accumulation in different rice varieties under salinity stress. *PLOS One*, *16*(12), e0260869. https://doi.org/10.1371/journal.pone.0260869

Singh, A. K., Velmurugan, A., Gupta, D. S., Kumar, J., Kesari, R., Konda, A., Singh, N. P., Roy, S. D., Biswas, U., Kumar, R. R., & Singh, S. (2019). Draft genome sequence of a less-known wild *Vigna*: Beach pea (*V. marina* cv. ANBp-14-03). *The Crop Journal*, *7*(5), 660-666. https://doi.org/10.1016/j.cj.2019.05.007

Squires, V. R., & Glenn, E. P. (2011). Salination, desertification, and soil erosion. In *The role of food, agriculture, forestry, and fisheries in human nutrition* (Vol. 3, pp. 102-123). Encyclopedia of Life Support Systems Publications.

Ullah, A., Bano, A., & Khan, N. (2021). Climate change and salinity effects on crops and chemical communication between plants and plant growth-promoting microorganisms under stress. *Frontiers in Sustainable Food Systems*, *5*, 618092. https://doi.org/10.3389/fsufs.2021.618092

Wakeel, A., Sümer, A., Hanstein, S., Yan, F., & Schubert, S. (2011). *In vitro* effect of different Na^+/K^+ ratios on plasma membrane H^+-ATPase activity in maize and sugar beet shoot. *Plant Physiology and Biochemistry*, *49*(3), 341-345. https://doi.org/10.1016/j.plaphy.2011.01.006

Wan, W., Liu, Q., Zhang, C., Li, K., Sun, Z., Li, Y., & Li, H. (2023). Alfalfa growth and nitrogen fixation constraints in salt-affected soils are in part offset by increased nitrogen supply. *Frontiers in Plant Science*, *14*, 1126017. https://doi.org/10.3389/fpls.2023.1126017

Wang, N., Fu, F., Wang, H., Wang, P., He, S., Shao, H., Ni, Z., & Zhang, X. (2021). Effects of irrigation and nitrogen on chlorophyll content, dry matter, and nitrogen accumulation in sugar beet (*Beta vulgaris* L.). *Scientific Reports*, *11*, 16651. https://doi.org/10.1038/s41598-021-95792-z

Witcombe, J. R., Hollington, P. A., Howarth, C. J., Reader, S., & Steele, K. A. (2008). Breeding for abiotic stresses for sustainable agriculture. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*, 703-716. https://doi.org/10.1098/rstb.2007.2179

Yadav, S., Modi, P., Dave, A., Vijapura, A., Patel, D., & Patel, M. (2020). Effect of abiotic stress on crops. In M. Hasanuzzaman, M. C. M. T. Filho, M. Fujita, & T. A. R. Nogueira (Eds.), *Sustainable crop production*. IntechOpen. https://doi.org/10.5772/intechopen.88434

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The Effect of Inulin Substitution as A Fat Replacer on Physicochemical and Sensory Properties of Muffins

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ABSTRACT

The increasing rates of obesity and related health problems are largely attributed to the excessive consumption of high-fat foods. Thus, a fat substitute is proposed to replace the same functions of fats in food products. This study explored the potential of inulin as a fat substitute to produce low-fat muffins. Five batches of muffins, each with varying levels of inulin replacing oil (ranging from 0 to 100%), were prepared to examine how this substitution would impact the physicochemical and sensory properties of the muffins. Calorie content was determined using a bomb calorimeter, whilst the moisture, fat and fibre content were determined based on the AOAC International standard method. The muffin's texture was analysed using a texture analyser, and the height was measured by calculating the average of different parts of the muffin. The acceptance level of muffins was conducted using a 9-point Hedonic scale. The addition of 15% inulin reduced the fat content by 68.05% and calories by 12.63% compared to the control without significantly affecting the physicochemical properties and sensory acceptability. Additionally, inulin provided the advantage of increasing fibre content by 82.76% when compared to the control sample. Increasing the amount of inulin also increased height and improved the aerated structure of muffins. The study provides evidence for the effectiveness of inulin as a fat replacer, which can help produce low-fat food products with good functional properties and nutritive value for health.

Keywords: Bakery products, fat replacer, nutritional content, physicochemical, sensory properties

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INTRODUCTION

The overconsumption of energy contributes to obesity and heightens the susceptibility to non-communicable diseases, such as diabetes, cancer, and cardiovascular conditions, in multiple countries. A significant global health concern arises from the threefold increase in obesity rates from 1975 to 2016, as highlighted by NCD Risk Factor Collaboration (NCD-RisC) (2017). Numerous countries have adopted policies to encourage healthier eating habits in response to this challenge. Consequently, consumers are displaying an escalating inclination toward reduced-fat products and an expanding curiosity about natural fat replacers renowned for their superior functionalities (Mozaffarian et al., 2018)

Bakery products are among the foods that contain relatively high fat. This fat is needed to improve the product's sensory characteristics and quality, including physical characteristics and textures that contribute to overall palatability. Fat serves multiple crucial purposes, such as enhancing lubrication, influencing sensory perception, and ensuring product stability and shape (Renzyaeva, 2013; Rios et al., 2014). However, frequent consumption of high-fat foods causes consumers to worry about excess energy intake, which can cause weight gain. However, fat is an important macronutrient for human health; too many leads to obesity, diabetes type II, cardiovascular diseases (CVDs) and metabolism disorders (Barroso et al., 2017).

Saturated and trans-fat replacement ingredients have become more common in producing healthy food products. A fat replacer serves as a substance that can mimic some or all the fat activities while using fewer calories. It is important for reducing the risks associated with fat as well as for reaching the desired properties of the finished product without degrading its inherent qualities (Colla et al., 2018). Moreover, the ideal fat replacer would not only indirectly mitigate risks but also enhance the functional properties of the food, contributing to health benefits upon consumption (Kraus, 2015).

Inulin has been part of daily food intake for centuries and contributes to nutritional benefits as well as exhibits important technological properties (Moghadam et al., 2019; Wang et al., 2019; Wei et al., 2023). Inulin also has numerous biological activities, such as reducing the risk of colon cancer, arteriosclerosis, and osteoporosis, regulating food intake by reducing appetite, controlling diabetes and obesity, stimulating the immune system, enhancing absorption of calcium, magnesium, and iron, and maintaining low levels of triglycerides and cholesterol (Shoaib et al., 2016).

Short-chain inulin has a good, mildly sweet mouthfeel and is highly soluble. Meanwhile, long-chain inulin is used as a texture modifier or can act as a fat replacer due to its poor solubility and viscosity (Meyer et al., 2011). It is highlighted that long-chain inulin molecules possess a distinctive capacity to imitate fat, rendering them valuable as fat replacers, as well as exhibiting the capability to form microcrystals (Ahmed & Rashid,

2019). These microcrystals establish a network structure through effective water retention, forming minor aggregates that can subsequently combine to create an expansive gel network (Bayarri et al., 2011).

Many previous studies have been conducted to replace some of the fat in various baked products (Rodríguez-García et al., 2014; Sayed & Khalil, 2017; Zahn et al., 2010), which reduced significant reduction of fat content in the products. Inulin emerged as the most efficient option for substituting dietary fat in baked goods, reducing overall energy content without compromising consumer satisfaction. Fat replacement by up to 50% inulin did not affect consumer acceptability (Rodríguez-García et al., 2014). Adding inulin at 8% resulted in acceptable attributes comparable to muffins made with full fat (Ren et al., 2020).

Consumers highly value muffins due to their pleasurable taste and resilient consistency. Nevertheless, the increased caloric content from incorporating fats or oils has resulted in heightened consumer concerns regarding the product (Martínez-Cervera et al., 2013). A muffin normally contains approximately 50% fat, providing a smooth and soft texture. Therefore, there is a need to provide consumers with low-calorie foods without compromising the quality and acceptance of the food product. This study aims to determine the influence of replacing fat with inulin in muffins on nutritional composition, textural properties, and sensory acceptability.

MATERIALS AND METHODS

Muffin Butter Preparation

The formulation and procedure to prepare muffin butter were taken from Gisslen (2016) with slight modifications. The muffin batter mixture was prepared by weighing wheat flour, icing sugar, baking powder, salt, egg, fresh milk, vanilla extract, and oil (Table 1). The dry ingredients of wheat flour, sugar, baking powder, and salt were shifted into a bowl to prepare the control muffin sample. Meanwhile, in a separate bowl, all liquid elements encompassing egg, fresh milk, vanilla extract, and oil were vigorously whisked using a handheld wire whisk. Subsequently, the liquid constituents were combined with the blend of dry ingredients, and the mixture was carefully mixed using a spatula. This process continued until all the flour moistened, forming a batter with a slightly lumpy texture. In muffins containing inulin, the powder was included in the dry components and blended using a whisk to ensure uniform dispersion, followed by slow incorporation with the wet ingredients. Each formulation containing inulin employed a specific quantity of water, as indicated in Table 2.

The batter was then panned and baked immediately in an oven with a temperature of 180°C for 20 min; otherwise, the muffin would lose its volume. The process was repeated for three replications with the addition of inulin levels, as stated in Table 2. Water content was adjusted in all formulations to allow inulin to act as a fat mimetic and obtain a constant batter viscosity during the preparation of muffins.

Table 1

The weight and baker's percentage of ingredients for preparation of control muffin

Ingredients	Weight of ingredients (g)	Baker's percentage (%)
Wheat flour	260	100
Sugar	120	46.2
Baking powder	10	3.8
Salt	6	2.3
Eggs	50	19.2
Milk	200	76.9
Vanilla extract	5	1.9
Oil *	130	50
Total	751	300.3

Note. *The oil replacement with inulin solution was varied at 0, 25, 50, 75, and 100%, respectively

Table 2

Variation in the amount of oil, inulin, and water in the formulated muffin

% Fat replacement level	% Corn oil (Weight, g)	% Inulin solution	
		Inulin (g)	Water (g)
0	50.0 (130)	0 (0)	0 (0)
25	37.5 (97.5)	5 (13)	7.5 (19.5)
50	25.0 (65)	10 (26)	15.0 (39)
75	12.5 (32.5)	15 (39)	22.5 (58.5)
100	0 (0)	20 (52)	30.0

Note. Expressed as baker's percentage, inulin is added in a solution form

Determination of Texture

The muffin's consistency was evaluated through applying texture profile analysis (TPA) utilising a TA.XT Plus texture analyser (Stable Micro Systems, United Kingdom). The TPA protocol utilised in this investigation was adapted from the methodology proposed by Bender et al. (2017) with modest modifications. Three replicates were prepared for each muffin formulation. A cylindrical probe made of aluminium with a diameter of 75 mm (referred to as P/75) was utilised to compress the muffin according to its vertical dimension to conduct the analysis. The TPA test employed a dual compression cycle, with compression levels reaching up to 40%. The experimental circumstances consisted of a pre-test velocity of 5 mm/s, a compression velocity of 1 mm/s, and a post-test velocity of 5 mm/s. The texture profile was evaluated to assess its firmness,

adhesiveness, springiness, cohesiveness, and chewiness.

Determination of Muffin Height

Muffin height was measured using a centimetre (cm) ruler. The height of the muffin was measured specifically from the highest point (referred to as the pinnacle) to the lowest point (the base of the muffin cup). These measurements were taken before the muffin was baked and after it had cooled for one hour at room temperature, which was maintained at 25°C (Arifin et al., 2019). Thirty muffins were analysed, with each formulation consisting of three muffin samples. For each muffin, ten measurements were recorded from various sides.

Determination of Nutritional Composition

The moisture, fat, and fibre content of the produced muffins were assessed in accordance with Horwitz (2006).

Determination of Calorie Value

The calorie value in the muffin was determined by using a bomb calorimeter (IKA C2000, Germany) to measure the heat released on the complete combustion of the food samples. Following the procedure by Basolo et al. (2020), approximately 1 g of the sample was weighed and placed in a combustion capsule, then inserted in the oxygen-bomb head. For thorough combustion, the fuse wire was connected to the bomb head and subsequently brought into contact with the pellet. Prior to inserting the bomb head, 1 ml of water was introduced into the bomb calorimeter. A temperature-controlled water volume of 2 L was poured into an associated container and then positioned within the calorimeter. The oxygen bomb was carefully placed into the container after ensuring an airtight seal and the calorimeter electrodes were affixed to the oxygen bomb head. Once the pellet weight was recorded, the samples underwent combustion. The samples were run in triplicate, and the average results were calculated. The total calories of the muffin were calculated below:

Total calories of muffin = Total pellet calories (kcal/g) x Total dry weight of samples

Determination of Sensory Evaluation

A hedonic test determined the sensory acceptability of the different muffin formulations. The muffin was cut into quarters and packed in plastic for each formulation. For each panellist (n = 30), the sample of muffins with different levels of inulin (0, 12.5, 25, 37.5, and 50% expressed as baker's per cent) was presented on a tray. A 9-point hedonic scale for odour, moistness, softness, sweetness, taste, and overall flavour was used in the sensory evaluation. A balanced presentation order was used, and all panellists were provided drinking water to cleanse their palettes between the samples.

Statistical Analysis

The data was analysed through a one-

way analysis of variance (ANOVA), and subsequently, Tukey's test was employed to compare the means of the samples. This analysis was carried out at a significance level of 0.05 ($p < 0.05$). The statistical software utilised for data analysis was IBM SPSS (version 25). The results were expressed as mean values of the three replicated samples ± standard deviation.

RESULTS AND DISCUSSION

Textural Properties

In general, substituting inulin into the formulation did not affect the textural properties of vanilla muffins. Table 3 shows the textural properties of muffins for different levels of inulin. Having similar textural properties of muffins between different formulations gives us a sign that inulin was able to produce acceptable qualities as a full-fat muffin. Long-chain inulin was reported as having the capability to produce microcrystals, which aggregate together, interact with water, and finally agglomerate to generate a gel network (Bayarri et al., 2011), which could mimic the functions of fat in baked by lubricating the dry ingredients.

Table 3
Texture properties for different concentrations of inulin

Fat replacement level (%)	Firmness	Adhesiveness	Springiness	Cohesiveness	Chewiness
0	3363.78 ± 256.06 ^{a}	-4.45 ± 6.18 ^{a}	0.86 ± 0.01 ^{a}	0.61 ± 0.01 ^{ab}	2063.16 ± 145.54 ^{a}
25	3391.93 ± 793.60 ^{a}	-19.73 ± 17.66 ^{a}	0.86 ± 0.01 ^{a}	0.59 ± 0.04 ^{ab}	1986.44 ± 459.21 ^{a}
50	3869.44 ± 319.20 ^{a}	-52.92 ± 8.09 ^{ab}	0.87 ± 0.01 ^{a}	0.56 ± 0.01 ^{b}	2219.12 ± 274.62 ^{a}
75	3917.79 ± 143.46 ^{a}	-36.443 ± 27.40 ^{a}	0.87 ± 0.01 ^{a}	0.58 ± 0.03 ^{ab}	2239.49 ± 84.75 ^{a}
100	3866.20 ± 123.60 ^{a}	-131.73 ± 69.22 ^{b}	0.84 ± 0.11 ^{a}	0.64 ± 0.04 ^{a}	2463.46 ± 94.23 ^{a}

Note. Different superscript letters in the same column represent significant differences ($p < 0.05$). Values are mean ± standard deviation

Although there is no significant difference in the firmness of the muffin, the trend shows that the addition of inulin increased the firmness of the muffin (Table 3). This finding is similar to Harastani et al. (2021) in that they used inulin and green banana flour to reform muffins. The replacement of 50% inulin leads to the lowest cohesiveness of the muffin. The results also showed an increase in the cohesion trend in both directions, whether full fat or one 100% inulin replacement.

This situation may be caused by the ability of either fat or inulin to retain water, which in turn increases the cohesiveness of the muffin. Bojnanska et al. (2015) found that increasing the level of inulin in bread dough (15–25%) resulted in higher water absorption compared to lower levels of inulin. This increase in water absorption contributed to enhanced cohesiveness in the bread. Meanwhile, fat in baked products is a binding agent, holding ingredients together and preventing them from falling apart (Colla et al., 2018).

The most obvious effect is shown when 100% fat replacement has caused a significant decrease in adhesiveness. It means that increasing inulin substitution might weaken the strength and integrity of the product, but overall, the substitution revealed that inulin improves the textural properties of muffins. This finding is almost the same as that of Liu et al. (2016). The previous study showed that substituting fat with inulin may significantly influence texture parameters in bakery products (Harastani et al., 2021; Sayed & Khalil, 2017; Zahn et al., 2010). Since adding a fat replacer at a certain level can cause significant changes in texture and physical properties, complete fat replacement is not recommended in baked products.

Muffins Height and Volume

The height and cross-section measurements of baked muffins for each formulation are shown in Table 4 and Figure 1, respectively. This study shows that the height of the muffin increased with the increase of inulin. There is a significant increment in the height of muffins added, with 15% and 20% inulin showing values of 61.46% and 62.89%, respectively. The addition of 5% and 10% inulin did not have much effect on its height compared to the control (Table 4). This result differs from Liu et al. (2016), in which the specific volume and the height decreased after certain levels of inulin substitution. The height or volume of muffins significantly increased by more than 60% when 15% and 20% of inulin were added. The degree of polymerisation (DP) of certain inulin can impact the volume of baked items. According to Ziobro et al. (2013), the loaf volume increased with the application of inulin with an average DP of 10. In contrast, the volume decreased when inulin was used with an average DP of 23. Their study also showed a similar trend to the current finding in which, although the volume increased, the internal structure was less uniform and more open.

The cross-sectional diagram depicted in Figure 1 demonstrates that incorporating inulin can potentially enhance the aerated structure of muffins. The analysis of muffin cross sections reveals that increasing the inulin content from 5 to 20% (equivalent to a complete substitution of fat) leads to an enlargement of the bubble size. The number of bubbles exhibited a decline, simultaneously with an expansion in the dimensions of the bubbles, as the concentration of inulin increased. This discovery aligns with a research study by Rodríguez-García et al. (2014). Muffins that contain 100% fat replacement level clearly

Table 4
Height measurement for different concentrations of muffin after baking

Fat replacement level (%)	0	25	50	75	100
Muffin height (cm)	4.68 ± 0.19 [b]	4.40 ± 0.08 [b]	4.40 ± 0.14 [b]	5.19 ± 0.22 [a]	5.39 ± 0.08 [a]
Per cent of the increment (%)	57.27	54.54	54.54	61.46	62.89

Note. Initial height = 2 cm, n = 30; Different superscript letters in the same row represent significant differences ($p < 0.05$)

(a) (b) (c) (d) (e)

Figure 1. Muffins with different percentages of inulin cut in half after cooling: (a) 0% fat replacement, (b) 25% fat replacement, (c) 50% fat replacement, (d) 75% fat replacement, and (e) 100% fat replacement

showed uneven bubble size. It shows that fat replacement can be added to the muffin with only a maximum of 75%. The internal texture of the muffin would depend on the type of fat replacer used in the formulation in which inulin was found to give an irregular porous structure as compared to hydroxypropyl methylcellulose (HPMC) (Ren et al., 2020).

The present analysis reveals that the control sample containing 100% corn oil exhibits smaller, evenly distributed bubbles than formulations supplemented with inulin. Nevertheless, using 100% oil in the muffin exhibits a limited specific volume within a highly condensed and tightly knit grain structure. The cross-section of muffins containing inulin also showed heterogeneous distributions of air cells in the crumb. The irregular crumb in the muffin containing inulin, especially at the higher level, might be due to high hydration dough in which part of the oils was substituted with a certain amount of water. In this case, the water-to-flour weight ratio increased as the inulin increased. Higher water addition would significantly increase specific volume and pore size in the experimental bread, thus simultaneously decreasing the number of pores (Schoenlechner et al., 2010).

Nutritional Composition

There was an increase in moisture content in the muffin with the increase of inulin. Replacing oils with inulin in the muffin significantly increased moisture content from 24.04 ± 0.30% in the control sample to 32.70% ± 0.36% in 100% fat replacement (Table 5). A similar result was reported by Zahn et al. (2010) when 50% margarine was replaced with inulin in muffins. The increase in trend is due to the use of carbohydrate-based fat replacers that possess high water binding capacity and require a higher amount of water in the formulation. These features provide benefits in creating a paste that can mimic the texture and consistency of fats by offering flow properties or lubricants that closely resemble those found in the food system (Colla et al., 2018). Water binding capacity can be influenced by the inulin's polymerisation degree. The inclusion of High-Soluble Inulin (HSI) (DP < 10) and Granulated Inulin (GR) (DP ≥ 10) preparations reduced the dough's water absorption by 1.6–4.0% compared to the control, whereas High Performance Inulin (HPX) (DP > 23) enhanced water absorption by 1.6–4.8% (Ziobro et al., 2013).

The muffins that underwent a 75% fat replacement demonstrated a significant reduction of 68% in fat content compared to the control sample, which consisted of corn oil muffins. This reduction in fat content did not have any discernible impact on the degree of acceptability indicated by the panellists. Although the 100% fat replacement can reduce up to 95% of fat (Table 5), the resulting muffins are less acceptable compared to formulations with less fat replacement (Table 6). Majzoobi et al. (2018) have reported similar results, indicating that complete fat replacement in cake leads to diminished quality, as evidenced by asymmetric structure, reduced volume, light crust, and a tougher texture.

Including inulin in the formulations resulted in a notable increase in the fibre content in the muffin, which aligns with the research conducted by Ng et al. (2021) and Zahn et al. (2010). The use of inulin in food products could influence organoleptic attributes, affecting consumer acceptability. Inulin found in foods serves as both soluble dietary fibre and prebiotics. Furthermore, it is worth noting that inulin possesses the additional benefit of being resistant to enzymatic digestion within the human small intestine. This characteristic renders it highly desirable as a dietary fibre source in numerous processed food items (Chaito et al., 2016).

Calorie Value of Muffins

Inulin has been recognised as a fat substitute capable of emulating the functional and sensory characteristics of unaltered high-fat food items while not adding to the overall caloric content. This study found that increasing the amount of inulin in muffins decreased their calorie content (Table 5), potentially making them a healthier food option. Fat replacement at 50%, 75%, and 100% significantly reduced calories by 12.37%, 12.63%, and 30.96%, respectively, compared to the control sample (100% oil). Although calorie content was successfully

Table 5
Nutritional composition and calorie content per 100 g muffin at different concentrations of inulin

Inulin content (%)	Fat replacement level (%)	Moisture content (%)	Fat content (%)	Fibre content (%)	Calorie content (kcal)
0	0	24.04 ± 0.30 ^{c}	17.37 ± 0.60 ^{a}	0.05 ± 0.03 ^{b}	388 ± 22 ^{a}
5	25	25.30 ± 0.48 ^{c}	12.73 ± 0.55 ^{b}	0.12 ± 0.05 ^{b}	386 ± 24 ^{ab}
10	50	29.08 ± 0.02 ^{b}	10.52 ± 0.06 ^{c}	0.17 ± 0.07 ^{b}	340 ± 20 ^{bc}
15	75	31.32 ± 0.87 ^{a}	5.55 ± 0.53 ^{d}	0.29 ± 0.06 ^{a}	339 ± 9 ^{c}
20	100	32.70 ± 0.36 ^{a}	0.90 ± 0.21 ^{e}	0.32 ± 0.05 ^{a}	323 ± 18 ^{c}

Note. Different superscript letters in the same column represent significant differences ($p < 0.05$)

reduced by 30.96% in the muffin with full-fat replacement (20% inulin), this formulation has affected the texture and texture acceptability of the muffin. Therefore, it is advisable to substitute the fat by using a maximum of only 15% inulin (75% fat replacement) in the muffins.

It is important to note that calorie content is not the only factor to consider when determining the healthfulness of food; other nutrients like fibre, vitamins, minerals, and overall diet should also be considered. Various food products have employed inulin as a sugar or fat replacement, which can assist in lowering the calorie level (Gao et al., 2016; Khramova et al., 2021; Soh et al., 2021). Adding inulin from chicory root effectively replaced dietary fat in sponge cake, reducing total energy and producing a softer texture without affecting consumer acceptance (Rodríguez-García et al., 2012).

Sensory Properties

Sensory evaluation was conducted to determine if muffins containing inulin affected sensory acceptance among panellists. Table 6 represents the sensory acceptability of muffins formulated with different levels of inulin. This investigation reveals that the complete substitution of oil with inulin did not impact the sensory acceptance of the muffins in terms of their aroma, taste, and sweetness features. However, the acceptance levels pertaining to softness and moistness showed a notable decrease in a muffin that underwent a substitution of 20% inulin or a complete replacement of fat. The result also showed that inulin can replace fat for up to 75% without affecting the overall acceptance level. Nevertheless, substituting inulin up to 100% resulted in a significant reduction compared to other muffins. The 100% substitution of fat with inulin had a detrimental impact on the overall acceptability, indicating that inulin cannot fully replace the functional role of fat. In their review on fat replacers, Colla et al. (2018) did not recommend replacing 100% of inulin in baked products because it might cause a significant decrease in consumer acceptance.

Table 6
Sensory acceptability of muffins for different concentrations of inulin

Inulin content (%)	Odour	Moistness	Softness	Sweetness	Taste	Overall acceptability
0	6.87 ± 1.55 [a]	5.60 ± 1.83 [ab]	5.47 ± 1.74 [ab]	6.333 ± 1.47 [a]	5.97 ± 1.56 [a]	6.07 ± 1.66 [ab]
5	6.67 ± 1.58 [a]	5.80 ± 1.61[ab]	5.77 ± 1.72 [ab]	6.03 ± 1.97 [a]	6.13 ± 1.81 [a]	6.27 ± 1.78 [ab]
10	6.37 ± 1.73 [a]	6.23 ± 1.65 [a]	6.30 ± 1.68 [a]	6.50 ± 1.503 [a]	6.40 ± 1.38 [a]	6.70 ± 1.47 [a]
15	6.73 ± 1.80 [a]	5.47 ± 2.26[ab]	5.33 ± 2.41 [ab]	6.10 ± 2.37 [a]	5.70 ± 2.23 [a]	5.87 ± 2.19 [ab]
20	5.80 ± 1.88 [a]	4.83 ± 1.97 [b]	4.80 ± 2.04 [b]	5.37 ± 2.08 [a]	5.43 ± 1.96 [a]	5.23 ± 2.03 [b]

Note. Different superscript letters in the same column represent significant differences ($p < 0.05$). Values are mean ± standard deviation

The impact of using inulin as a substitute for fat in baked goods can exhibit variability contingent upon the specific food item and the quantity of inulin employed. A study by Liu et al. (2016) showed that the inclusion of 6% inulin as a substitute for fat in a prebiotic cake did not significantly impact sensory characteristics compared to a muffin containing 10% inulin. However, a study by Ren et al. (2020) revealed that the incorporation of inulin as a substitute for fat in muffins yielded an improved sensory experience characterised by reduced oiliness in the end product.

CONCLUSION

This study proves that incorporating inulin into muffin recipes yields notable fat and calorie content reductions while enhancing fibre. Importantly, these modifications do not adversely affect the physical and chemical characteristics of the sensory appeal of the end product. The study's results demonstrated that replacing oil with inulin led to a significant decrease in fat and calorie levels compared to the control group. Moreover, the augmentation of inulin content positively impacted the height and texture of muffins, resulting in an enhanced aerated structure. The utilisation of inulin as a substitute for fat also presents a potential avenue for enhancing the dietary fibre composition of the muffin. This study provides evidence that inulin is a promising and effective fat replacer in baked goods, which can help produce low-fat, low-calorie food products with good functional properties and nutritive value for health. Further investigation is needed to examine the prospective utility of inulin as a replacement for fat in various food items and assess its long-term effects on human health.

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REFERENCES

Ahmed, W., & Rashid, S. (2019). Functional and therapeutic potential of inulin: A comprehensive review. *Critical Reviews in Food Science and Nutrition*, *59*(1), 1–13. https://doi.org/10.1080/10408398.2017.1355775

Arifin, N., Siti Nur Izyan, M. A., & Huda-Faujan, N. (2019). Physical properties and consumer acceptability of basic muffin made from pumpkin puree as butter replacer. *Food Research*, *3*(6), 840–845. https://doi.org/10.26656/fr.2017.3(6).090

Barroso, T. A., Marins, L. B., Alves, R., Gonçalves, A. C. S., Barroso, S. G., & de Souza Rocha, G. (2017). Association of central obesity with the incidence of cardiovascular diseases and risk factors. *International Journal of Cardiovascular Sciences*, *30*(5), 416-424. https://doi.org/10.5935/2359-4802.20170073

Basolo, A., Parrington, S., Ando, T., Hollstein, T., Piaggi, P., & Krakoff, J. (2020). Procedures for measuring excreted and ingested calories to assess nutrient absorption using bomb calorimetry. *Obesity*, *28*(12), 2315–2322. https://doi.org/10.1002/oby.22965

Bayarri, S., González-Tomás, L., Hernando, I., Lluch, M. A., & Costell, E. (2011). Texture perceived on inulin-enriched low-fat semisolid dairy desserts. rheological and structural basis. *Journal of Texture Studies*, *42*(3), 174–184. https://doi.org/10.1111/j.1745-4603.2010.00280.x

Bender, A. B. B., Speroni, C. S., Salvador, P. R., Loureiro, B. B., Lovatto, N. M., Goulart, F. R., Lovatto, M. T., Miranda, M. Z., Silva, L. P., & Penna, N. G. (2017). Grape pomace skins and the effects of its inclusion in the technological properties of muffins. *Journal of Culinary Science and Technology*, *15*(2), 143–157. https://doi.org/10.1080/15428052.2016.1225535

Bojnanska, T., Tokar, M., & Vollmannova, A. (2015). Rheological parameters of dough with inulin addition and its effect on bread quality. *Journal of Physics: Conference Series* (Vol. 602, No. 1, p. 012015). IOP Publishing. https://doi.org/10.1088/1742-6596/602/1/012015

Chaito, C., Judprasong, K., & Puwastien, P. (2016). Inulin content of fortified food products in Thailand. *Food Chemistry*, *193*, 102–105. https://doi.org/10.1016/j.foodchem.2014.10.140

Colla, K., Costanzo, A., & Gamlath, S. (2018). Fat replacers in baked food products. *Foods*, *7*(12), 192. https://doi.org/10.3390/foods7120192

Gao, J., Brennan, M. A., Mason, S. L., & Brennan, C. S. (2016). Effect of sugar replacement with stevianna and inulin on the texture and predictive glycaemic response of muffins. *International Journal of Food Science and Technology*, *51*(9), 1979–1987. https://doi.org/10.1111/ijfs.13143

Gisslen, W. (2016). *Professional baking* (7th ed.). John Wiley & Sons.

Harastani, R., James, L. J., Ghosh, S., Rosenthal, A. J., & Woolley, E. (2021). Reformulation of muffins using inulin and green banana flour: Physical, sensory, nutritional and shelf-life properties. *Foods*, *10*(8), 1883. https://doi.org/10.3390/foods10081883

Horwitz, W. (Ed.). (2006). *Official Methods of Analysis of AOAC International* (18th ed.). AOAC.

Khramova, V. N., Khrapova, E. V., Gorlov, I. F., Knyazhechenko, O. A., Khramova, Y. I., Burdina, A. N., & Chekhova, E. A. (2021). Development of prebiotic-rich sausage breads with reduced calories. In *IOP Conference Series: Earth and Environmental Science* (Vol. 677, No. 3, p. 032036). IOP Publishing. https://doi.org/10.1088/1755-1315/677/3/032036

Kraus, A. (2015). Development of functional food with the participation of the consumer. Motivators for consumption of functional products. *International Journal of Consumer Studies*, *39*(1), 2–11. https://doi.org/10.1111/ijcs.12144

Liu, J., Luo, D., Chen, R., Xu, B., & Liu, J. (2016). Effects of short-chain inulin on quality of chinese steamed bread. *Journal of Food Quality*, *39*(4), 255–263. https://doi.org/10.1111/jfq.12201

Majzoobi, M., Mohammadi, M., Mesbahi, G., & Farahnaky, A. (2018). Feasibility study of sucrose and fat replacement using inulin and rebaudioside A in cake formulations. *Journal of Texture Studies*, *49*(5), 468–475. https://doi.org/10.1111/jtxs.12330

Martínez-Cervera, S., de la Hera, E., Sanz, T., Gómez, M., & Salvador, A. (2013). Effect of nutriose on rheological, textural and sensorial characteristics of spanish muffins. *Food and Bioprocess Technology*, *6*, 1990–1999. https://doi.org/10.1007/s11947-012-0939-x

Meyer, D., Bayarri, S., Tárrega, A., & Costell, E. (2011). Inulin as texture modifier in dairy products. *Food Hydrocolloids*, *25*(8), 1881–1890. https://doi.org/10.1016/j.foodhyd.2011.04.012

Moghadam, B. E., Keivaninahr, F., Fouladi, M., Mokarram, R. R., & Nazemi, A. (2019). Inulin addition to yoghurt: Prebiotic activity, health effects and sensory properties. *International Journal of Dairy Technology*, *72*(2), 183–198. https://doi.org/10.1111/1471-0307.12579

Mozaffarian, D., Rosenberg, I., & Uauy, R. (2018). History of modern nutrition science — Implications for current research, dietary guidelines, and food policy. *BMJ*, *361*, k2392. https://doi.org/10.1136/bmj.k2392

NCD Risk Factor Collaboration. (2017). Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: A pooled analysis of 2416 population-based measurement studies in 128·9 million children, adolescents, and adults. *Lancet*, *390*(10113), 2627–2642. https://doi.org/10.1016/S0140-6736(17)32129-3

Ng, F. S. K., Chiang, J. H., Ng, G. C. F., Lee, C. S. H., & Henry, C. J. (2021). Influence of inulin–konjac suspension as a fat replacer in baked muffins and its impact on textural and oxidative stability upon storage. *Journal of Food Processing and Preservation*, *45*(10), e15769. https://doi.org/10.1111/jfpp.15769

Ren, Y., Song, K.-Y., & Kim, Y. (2020). Physicochemical and retrogradation properties of low-fat muffins with inulin and hydroxypropyl methylcellulose as fat replacers. *Journal of Food Processing and Preservation*, *44*(10), e14816. https://doi.org/10.1111/jfpp.14816

Renzyaeva, T. V. (2013). On the role of fats in baked flour goods. *Food and Raw Materials*, *1*(1), 19-25. https://doi.org/10.12737/1513

Rios, R. V., Pessanha, M. D. F., de Almeida, P. F., Viana, C. L., & da Silva Lannes, S. C. (2014). Application of fats in some food products. *Food Science and Technology*, *34*(1), 3–15. https://doi.org/10.1590/s0101-20612014000100001

Rodríguez-García, J., Puig, A., Salvador, A., & Hernando, I. (2012). Optimization of a sponge cake formulation with inulin as fat replacer: Structure, physicochemical, and sensory properties. *Journal of Food Science*, *77*(2), C189–C197. https://doi.org/10.1111/j.1750-3841.2011.02546.x

Rodríguez-García, J., Salvador, A., & Hernando, I. (2014). Replacing fat and sugar with inulin in cakes: Bubble size distribution, physical and sensory properties. *Food and Bioprocess Technology*, *7*, 964–974. https://doi.org/10.1007/s11947-013-1066-z

Sayed, H. S., & Khalil, S. R. (2017). Effect of chicory inulin extract as a fat replacer on texture and sensory properties of cookies. *Middle East Journal of Applied Sciences*, *7*(1), 168–177.

Schoenlechner, R., Mandala, I., Kiskini, A., Kostaropoulos, A., & Berghofer, E. (2010). Effect of water, albumen and fat on the quality of gluten-free bread containing amaranth. *International Journal of Food Science and Technology*, *45*(4), 661–669. https://doi.org/10.1111/j.1365-2621.2009.02154.x

Shoaib, M., Shehzad, A., Omar, M., Rakha, A., Raza, H., Sharif, H. R., Shakeel, A., Ansari, A., & Niazi, S. (2016). Inulin: Properties, health benefits and food applications. *Carbohydrate Polymers*, *147*, 444–454. https://doi.org/10.1016/j.carbpol.2016.04.020

Soh, J. I. X., Wilian, M., & Yan, S. W. (2021). Inulin enhances nutritional, sensorial and technological characteristics of synbiotic yogurt drink. *British Food Journal*, *123*(7), 2571–2581. https://doi.org/10.1108/bfj-11-2020-1044

Wang, L., Yang, H., Huang, H., Zhang, C., Zuo, H.-X., Xu, P., Niu, Y.-M., & Wu, S.-S. (2019). Inulin-type fructans supplementation improves glycemic control for the prediabetes and type 2 diabetes populations: Results from a GRADE-assessed systematic review and dose–response meta-analysis of 33 randomized controlled trials. *Journal of Translational Medicine*, *17*, 410. https://doi.org/10.1186/s12967-019-02159-0

Wei, W., Wong, C. C., Jia, Z., Liu, W., Liu, C., Ji, F., Pan, Y., Wang, F., Wang, G., Zhao, L., Chu, E. S. H., Zhang, X., Sung, J. J. Y., & Yu, J. (2023). *Parabacteroides distasonis* uses dietary inulin to suppress NASH via its metabolite pentadecanoic acid. *Nature Microbiology*, *8*, 1534–1548. https://doi.org/10.1038/s41564-023-01418-7

Zahn, S., Pepke, F., & Rohm, H. (2010). Effect of inulin as a fat replacer on texture and sensory properties of muffins. *International Journal of Food Science and Technology*, *45*(12), 2531–2537. https://doi.org/10.1111/j.1365-2621.2010.02444.x

Ziobro, R., Korus, J., Juszczak, L., & Witczak, T. (2013). Influence of inulin on physical characteristics and staling rate of gluten-free bread. *Journal of Food Engineering*, *116*(1), 21–27. https://doi.org/10.1016/j.jfoodeng.2012.10.049

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Overexpression of amiR2937 and amiR854e in Transgenic *Arabidopsis thaliana* Indirectly Impacts the Photosynthesis Performances by Targeting Specific Target Transcripts in the MEP Pathway

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ABSTRACT

Artificial miRNAs (amiRNAs) are artificial small RNAs engineered to silence specific plant mRNA transcripts. They are generated by expressing a functional microRNA (miRNA) with modified sequences *in planta*. Two miRNAs, miR2937 and miR854e, were selected based on their predicted target transcript, *GGPS2* (geranylgeranyl pyrophosphate synthase 2) and *TPS13* (terpenoid synthase 13). In the methylerythritol phosphate pathways, *GGPS2* and *TPS13* enzymes play a role in synthesizing sesquiterpenes, triterpenes, diterpenoids, carotenoids, gibberellins, and chlorophyll, respectively. Therefore, in this study, these two miRNAs were overexpressed in *Arabidopsis thaliana* in single and co-overexpression to analyze the change in the abundance of phytol and trans-beta-lone compounds. Through real-time quantitative polymerase chain reaction (RT-qPCR) analysis, a fold-up regulation of amiR2937 and amiR854e was observed in both transgenic plants harboring single and double constructs. Meanwhile, the *GGPS2* and *TPS13* enzymes showed a decreasing pattern in all transgenic plants, indicating that the miRNAs had successfully suppressed the target transcripts. Solid-phase microextraction-gas chromatography-mass spectrometry analysis revealed that the number of phytols was decreased in all transgenic plants but was significant in plants harboring construct miR854e. Meanwhile, there is an increasing pattern of trans-beta-ionone in all transgenic plants

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compared to wild-type plants. Consistently, with the decrease in phytol content, soil plant analysis development value, and total chlorophyll content, the photosynthesis rate decreased in the transgenic plants compared to the wild type. Indeed, the overexpression of these two miRNAs affects the production of target transcript and changes the plant development.

Keywords: amiRNAs, *Arabidopsis thaliana*, gene-silencing, post-transcriptional regulation, sesquiterpenoid and phytol biosynthesis

INTRODUCTION

In plants, metabolites from both primary and secondary metabolism play significant roles in development and growth. The primary metabolism of plants produces important molecules: carbohydrates, lipids, proteins, and nucleic acids, essential metabolites for general plant development and growth. In contrast, the secondary metabolism of plants produces many small biomolecules known as secondary metabolites (SMs). These SMs include phenolics, phytols, terpenoids, nitrogen-containing compounds, oligosaccharides, and alkaloids. In addition, SMs have been classified into terpenoids, phenylpropanoids, and alkaloids based on their biosynthetic pathway site and natural chemical properties. However, the production of SMs in the plant is very low, and the plant releases the compound only when needed. The plant species affect how secondary metabolites are produced and also on certain conditions such as weather, environmental stress, and nutritional sources (Akula & Ravishankar, 2011; Ramirez-Estrada et al., 2016).

Artificial microRNA (amiRNAs) are artificial small RNAs (sRNAs) engineered to silence specific plant mRNA transcripts. The expression of a functional miRNA with modified sequences *in planta* generates them. They are derived from the Dicer-Like1 (DCL1) protein cleavage of miRNA precursors with fold-back structures. MicroRNA (miRNAs) are short (19–24 nucleotides) non-coding RNAs influencing key plant processes like growth, development, and stress response (Ameres & Zamore, 2013; Martin et al., 2010). Research on miRNA biogenesis laid the foundation for amiRNA design, and in recent years, there has been a proliferation of new information on the role of sequence and structure in pri-miRNA processing (Jin et al., 2020; Kwon et al., 2019; Liu et al., 2018; Wang et al., 2020). In wheat, miRNAs and their targets have a complex role in controlling the growth of grains (Li et al., 2015).

Sesquiterpenes (SQs) are essential for the growth and development of plants. They function as defense agents or pheromones and were created in stressed plants. They are the most prevalent volatile secondary metabolites of plants found in nature and are part of the terpene family. They make up 28% of all terpenes found in plants. Due to their extensive involvement in the food, cosmetics, chemical, and pharmaceutical industries, they are well known. It appears that the sesquiterpene (E)-caryophyllene also acts as a defense against a bacterial disease

since the bacterial growth on the stigma of flowers without (E)-caryophyllene emission was larger than that of wild-type flowers (Huang et al., 2012). Some SQs also have a role in signal biocommunication; research disclosed a root growth stimulation caused by volatile SQ emission from adjacent fungi (Ditengou et al., 2015). SQs are well-known in the pharmaceutical industry for helping people in many ways, such as by reducing anxiety and depression (Bahi et al., 2014). Besides, current research has discovered that sesquiterpene prevents colon and skin cancer by acting as a chemopreventive agent (Picaud et al., 2006). Considering its importance in plant defense systems and commercial significance, the mechanism of sesquiterpene biosynthesis has been the focus of numerous studies. Both cosmetic and non-cosmetic items may contain the aromatic component phytol, used in many scent compounds (McCourt & Benning, 2010). However, in the medical area, phytol has demonstrated antinociceptive, antioxidant, anti-inflammatory, and antiallergenic properties (de Menezes Patrício Santos et al., 2013; Ryu et al., 2011). Recent studies have shown that phytol is an effective immunostimulant that activates innate and acquired immunity and induces long-term memory more effectively than several types of commercial adjuvants (Lim et al., 2006). Additionally, even in immune-compromised mice, phytol and its derivatives show no cumulative inflammatory or toxic consequences (Chowdhury & Ghosh, 2012).

The formation of a terpenoid usually undergoes a continuous head-to-tail addition of its building blocks, which are isoprene diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Berthelot et al., 2012; Laskovics & Poulter, 1981). First, a head-to-tail condensation of IPP and DMAPP produces geranyl diphosphate (GPP), the precursor of monoterpenoid. Then, the successive addition of IPP results in the formation of precursors of sesquiterpenoid and diterpenoid, which are farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), respectively. IPP and DMAPP are produced by two distinct processes, the predominantly cytosolic mevalonic acid (MVA) system and the plastidial methylerythritol phosphate (MEP) pathway, which both implicated the target genes *GGPS2* and *TPS13*. Figure 1 illustrates how miRNA target transcripts are involved in the terpenoid pathway. Precursors from the MVA route can be used to biosynthesize sesquiterpenoids, polyphenols, phytosterols, brassinosteroids, and triterpenoids in the cytosol, as well as terpenoids in the mitochondria. When the major isoprenoid pathway genes are overexpressed or underexpressed, this results in metabolic perturbations and changes in metabolic flux, which in turn cause feedback or feedforward signals to change the expression of upstream or downstream genes. All-trans-GGPP, produced by all-trans-GGPSs, is similar to (E, E)-FPP. It is a significant branching point for several downstream terpenoid pathways in primary and specialized

metabolism. These include the production of poly-oligoprenols, abscisic acid, strigolactones, gibberellins, plastoquinones, and diterpenoids, as well as the biosynthesis of carotenoids and the byproducts of their breakdown. Geranylgeranylated proteins are also produced. Overexpression or reduction expression of the enzyme of *GGPS2* indeed can affect the development of the plant. For instance, the phenotypes of *Ggps1* mutants show that this gene is essential for development and chlorophyll production, including seedling-lethal albinism and embryo-lethality (Ruppel et al., 2013).

Therefore, gene manipulation using miRNA or artificial miRNA has been introduced in this research. Overexpression of the selected miRNA in the transgenic plant has been proven to successfully affect the abundance of target transcripts, which are the enzymes involved in the secondary metabolite pathway. As an example, it has been reported by X. Chen (2004) as well as Tholl and Lee (2011), that even in the absence of a Class II-type 1-deoxy-D-xylose-5-phosphate synthase (DXS) enzyme, the plastidial production of monoterpene and diterpene specialized metabolites was observed at very low concentrations in *Arabidopsis* tissues. By using the metabolomic approach, the effect of miRNA overexpression on the production of secondary metabolites has been studied, specifically on sesquiterpene and phytol. Besides, the changes in the abundance of miRNAs and their target transcripts and the photosynthesis rates were also measured. Therefore, obtaining the information from this research will assist in further understanding the miRNA functions and their mechanism work on silencing the target transcript involved in the secondary metabolites pathway. Finally, this knowledge will pave the road for manipulating secondary metabolite production in plants and, ultimately, using miRNA as a tool in industry.

MATERIALS AND METHODS

Plant Materials, Plant Vectors, and Bacterial Cultures

Arabidopsis thaliana ecotype Columbia-0 seeds were used in this experiment. Wild-type plants were grown for uniformity under the standard growth condition (light intensity: 100–150 µmol/m^2/s, light period: 16 hr days/8 hr night, temperature: 22°C, relative humidity: 60%) in plant growth chambers. Plant vectors, pMDC32B-AtMIR390a-B/C, and chemically competent cells, *Escherichia coli* TOP10 and STELLAR strain (ThermoFisher Scientific, USA), and *Agrobacterium tumefaciens*, GV3101 strain, were obtained from Plant Biotechnology Lab, Universiti Kebangsaan Malaysia (UKM).

Development of amiRNAs Over-expression Constructs Using PMDC32b-AtmiR390a-b/c and pB2GW7 Vector. The amiRNAs oligonucleotides were designed by the p-sams web tool and are available on the Carrington Laboratory home page (http://p-sams.carringtonlab.org). Both oligonucleotide primers (75 bases long) had an overhang facilitating

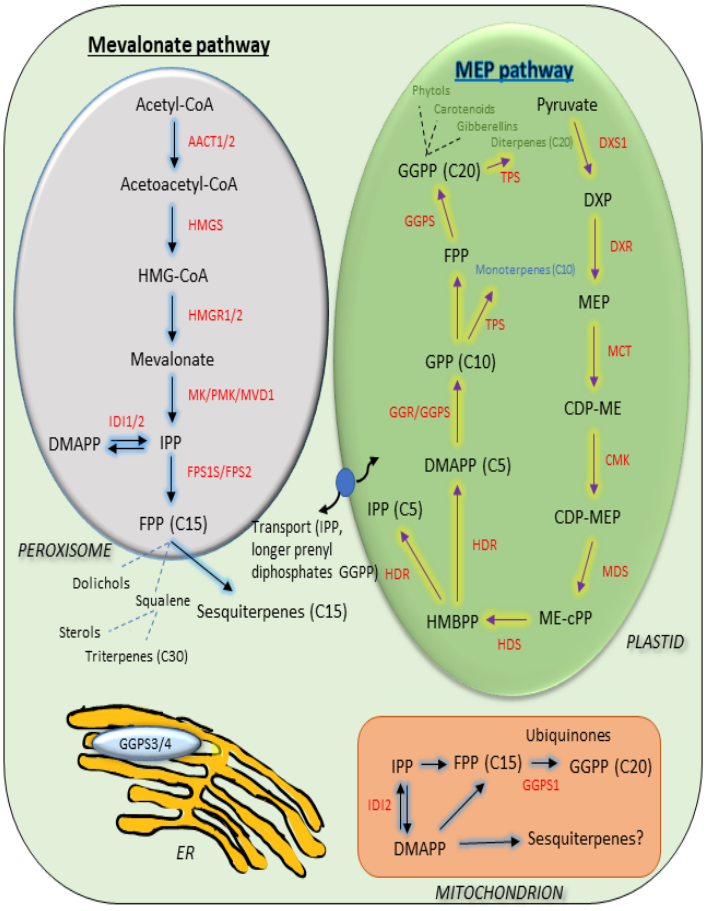

Figure 1. The subcellular structure of the terpene production pathways in *Arabidopsis*

Note. According to Sapir-Mir et al. (2008), the dashed line denotes a potential partial or complete placement of mevalonic acid pathway enzymes in peroxisomes. Involvement of the predicted target transcript *GGPS2* and *TPS13* in the terpenoid pathway (Modified from Tholl and Lee, 2011)

Note. Red terms = Enzymes: CoA = coenzyme-A; AACT1/2 = Acetyl-CoA acetyltransferase 1; HMGS = Hydroxymethylglutaryl-CoA synthase; HMGR1/2 = HMG-CoA reductase; MK = Mevalonate kinase; PMK = Phosphomevalonate kinase; MVD1 = Mevalonate-5-pyrophosphate decarboxylase; IDI1/2 = Isopentenyl-diphosphate delta-isomerase; FPS1/2 = Farnesyl pyrophosphate synthase ½; DXS1 = 1-deoxy-D-xylulose 5-phosphate synthase 1; DXR = 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MCT = Medium-chain triglyceride; CMK = 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase; MDS = 4-diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS = 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HDR = 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; GGR = Geranylgeranyl reductase; GGPS = Geranylgeranyl pyrophosphate synthase; TPS = Terpenoid synthase; IDI2 = Isopentenyl-diphosphate delta isomerase 2; GGPS1 = Geranylgeranyl pyrophosphate synthase 1Black terms = Molecules; HMG-CoA = Hydroxymethylglutaryl-CoA; DMAPP = Dimethylallyl pyrophosphate; IPP = Isopentenyl pyrophosphate; FPP = Farnesyl diphosphate; DXP = 1-Deoxy-D-xylulose 5-phosphate; MEP = 2-C-methyl-D-erythritol 4-phosphate (methylerythritol phosphate); CDP-ME = 4-Diphosphocytidyl-2-C-methyl-D-erythritol; CDP-MEP = 4-Diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; ME-cPP = 2-C-methyl-D-erythritol-2,4-cyclodiphosphate; HMBPP = (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; GPP = Geranyl diphosphate; ER = Endoplasmic reticulu

ligation for cloning into the miRNA overexpression vector. The oligonucleotides pair for amiR2937 were amiR2937F: TGTAATAAGAGCTGTTGAA GGAGT CATGATGA TCACATTCG TTATCTATTTTTTGACT CCTTCACCAGCTCTTAT and amiR2937R: AATGATAA GAGCTGGTG AAGGAGTCAAAAAATAGA TAACGAATGTGATCATCATGA CTCCTTCAACAGCTCTTAT while for amiR854e, amiR854eF: TGTAGATGAG GATAGGGAGGA GGAGATGATGA TCACATTCGTT ATCTATTTTTTCTC CTCCTCCATATCCTCATC and amiR854eR: AATGGATGAGGA TATGGAGGAGGAGAAAAAATAGAT AACGAATGTGATCATCATCT CCTCCTCCCTATCCTCATC. Each amiRNA insert was synthesized by annealing the two partially complementary oligonucleotides (Carbonell et al., 2014). The pMDC32b-AtMIR390a B/c vector was joined to the annealed amiRNA inserts using the T4 DNA ligase enzyme (New England Biolabs, USA). Since this study also focuses on the co-overexpression of miRNA in *A. thaliana*, two plasmids with different markers are needed to select transgenic plants. Therefore, miR854e was cloned into the pB2GW7 vector using the Gateway Cloning technology kit from Thermo Fisher Scientific (Malaysia). The DNA fragment of interest was added with attB1/attB2 sites by polymerase chain reaction (PCR), and then the DNA fragment was cloned into pDONR221 vectors through BP Clonase procedures. PCR fragments containing the appropriate attL site were produced using a particular primer and universal primer M13. An LR Clonase recombination reaction immediately cloned the PCR fragments into the intended target vector (pB2GW7) after being gel-purified.

Developing Transgenic *A. thaliana* Plants

The GV3101 strain of *Agrobacterium* was used for the heat shock transformation method. Once the wild type of *A. thaliana* plants reached the anthesis stage, the transformation was done using the flora dip transformation method. The solution was prepared using a 100 ml Luria Betani (LB) media containing *Agrobacterium* construct colony. It was grown at 28°C until it reached an optical density (OD) of around 0.5 and was finally centrifuged to collect the cell. In 200 ml autoclaved water, 15 g sucrose (Duchefa Biochemie, Netherlands), well-grown *Agrobacterium* cell culture, and 0.025% of silwet L-77 (PhytoTech Labs, USA) were added. The plant inflorescences were then submerged for 15 to 20 s with mild agitation in the liquid *Agrobacterium* culture (Bent, 2006). The transformation was repeated twice to increase efficiency. After the plant reached maturity and was ready to be harvested, the seeds were screened through antibiotics and genomic PCR. The transgenic plant's seeds were called the T1 generation, and they were grown until the T3 generation to get a stable homozygous generation.

Growing and Sampling of *A. thaliana* Plants for Analysis

One line of T3 generation of each construct (pMDC32b_amiR2937, pB2GW7_amiR854e, and co-overexpression: pMDC32b_amiR2937 + pB2GW7_amiR854e) of the transgenic plant was selected for final analysis. The seeds were rinsed with autoclaved distilled water after being cleaned with 70% ethanol (Hamburg Chemical HmbG, Germany) for 1 min, 50% Clorox (Clorox®, Malaysia), and Tween® 20 (Duchefa Biochemie, Netherlands) for 10 min. The seeds were plated on a Murashige and Skoog (MS) media (Duchefa Biochemie, Netherlands) plate containing antibiotic (pMDC32b_amiR2937: 25 µg/ml hygromycin (Thermo Fisher Scientific, Malaysia); pB2GW7_amiR854e: 10 µg/ml BASTA (Thermo Fisher Scientific, Malaysia); and co-overexpression pMDC32b_amiR2937 + pB2GW7_amiR854e: 25 µg/ml hygromycin (Thermo Fisher Scientific, Malaysia) and 10 µg/ml BASTA [Thermo Fisher Scientific, Malaysia]). The seedlings were planted in the soil after 14 days and grown there until six weeks old. The sampling was carried out once the plants reached six weeks of age. The leaves were chopped using a sterilized scissor into a prelabeled 50 ml Rnase-free Falcon tube. Each plant's healthy leaves were collected, immediately frozen in liquid nitrogen, and kept at -80°C until analysis.

Gene Expression Analyses

RNA Extraction and First-strand cDNA Synthesis. Total RNA from each line was isolated using PureLink Plant RNA Reagent (Life Technologies, USA) according to the manufacturer's protocol. The extracted, purified RNA samples were further diluted, and NanoDrop (Thermo Fisher Scientific, USA) was used to determine the RNA's purity. Following the manufacturer's instructions, the PhotoScript II First Strand cDNA Synthesis Kit (New England Biolabs, USA) was used to synthesize first-strand cDNA. The synthesized cDNAs were stored at -80°C for further analysis.

Validation and Expression Profile using RT-qPCR. RT-qPCR was carried out to quantify the abundance and expression of the miRNAs and their target transcripts in the terpenoid pathway under *A. thaliana* inoculation using Luna® Universal qPCR Master Mix (New England Biolabs, USA). Specific primers were designed to amplify *miR* genes and their target transcript where the expected cleavage site was between the forward and reverse primers. *Act 20* gene was used as an internal control in this analysis. Table 1 lists the designed primers used in the RT-qPCR analysis.

Secondary Metabolites Profiling using SPME-GCMS Analysis

Using solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GCMS) in three biological replicates, metabolite profiling of transgenic *A. thaliana* was performed during the sixth week of growth. *Arabidopsis thaliana* leaves were harvested (approximately 1 g) in liquid nitrogen and ground into small pieces. The pulverized tissues were then immediately put into labeled solid-phase microextraction (SPME) vials to prevent the evaporation of

Table 1
List of targeted gene primers for real-time quantitative polymerase chain reaction

Targeted genes primers	Sequence (5' – 3')	Amplicon size
ACT20-F	GAAAGTCTTGCGTTCACAAAGT	104 bp
ACT20-R	TGTAATAGCGTCTAACTCCGAATC	
MIR2937-F	AAGGAGTCATGATGATCACATTCG	70 bp
MIR2937-R	GAAGAGCCAATGATAAGAGCTGG	
GGPS2-F	GGTTGCTCCTTTAGTAGCTCTTA	90 bp
GGPS2-R	ACATAAACCCAACACACACAAACA	
MIR854e-F	GGATAGGGAGGAGGAGATGATGA	103 bp
MIR854e-R	GCCTCGGCCTTTTTCATTGTAG	
TPS13-F	TGCGTTCACAAAGTTGTCTCACT	70 bp
TPS13-R	CTCCGAATCATCGATGGGAACAGAG	

volatile chemicals. Screw caps were used to close the vials, which were then heated in a water bath to 65°C for 15 min. A septum cap introduced an SPME needle to gather volatile chemicals. To absorb the volatiles, SPME fiber (100 M polydimethylsiloxane, Sigma-Aldrich, USA), which was housed inside the SPME needle, was injected into the vials (Câmara et al., 2006). The collecting vial's SPME fiber equilibrium time was 30 min. at 55 to 60°C. The non-polar column was a diameter of HP-5MS (Agilent, USA, 30 m x 0.25 mm x 0.25 m). A splitless injection was set at 50°C held for 3 min, increased to 100°C at a rate of 20°C/min, and held at 250°C for 3 min. A GC-MS chromatogram was created using the results of a search of the NIST/EPA/NIH spectrum library (version 11) to locate the peaks.

Total Chlorophyll Content Analysis using SPAD-METER 502

When the plant reaches the age of 6 weeks, the chlorophyll content in the leaves is read using a SPAD-METER 502 (DS Technology, Malaysia). Readings were taken on 12 individual *A. thaliana* plants from the same line. The reading method was taken by reading two leaves on each plant three times, and the average reading was taken.

Total Chlorophyll Content Analysis Using Acetone Extraction Method

Chlorophyll extraction was performed following a protocol developed by (Liang et al., 2017). Two leaves from the previous analysis were cut into a circle using a cork borer. For at least 24 hr, the leaves were incubated in a 2 ml Eppendorf tube containing 1 ml of an 80% acetone solution (1Malaysia Bio Lab, Malaysia). The solution was then centrifuged at 1,957 x *g* for 5 min. At wavelengths of 646 and 663 nm (A_{646} and A_{663}), the absorbance of the supernatant was determined in this investigation. Samples with absorbance greater than 1 had their concentrations of 80% acetone cut in half before being re-analyzed. Following Arnon's equation, the amount of chlorophyll was calculated as follows:

Chlorophyll *a* (µg/ml) = 12.7 (A_{663}) – 2.69 (A_{645})

Chlorophyll *b* (µg/ml) = 22.9 (A_{645}) – 4.68 (A_{663})

Total chlorophyll (µg/ml) = 20.2 (A_{645}) + 8.02 (A_{663})

The area of the leaves was kept constant, and chlorophyll content and the total chlorophyll content per leaf area were expressed as ng/mm^2.

Gaseous Exchange

Gas exchange measurements were conducted using the Li-6800 portable gas exchange device (LI-COR, Malaysia). Photosynthetic rate readings were read on six plants for each line of transgenic and wild-type plants. All measurement data were collected between 8:00 a.m. and 12:00 p.m. Light saturation was set at 120 µmol photons/m^2/s, and carbon dioxide (CO_2) surrounding the leaves was set at 400 µmol/mol to induce photosynthesis rates. In addition, to maximize the stomatal aperture, the blue light was set to 10% of the active photosynthetic photon flux density. Measurements were made when the leaf temperature was ~25°C.

Statistical Analyses

A *t*-test was performed for statistical analysis to determine significant differences among the samples. Differences were taken as significant when the *P*-value was < 0.05.

RESULTS AND DISCUSSION

Selection and Confirmation of Transgenic Plants through Genomic PCR

Genomic PCR analysis was done when the plant had grown true leaves. The genomic DNA was extracted following the protocol prepared by (Kasajima et al., 2004). As shown in Figures 2, 3, and 4, the tested plants showed the correct amplicon size for the inserts: miR2937 insert with a band of 366 bp, and miR854e insert with a band of 312 bp. Meanwhile, the transgenic plant co-overexpressed both constructs and showed the correct amplicon size for both inserts, 312 bp and 366 bp. This step was crucial to ensure that the desired insert was copied into the genome of the transgenic plants.

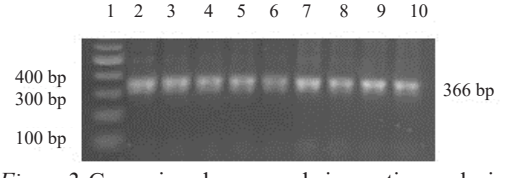

Figure 2. Genomic polymerase chain reaction analysis of transgenic plant harboring construct pMDC32b_ amiR2937 with the size of amplification 366 bp

Note. Lane 1 = 100 bp ladder (Invitrogen, USA); Lanes 2-10 = Transgenic plant harboring construct pMDC32b_amiR2937

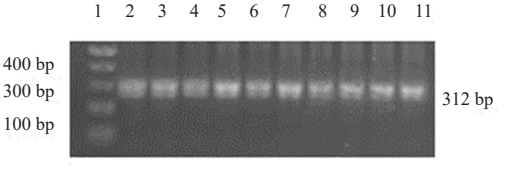

Figure 3. Genomic polymerase chain reaction analysis of transgenic plant harboring constructs pB2GW7_ miR854e with a size of amplification 312 bp

Note. Lane 1 = 100 bp ladder (Invitrogen, USA); Lanes 2-11 = Transgenic plant harboring construct pB2GW7_ miR854e

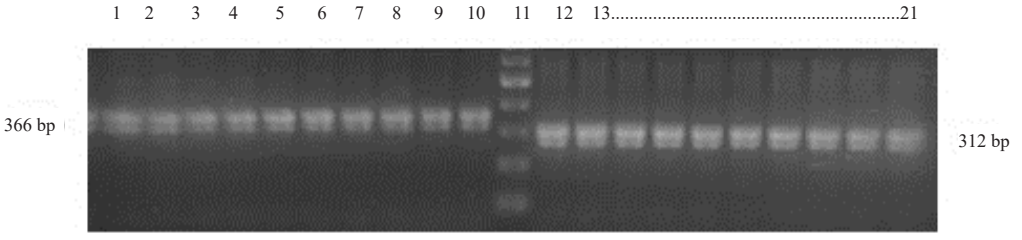

Figure 4. Genomic polymerase chain reaction analysis of transgenic plant harboring both constructs pMDC32b_amiR2937 and pB2GW7_amiR854e with a size of amplification 366 bp and 312 bp, respectively

Note. Lane 11 = 100 bp ladder (Invitrogen, USA); Lanes 1-10 and lanes 12-21 = Transgenic plant harboring both constructs pMDC32b_amiR2937 and pB2GW7_amiR854e, respectively

Expression Profiles of miRNAs and Their Target Transcripts by RT-qPCR

Quantitative expression analysis of pMDC32b_amiR2937 and pB2GW7_amiR854e and their target transcript, *GGPS2* and *TPS13*, were investigated in leaves of the *Arabidopsis* compared to the wild type. The specified miRNA's expression and target transcripts were experimentally validated using an RT-qPCR. The expression profile was shown in Figure 5, whereas pMDC32b_amiR2937 was upregulated by 0.89-fold. Meanwhile, co-overexpression of amiR2937 + amiR854e was significantly upregulated by 1.88-fold. Its target transcript, *GGPS2*, was significantly downregulated in both transgenic lines by 0.89- and 0.92-folds, respectively. The decrease in the accumulation of *GGPS2* mRNA in the transgenic plants overexpressing amiR2937 shows that amiR2937 has successfully suppressed the target (*GGPS2*) as compared to the wild type, where the abundance of amiR2937 and *GGPS2* was unaffected. The simplest outcome of the condensation reaction between DMAPP and IPP is C10 geranyl diphosphate (GPP), the precursor of monoterpenoids, which is controlled by GPPS present in plastids (Beck et al., 2013; Chen et al., 2015). An RT-qPCR study revealed that, among 17 different miRNAs, miR2937 showed a change in abundance in the pollen as compared to the leaves and had targeted the activator of spomin: luc2 (ASML2), which activates the promoter of sugar-inducible genes (Grant-Downton et al., 2009).

In Figure 6, the relative gene expression of amiR854e was significantly increased in both transgenic plants compared to the wild type. There was an upregulation by 1.81-fold in pB2GW7_amiR854e and 2.52-fold in amiR2937 + amiR854e. The transgenic lines that overexpress amiR854e were predicted to target *TPS13* genes. Accordingly, the result showed a downregulation of the target transcript by 0.84-fold in pB2GW7_amiR854e and 0.87-fold in amiR2937 + amiR854e. There is no such study where *TPS13* has yet been reported as target transcripts of miR854e. It is the first study to report that amiR854e overexpression caused the target transcript, *TPS13*, to be downregulated. The target, *TPS13*, was a type-a *Arabidopsis* TPS and a Class I protein. This Class I protein lacks

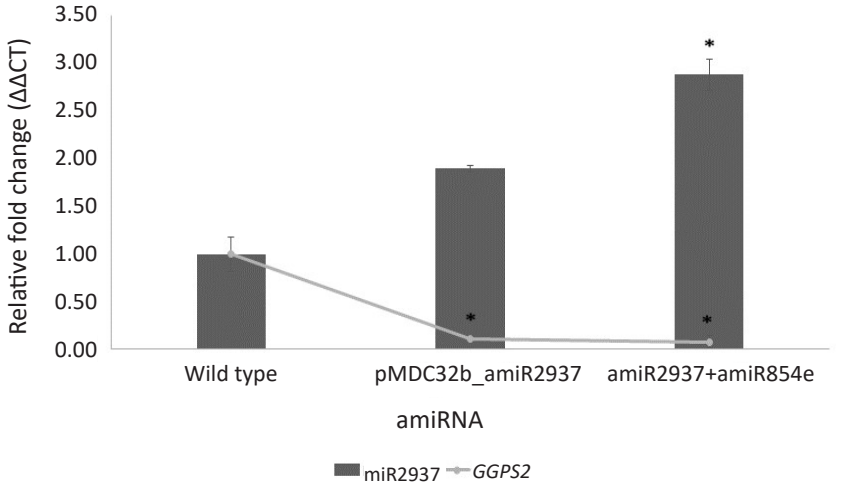

Figure 5. Graphical representation of relative gene expression of amiR2937 and *GGPS2* target transcripts in control and transgenic plants

Note. Asterisk(*) indicates the significant difference in gene expression towards control (*t*-test, $P < 0.05$)

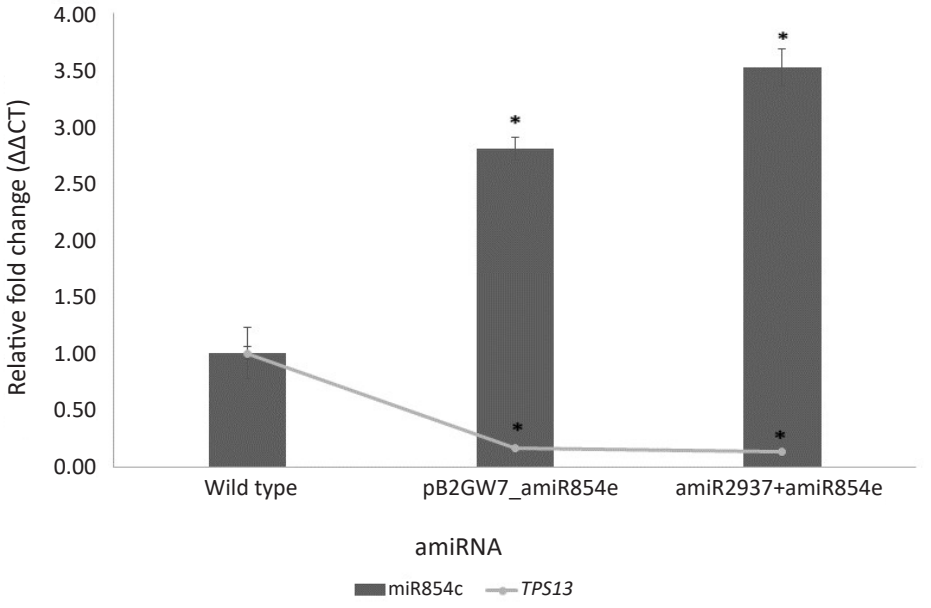

Figure 6. Graphical representation of relative gene expression of amiR854e and *TPS13* target transcripts in control and transgenic plants

Note. Asterisk(*) indicates the significant difference in gene expression towards the control (*t*-test, $P < 0.05$)

a y-domain and carries a non-functional N-terminal domain (Tholl & Lee, 2011). According to Tholl and Lee (2011), *TPS13* was included in four type-a *Arabidopsis* TPS genes (*TPS11*, *TPS12*, *TPS13*, and *TPS21*). Sesquiterpene synthases are proteins encoded by these TPS genes, which lack plastidial transit peptides.

Both *TPS12* and *TPS13* enzymes catalyzed the synthesis of the minor products (E)-nerolidol and (Z)-y-bisabolene, which are both derivatives of bisabolol (Ro et al., 2006). It was discovered that the animal and plant realms possess the miR854 gene. Previous research identified that miR854 in *A. thaliana* had homologs in animal and human genomes by one nucleotide difference. The miR894 targeted the UBP1 protein, which was involved in transcription (Arteaga-Vázquez et al., 2006). Besides, in *A. thaliana*, family miR854 consists of another four family members beginning with miR854a, miR854b, miR854c, and miR854d (Kozomara & Griffiths-Jones, 2014).

Terpenoid Profiling in Transgenic *A. thaliana* by SPME-GCMS Analysis

SPME-GCMS analysis was carried out to reveal the terpenoid content in transgenic *A. thaliana* compared to the wild type. The GC-MS result in Figure 7 above shows that the amount of phytol (a diterpenoid) in transgenic plants overexpressing pMDC32b_amiR2937 was reduced by 1.86%. The reduction was not significant, which may be due to the stability of the transgene and its ability to be expressed in plants. Many factors can affect how transgene expression varies between individuals. For instance,

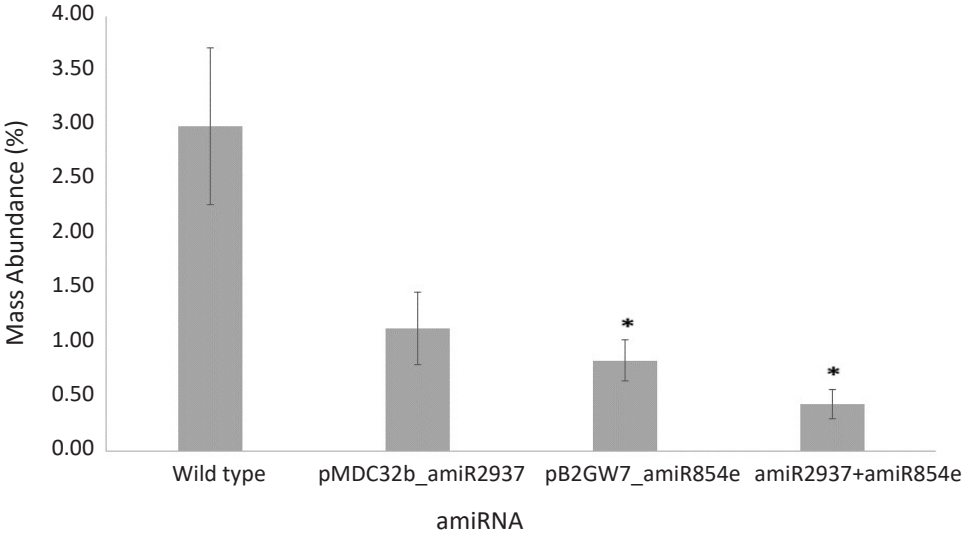

Figure 7. Graphical representation of changes in mass abundance of phytol in transgenic plants as compared to the wild-type

Note. Asterisk(*) indicates the significant difference in the abundance of phytol towards the control (*t*-test, $P < 0.05$)

promoters can influence the degree and diversity of transgene expression in different transformants (De Bolle et al., 2012). On the other hand, the amount of phytol in both transgenic plants harboring construct pB2GW7_amiR854e and amiR2937 + amiR854e were significantly downregulated by 2.16 and 2.56%, respectively.

Interestingly, based on the result, the abundance of phytol was greatly reduced in transgenic plants harboring amiR854e, even though the level of phytol is dependent on the level of *GGPP* transcript, which is regulated by amiR2937. According to Tholl (2015), TPS enzymes vary in their substrate specificity from using only a single prenyl diphosphate substrate to converting two or more substrates (e.g., GPP and FPP) *in vitro*. However, it depended on their subcellular localization. TPS enzymes located in plastids generally produce monoterpenes or diterpenes from the predominantly plastidial pools of GPP and GGPP, respectively. Meanwhile, TPSs in the cytosol primarily convert FPP to sesquiterpenes (or squalene in the biosynthesis of C_{30} terpenes) (Tholl, 2015).

The trans-β-ionone was a sesquiterpenoid compound with an increasing pattern compared to the wild-type plant. This compound is a precursor of carotenoids. This compound is of great biological value as it possesses anti-cancerous, antimicrobial, and anti-mutagenetic activities (Kang et al., 2013). From Figure 8, there was an up-regulation of the beta-ionone compound in all transgenic plants, and it was highest in transgenic plants overexpressing both

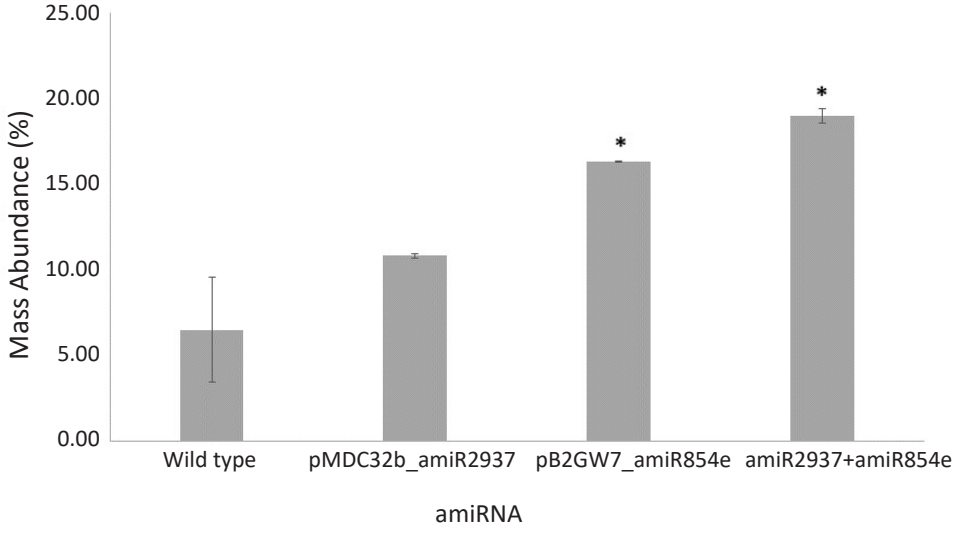

Figure 8. Graphical representation of changes in mass abundance of trans-beta-ionone in transgenic plants as compared to the wild-type

Note. Asterisk(*) indicates the significant difference in the abundance of trans-beta-ionone towards the control (*t*-test, $P < 0.05$)

constructs, amiR2937 + amiR854e, which was 12.49%. Based on the graph, trans-beta-ionone was slightly upregulated in the mutant, pMDC32b_amiR2937, by 4.3%. Meanwhile, in transgenic plants harboring construct pB2GW7_amiR854e, trans-beta-ionone was upregulated by 9.82%. From the analysis, transgenic plants that overexpressed amiR854e showed more upregulated trans-beta-ionone compounds compared to amiR2937. To our knowledge, *TPS13* was involved downstream of the MEP pathway of terpenoid biosynthesis. Theotherically, overexpression of miRNA in the plant would downregulate the production of the adjacent secondary metabolites. For example, the downregulation of sesquiterpene levels in *Pogostemon cablin* and *A. thaliana* is caused by the overexpression of miR-156.

Similarly, the overexpression of miR-393 alters glucosinolate and camalexin levels by disrupting the auxin signaling system (Yu et al., 2015). Therefore, the overexpression of miRNA in the plant definitely can change the production of the metabolites. However, whether the amount is up- or down-regulated depends on the pathway, which is still unknown. According to Tholl (2015), combinatorial mutations in downstream terpene synthases and prenyl diphosphate synthase are one method to increase pathway productivity. For instance, the level of the levopimaradiene product increased more than 2,000-fold when a route variation of a GGPPS and a terpene synthase that produces a levopimaradiene diterpene precursor was expressed in prokaryotes (Leonard et al., 2010).

Quantifying the Amount of Chlorophyll Content in *A. thaliana* Transgenic Plants by Using SPAD-METER 502 Reading

The relative soil plant analysis development (SPAD) values generated by the SPAD-502 meter are proportionate to the amount of chlorophyll in the leaf. In addition, the amount of chlorophyll in leaves is a general measure of chloroplast proliferation, photosynthetic ability, and leaf nitrogen content (Ling et al., 2011). This analysis aims to study the effect of miRNA overexpression in transgenic lines of *A. thaliana* on chlorophyll content. It should be noted that altering growing conditions, such as those that can cause a redistribution of chloroplasts inside mesophyll cells, can impact SPAD meter readings (Nauš et al., 2010). As a result, it is advised to refrain from comparing tests performed at different times and to always include an internal control as a point of comparison in every experiment. However, as the plants being studied are grown under the common, strict, and controlled circumstances employed here, the results should be accurate. The measuring area of the meter, which is 2 mm x 3 mm, is another thing to keep in mind.

However, by taking numerous measurements using various sections of the same leaf, this issue might be at least partially alleviated. From Figure 9, the SPAD value reading and chlorophyll content in pMDC32b_amiR2937 was significantly decreased by 1.644 nmol chl/cm^2. This transgenic plant overexpressed amiR2937, which was predicted to target *GGPS2*.

Theoretically, when the abundance of the miRNA increases, the *GGPS2* is predicted to decrease. As the *GGPS2* enzyme was involved in the MEP pathway and produced the product of chlorophyll, this result showed that the amount of chlorophyll content was decreased. It had been reported by Beck et al. (2013), in *A. thaliana*, there were twelve members of the GGPS reported being positioned in a variety of subcellular compartments, including the mitochondria, endoplasmic reticulum (ER), or chloroplast. The enzymatic product of trans-geranylgeranyl diphosphate synthase, (E,E,E)-GGPP was an important branch point enzyme because it served as a key precursor for a wide range of primary and specialized isoprenoid compounds, such as carotenoids and carotenoid breakdown products. Besides, for pB2GW7_amiR854e, the SPAD value was significantly decreased by 1.203 nmol chl/cm^2. These transgenic lines had overexpressed miR854e, which was predicted to target the *TPS13* gene. Meanwhile, in the transgenic lines that harbored both constructs, amiR2937 amiR854e, the SPAD value reading was decreased by 1.139 nmol chl/cm^2.

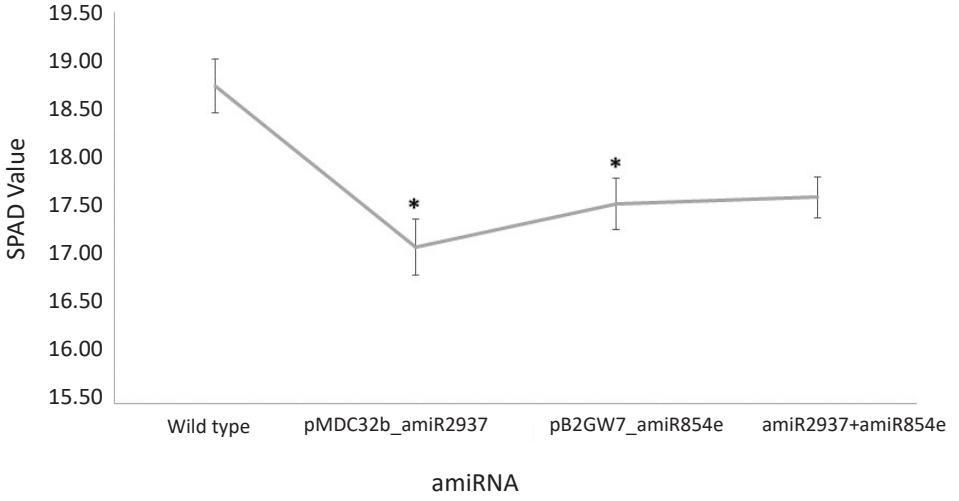

Figure 9. Graphical representation of soil plant analysis development (SPAD) value and chlorophyll content per leaf area in the transgenic plants compared to the wild-type

Note. Asterisk(*) indicates the significant difference in the chlorophyll content towards the control (*t*-test, $P < 0.05$)

Chlorophyll *a*/*b* and Total Chlorophyll Content by Acetone Extraction

This study analyzed the differences in chlorophyll *a*/*b* and total chlorophyll content between the transgenic plants and the wild type. The main photosynthetic pigment is chlorophyll *a* (chl *a*), while chlorophyll *b* (chl *b*) is merely an auxiliary pigment. Therefore, there was a huge difference between the content of chl *a* and chl *b* in

Figure 10. Chl *a* present in all plants, algae, bacteria, cyanobacteria, and phototrophs, but chl *b* only exists in green algae and plants. Figure 10 shows that all transgenic plants had much lower chl *a* and *b* levels than the wild-type plants. There was down-regulation of chl *a* in pMDC32b_amiR2937, pB2GW7_amiR854e, and amiR2937 + amiR854e by 2.661, 0.99, and 2.133 µg/ml, respectively. Meanwhile, chl *b* was downregulated by 0.770, 0.394, and 0.643 µg/ml.

Total chlorophyll content per leaf area in Figure 11 showed a decreasing pattern in all transgenic lines and was highest in pMDC32b_amiR2937 by 244.09 ng/mm^2. This result was consistent with the SPAD value above, showing a decreasing pattern in the same transgenic plants. Meanwhile, transgenic plants that harbor construct pB2GW7_amiR854e indicated the lowest total chlorophyll content by 81.48 ng/mm^2. Interestingly, total chlorophyll content in transgenic plants harboring double miRNA construct, amiR2937 + amiR854e, also decreased but was higher than pB2GW7_amiR854e by 192.37 ng/mm^2. Overall, the result indicated that amiR2937 may influence the total chlorophyll in the transgenic plant compared to the wild type. Moreover, the SPAD value result in Figure 9 also revealed that the transgenic plant harboring miR2937 construct has the lowest chlorophyll content per leaf area. As the target transcript, *GGPS2* was indirectly involved in the production of phytol in the terpenoid pathway; the overexpression of amiR2937 may affect the total chlorophyll production in the transgenic plant.

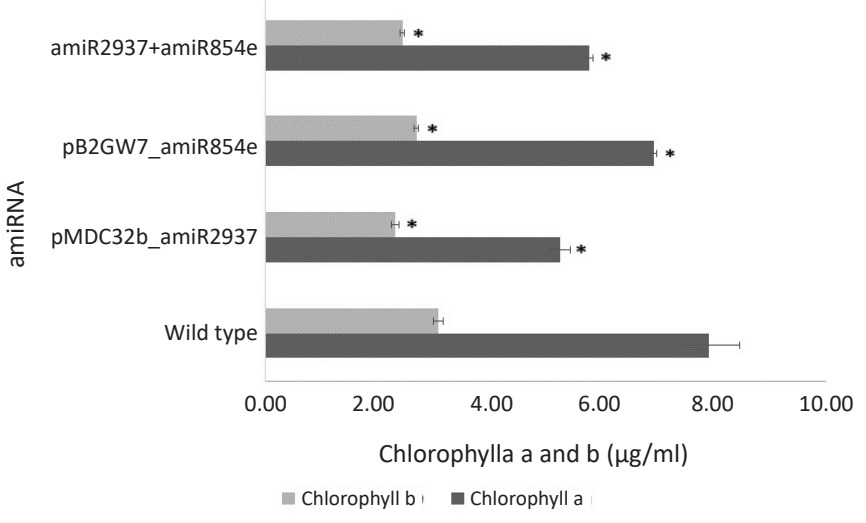

Figure 10. Graphical representation of chlorophyll *a* and *b* in transgenic plants compared to the wild type
Note. Asterisk (*) indicates the significant difference in the chlorophyll a/b towards the control (*t*-test, $P < 0.05$)

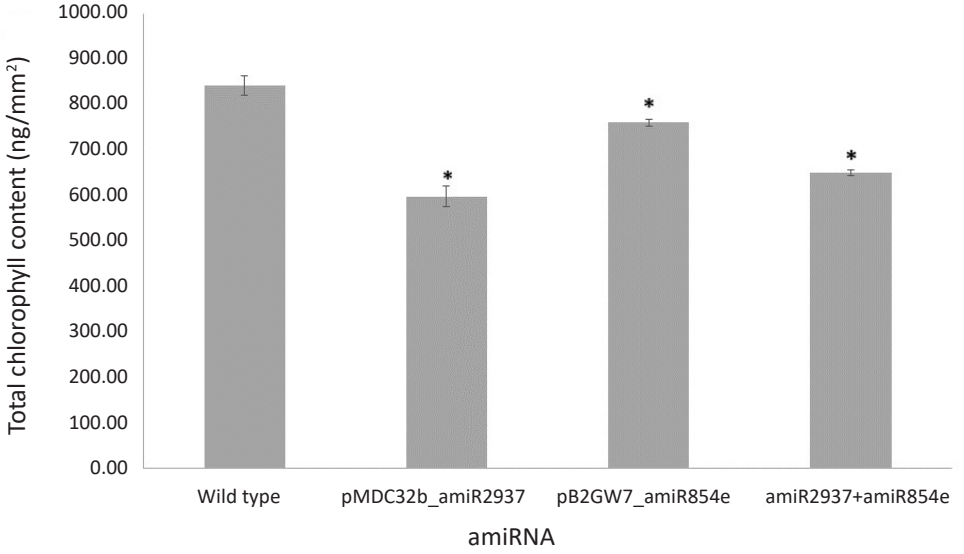

Figure 11. Graphical representation of total chlorophyll content in the transgenic plants compared to the wild-type

Note. Asterisk(*) indicates the significant difference in the total chlorophyll content towards the control (*t*-test, $P < 0.05$)

Effect of Phytol Content Degradation in *A. thaliana* Transgenic Plants Towards Gas Exchange Performances

As photosynthesis in plants occurs at its maximum during those hours, gaseous exchange analysis was carried out between 8 a.m. and 12 p.m. The rate of photosynthesis could be influenced by stomatal conductance, light intensity, and carbon dioxide concentration in the leaves (Feng et al., 2019). The plants were grown under constant conditions, light intensities, controlled surrounding temperature, relative humidity, and water supply throughout the analysis. These variables can affect the rate of photosynthesis in plants because diverse processes associated with growth and development depend on the interaction of intracellular organelles. In addition, the primary location for photosynthesis, the chloroplast, is extremely susceptible to a variety of adverse situations, including salinity, drought, extremely high or low temperatures, flooding, fluctuating light levels, and UV radiation (Saravanavel et al., 2011). Plus, phytol, a chlorophyll component, can surely influence gas exchange performances in plants.

Consistent with the degradation of phytol content, all transgenic plants showed a decreasing photosynthesis rate pattern, as shown in Figure 12. The gas exchange in pMDC32b_amiR2937, pB2GW7_amiR854e, and amiR2937 + amiR854e was slightly decreasing by 1.262, 1.364, and 1.144 µmol/m²/s, respectively. The photosynthesis rate was also consistent with the total chlorophyll content and

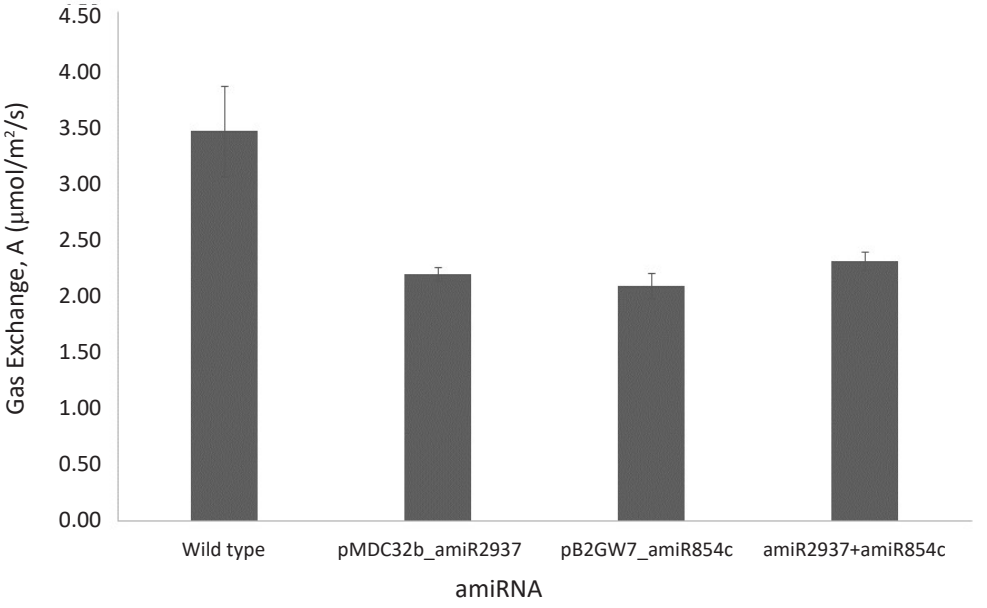

Figure 12. Graphical representation of gaseous exchange in transgenic plants compared to the wild-type
Note. Asterisk(*) indicates the significant difference in gaseous exchange towards the control (*t*-test, $P < 0.05$)

SPAD value result, which showed a decreasing pattern in all transgenic plants. Besides chlorophyll content, leaf stomatal conductance is essential for CO_2 uptake and desiccation prevention. Reduced leaf turgor, atmospheric vapor pressure, and chemical signals created by roots are the main causes of stomata closure in response to drought and salinity stress. Due to stomatal transport restrictions for gases, salt, and drought adversely influence photosynthesis and mesophyll metabolism (Chaves et al., 2009; Parida et al., 2005). Therefore, throughout the analysis, the stomata conductance was maintained and stable to minimize the technical error.

CONCLUSION

This study demonstrates that overexpression of amiR2937 and amiR854e in the transgenic plant of *A. thaliana* had successfully suppressed their target transcript, *GGPS2* and *TPS13*. All transgenic plants drastically reduced the target transcript levels compared to the wild type. The change in the abundance of phytol, which was the component of chlorophyll, is analyzed through GCMS analysis. Consistent with the downregulation of the target transcripts, the abundance of phytol in all transgenic plants showed a decreasing pattern compared to the wild type. Meanwhile, trans-β-ionone, a sesquiterpenoid compound, had increased abundance compared to the wild-type plant. The overexpression

of amiR854e revealed that it could affect trans-beta-ionone production when the amount was significantly increased in single and co-overexpression plants. The SPAD value analysis was consistent with the phytol metabolite result, which showed a decreasing pattern in the transgenic plant. SPAD value indicates chlorophyll content in the plant, and the overexpression of both miRNA, amiR2937 and amiR854e, had decreased its amount. Meanwhile, the photosynthetic activity and total chlorophyll content in the transgenic plants also decreased compared to the wild type. Moreover, both target transcripts, *GGPS2* and *TPS13*, were directly involved in the production of phytol (diterpenoid) in plants, and the overexpression of these miRNAs can affect its production. Based on this study, the expression of the downstream miRNA amiR854e has shown a significant change pattern in all the analyses carried out because the position of the downstream target transcript is more specific.

Next, this study suggests studying the functionality of other miRNAs involved in downstream pathways, such as miR5631, miR835-5p, and miR393a-5p. Furthermore, there is no other report on the functionality of the miRNA that has been done, so this research will pave the way to understanding the role of the miRNA in the terpenoid biosynthesis pathway. Many more secondary metabolites with high value in the market can be explored. Besides, the *Arabidopsis* genome contains a gene family of 11 predicted GGPSs (GGPS1-11) (Lange & Ghassemian, 2003) that were in different organelles: plastids, cytosol, endoplasmic reticulum, peroxisome, and mitochondria (Tholl & Lee, 2011). Using different algorithms, analysis of putative targeting sequences suggested five other *GGPS* isoforms to be plastidial proteins (*GGPS2*, 5, 10, 11). As the *GGPS2* enzymes were in the plastid, it is suggested that the other plastidial GGPS isoforms be studied as well. Moreover, an in-depth study of the GGPS function should be carried out by allocating the GGPP precursors and controlling the metabolic flux to distinguish terpene biosynthetic pathways.

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REFERENCES

Akula, R., & Ravishankar, G. A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling and Behavior*, *6*(11), 1720–1731. https://doi.org/10.4161/psb.6.11.17613

Ameres, S. L., & Zamore, P. D. (2013). Diversifying microRNA sequence and function. *Nature Reviews Molecular Cell Biology*, *14*, 475–488. https://doi.org/10.1038/nrm3611

Arteaga-Vázquez, M., Caballero-Pérez, J., & Vielle-Calzada, J. P. (2006). A family of microRNAs present in plants and animals. *The Plant Cell*, *18*(12), 3355–3369. https://doi.org/10.1105/tpc.106.044420

Bahi, A., Al Mansouri, S., Al Memari, E., Al Ameri, M., Nurulain, S. M., & Ojha, S. (2014). β-Caryophyllene, a CB_2 receptor agonist produces multiple behavioral changes relevant to anxiety and depression in mice. *Physiology and Behavior*, *135*, 119–124. https://doi.org/10.1016/j.physbeh.2014.06.003

Beck, G., Coman, D., Herren, E., Águila Ruiz-Sola, M., Rodríguez-Concepción, M., Gruissem, W., & Vranová, E. (2013). Characterization of the GGPP synthase gene family in *Arabidopsis thaliana*. *Plant Molecular Biology*, *82*, 393–416. https://doi.org/10.1007/s11103-013-0070-z

Bent, A. (2006). *Arabidopsis thaliana* floral dip transformation method. In K. Wang (Ed.), Agrobacterium *protocols: Methods in molecular biology* (Vol. 343, pp. 87–104). Humana Press. https://doi.org/10.1385/1-59745-130-4:87

Berthelot, K., Estevez, Y., Deffieux, A., & Peruch, F. (2012). Isopentenyl diphosphate isomerase: A checkpoint to isoprenoid biosynthesis. *Biochimie*, *94*(8), 1621–1634. https://doi.org/10.1016/j.biochi.2012.03.021

Câmara, J. S., Arminda Alves, M., & Marques, J. C. (2006). Development of headspace solid-phase microextraction-gas chromatography-mass spectrometry methodology for analysis of terpenoids in Madeira wines. *Analytica Chimica Acta*, *555*(2), 191–200. https://doi.org/10.1016/j.aca.2005.09.001

Carbonell, A., Takeda, A., Fahlgren, N., Johnson, S. C., Cuperus, J. T., & Carrington, J. C. (2014). New generation of artificial microRNA and synthetic trans-acting small interfering RNA vectors for efficient gene silencing in *Arabidopsis*. *Plant Physiology*, *165*(1), 15–29. https://doi.org/10.1104/pp.113.234989

Chaves, M. M., Flexas, J., & Pinheiro, C. (2009). Photosynthesis under drought and salt stress: Regulation mechanisms from whole plant to cell. *Annals of Botany*, *103*(4), 551–560. https://doi.org/10.1093/aob/mcn125

Chen, Q., Fan, D., & Wang, G. (2015). Heteromeric geranyl (geranyl) diphosphate synthase is involved in monoterpene biosynthesis in *Arabidopsis* flowers. *Molecular Plant*, *8*(9), 1434–1437. https://doi.org/10.1016/j.molp.2015.05.001

Chen, X. (2004). A microRNA as a translational repressor of *APETALA2* in *Arabidopsis* flower development. *Science*, *303*(5666), 2022–2025. https://doi.org/10.1126/science.1088060

Chowdhury, R. R., & Ghosh, S. K. (2012). Phytol-derived novel isoprenoid immunostimulants. *Frontiers in Immunology*, *3*, 49. https://doi.org/10.3389/fimmu.2012.00049

De Bolle, M., Beyers, W., De Clercq, B., & De Fruyt, F. (2012). General personality and psychopathology in referred and nonreferred children and adolescents: An investigation of continuity, pathoplasty, and complication models. *Journal of Abnormal Psychology*, *121*(4), 958–970. https://doi.org/10.1037/a0027742

de Menezes Patrício Santos, C. C., Salvadori, M. S., Mota, V. G., Costa, L. M., de Almeida, A. A. C., de Oliveira, G. A. L., Costa, J. P., de Sousa, D. P., de Freitas, R. M., & de Almeida, R. N. (2013). Antinociceptive and antioxidant activities of phytol *in vivo* and *in vitro* models. *Neuroscience Journal*, *2013*, 949452. https://doi.org/10.1155/2013/949452

Ditengou, F. A., Müller, A., Rosenkranz, M., Felten, J., Lasok, H., van Doorn, M. M., Legué, V., Palme, K., Schnitzler, J.-P., & Polle, A. (2015). Volatile signaling by sesquiterpenes from ectomycorrhizal fungi reprogrammes root architecture. *Nature Communications*, *6*, 6279. https://doi.org/10.1038/ncomms7279

Feng, L., Raza, M. A., Li, Z., Chen, Y., Khalid, M. H. Bin, Du, J., Liu, W., Wu, X., Song, C., Yu, L., Zhang, Z., Yuan, S., Yang, W., & Yang, F. (2019). The influence of light intensity and leaf

movement on photosynthesis characteristics and carbon balance of soybean. *Frontiers in Plant Science*, *9*, 1952. https://doi.org/10.3389/fpls.2018.01952

Grant-Downton, R., Trionnaire, G. L., Schmid, R., Rodriguez-Enriquez, J., Hafidh, S., Mehdi, S., Twell, D., & Dickinson, H. (2009). MicroRNA and tasiRNA diversity in mature pollen of *Arabidopsis thaliana*. *BMC Genomics*, *10*, 643. https://doi.org/10.1186/1471-2164-10-643

Huang, M., Sanchez-Moreiras, A. M., Abel, C., Sohrabi, R., Lee, S., Gershenzon, J., & Tholl, D. (2012). The major volatile organic compound emitted from *Arabidopsis thaliana* flowers, the sesquiterpene (*E*)-β-caryophyllene, is a defense against a bacterial pathogen. *New Phytologist*, *193*(4), 997–1008. https://doi.org/10.1111/j.1469-8137.2011.04001.x

Jin, W., Wang, J., Liu, C.-P., Wang, H.-W., & Xu, R.-M. (2020). Structural basis for pri-miRNA recognition by Drosha. *Molecular Cell*, *78*(3), 423-433.e5. https://doi.org/10.1016/j.molcel.2020.02.024

Kang, J., Liu, C., & Kim, S.-H. (2013). Environmentally sustainable textile and apparel consumption: The role of consumer knowledge, perceived consumer effectiveness and perceived personal relevance. *International Journal of Consumer Studies*, *37*(4), 442–452. https://doi.org/10.1111/ijcs.12013

Kasajima, I., Ide, Y., Ohkama-Ohtsu, N., Hayashi, H., Yoneyama, T., & Fujiwara, T. (2004). A protocol for rapid DNA extraction from *Arabidopsis thaliana* for PCR analysis. *Plant Molecular Biology Reporter*, *22*, 49–52. https://doi.org/10.1007/BF02773348

Kozomara, A., & Griffiths-Jones, S. (2014). MiRBase: Annotating high-confidence microRNAs using deep sequencing data. *Nucleic Acids Research*, *42*(D1), D68–D73. https://doi.org/10.1093/nar/gkt1181

Kwon, S. C., Baek, S. C., Choi, Y.-G., Yang, J., Lee, Y-S., Woo, J.-S., & Kim, V. N. (2019). Molecular basis for the single-nucleotide precision of primary microRNA processing. *Molecular Cell*, *73*(3), 505-518.e5. https://doi.org/10.1016/j.molcel.2018.11.005

Lange, B. M., & Ghassemian, M. (2003). Genome organization in *Arabidopsis thaliana*: A survey for genes involved in isoprenoid and chlorophyll metabolism. *Plant Molecular Biology*, *51*, 925-948. https://doi.org/10.1023/A:1023005504702

Laskovics, F. M., & Poulter, C. D. (1981). Prenyltransferase: Determination of the binding mechanism and individual kinetic constants for farnesylpyrophosphate synthetase by rapid quench and isotope partitioning experiments. *Biochemistry*, *20*(7), 1893-1901. https://doi.org/10.1021/bi00510a027

Leonard, E., Ajikumar, P. K., Thayer, K., Xiao, W.-H., Mo, J. D., Tidor, B., Stephanopoulos, G., & Prather, K. L. J. (2010). Combining metabolic and protein engineering of a terpenoid biosynthetic pathway for overproduction and selectivity control. *PNAS*, *107*(31), 13654-13659. https://doi.org/10.1073/pnas.1006138107

Li, T., Hasegawa, T., Yin, X., Zhu, Y., Boote, K., Adam, M., Bregaglio, S., Buis, S., Confalonieri, R., Fumoto, T., Gaydon, D., Marcaida, M., Nakagawa, H., Oriol, P., Ruane, A. C., Ruget, F., Singh, B., Singh, U., Tang, L., … Bouman, B. (2015). Uncertainties in predicting rice yield by current crop models under a wide range of climatic conditions. *Global Change Biology*, *21*(3), 1328–1341. https://doi.org/10.1111/gcb.12758

Liang, Y., Urano, D., Liao, K.-L., Hedrick, T. L., Gao, Y., & Jones, A. M. (2017). A non-destructive method to estimate the chlorophyll content of *Arabidopsis* seedlings. *Plant Methods*, *13*, 26. https://doi.org/10.1186/s13007-017-0174-6

Lim, S.-Y., Meyer, M., Kjonaas, R. A., & Ghosh, S. K. (2006). Phytol-based novel adjuvants in vaccine formulation: I. assessment of safety and efficacy during stimulation of humoral and cell-mediated immune responses. *Journal of Immune Based Therapies and Vaccines*, *4*, 6. https://doi.org/10.1186/1476-8518-4-6

Ling, Q., Huang, W., & Jarvis, P. (2011). Use of a SPAD-502 meter to measure leaf chlorophyll concentration in *Arabidopsis thaliana*. *Photosynthesis Research*, *107*, 209–214. https://doi.org/10.1007/s11120-010-9606-0

Liu, Z., Wang, J., Cheng, H., Ke, X., Sun, L., Zhang, Q. C., & Wang, H.-W. (2018). Cryo-EM structure of human dicer and its complexes with a pre-miRNA substrate. *Cell*, *173*(5), 1191-1203.e12. https://doi.org/10.1016/j.cell.2018.03.080

Martin, R. C., Liu, P.-P., Goloviznina, N. A., & Nonogaki, H. (2010). MicroRNA, seeds, and Darwin?: Diverse function of miRNA in seed biology and plant responses to stress. *Journal of Experimental Botany*, *61*(9), 2229–2234. https://doi.org/10.1093/jxb/erq063

McCourt, P., & Benning, C. (2010). *Arabidopsis*: A rich harvest 10 years after completion of the genome sequence. *Plant Journal*, *61*(6), 905–908. https://doi.org/10.1111/j.1365-313X.2010.04176.x

Nauš, J., Prokopová, J., Řebíček, J., & Špundová, M. (2010). SPAD chlorophyll meter reading can be pronouncedly affected by chloroplast movement. *Photosynthesis Research*, *105*, 265–271. https://doi.org/10.1007/s11120-010-9587-z

Parida, A. K., Mittra, B., Das, A. B., Das, T. K., & Mohanty, P. (2005). High salinity reduces the content of a highly abundant 23-kDa protein of the mangrove *Bruguiera parviflora*. *Planta*, *221*, 135–140. https://doi.org/10.1007/s00425-004-1415-2

Picaud, S., Olsson, M. E., Brodelius, M., & Brodelius, P. E. (2006). Cloning, expression, purification, and characterization of recombinant (+)-germacrene D synthase from *Zingiber officinale*. *Archives of Biochemistry and Biophysics*, *452*(1), 17–28. https://doi.org/10.1016/j.abb.2006.06.007

Ramirez-Estrada, K., Vidal-Limon, H., Hidalgo D., Moyano, E., Golenioswki, M., Cusidó, R. M., & Palazon, J. (2016). Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. *Molecules*, *21*(2), *182*. https://doi.org/10.3390/molecules21020182

Ro, D.-K., Paradise, E. M., Quellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C. Y., Withers, S. T., Shiba, Y., Sarpong, R., & Keasling, J. D. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature*, *440*, 940–943. https://doi.org/10.1038/nature04640

Ruppel, N. J., Kropp, K. N., Davis, P. A., Martin, A. E., Luesse, D. R., & Hangarter, R. P. (2013). Mutations in *GERANYLGERANYL DIPHOSPHATE SYNTHASE 1* affect chloroplast development in *Arabidopsis thaliana* (Brassicaceae). *American Journal of Botany*, *100*(10), 2074–2084. https://doi.org/10.3732/ajb.1300124

Ryu, K.-R., Choi, J.-Y., Chung, S., & Kim, D.-H. (2011). Anti-scratching behavioral effect of the essential oil and phytol isolated from *Artemisia princeps* Pamp. in mice. *Planta Medica*, *77*(1), 22–26. https://doi.org/10.1055/s-0030-1250119

Sapir-Mir, M., Mett, A., Belausov, E., Tal-Meshulam, S., Frydman, A., Gidoni, D., & Eya, Y. (2008). Peroxisomal localization of *Arabidopsis* isopentenyl diphosphate isomerases suggests that part of the plant isoprenoid mevalonic acid pathway is compartmentalized to peroxisomes. *Plant Physiology*, *148*(3), 1219–1228. https://doi.org/10.1104/pp.108.127951

Saravanavel, R., Ranganathan, R., & Anantharaman, P. (2011). Effect of sodium chloride on photosynthetic pigments and photosynthetic characteristics of *Avicennia officinalis* seedlings. *Recent Research in Science and Technology, 3*, 177-180.

Tholl, D. (2015). Biosynthesis and biological functions of terpenoids in plants. In J. Schrader & J. Bohlmann (Eds.), *Biotechnology of isoprenoids: Advances in biochemical engineering/ biotechnology* (Vol. 148, pp. 63–106). Springer. https://doi.org/10.1007/10_2014_295

Tholl, D., & Lee, S. (2011). Terpene specialized metabolism in *Arabidopsis thaliana*. *The Arabidopsis Book*, *9*, e0143. https://doi.org/10.1199/tab.0143

Wang, J., Lee, J. E., Riemondy, K., Yu, Y., Marquez, S. M., Lai, E. C., & Yi, R. (2020). XPO5 promotes primary miRNA processing independently of RanGTP. *Nature Communications*, *11*, 1845. https://doi.org/10.1038/s41467-020-15598-x

Yu, Z.-X., Wang, L.-J., Zhao, B., Shan, C.-M., Zhang, Y.-H., Chen, D.-F., & Chen, X.-Y. (2015). Progressive regulation of sesquiterpene biosynthesis in arabidopsis and patchouli (*Pogostemon cablin*) by the MIR156-targeted SPL transcription factors. *Molecular Plant*, *8*(1), 98–110. https://doi.org/10.1016/j.molp.2014.11.002

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Proximate Composition and Phytochemical Analysis of Malaysian *Liberica* sp. Coffee Bean and Its Pulp

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ABSTRACT

Arabica, Robusta, and Liberica are the three main coffee species cultivated globally. Liberica coffee is a minor species, accounting for less than 1% of global cultivation. Due to favorable climatic conditions in Malaysia, Liberica coffee dominates coffee production, accounting for 73%, while Robusta makes up the remaining 27%. Nevertheless, the substantial coffee production resulted in approximately 15 million tons of discarded skin and pulp, contributing to environmental pollution. This study was conducted due to insufficient information and research on the proximate composition and phytochemical compounds of the coffee bean and pulp from *Liberica* sp. This study aims to determine the proximate composition of coffee beans and pulp extracts from *Liberica* sp. and to identify the phytochemical composition using liquid chromatography-mass spectrometry (LC-MS) analysis. The nutritional values of carbohydrates, protein, crude fiber, crude fat, and ash were obtained using proximate analysis. Coffee beans exhibited the highest value for crude protein (11.96%) and crude fiber (11.83%), whereas coffee pulp has the highest significant value for moisture content (68.81%) and ash (7.31%). LC-MS analysis shows emmotin A and

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deoxymiroestrol were the major phytochemical compounds. These findings contribute to understanding the nutritional value and phytochemical compounds of coffee beans and pulp from *Liberica* sp. that may contribute to sustainable waste management and other applications in the food and beverage industry.

Keywords: Coffee bean, *Liberica* sp., phytochemical composition, proximate, pulp

INTRODUCTION

Coffee is the most demanding beverage worldwide, mostly cultivated at 600 and 2,500 m a.s.l. in Africa, South-East Asia, and Central and South America (Schenker et al., 2002). Arabica and Robusta, the main coffee species, constitute about 80 and 20% of the world's production, respectively, while Liberica is the least grown, with only 1% of cultivation. According to Ismail et al. (2014), the optimal growing temperature of the coffee plant in Malaysia ranges from 18 to 28°C, where Arabica is found as the minor species, while Liberica dominates about (73%), and Robusta (27%) are found to be the main cultivated species. Liberica is known for its robustness and flavor, similar to Robusta, while Arabica coffee has a sweet and caramel flavor profile.

Two primary methods are normally used for processing coffee cherries, including dry and wet. Coffee farmers often use the wet processing method to meet market demand and improve coffee production quality (Campos et al., 2020). In this approach, the coffee cherries underwent pulping, fermented, washed, and dried, with the skin and pulp removed and discarded as waste. The coffee cherries must be processed before being sold commercially. However, the widespread commercialization of coffee production can lead to ecological damage, as the waste from coffee production can cause environmental pollution (Geremu et al., 2016). Coffee pulp is usually disposed of in large landfills without undergoing any treatments. Consequently, this issue has received greater attention in recent years as researchers have sought ways to reduce environmental pollution by finding alternative uses for waste. Despite the significant quantities produced, numerous studies have proposed strategies to reduce environmental pollution from coffee production waste.

In Malaysia, *Liberica* sp. is the main species cultivated and is native to southern Johor. The soil in Johor makes it suitable for cultivation due to the humidity, heat, and clay-like soil conditions. The coffee industry generates a significant amount of solid and liquid byproducts, approximately 15 million tons globally (Kovalcik et al., 2018), which are sources of pollution if not properly disposed of (Nabais et al., 2008). The primary source of solid byproducts in the industry was coffee pulp and ground coffee. Coffee cherries contain about 43% of pulp, and of the 15 million tons of waste generated annually, 8.5 million tons are pulp. The residues may contain bioactive compounds that have potential

value for some alternatives to reuse the waste, including fertilizers, livestock feed, compost, and mushroom cultivation, as reported by Heeger et al. (2017).

In accordance with Al-Dhabi et al. (2017), coffee beans consist of a diverse range of chemicals associated with health, including antioxidants, melanoidins, diterpenes, phenolic compounds, vitamin precursors, and xanthine. Antioxidants are substances that safeguard living tissues against free radical damage. Adam et al. (2009) have reported that free radicals are molecules produced by incomplete oxygen reduction during aerobic respiration and have been associated with oxidative stress in several illnesses, including cancer, inflammatory, cardiovascular, and neurological disorders. The coffee extract showed high antioxidant and metal-chelating properties and has gained much attention recently. In Malaysia, some previous studies have been done on *Liberica* sp. but were focused on the physical properties of coffee beans, the antioxidant activity of coffee pulp, and the physicochemical properties of coffee silver skin (Buyong & Nillian, 2023; Ismail et al., 2014; Nillian et al., 2020). However, no study is still focusing on proximate analysis and phytochemical composition of coffee beans and pulp from *Liberica* sp., thus leading to this study. Hence, this research intends to determine the proximate analysis of coffee beans and pulp from *Liberica* sp. and identify the phytochemical composition of this coffee species using LC-MS analysis.

MATERIALS AND METHODS

Preparation of Samples

The coffee fruits were obtained from the Jabatan Pertanian Batu Pahat, Johor. The collected fruits were thoroughly washed and separated to obtain the bean and pulp, as shown in Figures 1 (a) and (b). The bean and pulp were dried and ground into fine powder with approximately 200 µm particle size. The fresh sample of coffee beans and pulp was used to analyze moisture. In contrast, the ground sample was used to analyze protein, crude fat, crude fiber, carbohydrate, and ash using the method described by AOAC INTERNATIONAL (1990), Chang (2003), James (1995), as well as Kirk and Sawyer (1991). Each sample was repeated thrice for each analysis.

(a) (b)

Figure 1. (a) Coffee bean; and (b) coffee pulp after being separated from coffee fruit of *Liberica* sp.

Proximate Analysis of Coffee Bean and Coffee Pulp

Determination of Moisture Content. This procedure used the gravimetric method described by AOAC INTERNATIONAL (1990). The 5 g sample was measured and oven-dried at 105°C for six days until a constant weight was obtained. It was weighed after being cooled to room

temperature at 25°C. The drying, cooling, and weighing process were repeated until stable weight was achieved. The moisture percentage was calculated based on the difference in weight between the samples before and after drying.

$$\text{Moisture (\%)} = \frac{\text{(Weight of sample before drying)} - \text{(Weight of sample after drying)}}{\text{Weight of sample before drying}} \times 100\%$$

Determination of Protein. Kjedahl's method was carried out according to Chang (2003). The total nitrogen content was multiplied by a factor of 6.25. In a digestion flask, 10 ml of concentrated sulfuric acid (H_2SO_4, R&M Chemicals, United Kingdom) was mixed with 5 g of the sample and a selenium catalyst tablet was added. Beneath the fume cupboard, the solution was subsequently heated until a clear solution was achieved. The digest was diluted in a volumetric flask to a volume of 100 ml prior to starting the analysis. In the Kjedahl distillation apparatus, an equivalent volume of 45% sodium hydroxide (NaOH, R&M Chemicals, United Kingdom) was mixed with 10 ml of the digest, and three drops of a mixed indicator (bromocresol green/methyl red, Sigma-Aldrich, USA) were added afterward upon collection of the distillate into 10 ml of 40% boric acid (H_3BO_3, R&M Chemicals, United Kingdom). In total, 50 ml of distillates were collected and titrated against 0.02 N ethylenediamine tetraacetic acid (EDTA, Sigma-Aldrich, USA), starting from a green endpoint to a deep red endpoint. A blank reagent was further digested, distilled, and titrated. The nitrogen content was calculated using the following formula:

$$\text{Nitrogen (\%)} = \frac{100\%}{w} \times \frac{N \times 14}{1000} \times \frac{Vt}{Va} \times t.b$$

where, w = Sample weight (0.5 g), N = Titrant normality (0.02 N H_2SO_4), Vt = Total digest volume (100 ml), Va = Volume of digest analyzed (10 ml), t = Titrate sample value, and b = Blank titer value.

Determination of Total Ash Content. According to AOAC INTERNATIONAL (1990) and James (1995), this study used a furnace incinerating process, where 5 g of the sample was placed into a weighed porcelain crucible and was burnt at 550°C in a muffle furnace until completely burned to ash. The weight of the ash was estimated as a percentage of the sample weight after cooling and weighing.

$$\text{Ash (\%)} = \frac{\text{(Weight of crucible with ash)} - \text{(Weight of crucible)}}{\text{Weight of sample}} \times 100\%$$

Determination of Crude Fiber. The crude fiber was analyzed using the procedure of James (1995), which involved refluxing a 5 g sample in 150 ml of 1.25% H_2SO_4 solution

(R&M Chemicals, United Kingdom) for 30 min. A double cloth was used to collect the particles, which were washed with hot water in several portions. Under the same condition, it was mixed with 150 ml of 1.25% NaOH (R&M Chemicals, United Kingdom) in the flask and boiled again for another 30 min. The sample was rinsed with several parts of water and then allowed to drain until dry. It was then quantitatively transferred to a weighed crucible, where it was dried in the oven at 105℃ until no further changes in weight. The sample burnt where it was, leaving only ashes, and was transferred to a muffle furnace. The difference in the samples' weight determined the crude fiber's weight.

$$\text{Crude fibre (\%)} = \frac{w2 - w3}{\text{Weight of sample}} \times 100\%$$

where $w2$ = Weight of the crucible with the sample after washing, boiling, and drying, and $w3$ = Weight of the crucible with a sample of ash.

Determination of Crude Fat. The determination of crude fat was done using a previous method by Kirk and Sawyer (1991). A gravimetric solvent extraction procedure was used where 5 g of samples were placed in the thimble after being wrapped in porous paper (Whatman filter paper). The thimble was put in a Soxhlet extractor (Gerhardt, United Kingdom) and 200 ml of petroleum ether (R&M Chemicals, United Kingdom) as solvent. The process of solvent was heating, evaporating, and condensing in the reflux flask, where the water condenser was connected to the top part of the reflux. The oil extract was then poured into the boiling flask after the sample in the thimble was submerged in the solvent until the reflux flask was full. This process was repeated for 8 hr. The oil extracts flask was oven-dried at 60℃ for 24 hr to remove the residual solvent. Once the flask was cooled to room temperature, the flask was weighed to determine the percentage of crude fat according to the formula:

$$\text{Fat (\%)} = \frac{(\text{Weight of flask with oil extracts}) - (\text{Weight of empty flask})}{\text{Weight of sample}} \times 100\%$$

Determination of Carbohydrate. The percentage of carbohydrates was determined by the difference of moisture, protein, ash, crude fat, and crude fiber of 100% using the following equation below:

$$\text{Carbohydrate (\%)} = 100\% - (\text{Moisture} + \text{protein} + \text{ash} \_ \text{crude fiber} + \text{crude fat})\%$$

Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis of Coffee Pulp and Coffee Bean

Sample Extraction Preparation. Methanol was used as a solvent to extract compounds in the coffee beans and coffee pulp according to the method previously described by Redfern et al. (2014). Initially, both samples

were dried, and mortar and pestle were used to acquire powdered samples. The resulting powder was placed in a thimble and put into a Soxhlet extractor apparatus (Gerhardt, United Kingdom). A quantity of 200 ml of methanol (R&M Chemicals, United Kingdom) as solvent was added to the apparatus. It was subjected to heat with isomantle and allowed to evaporate. The evaporated solvent was poured back, and the cycle was repeated for 8 hr. Upon completion of the process, the solvent was removed, leading to a yield of approximately 2 to 3 ml of extracted plant material.

LC-MS Analysis. The LC-MS system, which consists of an Agilent 1290 Infinity LC system linked to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source (Agilent Technologies, USA), was used in this study. The instrument was run using an electrospray (ES) interface in positive ion mode. The Agilent Zorbax Eclipse XDB-C18 Analytical, 150 mm x 4.6 mm, 5 microns (P/N: 993967-902) (Thermo Fisher Scientific, USA) was utilized with a 40℃ of column temperature and flow rate of 0.4 ml/min, and liquid chromatography was carried out on 3.0 µl of the sample at a concentration 1 mg/ml. The mobile phase was made up of 0.1% formic acid (R&M Chemicals, United Kingdom) in water as Solvent A, while 0.1% formic acid (R&M Chemicals, United Kingdom) in methanol (R&M Chemicals, United Kingdom) as Solvent B with a 24-min runtime was operated in the gradient mode.

The mass spectra were obtained in positive ionization mode with a range of m/z 100 to 3,200. The parameters of the MS scan were the positive ion capillary voltage of 4,000 V, the voltage of the fragmentor was 125 V, and the voltage of the skimmer was 65 V. The nebulizer pressure and flow rate were adjusted to 45 psi and 10 L/min, respectively, and a temperature of 300℃ drying gas was used. The software used to analyze the MS data was Agilent Masshunter Qualitative Analysis (version B.07.00, Agilent Technologies, Germany), which generated a list of potential compounds with more than 100 counts of peak-only using the molecular feature extraction (MFE) algorithm. The compounds with a relative height of more than 2.5% and an absolute height greater than 5,000 counts were included. The permitted ionic species for positive ions were hydrogen (H^+), sodium (Na^+), potassium (K^+), and ammonium (NH_4^+), whereas chloride (Cl^-) was allowed for negative ions. With a high score indicating a closer resemblance, the software also provided a score (%) to demonstrate how closely the software matched the actual formula with the created molecular formula of the compound.

Statistical Analysis

The obtained data were statistically analyzed using an analysis of variance (ANOVA) *t*-test, and the result was subsequently presented in mean ± standard deviation (SD) using SPSS Statistic 27.

RESULTS AND DISCUSSION

Proximate Analysis of Coffee Bean and Coffee Pulp from *Liberica* sp.

The proximate composition of coffee bean and pulp extracts is shown in Table 1. The data shows that coffee bean and pulp extracts have the highest value of moisture (60.39 and 68.81%) and crude protein (11.96 and 9.07%) but the lowest value of fat content (4.28 and 0.80%). The moisture content level is important for the preservation of coffee and affects the quality of coffee (Ismail et al., 2013). In the previous study reported by Zainol et al. (2020), the moisture content of three different species of coffee beans ranged from 61.83 to 66.51%, where the moisture content of Liberica coffee was 61.83%. Similar research has been conducted by Rohaya et al. (2023), who reported that the moisture content of coffee pulp from Indonesia from three different species ranged from 67.61 to 79.49%, where Arabica coffee has the highest moisture content. The findings show that the moisture level was influenced by the conditions of the location cultivation, species variety and the processing technique (Harahap, 2017).

The ash content of coffee beans and pulp found in this study was 3.42 and 7.31%. The ash content is commonly associated with many mineral compounds, which is crucial in determining the nutritional value. Coffee represents the number of essential minerals such as magnesium (6.4 to 7.5%), potassium (6.6%), phosphorus (2.2%), sodium (2.2%), and calcium (0.7%), which play an important role in the body metabolism (Olechno et al., 2021; Sena et al., 1998). The ash content of coffee beans from this study was higher than that of three different coffee species from a previous study by Zainol et al. (2020). Meanwhile, the previous study by Rohaya et al. (2023) reported that Liberica coffee (1.74%) has the highest ash content than Arabica (0.25%) and Robusta (0.52%). The differences in the ash content are due to the nutrients and minerals available in the environmental climate (Oliveira et al., 2013). Due to the different roles and purposes, coffee beans serve as the energy-rich seed for plant growth, while coffee pulp provides nutrients and moisture to support the bean's development.

The results show that coffee bean has a high amount of fat compared to coffee

Table 1
Proximate analysis of coffee bean and coffee pulp

Composition	Coffee pulp (w/w, %)	Coffee bean (w/w, %)
Moisture	68.81 ± 0.10	60.39 ± 1.23
Ash	7.31 ± 0.05	3.42 ± 0.02
Fat	0.80 ± 0.20	4.28 ± 0.23
Crude protein	9.07 ± 0.55	11.96 ± 0.27
Crude fiber	7.48 ± 0.06	11.83 ± 0.06
Carbohydrate	6.77 ± 0.82	8.12 ± 1.39

Note. Values are presented as mean ± SD

pulp, which is 4.28 and 0.80%, respectively. Wibowo et al. (2022) have stated that fat found in the coffee bean serves as a protective bean and will form a layer of oil when roasted. Fat content is one of the chemical compositions that influence the coffee flavor. This finding on the ash content of coffee pulp is aligned with the previous study by Rohaya et al. (2023), where the fat content of coffee pulp from three different species ranged from 0.30 to 1.01%. In the study, it was reported that Robusta coffee pulp has the highest amount of fat content. Meanwhile, the previous study by Zainol et al. (2020) reported that Liberica coffee has the highest fat content, which is 2.79%, comparable to Arabica with 1.38% and Robusta with 1.67%. The differences in fat content can be caused by various factors, including species variety, parts of the plants, processing methods, and storage conditions (Mussatto et al., 2011; Wibowo et al., 2022). Coffee beans from *Liberica* sp. have higher protein content than coffee pulp, which is 11.96 and 9.07%.

Protein is also one of the chemical components that influence the taste and the nutritional value. The study reported that the protein content of coffee beans from three different species ranged from 5.15 to 7.20%, with Liberica coffee having the highest protein content while Arabica coffee has the lowest value of protein content of 5.15% (Zainol et al., 2020). According to a previous study by Rohaya et al. (2023), the amount of protein ranged from 4.08 to 7.88%, where Robusta coffee pulp has the highest protein content. This finding shows that coffee pulp from Liberica coffee can be used in animal feed such as ruminants, supported by Núñez et al. (2015).

The amount of crude fiber in this study was lower than that of coffee beans and pulp reported by the previous study. Zainol et al. (2020) reported that the crude fiber of coffee beans from three different species was 19%, with Arabica coffee having the highest amount at 19.82%. Meanwhile, the crude fiber of coffee pulp from Rohaya et al. (2023) ranged from 13.79 to 38.51%, whereas Liberica coffee has the highest amount of crude fiber. Crude fiber is a residue derived from materials containing cellulose with a small amount of lignin and pentose (Sitohang & Pandiangan, 2022). The differences in the amount of crude fiber of the coffee bean and pulp were influenced by the type of coffee species and the elevation of coffee cultivation (Rohaya et al., 2023).

The number of carbohydrates in coffee beans in this study was similar to the previous study by Zainol et al. (2020), in which the range of carbohydrates from three different species was 6.22 to 8.91%, where Liberica coffee was the highest. Carbohydrates, on the other hand, contribute to bodily metabolism by providing the requisite energy. The amount of carbohydrates in coffee beans was higher than in coffee pulp. Consequently, the coffee bean has the potential to serve as a significant reservoir of both energy and fiber essential for bodily functions (Mohd Jailani et al., 2020; Zaidan et al., 2019).

LC-MS Analysis of Coffee Bean and Coffee Pulp

The LC-MS chromatogram shows a total of 11 compounds in both coffee bean and pulp extracts, including several phytochemicals that comprised numerous phytochemicals displaying mass that matched (5 ppm tolerance) in the database. The compound classes in both extracts dominantly belonged to phenolic acids, terpenes, coumarins, and stilbenes. As shown in Tables 2 and 3, emmotin A is the highest compound found in coffee bean and pulp extracts, as shown by peak 10 in Figure 2 and peak 8 in Figure 3. Emmotin A belongs to the terpenes compound class, and Chu et al. (2016) have reported that Arabica coffee contains a large number of terpenes containing 16-O-methyl coffee, which has been used as a marker to distinguish small and medium coffee. According to Saleem et al. (2020), emmotin A has been identified as a primary source of natural antioxidants and enzyme inhibitor compounds, which is most likely contributing to the antioxidant properties in both coffee bean and pulp extracts. Deoxymiroestrol shows the highest number of compounds after emmotin A in the coffee bean and pulp extracts, according to Tables 2 and 3, as shown by peak 9 in Figure 2 and peak 7 in Figure 3. Deoxymiroestrol is a phytoestrogen compound class with a health benefit, including reproductive health, heart health, weight loss, hormone-dependent tumors, and immune systems (Desmawati & Sulastri, 2019). Based on the findings, Liberica coffee would be a useful alternative as animal feed because coffee beans and pulp have phytoestrogen compounds, which have beneficial effects such as stimulating

Table 2
Bioactive compounds of coffee bean extract from Liberica sp. *were identified using liquid chromatography-mass spectrometry*

Peak	Name	Formula	Difference (MFG. ppm)	RT	Vol (%)	Score (MFG)	Score (DB)	Difference (DB. ppm)
1	2-amino-3-methyl-1-butanol	$C_5H_{13}NO$	0.86	3.684	2.47	99.82	99.82	0.86
2	Nigerose	$C_{12}H_{22}O_{11}$	1.11	4.115	0.54	94.18	89.45	1.11
3	Scopolin	$C_{16}H_{18}O_9$	-0.01	6.761	0.69	99.62	99.62	-0.01
4	Scopolin	$C_{16}H_{18}O_9$	-0.15	7.361	6.46	99.84	99.84	-0.15
5	Cis-5-caffeoylquinic acid	$C_{16}H_{18}O_9$	1.15	7.37	0.63	99.06	99.03	1.15
6	3-O-feruloylquinic acid	$C_{17}H_{20}O_9$	1.49	7.971	4.35	98.93	98.91	1.49
7	3-O-feruloylquinic acid	$C_{17}H_{20}O_9$	0.35	8.102	1.39	99.31	99.31	0.35
8	Hydroxyamobarbital	$C_{11}H_{18}N_2O_4$	-1.06	11.557	0.33	91.33	91.33	-1.06
9	Deoxymiroestrol	$C_{20}H_{22}O_5$	0.46	11.829	9.06	99.85	99.84	0.46
10	Emmotin A	$C_{16}H_{22}O_4$	0.34	11.93	23.55	99.83	99.83	0.34
11	Trp Ile Lys	$C_{23}H_{35}N_5O_4$	0.02	12.589	0.63	95.11	95.11	0.02

Note. MFG = Molecular Formula Generator; RT = Retention Time; Vol = Volume; DB = Database

Table 3
Bioactive compounds of coffee pulp extract from Liberica sp. *were identified using liquid chromatography-mass spectrometry*

Peak	Name	Formula	Difference (MFG. ppm)	RT	Vol (%)	Score (MFG)	Score (DB)	Difference (DB. ppm)
1	D-glucoside	$C_7H_{14}O_6$	1.63	3.78	3.82	98.51	97.64	1.62
2	Quinic acid	$C_7H_{12}O_6$	-0.46	4.048	4.39	98.51	98.51	-0.46
3	(2S,3S)-2,3-Dihydro-2,3-dihydroxybenzoate	$C_7H_8O_4$	1.65	4.05	0.23	91.07	91.06	1.66
4	Penicilic acid	$C_8H_{10}O_4$	1.70	5.446	0.28	97.47	97.47	1.70
5	L-galactose	$C_6H_{12}O_6$	-1.84	6.882	0.56	95.76	95.76	-1.84
6	Lucuminic acid	$C_{19}H_{26}O_{12}$	0.52	7.995	0.17	99.04	99.03	0.52
7	Deoxymiroestrol	$C_{20}H_{22}O_5$	0.77	11.833	6.68	99.72	99.71	0.77
8	Emmotin A	$C_{16}H_{22}O_4$	1.37	11.934	25.63	99.39	99.36	1.37
9	Nonoxynol-9	$C_{33}H_{60}O_{10}$	0.38	12.539	0.51	99.10	99.09	0.38
10	Digitoxigenin	$C_{23}H_{34}O_4$	1.69	12.577	0.42	95.66	95.66	1.69
11	Trp Ile Lys	$C_{23}H_{35}N_5O_4$	-0.69	12.595	0.98	99.29	99.28	-0.69

Note. MFG = Molecular Formula Generator; RT = Retention Time; Vol = Volume; DB = Database

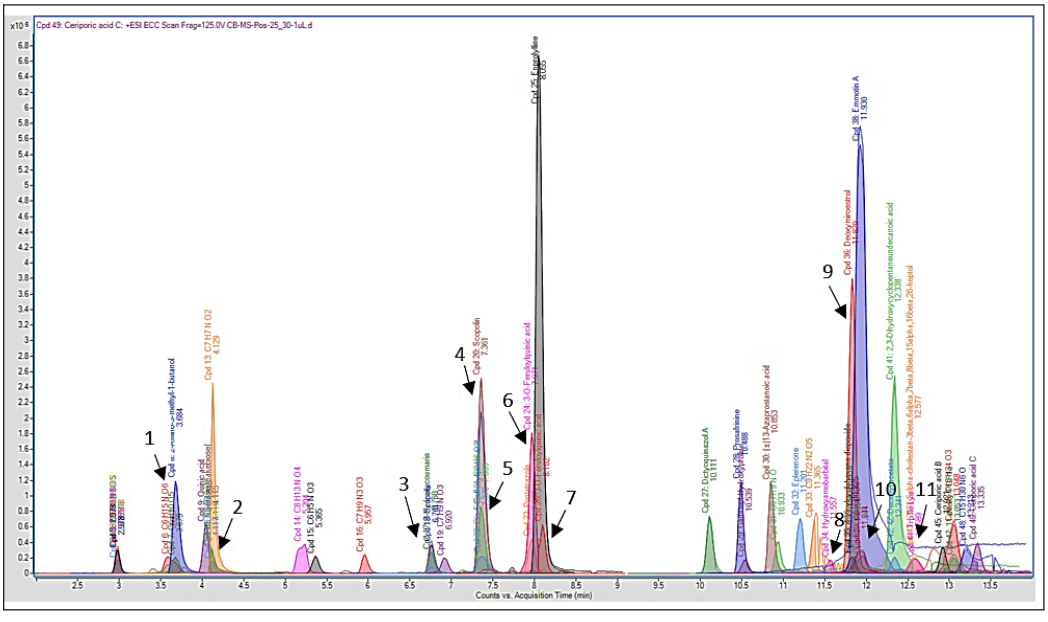

Figure 2. Total ion chromatogram from coffee bean extract of *Liberica* sp.

growth rate and increasing weight gain in livestock (Pace et al., 2006).

Based on Table 2, scopolin was a major compound in the coffee bean extracts, as shown by peaks 3 and 4 in Figure 2. Scopolin is a derivative of coumarins, which plays an important role in being present in high concentrations in several dietary

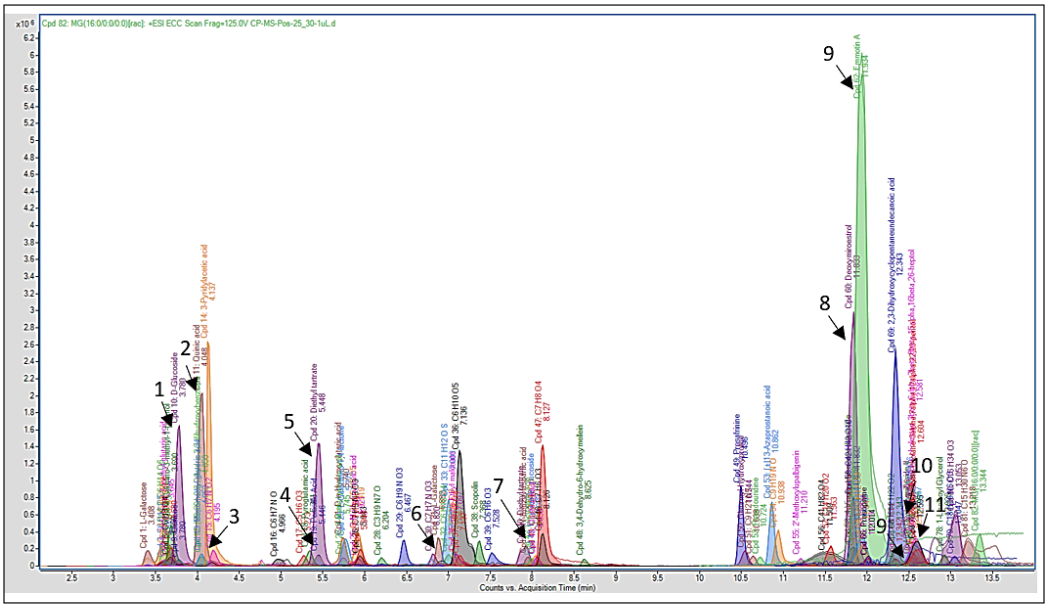

Figure 3. Total ion chromatogram from coffee pulp extract of *Liberica* sp.

plant species. Coumarin is a phytochemical compound tested for pharmacological properties such as anti-inflammatory, antioxidant, antimicrobial, antidepressant, neuroprotective, or antitumoral effects (Srikrishna et al., 2018). According to this finding, coffee beans from Liberica can be used as a nutraceutical product as coumarins were found in some widely used foods such as tea and coffee (Lončar et al., 2020). According to Table 2, coffee bean extract from Liberica has chlorogenic acids, which are cis-5-caffeoylquinic acid and 3-O-feruloylquinic acid, as shown in Figure 2 by peaks 5, 6 and 7. Chlorogenic acid is the main phenolic compound, which has the biological functions of lowering blood lipid, antioxidant, and antibacterial (Asamenew et al., 2019).

Table 3 shows that coffee pulp has a D-glucoside bioactive compound, as shown by peak 1 in Figure 3. The glucoside is a glycoside which is chemically derived from glucose. This finding was aligned with the previous study by Murthy et al. (2012), which reported that Arabica coffee pulp also has glucoside compounds. The findings of this study align with the previous research by Yulianti et al. (2023), which focuses on green beans in Arabica coffee and possesses reported similar compound classes. To be hypothesized, coffee bean and pulp extracts may contain bioactive compounds similar to those of the same species. Different coffee bean and pulp compounds resulted from their specialized roles, biochemical processes, and environmental interaction.

Using coffee beans and pulp from *Liberica* sp. as a food ingredient is essential to characterize these compounds. In this aspect, the application of chromatographic techniques capable of differentiating the

bioactive compounds in the methanolic extracts of coffee bean and pulp from *Liberica* sp. positive ionization modes was performed with a quadrupole time-of-flight mass spectrometry (Q-TOF-MS) coupled to LC system equipped with dual electrospray ionization source (ESI) of methanolic extracts.

CONCLUSION

The proximate and phytochemical compounds of coffee bean and pulp extracts provide valuable insights into these two components' distinct composition and functional roles within the coffee fruit. Based on the findings, coffee beans exhibited high contents of crude protein, crude fiber, carbohydrates, and fats, emphasizing the beans' energy reserves. In contrast, coffee pulp shows a high content of moisture and ash as the supportive nutritional role for the growth of the bean. On the other hand, LC-MS analysis determines that emmotin A and deoxymiroestrol are the major phytochemical compounds in coffee beans and pulp, contributing to coffee's overall taste, aroma, and nutritional value. Overall, these findings enhance the understanding of the nutritional value and phytochemical compounds in coffee beans and pulp from *Liberica* sp., which could be beneficial for use as a food ingredient or other applications in the food and beverages industry, contributing to sustainable waste management.

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REFERENCES

Adam, M., Dobiáš, P., Eisner, A., & Ventura, K. (2009). Extraction of antioxidants from plants using ultrasonic methods and their antioxidant capacity. *Journal of Separation Science*, *32*(2), 288-294. https://doi.org/10.1002/jssc.200800543

Al-Dhabi, N. A., Ponmurugan, K., & Jeganathan, P. M. (2017). Development and validation of ultrasound-assisted solid-liquid extraction of phenolic compounds from waste spent coffee grounds. *Ultrasonics Sonochemistry*, *34*, 206-213. https://doi.org/10.1016/j.ultsonch.2016.05.005

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

Asamenew, G., Kim, H.-W., Lee, M.-K., Lee, S.-H., Lee, S., Cha, Y.-S., Lee, S. H., Yoo, S. M., & Kim, J.-B. (2019). Comprehensive characterization of hydroxycinnamoyl derivatives in green and roasted coffee beans: A new group of methyl hydroxycinnamoyl quinate. *Food Chemistry: X*, *2*, 100033. https://doi.org/10.1016/j.fochx.2019.100033

Buyong, N. L., & Nillian, E. (2023). Physiochemical properties of Sarawak's adapted Liberica coffee silver skin utilizing varying solvents. *Food Science and Nutrition*, *11*(10), 6052-6059. https://doi.org/10.1002/fsn3.3541

Campos, G. A. F., Sagu, S. T., Celis, P. S., & Rawel, H. M. (2020). Comparison of batch and continuous wet-processing of coffee: Changes in the main compounds in beans, by-products and wastewater. *Foods*, *9*(8), 1135. https://doi.org/10.3390/foods9081135

Chang, S. K. C. (2003). Protein analysis. In S. S. Nielsen (Ed.), *Food analysis* (pp. 133-146). Springer. https://doi.org/10.1007/978-1-4419-1478-1_9

Chu, R., Wan, L.-S., Peng, X.-R., Yu, M.-Y., Zhang, Z.-R., Zhou, L., Li, Z.-R., & Qiu, M.-H. (2016). Characterization of new *ent*-kaurane diterpenoids of Yunnan Arabica coffee beans. *Natural Products and Bioprospecting*, *6*, 217-223. https://doi.org/10.1007/s13659-016-0099-1

Desmawati, D., & Sulastri, D. (2019). Phytoestrogens and their health effect. *Open Access Macedonian Journal of Medical Sciences*, *7*(3), 495-499. https://doi.org/10.3889%2Foamjms.2019.086

Geremu, M., Tola, Y. B., & Sualeh, A. (2016). Extraction and determination of total polyphenols and antioxidant capacity of red coffee (*Coffea arabica* L.) pulp of wet processing plants. *Chemical and Biological Technologies in Agriculture*, *3*, 25. https://doi.org/10.1186/s40538-016-0077-1

Harahap, M. R. (2017). Identifikasi daging buah kopi Robusta (*Coffea robusta*) berasal dari provinsi Aceh [Identification of the pulp of Robusta coffee (*Coffea robusta*) originating from Aceh province]. *Elkawnie: Journal of Islamic Science and Technology*, *3*(2), 201-210. https://doi.org/10.22373/ekw.v3i2.2770

Heeger, A., Kosińska-Cagnazzo, A., Cantergiani, E., & Andlauer, W. (2017). Bioactives of coffee cherry pulp and its utilisation for production of Cascara beverage. *Food Chemistry*, *221*, 969-975. https://doi.org/10.1016/j.foodchem.2016.11.067

Ismail, I., Anuar, M. S., & Shamsudin, R. (2013). Effect on the physico-chemical properties of Liberica green coffee beans under ambient storage. *International Food Research Journal*, *20*(1), 255-264.

Ismail, I., Anuar, M. S., & Shamsudin, R. (2014). Physical properties of Liberica coffee (*Coffea liberica*) berries and beans. *Pertanika Journal of Science and Technology*, *22*(1), 65-79.

James, C. S. (1995). *Analytical chemistry of foods*. Blackie Academic & Professional.

Kirk, R. S., & Sawyer, R. (1991). *Pearson's composition and analysis of foods* (9th ed.). Longman.

Kovalcik, A., Obruca, S., & Marova, I. (2018). Valorization of spent coffee grounds: A review. *Food and Bioproducts Processing*, *110*, 104-119. https://doi.org/10.1016/j.fbp.2018.05.002

Lončar, M., Jakovljević, M., Šubarić, D., Pavlić, M., Služek, B. V., Cindrić, I., & Molnar, M. (2020). Coumarins in food and methods of their determination. *Foods*, *9*(5), 645. https://doi.org/10.3390/foods9050645

Mohd Jailani, F. N. A., Zaidan, U. H., Abdul Rahim, M. B. H., Abd Gani, S. S., & Halmi, M. I. E. (2020). Evaluation of constituents and physicochemical properties of Malaysian underutilized *Ziziphus mauritiana* (Bidara) for nutraceutical potential. *International Journal of Fruit Science*, *20*(3), 394-402. https://doi.org/10.1080/15538362.2019.1641458

Murthy, P. S., Manjunatha, M. R., Sulochannama, G., & Naidu, M. M. (2012). Extraction, characterization, and bioactivity of coffee anthocyanins. *European Journal of Biological Sciences*, *4*(1), 13-19.

Mussatto, S. I., Ballesteros, L. F., Martins, S., & Teixeira, J. A. (2011). Extraction of antioxidant phenolic compounds from spent coffee grounds. *Separation and Purification Technology*, *83*, 173-179. https://doi.org/10.1016/j.seppur.2011.09.036

Nabais, J. M. V., Nunes, P., Carrott, P. J., Carrott, M. M. L. R., García, A. M., & Díaz-Díez, M. A. (2008). Production of activated carbons from coffee endocarp by CO_2 and steam activation. *Fuel Processing Technology*, *89*(3), 262-268. https://doi.org/10.1016/j.fuproc.2007.11.030

Nillian, E., Ismail, N. S., Boli, M. E., Buyong, N. L., Sng, N. N., Adeni, D. S. A., & Hussini, A. A. S. A. (2020). The feasibility study of physicochemical properties of Sarawak *Liberica* sp. coffee pulp. *Pertanika Journal of Tropical Agricultural Science*, *43*(4), 477-490. https://doi.org/10.47836/pjtas.43.4.05

Núñez, A. C., Rentería, I. D. C. D., Villagómez, M. Á. L., Elorza, P., Martínez, C. E. M. S., Pulido, S. A. A., & Rojas-Ronquillo, R. (2015). The use of coffee pulp as a potential alternative supplement in ruminant diets. *Journal of Agriculture, Science and Technology* A, *5*(3), 214-217. https://doi.org/10.17265/2161-6256/2015.03.010

Olechno, E., Puścion-Jakubik, A., Socha, K., & Zujko, M. E. (2021). Coffee brews: Are they a source of macroelements in human nutrition? *Foods*, *10*(6), 1328. https://doi.org/10.3390/foods10061328

Oliveira, P. D., Borém, F. M., Isquierdo, E. P., da Silva Giomo, G., de Lima, R. R., & Cardoso, R. A. (2013). Physiological aspects of coffee beans, processed and dried through different methods, associated with sensory quality. *Coffee Science*, *8*(2), 211-220.

Pace, V., Carbone, K., Spirito, F., Iacurto, M., Terzano, M. G., Verna, M., Vincenti, F., & Settineri, D. (2006). The effects of subterranean clover phytoestrogens on sheep growth, reproduction and carcass characteristics. *Meat Science*, *74*(4), 616-622. https://doi.org/10.1016/j.meatsci.2006.05.006

Redfern, J., Kinninmonth, M., Burdass, D., & Verran, J. (2014). Using Soxhlet ethanol extraction to produce and test plant material (essential oils) for their antimicrobial properties. *Journal of Microbiology and Biology Education*, *15*(1), 45-46. https://doi.org/10.1128%2Fjmbe.v15i1.656

Rohaya, S., Anwar, S. H., Amhar, A. B., Sutriana, A., & Muzaifa, M. (2023). Antioxidant activity and physicochemical composition of coffee pulp obtained from three coffee varieties in Aceh, Indonesia. In *IOP Conference Series: Earth and Environmental Science* (Vol. 1182, No. 1, p. 012063). IOP Publishing. https://doi.org/10.1088/1755-1315/1182/1/012063

Saleem, H., Sarfraz, M., Khan, K. M., Anwar, M. A., Zengin, G., Ahmad, I., Khan, S.-U., Mahomoodally, M. F., & Ahemad, N. (2020). UHPLC-MS phytochemical profiling, biological propensities, and *in-silico* studies of *Alhagi maurorum* roots: A medicinal herb with multifunctional properties. *Drug Development and Industrial Pharmacy*, *46*(5), 861-868. https://doi.org/10.1080/03639045.2020.1762199

Schenker, S., Heinemann, C., Huber, M., Pompizzi, R., Perren, R., & Escher, R. (2002). Impact of roasting conditions on the formation of aroma compounds in coffee beans. *Journal of Food Science*, *67*(1), 60-66. https://doi.org/10.1111/j.1365-2621.2002.tb11359.x

Sena, L. P., Vanderjagt, D. J., Rivera, C., Tsin, A. T. C., Muhamadu, I., Mahamadou, O., Millson, M., Pastuszyn, A., & Glew, R. H. (1998). Analysis of nutritional components of eight famine foods of the Republic of Niger. *Plant Foods for Human Nutrition*, *52*, 17-30. https://doi.org/10.1023/a:1008010009170

Sitohang, A., & Pandiangan, M. (2022). Formulasi limbah kulit Ari kopi pembuatan pellet ikan [Formulation of Ari coffee skin waste for makin fish pellets]. *Jurnal Riset Teknologi Pangan Dan Hasil Pertanian (RETIPA)*, *3*(1), 1-12. https://doi.org/10.54367/retipa.v3i1.2240

Srikrishna, D., Godugu, C., & Dubey, P. K. (2018). A review on pharmacological properties

of coumarins. *Mini Reviews in Medicinal Chemistry*, *18*(2), 113-141. https://doi.org/10.2174/1389557516666160801094919

Wibowo, N. A., Djufry, F., Syafaruddin., Iflah, T., & Dani. (2022). The quality of Arabica coffee beans evaluation at various processing in Luwu Regency South Sulawesi, Indonesia. In *IOP Conference Series: Earth and Environmental Science* (Vol. 1038, No. 1, p. 012068). IOP Publishing. https://doi.org/10.1088/1755-1315/1038/1/012068

Yulianti, Y., Adawiyah, D. R., Herawati, D., Indrasti, D., & Andarwulan, N. (2023). Detection of markers in green beans and roasted beans of Kalosi-Enrekang Arabica coffee with different postharvest processing using LC-MS/MS. *International Journal of Food Science*, *2023*, 6696808. https://doi.org/10.1155/2023/6696808

Zaidan, U. H., Ab Karim, N., Ahmad, S., Abd Ghani, S. S., & Halmi, M. I. E. (2019). Nutraceutical evaluation and antioxidant potential of red kidney bean (*Phaseolus vulgaris*) and chickpea (*Cicer arietenum*) seed coats. *Asian Journal of Agriculture and Biology*, *7*(1), 19-26.

Zainol, M. K., Mohd Subri, I., Zamri, A. I., Mohd Zin, Z., Fisal, A., & Mamat, H. (2020). Antioxidative properties and proximate analysis of spent coffee ground (SCG) extracted using ultrasonic-methanol assisted technique as a potential functional food ingredient. *Food Research*, *4*(3), 636-644. https://doi.org/10.26656/fr.2017.4(3).358

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Short Communication

Development of an In-house aPPD ELISA for *Mycobacterium avium* Complex (MAC) Antibodies Detection in Zoo Primates

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ABSTRACT

In non-human primates (NHPs), *Mycobacterium avium* complex (MAC) species are the major source of non-tuberculous mycobacteriosis, causing tuberculous-like lesions in lymph nodes and parenchymatous organs in zoo and wildlife animals. Poor species-specific detection by serological diagnosis has negatively impacted the surveillance of MAC on non-human primates. Serum was collected from suspected twelve (n = 12) NHPs with no record of health monitoring, including gibbon (n = 5), capuchins (n = 2), siamang (n = 2), mandrill (n = 1), and orangutan (n = 2). An in-house avian purified protein derivative (aPPD) enzyme-linked immunosorbent assays (ELISA) antibody detection was developed and modified based on the established protocols. The aPPD ELISA for MAC antibodies detection at serum and Protein-G dilutions of 1:200-0.5µg/ml, respectively, detected 3/12 (25%) positive serum. At both serum and Protein-G dilutions of 1:100-0.05 and 1:300-1 µg/ml, the aPPD ELISA detected 12/12 (100%), respectively. The antibody was not detected for an in-house aPPD ELISA with serum and anti-monkey immunoglobulin G (IgG) dilutions at 1:100-0.5 and 1:300-1 µg/ml. However, 2/12 (16%)

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was detected using serum and anti-monkey IgG dilutions at 1:200-0.05 µg/ml. An in-house aPPD ELISA procedure for MAC antibodies detection in primates, at serum and Protein-G dilutions of 1:100-0.05 and 1:300-1 µg/ml, both have shown sensitivity and specificity of 100%, positive predictive value and negative predictive value of 100%, respectively. The serum and anti-monkey IgG have shown extremely low sensitivity and specificity. In conclusion, the performance of an in-house aPPD ELISA using three different dilutions on serum and conjugates in detecting MAC in a primate has shown that Protein-G horseradish peroxidase, as secondary conjugates were able to detect MAC antibodies.

Keywords: Antibodies, ELISA, *Mycobacterium avium* complex, non-human primates, Protein-G

INTRODUCTION

Non-human primates (NHPs) are susceptible to non-tuberculous mycobacteriosis caused by *Mycobacterium avium* complex (MAC) organisms that are slow-growing mycobacterium that are found in different sources, such as water, soil, and foodstuffs. Members of MAC are known to cause various diseases, which include similar diseases like tuberculosis in human beings and birds, lymphadenitis in humans and mammals, and Johne's disease in ruminants (Biet et al., 2005). These mycobacteria pose a serious risk to zoos and wildlife sanctuaries (Roller, Hansen, Böhlken-Fascher et al., 2020). It has become an important part of disease prevention and control in zoos and wildlife sanctuaries (Roller, Hansen, Knauf-Witzens et al., 2020).

Different categories of tuberculosis (TB) antibody detection tests have been developed for humoral response in different species, such as lateral flow test and ELISA (Bezos et al., 2014). Antibody ELISA kits against MAC in some species are usually not permitted for use with sera from another animal (Manning, 2011). Some tests could give different results even from animals of the same species (Pruvot et al., 2013). Species differences exist with respect to serological results because of the specificity of the reagents inside the different ELISA kits sold commercially. The secondary antibodies included in the test kits have different capabilities in binding to IgG from different species (Pruvot et al., 2013). Some research has reported disparities in binding levels to IgG by different conjugates on different wildlife species (Kramsky et al., 2003; Pruvot et al., 2013).

Protein G is derived from the C and G groups of streptococci, an important immunoglobulin (Ig) for bacterial cell wall receptor binding. The Fc part of IgG and IgG subclasses is binding at different degrees. An example is the mouse IgG2, which is strongly attached to protein G, while mouse IgG1 is weakly bound (Kramsky et al., 2003). Protein G has recombinant forms that are used as laboratory reagents. These forms have removed the non-Ig binding part, which is the albumen, reducing its nonspecific

protein binding. The recombinant protein G has purification and Ig detection properties. As such, it is used as a laboratory reagent in various domestic and laboratory species. The use of recombinant protein G has not been well established beyond ruminant species of cattle, sheep, and goats (Kramsky et al., 2003). The development of a protein G ELISA for antibody detection was experimented with for use in different nondomestic artiodactylid species. Protein G exhibits a greater species specificity with little differentiation in isotype-binding cell wall proteins, e.g., protein A. Several research studies have reported a strong binding affinity between protein G and Ig from different species, as well as selective reactivity to IgG. This unique characteristic has led to our choice of protein G as the conjugate or secondary antibody for the use in this species in our study (Kramsky et al., 2000, 2003). The horseradish peroxidase (HRP)-conjugated anti-monkey IgG was produced by immunoelectrophoresis and reacts especially with monkey IgG and light chains common immunoglobulins of other monkeys. No antibody was detected against non-immunoglobulins serum proteins. It can cross-react with IgG from other species (Frost et al., 2014).

Antemortem diagnosis of tuberculosis in wildlife using serology is critical in monitoring the effects of *Mycobacterium tuberculosis* complex (MTBC) and MAC-associated infections for better surveillance and control measures. To our understanding, no ELISA kits exist for MAC detection in NHPs. Thus, an attempt to modify and develop an in-house ELISA protocol for MAC detection in NHPs by implementing a modified protocol as described by Boadella et al. (2011) and Pruvot et al. (2013), which had been evaluated in wild boar and wild ruminants in their study, respectively. Therefore, the study aims to develop an in-house aPPD ELISA protocol for detecting IgG antibodies to MAC in non-human primates.

MATERIALS AND METHODS

Ethical Approval

This research was approved following the review by the Department of Wildlife and National Parks (PERHILITAN, Malaysia) regulations and the university animal ethics committee approval (UPM/AICUC/AUP-U040/2019). Written informed consent was obtained from the zoological centres for the involvement of these animals in this study.

Study Animals

The study was carried out in Zoo Melaka and A'Famosa Safari Wonderland in central-south of Peninsular Malaysia after being requested by the resident veterinarians for clinical service and conducting TB and non-tuberculous mycobacteria screening on their captive primates. Animals were selected based on reduced activity and performance. There was no history of TB screening of the animals on the premises. Blood samples were collected from twelve (n = 12) NHPs, including gibbon (n = 5), capuchins (n = 2), siamang (n = 2), mandrill (n = 1), and orangutan (n = 2).

Sample Collection and Processing

The zoo personnel managed all the animals. Zoo veterinarians sedated animals before conducting the sampling procedures, and the animals were monitored throughout the procedure until recovery. Blood samples were collected using a 23-gauge syringe and needle, stored in well-labelled plain tubes, and later centrifuged at the clinical studies laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia for 252 x *g* for 15 min within 3 hr of collection. The extracted serum was poured into Citadel Deep-well Plates and Cluster tube (SSIbio, USA), and the sera were stored at -20°C, which was used to determine antibodies. The skin test was conducted as described in the previous study (Lekko et al., 2022).

Development of An In-house ELISA aPPD Using HRP-conjugated Anti-monkey IgG for MAC Detection in Primates

The aPPD ELISA trials with modification were developed based on the established previous protocols (Boadella et al., 2011; Pruvot et al., 2013). Briefly, the 96-well ELISA plate was coated with antigen, aPPD at 5 µg/ml for at least 18 hr at 4ºC or overnight. Then, the wells were washed three times with 300 µl of phosphate-buffered saline (PBS) solution containing 0.05% Tween 20 (PBST, Abcam, United Kingdom) and blocked for 1 hr with 140 µl of 5% skim milk (Sunlac, Malaysia) in PBST at room temperature. After emptying the plate, the NHPs sera were added at 1:100, 1:200, and 1:300 dilutions and incubated at 37ºC for 30 min. IgG antibody detection was performed by adding 100 µl of HRP-conjugated anti-monkey IgG antibodies (Abcam, United Kingdom) at 0.5 µg/ml 1:500 dilutions and incubating the plate at room temperature for 1 hr. The plate was removed and washed three times with PBST, then tetramethylbenzidine (TMB, Promega Corp., USA) at 50 µl 3,3',5,5' was added to the plate and prevented from light for 15 min at room temperature. Fifty (50) µl 2M sulphuric acid (H_2SO_4, Sigma-Aldrich, USA) was used as a stop reaction. The optical densities (OD) were measured by an ELISA plate reader (Tecan Austria GmbH, Austria) at 450 nm.

Development of An In-house ELISA aPPD Using HRP-recombinant Protein G for MAC Detection in Primates

For aPPD ELISA (HRP-recombinant Protein G), the protocol followed as previously described by Boadella et al. (2011) and Che' Amat et al. (2015) with modification. Sera were added to the plate at a dilution rate of 1:100, 1:200, and 1:300 in PBS, and incubation was done for 1 hr at 37ºC, and then washing of plates was performed three times with PBST. Protein G conjugates (Abcam, United Kingdom) were added at a dilution of 0.5 µg/ml (1:10,000) in PBST and incubated for 80 min at 37ºC. The OD was measured using an ELISA plate reader at 450 nm.

Statistical Analyses

The sensitivity (Se) and specificity (Sp) were determined based on the polymerase chain reaction (PCR) positive results from

blood and pharyngeal swabs and were used as the reference standard. Chi-square (Fisher's exact test) was computed for sensitivity, specificity, and predictive values using Graph Pad Prism (version 8.2).

RESULTS

PCR Samples

Conventional PCR was used on blood and pharyngeal swabs. All twelve samples from blood and pharyngeal swabs from NHPs (two orangutans, five gibbons, two capuchins, two siamangs, and one mandrill) were positive for MAC (Table 1).

Development of An In-house ELISA aPPD for Antibody Detection Against MAC Using HRP-conjugated Anti-monkey IgG and Recombinant Protein G in Primates

The aPPD ELISA for MAC antibodies detection at serum and protein G dilutions of 1:200-0.5 µg/ml, respectively, detected 3/12 (25%) of NHPs serum sample. At serum and protein G dilutions of 1:100-0.05 µg/ml, the aPPD ELISA detected 12/12 (100%). The app ELISA at serum and protein G dilutions of 1:300-1 µg/ml detected 12/12 (100%) of NHPs positive serum samples. The antibody was not detected for an in-house aPPD ELISA with serum and anti-monkey IgG dilutions at 1:100-0.5 and 1:300-1 µg/ml. However, it was detected from 2/12 (16%) serum and anti-monkey IgG dilutions at 1:200-0.05 µg/ml (Table 2).

Sensitivity and Specificity of In-house ELISA aPPD for MAC Antibody Detection in Primates

Se and Sp were determined based on the PCR positive results as the reference method. The development of an in-house ELISA aPPD procedure for MAC antibodies detection in zoo primates, at serum and

Table 1
Results from different diagnostic techniques applied to captive non-human primates in zoological parks

NHPs species	Skin test	Tuberculin reaction scoring	Serology	PCR MAC blood	PCR MAC pharyngeal swab
Orangutan	Reactive	4	Positive	Positive	Positive
Orangutan	Reactive	3	Positive	Positive	Positive
Gibbon	Non-reactive	0	Negative	Positive	Positive
Gibbon	Non-reactive	0	Negative	Positive	Positive
Gibbon	Non-reactive	0	Negative	Positive	Positive
Gibbon	Non-reactive	0	Negative	Positive	Positive
Gibbon	Non-reactive	0	Negative	Positive	Positive
Capuchin	Non-reactive	0	Negative	Positive	Positive
Capuchin	Non-reactive	0	Negative	Positive	Positive
Siamang	Non-reactive	0	Negative	Positive	Positive
Siamang	Non-reactive	0	Negative	Positive	Positive
Mandrill	Non-reactive	0	Negative	Positive	Positive

Note. NHPs = Non-human primates; PCR MAC = Polymerase chain reaction *Mycobacterium avium* complex (Source: Lekko et al., 2022)

Table 2
Dilutions, sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV) of different enzyme-linked immunosorbent assays (ELISA) protocols in zoo primates

Test	Serum dilutions-conjugates dilutions (µg/ml)	Se (95% CI)	Sp (95% CI)	PPV (95% CI)	NPV (95% CI)
ELISA protein G HRP	1:200-0.5	25% (8.8-53.2)	57.1% (36.1-75.5)	25% (8.8-53.2)	57.1% (36.5-75.5)
ELISA anti-monkey IgG HRP	1:100-0.5	-	-	-	-
ELISA protein G HRP	1:100-0.05	100% (75.7-100)	100% (75.7-100)	100% (75.7-100)	100% (75.7-100)
ELISA anti-monkey IgG HRP	1:200-0.05	16.6% (29.6-44.8)	54.5% (34.6-73.0)	16.6% (29.6-44.8)	54.5% (34.6-73.0)
ELISA protein G HRP	1:300-1	100% (75.7-100)	100% (75.7-100)	100% (75.7-100)	100% (75.7-100)
ELISA anti-monkey IgG HRP	1:300-1	-	-	-	-

Note. CI = Confidence interval; IgG = Immunoglobulin G; HRP = Horseradish peroxidase

protein G dilutions of 1:200-0.5 µg/ml, showed a very low Se of 25% and Sp of 57.1%, positive predictive value (PPV) of 25% and negative predictive value (NPV) of 57.1%. While at 1:100-0.05 and 1:300-1 µg/ml dilutions, ELISA aPPD has both Se and Sp of 100% and PPV and NPV of 100%. The serum and anti-monkey IgG at 1:200-0.05 µg/ml dilutions, the ELISA aPPD have very low Se (16.6%) and 54.5% Sp, 16.6% PPV, and 54.5% NPV (Table 2).

DISCUSSION

This trial is among the several protocols developed for detecting serum antibodies against MAC in captive zoo primates. The performance of an in-house aPPD ELISA using three different dilutions on serum and conjugates in detecting MAC in primates showed that the protein G HRP, as secondary conjugates, was able to detect MAC antibodies better than anti-monkey IgG HRP conjugates. It showed that secondary antibodies have different capabilities in binding to IgG from different species (Kramsky et al., 2003; Pruvot et al., 2013). The aPPD ELISA at 1:100-0.05 and 1:300-1 µg/ml dilutions showed a good Se (100%) and 100% Sp. Other report using aPPD ELISA in wild ruminants showed 73% Se and 90% Sp; wild boar shows 79.2% Se and 100% Sp reported by Boadella et al. (2011), and 72.6% Se and 96.4% Sp were reported by Aurtenetxe et al. (2008). The OD obtained showed better discrimination of serum and conjugates dilutions at 1:100-0.05 and 1:300-1 µg/ml with protein G HRP. Decrease in OD values of serum and conjugates dilutions at 1:100-0.5 and 1:300-1 µg/ml was observed with anti-monkey IgG HRP conjugate. Aurtenetxe et al. (2008) reported that the serum dilutions above 1:500 yield decreased in OD values. This test's moderate sensitivity and specificity

could be used for comprehensive disease monitoring and surveillance of MAC in the host (Boadella et al., 2011). However, factors such as the cut-off values, sample types, status of infection or species examined and prevalence of cross-reacting environmental mycobacteria could affect the sensitivities of a test (Bezos et al., 2014; Downs et al., 2018).

The choice of secondary antibodies has an important effect on the result of an ELISA test due to species-associated differences in affinity. Species differences exist in the outcomes of serological results due to the specificity of the reagents inside the different ELISA kits. The secondary antibodies included in the test kits have different capabilities in binding to IgG from different species (Kramsky et al., 2003; Pruvot et al., 2013). A few research studies have reported disparity in binding levels to IgG by different conjugates in different wildlife species (Kramsky et al., 2003; Pruvot et al., 2013). The level of unspecific binding of serum immunoglobulins to the reagents of the ELISA kits remains unpredictable. It could affect the results by reducing the signal-to-noise ratio, the ratio of the OD of positive and negative control in each serum sample and conjugate concentration (Pruvot et al., 2013). Modifying these reagents is needed to help determine cut-off values when using an ELISA kit for wildlife animal sample testing.

Cross-reactions between MAC and MTBC could affect the diagnosis of TB, especially when there is lacked diagnostics on wildlife. For this reason, an in-house aPPD ELISA protocol for MAC antibody detection in NHPs was developed and evaluated. MAC infections in NHPs are important in terms of animal welfare, conservation, and disease monitoring. Various outbreaks of MAC infection have been reported from NHPs around the world (Roller, Hansen, Böhlken-Fascher et al., 2020; Roller, Hansen, Knauf-Witzens et al., 2020). Serological diagnosis of MAC infection, such as ELISA, would be useful in evaluating wildlife samples for MAC infection. More so, ELISA kits commercially available for MAC are mostly available for use in livestock ruminants and not available for wildlife species (Pruvot et al., 2013).

Similar studies were performed in wild ruminants following modification of commercial IDEXX ELISA kit for detecting MAP in elk (*Cervus elaphus*), bison (*Bison bison*), and caribou (*Rangifer tarandus*) by using protein G, anti-bovine, and anti-deer conjugates with Se of 73% and Sp of 90% (Kramsky et al., 2000, 2003; Pruvot et al., 2013) and in fallow deer (*Dama dama*)" with 72% Se and 100% Sp (Prieto et al., 2014). MAC antibodies were also detected using a commercial IDEXX kit for detecting MAP in Sumatran orangutan (*Pongo abelii*), bonobo (*Pan paniscus*), and gorillas (*Gorilla gorilla gorilla*) using protein G (Roller, Hansen, Böhlken-Fascher, et al., 2020). Although the conventional plate ELISA test is used, better detection of IgG was demonstrated by the use of protein G HRP as secondary conjugates for the development of in-house ELISA, and this is

similar to studies of Kramsky et al. (2003) and Pruvot et al. (2013) on wild animals. The major limitation of our research is the sample size used, as this was the number allowed to be evaluated by zoo authorities. The results of this study supported our hypothesis; despite the low sensitivity of the in-house ELISA, the use of protein G conjugates at some dilution could enhance the performance of the plate ELISA.

CONCLUSION

The performance of an in-house aPPD ELISA using three different dilutions on serum and conjugates in detecting MAC in primates showed that the protein G HRP, as secondary conjugates, was able to detect MAC antibodies better than anti-monkey IgG HRP conjugates. A developed protocol of an in-house aPPD ELISA for antibody detection against MAC in primates showed a moderate sensitivity on selected serum and conjugated dilutions. Thus, it may provide an option for better sensitivity for TB testing and diagnosis for MAC infection in primates. Therefore, to produce acceptable data results, it is necessary to make changes and modifications in ELISA kits, especially the secondary antibodies or conjugates specific to wildlife species. However, with the difficulty in producing wildlife diagnostics kits, it has been proven that modifications in plate ELISA could be used to improve confidence in test results.

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REFERENCES

Aurtenetxe, O., Barral, M., Vicente, J., de la Fuente, J., Gortázar, C., & Juste, R. A. (2008). Development and validation of an enzyme-linked immunosorbent assay for antibodies against *Mycobacterium bovis* in european wild boar. *BMC Veterinary Research*, *4*, 43. https://doi.org/10.1186/1746-6148-4-43

Bezos, J., Casal, C., Romero, B., Schroeder, B., Hardegger, R., Raeber, A. J., López, L., Rueda, P., & Domínguez, L. (2014). Current *ante-mortem* techniques for diagnosis of bovine tuberculosis. *Research in Veterinary Science*, *97*, S44–S52. https://doi.org/10.1016/j.rvsc.2014.04.002

Biet, F., Boschiroli, M. L., Thorel, M. F., & Guilloteau, L. A. (2005). Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium-intracellulare* complex (MAC). *Veterinary Research*, *36*(3), 411–436. https://doi.org/10.1051/vetres:2005001

Boadella, M., Lyashchenko, K., Greenwald, R., Esfandiari, J., Jaroso, R., Carta, T., Garrido, J. M., Vicente, J., de la Fuente, J., & Gortázar, C. (2011). Serologic tests for detecting antibodies against *Mycobacterium bovis* and *Mycobacterium avium* subspecies *Paratuberculosis* in Eurasian wild boar (*Sus scrofa scrofa*). *Journal of Veterinary Diagnostic Investigation*, *23*(1), 77–83. https://doi.org/10.1177/104063871102300111

Che' Amat, A., González-Barrio, D., Ortiz, J. A., Díez-Delgado, I., Boadella, M., Barasona, J. A., Bezos, J., Romero, B., Armenteros, J. A., Lyashchenko, K. P., Venteo, A., Rueda, P., & Gortázar, C. (2015). Testing Eurasian wild boar piglets for serum antibodies against *Mycobacterium bovis*. *Preventive Veterinary Medicine*, *121*(1–2), 93–98. https://doi.org/10.1016/j.prevetmed.2015.05.011

Downs, S. H., Parry, J. E., Upton, P. A., Broughan, J. M., Goodchild, A. V., Nuñez-Garcia, J., Greiner, M., Abernethy, D. A., Cameron, A. R., Cook, A. J., de la Rua-Domenech, R., Gunn, J., Pritchard, E., Rhodes, S., Rolfe, S., Sharp, M., Vordermeier, H. M., Watson, E., Welsh, M., … Clifton-Hadley, R. S. (2018). Methodology and preliminary results of a systematic literature review of ante-mortem and post-mortem diagnostic tests for bovine tuberculosis. *Preventive Veterinary Medicine*, *153*, 117–126. https://doi.org/10.1016/J.PREVETMED.2017.11.004

Frost, P. A., Calle, P. P., Klein, H., & Thoen, C. O. (2014). Zoonotic tuberculosis in nonhuman primates. In C. O. Thoen, J. H. Steele, & J. B. Kaneene (Eds.), *Zoonotic tuberculosis:* Mycobacterium bovis *and other pathogenic mycobacteria* (3rd ed., pp. 383–397). John Wiley & Sons. https://doi.org/10.1002/9781118474310.ch33

Kramsky, J. A., Manning, E. J. B., & Collins, M. T. (2003). Protein G binding to enriched serum immunoglobulin from nondomestic hoofstock species. *Journal of Veterinary Diagnostic Investigation*, *15*(3), 253–261. https://doi.org/10.1177/104063870301500306

Kramsky, J. A., Miller, D. S., Hope, A., & Collins, M. T. (2000). Modification of a bovine ELISA to detect camelid antibodies to *Mycobacterium paratuberculosis*. *Veterinary Microbiology*, *77*(3–4), 333–337. https://doi.org/10.1016/S0378-1135(00)00318-7

Lekko, Y. M., Ooi, P. T., Omar, S., Mazlan, M., Sivapalan, N., Ramanoon, S. Z., Jesse, F. F. A., Jasni, S., & Che-Amat, A. (2022). Surveillance and detection of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* complex in captive non-human primates in zoological parks. *Pakistan Veterinary Journal*, *42*(3), 340-345. https://doi.org/10.29261/pakvetj/2022.057

Manning, E. J. B. (2011). Paratuberculosis in captive and free-ranging wildlife. *Veterinary Clinics of North America: Food Animal Practice*, *27*(3), 621–630. https://doi.org/10.1016/j.cvfa.2011.07.008

Prieto, J. M., Balseiro, A., Casais, R., Abendaño, N., Fitzgerald, L. E., Garrido, J. M., Juste, R. A., & Alonso-Hearn, M. (2014). Sensitive and specific enzyme-linked immunosorbent assay for detecting serum antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in fallow deer. *Clinical and Vaccine Immunology*, *21*(8), 1077–1085. https://doi.org/10.1128/CVI.00159-14

Pruvot, M., Forde, T. L., Steele, J., Kutz, S. J., De Buck, J., van der Meer, F., & Orsel, K. (2013). The modification and evaluation of an ELISA test for the surveillance of *Mycobacterium avium* subsp. *paratuberculosis* infection in wild ruminants. *BMC Veterinary Research*, *9*, 5. https://doi.org/10.1186/1746-6148-9-5

Roller, M., Hansen, S., Böhlken-Fascher, S., Knauf-Witzens, T., Czerny, C.-P., Goethe, R., & Abd El Wahed, A. (2020). Molecular and serological footprints of *Mycobacterium avium* subspecies infections in zoo animals. *Veterinary Sciences*, *7*(3), 117. https://doi.org/10.3390/VETSCI7030117

Roller, M., Hansen, S., Knauf-Witzens, T., Oelemann, W. M. R., Czerny, C. P., Abd El Wahed, A., & Goethe, R. (2020). *Mycobacterium avium* subspecies *paratuberculosis* infection in zoo animals: A review of susceptibility and disease process. *Frontiers in Veterinary Science*, *7*, 572724. https://doi.org/10.3389/fvets.2020.572724

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Characterization of Liquid Organic Fertilizer (LOF) Derived from Unmarketable Vegetables and Fruits

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ABSTRACT

The Malaysian government has been challenged with handling unmarketable vegetables and fruit waste that pollutes the environment and emits greenhouse gases, mainly methane and nitrous oxide. These greenhouse gases have been contributing to climate change. In contrast, these wastes consist of high moisture and readily biodegradable nutrients that can serve as the perfect substrate rate for fermentation. The valuable nutrients contained in these wastes can produce liquid organic fertilizers (LOF), which help improve the soil's physical, chemical, and biological characteristics and reduce the demand for inorganic fertilizers and costs to farmers. In this regard, a study was conducted to produce and characterize LOF derived from unmarketable vegetables and fruit waste. The waste was identified from the nearest wet market, collected, and incubated in containers with a ratio of 1: 2: 0.1 (10 kg unmarketable vegetable and food waste: 20 L water: 1 kg inducer) for 30, 45, and 60 days. The unmarketable vegetables and fruits were fermented using three different types of inducers: yeast (Y), brown sugar (BS), and shrimp paste (SP). Unmarketable vegetables and fruit waste with no inducer were also included as a control. Samples from the produced LOF were taken after 30, 45, and 60 days of fermentation, filtered, and subjected to analysis for pH, electrical conductivity (EC), macro-, and micronutrients. These experiments were laid out in a randomized

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complete block design (RCBD) with three replications. The highest nitrogen (0.95%), phosphorus (0.31%), potassium (1.68%), copper (0.23 ppm), and manganese (9.03 ppm) were obtained from LOF fermented for 60 days using yeast, indicating that it improved the nutrient availability of agricultural waste. Moreover, this treatment provided optimum pH and EC values for the growth and development of plants. Thus, LOF derived from unmarketable vegetable and fruit waste can be considered an attractive alternative for supplementing chemical fertilizers.

Keywords: Fermentation, food security, inducer, liquid fertilizer, sustainable farming, unmarketable

INTRODUCTION

The ever-increasing population causes a pressing need to increase food production. Food insecurity may quickly become a primary national concern within the context of insufficient production. In 2019, self-sufficiency rates in Malaysia for the five primary food commodities, such as rice, vegetables, fruits, beef, and milk, were 63%, 44%, 78.2%, 22.3%, and 64%, respectively (Hazri, 2022). As in many countries, the agricultural sector is Malaysia's main food supply source. Therefore, developing the agricultural sector is very important to balance the demand and supply of food products to sustain food security.

Among many other factors, the role of fertilizers in boosting agricultural productivity and food production is substantial. The application of fertilizers helps to replace the nutrients that crops uptake from the soil in food production. The agricultural productivity and crop yield would significantly decrease if the nutrients in the soil were low. The source of plant nutrients can be grouped into two general categories, which are inorganic and organic fertilizers. In contrast to organic, inorganic fertilizers typically have quick-release formulae that make nutrients readily available to plants only days after application. A plant can only use nutrients to the level of its extraction capacity and during its growth period. Thus, when fertilizers are applied untimely and excessively, the unused or unabsorbed remainders have the propensity to alter soil physicochemical characteristics, leach deep into groundwater under intensive rain or irrigation, and contaminate water sources, resulting in several human and animal health issues (Ibrahim et al., 2014; Mohd Zaini et al., 2022). Another crucial factor is the manufacturing of nitrogen (N), phosphorus (P), and potassium (K) chemical fertilizers, resulting in waste contaminating soil and water resources.

Additionally, intensive chemical usage for the long term in agricultural practices could significantly impact food safety issues and environmental health, such as acidifying or salinizing soils, which imposes huge costs of soil amendments (Mohd Zaini et al., 2022). Though chemical fertilizers are essential for enhancing crop productivity, they are among the most expensive agricultural supplies due to the high manufacturing costs associated with

using imported inputs; thereby, they are unaffordable to poor farmers (da Silva et al., 2015). For that reason, the government of Malaysia has been requested to subsidize fertilizers for the sake of resource farmers, who are having difficulty coping with the ever-increasing prices, which have a very direct impact on increasing food production. Salahuddin Ayub, a former Minister of Agriculture and Agro-based Industries, claimed that subsidizing fertilizers is essential to enhance food production because the ongoing conflict between Russia and Ukraine is afraid to increase fertilizer costs globally (Bunyan, 2022).

The use of organic fertilizers for food production can be considered one of the best alternatives to the challenges associated with chemical fertilizers and is a more sustainable and environmentally friendly agricultural practice (Mohd Zaini et al., 2022). Organic fertilizers are generally considered an effective way to sustain soil fertility and plant growth. Vegetables and fruits are usually available in excess in every household and around local markets. Food and Agriculture Organization of the United Nations (FAO) (2019) estimated that about one-third of the food production in the world is wasted. Agricultural wastes are a by-product of agriculture, such as unmarketable vegetables and fruits, rotten vegetables and fruits, fisheries, and household waste that are not utilized or thrown away so that they have no sale value but can directly cause environmental pollution and disease and interfere with environmental cleanliness. Unmarketable vegetables and fruits refer to produce that cannot be sold or marketed for various reasons, such as dehydrated, rotten, or overripe, which do not meet the quality standards required for commercial distribution (Duarte et al., 2009). These wastes that have been wasted and polluting the environment can be decomposed or processed into LOF, be used for food production, and be one of the viable solutions to overcome the problems linked to chemical fertilizers. Apart from contributing towards solving the challenges with chemical fertilizers, organic fertilizers are aligned with the Responsible Consumption and Production of the Sustainable Development Goals (SDG 12).

The use of organic fertilizers enhances soil properties such as improving nutrient mobilization from an organic and chemical source, promotes the colonization of mycorrhizae, improves phosphorous supply, promotes root growth due to better soil structure, increases soil organic matter content, enhances the exchange capacity of nutrients, increases soil water retention, and promotes soil aggregates and buffering capacity (Kala et al., 2011; Lal et al., 1997). Organic fertilizers can be available both in liquid and solid states. LOF is a soluble solution that supplies one or more nutrients that meet the plant's needs. Liquid fertilizer is being studied as a possible method to lengthen the shelf life of fertilizer. The liquid form allows the manufacturer to increase the number of nutrients and inducers; besides, it can tolerate a high temperature of 55°C (Nhu et al., 2018). In addition, when applying liquid fertilizers,

the nutrients can be distributed more evenly, and their concentrations are set up to meet the needs of the plants (Ginandjar et al., 2019). Organic liquid fertilizer is produced after the anaerobic biological decomposition of organic materials, releasing some important plant nutrients in the form of complex and simple compounds. Research conducted by Martínez-Alcántara et al. (2016) demonstrated that applying LOF led to higher absorption of macro and micronutrients in citrus trees compared to utilizing mineral fertilizers. In addition, organic liquid fertilizer treatment yielded the highest measurements for plant height, number of branches per plant, leaf count, leaf area, fresh and dry weight, fruit quantity per plant, and overall chili yield (Deore et al., 2010). Pangaribuan et al. (2019) found consistent enhancement in sweet corn's growth, yield, and quality through LOF. The study concludes that LOF has the potential to serve as a supplementary addition to conventional inorganic fertilizers in tropical organic sweet corn cultivation.

The production of LOF can be enhanced by inducers such as brown sugar, yeast, and shrimp paste for the fermentation process. Brown sugar is added to waste to increase the fermentation rate of food waste because brown sugar contains glucose and can be used as a food resource by microorganisms (Zhang et al., 2013). Yeast assists in the process of degrading organic materials at the beginning of the fermentation process, *and Saccharomyces cerevisiae* also increases the amount of protein in liquid organic during the process (Hidayati et al., 2011). Shrimp paste constitutes a variety of fermented products derived from shrimp. Due to the effect of seafood and microbial protease on proteins, these fermented products are often higher in soluble amino acids and smaller peptide chains, which will help with plant nutrient availability (Mohd Zaini et al., 2022). Farmers can potentially mitigate environmental pollution's effects by transforming waste from dried shrimp paste into LOF. This fertilizer can be produced collaboratively within the community and utilized in a program promoting natural greening (Mohd Kamaruddin et al., 2021).

Despite the huge quantities of unmarketable vegetables and fruit in the farmers' fields and around markets in Malaysia, these wastes have not yet been exploited in organic fertilizer. As far as the author knows, only limited information is available in the literature regarding the usage of these unmarketable vegetable and fruit wastes in the form of LOF. To date, raw food wastes have been utilized in the agricultural system to boost plant growth; however, the nutritional compositions of fermented wastes have not yet been known. Thus, this study was carried out to produce and characterize LOF derived from vegetables and fruits under various inducer and fermentation times.

MATERIALS AND METHODS

Treatments and Experimental Design

This research was conducted in Ladang 10, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The unmarketable vegetables and fruits were collected from

the local market and cut into small pieces (±2 cm) before being filled into a 45-L container. The unmarketable vegetables and fruits consisted of 1.5 kg cabbage, 1.5 kg lettuce, 1.5 kg green spinach, 1.5 kg bok choy, 1.5 kg Chinese broccoli, 1.5 kg water spinach, 0.5 kg bananas, and 0.5 kg papayas. The unmarketable vegetables and fruits were mixed with a ratio of 9:1, weight by weight. The containers were filled with unmarketable vegetables and fruits, water, and inducer with a ratio of 1:2:0.1 (weight: volume: weight). All the ingredients were mixed well in a gallon container and covered with a lid for anaerobic fermentation. The gallon container was placed in the shade and stirred for three days once to accelerate microbial activities. The treatments of this study comprised factorial combinations of 3 inducers and three fermentation periods. Unmarketable vegetables and fruit waste with no inducer were also included as a control. The four inducers were no inducer (NO), yeast (Y), brown sugar (BS), and shrimp paste (SP). The unmarketable vegetables and fruits were allowed to ferment for three different periods. The three different fermentation periods were: 30 days (30D), 45 days (45D), and 60 days (60D). The two factors (4 inducers and three fermentation periods) were combined factorially and laid out in a randomized complete block design (RCBD) with three replications.

Analysis of LOF

At the end of each of the fermentation periods of 30, 45, and 60 days, liquid samples (leachate) were taken from the containers, filtered, and subjected to analyses for pH, EC, total nitrogen (N), phosphorous (P), potassium (K), magnesium (Mg), calcium (Ca), iron (Fe), manganese (Mn), copper (Cu), and zinc (Zn). The methods of devices employed to determine pH were pH meter, electrical conductivity (EC) with EC meter; total N was the Kjeldahl method (Campbell & Hanna, 1937); P was the spectrophotometric method (J. Murphy & Riley, 1962), K, Ca, Mg, Fe, Mn, Cu, and Zn were inductively coupled plasma atomic emission spectrometry (ICP) method (Hamalová et al., 1997).

Statistical Analysis

Two-way analysis of variance (ANOVA) was employed by using *R*-studio. When significant differences among treatments existed $p < 0.05$ and $p < 0.01$, means separations were performed using Tukey's test.

RESULTS AND DISCUSSION

Inducers and Fermentation Period Effects on Chemical Properties of LOF

Results showed that applying various inducers to produce LOF from unmarketable vegetables and the fermentation period significantly affected the N, P, K, Mg, Ca, Cu, Mn, Fe, and Zn contents, pH, and EC (Table 1). The interaction effects between inducers and the fermentation period were also significant for all the measured variables (Table 1).

The pH of the produced LOF ranged

from 3.1 to 7.0 with brown sugar and shrimp paste, respectively (Table 1). The highest pH of the LOF was obtained from the decomposition of unmarketable vegetables using shrimp paste for 60D (7.54) followed by 45D (7.27), which were not statistically different from the fermentation of organic materials for 60D without inducer (7.41). The lowest pH (2.9–3.3) was recorded from the fermentation of unmarketable vegetables using brown sugar regardless of the fermentation period (Table 1). The pH of LOF without inducer, using yeast and shrimp paste at different fermentation periods, was moderately acidic to neutral, while brown sugar was strongly acidic (H. F. Murphy, 1968). These acidic pH values with brown sugar were caused by the substrate's high sugar content, which microbes used to produce lactic or acetic acids (Phibunwatthanawong & Riddech, 2019). It showed that the higher the sugar concentration in the substrate, the higher the organic acids and the lower the pH. There were also significant differences in pH among all the inducers and fermentation periods. Generally, the tested inducers varied significantly among each other in their pH values, and pH increased as the fermentation period progressed from 30 to 60 days, indicating that all treatments had higher pH values at 60D of fermentation (Table 1). Similar results were also reported by Tan (2015), who documented that the pH trend of fermenting samples increased from the early period to the 90th day.

Results presented that the highest EC of the LOF (32.33 dS/m) was recorded from the fermentation of unmarketable vegetables using shrimp paste for 60D. In contrast, the lowest EC (3.35–3.85 dS/m) was attained from brown sugar, irrespective of the fermentation period (Table 1). The shrimp paste (29.26 dS/m) and brown sugar (3.11 dS/m) inducers had the maximum and minimum EC values, respectively, compared to LOF produced from yeast (16.86 dS/m) and no inducer (6.71 dS/m) at all fermentation periods (Table 1). Similar to the pH values, the EC values also varied greatly among inducers and increased consistently as the fermentation period advanced (Table 1).

Results exhibited that the highest N content in the LOF was recorded from the fermentation of food waste for 60D using yeast (0.95%), followed by 45D (0.91%). Whereas the lowest N contents of LOF (0.23–0.24%) were obtained from brown sugar inducer regardless of fermentation period (Table 1). LOF without inducer at any fermentation periods (0.23–0.25%) and shrimp pest at 30D of the fermentation period (0.24%) also resulted in lower N contents. Results generally showed a significant increase in N contents of LOF fermented with yeast and with progress in the fermentation period (Table 1). The N contents of LOF increased from 0.26% at 30D to 0.32% at 60D of fermentation, indicating that the more days of fermentation, the higher the N contents. The significantly higher N content of LOF fermented with yeast could be ascribed to the transformation of ammonia to nitrate due to the involvement of nitrifying bacteria.

As microorganisms, yeasts can successfully metabolize and ferment organic materials in this natural setting because they can access essential nutrients and substrates (Walker & Stewart, 2016). In terms of nutrition, yeasts are less demanding compared to other microorganisms, such as lactic acid bacteria. The growths of microorganisms are highly enabled in the presence of fundamental substances like fermentable carbohydrates, amino acids, vitamins, minerals, and oxygen. Microorganisms can improve nutrient release from organic materials and enhance nutrient absorption from carbohydrates and other elements (Maicas, 2020).

The significantly lower N content of LOF fermented with brown sugar (0.23%) occurred due to the limited availability of nutrients and oxygen (Aisyah et al., 2011). Because of the very strongly acidic medium, it could be difficult for the bacteria to ferment the organic materials in brown sugar inducer to rebuild N. Moreover, oxygen is not distributed uniformly on the pile when organic matter is decomposed, and N is released in the form of ammonia to the air. Fermentation is faster in airtight environments (anaerobic) that do not require oxygen (Aisyah et al., 2011). It prevents the ammonia from being converted to nitrate, which further downstream the release of N in the form of ammonia gas. The ammonia gas is released into the air when temperature and pH are high (Ayuningtias, 2014).

N is one of the essential plant nutrients because it plays the most important role in various physiological processes. N has a crucial role in plants' growth and development, including the production of chlorophyll (dark-green color), which promotes plants' leaves, stems, and roots. Rapid early growth and enhanced development of leafy vegetables improved fruit quality and enhanced protein content of fodder crops, among a few of the vital roles that N plays in plants (Leghari et al., 2016). A deficiency of N in soils or plants negatively affects metabolism and results in abnormal plant growth and development, ultimately leading to reduced yield. In the absence of adequate N, plants exhibit deficiency symptoms such as yellowish leaves, a warranting indicator that enough N needs to be applied to correct the problem.

The results of nutrient analyses showed that the highest P contents were found in LOF fermented using yeast for 60D (0.31%) followed by 45D (0.26%). In contrast, the lowest P contents, which were negligible, were found using shrimp paste inducer regardless of fermentation period (Table 1). The decomposition of organic materials using brown sugar also resulted in low P contents (0.01–0.05%), which were statistically not different from shrimp paste. The highest P content in the LOF fermented for 60D using yeast could be attributed to the rise in total P due to the microbial activities in the inducer. The current result is in line with the findings of Lesik et al. (2019), who stated that the activities of decomposing fungi such as *Lactobacillus* sp., *Streptoyces* sp., cellulose, and yeast could remodel P and enhance its content. The fermentation process contributes to accelerating the

breakdown and reorganization of organic materials. The higher P content in the LOF fermented for 60D using yeast could also be ascribed to the availability of N, which are directly correlated; the higher the N content, the higher the P content (Table 2). The availability of more N in the LOF decomposed for 60D by yeast could have enhanced the rapid multiplication of microorganisms that ultimately increased the P content (Hidayati et al., 2011).

The lowest P contents in the LOF fermented using shrimp paste and brown sugar could be linked to the limited availability of N, which inhibited microbial activities that consequently resulted in the formation of the reduced amount of P. The reduction in P levels could also be related to the limited availability of other nutrients required by bacteria for the fermentation process, which could have contributed to the slow rate of organic matter decomposition. The limited availability, in turn, resulted from organic matter not being fully converted to nutrients that are used for metabolism (Santi, 2008). Generally, the highest P contents in LOF were recorded from organic matter decomposition with yeast, and more P was produced as the fermentation period increased (Table 1).

P is an essential plant nutrient that plays vital roles in plant processes, including photosynthesis, respiration, energy production, and nucleic acid biosynthesis. It is also crucial to various plant structures, including phospholipids (Balemi & Negisho, 2012). In the absence of sufficient P in soils or plants, the older leaves change to yellowish or reddish color due to the formation of anthocyanin pigment, which is one of the symptoms of deficiency of P.

Like the analytical results for P, the highest K content (1.68%) was found in the LOF fermented for 60D using yeast, whereas the lowest K in the LOF (0.53%) was obtained from the decomposition of organic matter for 30D using brown sugar (Table 1). The highest K with a longer fermentation period could be due to the fact that yeast has a large amount of K, and it increases during active metabolism (aerobic or anaerobic respiration of certain other substrates) (Armstrong, 1961). This fact is consistent with the (United States Department of Agriculture [USDA], 2019), which stated that 100 g of yeast contains 955 mg of K. In the presence of bacteria, microorganisms use K as a biocatalyst, and their activity significantly impacts the rise in K levels (Lesik et al., 2019). Similar to N and P nutrients, the highest K content in the produced LOF was obtained from the decomposition of organic matter using yeast, and it increased with an advanced period of fermentation (Table 1). Similar results were also reported by Hastuti et al. (2022), who stated that the levels of N, P, and K in organic fertilizers enhanced as the fermentation period increased from 7 to 21 days: the longer the fermentation time, the higher the N, P, and K contents in the fertilizer.

K is one of the most important minerals because many biochemical and physiological processes that affect plant growth, development, and metabolism

depend on it. It plays a crucial function as a biocatalyst in synthesizing and breaking down carbohydrates and converting proteins and amino acids. Additionally, it helps plants to withstand various biotic and abiotic stresses, including diseases, pest attacks, drought, salinity, cold, and waterlogging (Wang et al., 2013).

The highest Mg (0.19%) and Ca (0.09%) contents in the LOF were recorded from the decomposition of organic matter for 60D using shrimp paste and brown sugar, respectively. At the same time, the lowest Mg and Ca were obtained from the fermentation of vegetable leachates without any inducer (Table 1). The richness in Mg sources is ascribed to the enhanced amount of Mg in the LOF fermented with shrimp (Team Phactual, 2019). Similarly, brown sugar contains high Ca due to its molasses content (O'Connor, 2007), resulting in the maximum amount of Ca in the LOF when the organic matter is fermented. Like other macronutrients, the contents of Mg and Ca in the LOF enhanced with an increasing fermentation period (Table 1).

The results of micronutrient analyses in LOF showed that organic matter decomposition for 60D using yeast gave the highest Mn (9.03 mg/kg) and Cu (0.233 mg/kg) contents (Table 1). These results showed that the synthesis of LOF derived from unmarketable vegetables using yeast as an inducer for 60D was the best source for most micronutrients. LOF produced using brown sugar as an inducer for 60D had the highest Fe (11.70 mg/kg) content. As the fermentation period increased from 30 to 60 days, the contents of Zn, Fe, Mn, and Cu in the LOF also consistently enhanced regardless of inducers, indicating the need to allocate sufficient time for organic matter to be fully decomposed by various microorganisms. Similar results were also reported by Ullah et al. (2019), who stated that fermentation with yeast proved more productive than molasses.

The status of the nutrient contents of the produced LOF was evaluated according to the available regulations in Malaysia and other Asian countries. According to the Decree of the Indonesian Minister of Agriculture (DIMA) number 70/Permentan/SR.140/10/2011, the normal range of pH in LOF needs to be 4-9. Thus, the pH of the produced LOF, except those made of brown sugar, met the quality standard for minimal technical requirements (Refilda et al., 2018). According to the standards set for LOF in Thailand (National Bureau of Agricultural Commodity and Food Standards [ACFS], 2005), good quality LOF should normally have an EC value of less than 20 dS/m (Phibunwatthanawong & Riddech, 2019). Accordingly, the LOF produced using shrimp paste, irrespective of the fermentation period, was found to be more than the optimum range; thereby, it could aggravate soil salinity due to its high level of EC.

Although the availability of the highest N, P, and K contents in yeast decomposed LOF for 60D of fermentation (0.95, 0.31, and 1.68%, respectively), results could not meet the quality standard of LOF set in the Agriculture Regulation No.28/Regulation of

Table 1

Effect of various inducers and fermentation period on pH, electrical conductivity (EC), macronutrients (nitrogen [N], phosphorus [P], potassium [K], magnesium [Mg], and calcium [Ca]) and micronutrients (zinc [Zn], iron [Fe], manganese [Mn], and copper [Cu]) in a liquid organic fertilizer

Inducer	Fermentation period (Day)	pH	EC (dS/m)	N (%)	P (%)	K (%)	Mg (%)	Ca (%)	Zn (mg/kg)	Fe (mg/kg)	Mn (mg/kg)	Cu (mg/kg)
None	30	5.33^{cd}	6.17^{f}	0.26^{de}	0.10^{c}	0.96^{ef}	0.01^{h}	0.01^{h}	0.23^{ef}	2.37^{f}	0.13^{e}	0.00^{c}
	45	5.25^{d}	6.49^{f}	0.28^{de}	0.10^{cd}	0.92^{fg}	0.02^{gh}	0.04^{de}	0.67^{cde}	2.67^{f}	0.30^{e}	0.00^{c}
	60	7.41^{a}	7.47^{f}	0.32^{cde}	0.03^{de}	1.00^{e}	0.05^{f}	0.07^{b}	0.80^{bcde}	4.07^{e}	3.37^{c}	0.00^{c}
Yeast	30	5.67^{bcd}	15.32^{e}	0.83^{b}	0.23^{b}	1.24^{c}	0.07^{e}	0.03^{g}	0.07^{f}	4.27^{e}	0.10^{e}	0.13^{abc}
	45	5.66^{bcd}	16.86^{de}	0.91^{ab}	0.26^{ab}	1.35^{b}	0.08^{d}	0.04^{d}	0.40^{def}	6.57^{d}	5.83^{b}	0.07^{bc}
	60	5.78^{bc}	18.40^{d}	0.95^{a}	0.31^{a}	1.68^{a}	0.08^{d}	0.06^{c}	0.43^{def}	7.57^{c}	9.03^{a}	0.23^{a}
Brown sugar	30	2.90^{e}	3.35^{g}	0.23^{e}	0.04^{cde}	0.53^{i}	0.02^{gh}	0.01^{h}	0.70^{cde}	0.57^{h}	0.20^{e}	0.07^{bc}
	45	3.12^{e}	3.49^{g}	0.23^{e}	0.05^{cde}	0.62^{h}	0.03^{g}	0.04^{ef}	1.20^{bc}	10.83^{b}	0.40^{e}	0.17^{ab}
	60	3.30^{e}	3.85^{g}	0.25^{de}	0.01^{e}	0.64^{h}	0.03^{gh}	0.09^{a}	2.40^{a}	11.70^{a}	2.00^{d}	0.13^{abc}
Shrimp paste	30	6.10^{b}	26.30^{c}	0.24^{e}	0.00^{e}	0.88^{g}	0.11^{c}	0.01^{h}	0.97^{bcd}	0.47^{h}	0.17^{e}	0.13^{abc}
	45	7.27^{a}	29.16^{b}	0.35^{cd}	0.01^{e}	1.14^{d}	0.12^{b}	0.03^{g}	1.17^{bc}	2.73^{f}	0.13^{e}	0.13^{abc}
	60	7.54^{a}	32.33^{a}	0.40^{c}	0.00^{e}	1.17^{cd}	0.19^{a}	0.04^{f}	1.33^{b}	1.23^{g}	0.20^{e}	0.13^{abc}
Fermentation period	*p*-value	***	***	***	ns	***	***	***	***	***	***	ns
inducer	*p*-value	***	***	***	***	***	***	***	***	***	***	***
Fermentation period × Inducer	*p*-value	***	***	*	**	***	***	***	***	***	***	*

Note. Means ± standard error with different letters is significantly different at $p < 0.05$ using Tukey, ns = No significant, *$p \leq .05$, **$p \leq .01$, ***$p \leq .001$

Table 2
Correlation coefficients (r) between nitrogen (N), phosphorus (P), and potassium (K) in different fermentation days

Variables measured	30 days N (%)	30 days P (%)	30 days K (%)	45 days N (%)	45 days P (%)	45 days K (%)	60 days N (%)	60 days P (%)
30 days P (%)	0.84***							
30 days K (%)	0.79**	0.73**						
45 days N (%)	0.98***	0.79***	0.82***					
45 days P (%)	0.94***	0.95***	0.78**	0.89***				
45 days K (%)	0.73**	0.52ns	0.91***	0.82***	0.60*			
60 days N (%)	0.97***	0.79**	0.84***	0.99**	0.88***	0.84***		
60 days P (%)	0.99***	0.89***	0.78**	0.97***	0.95***	0.71**	0.97***	
60 days K (%)	0.86***	0.68*	0.95***	0.91***	0.76**	0.97***	0.93***	0.85***

Note. ns = No significant, *$p \leq .05$, **$p \leq .01$, ***$p \leq .001$

the Minister of Agriculture/OT.140/2/200. The regulation stated that LOF's total N, P, and K contents should be greater than 2% (Wahida & Suryaningsih, 2016). Thus, the quality of the LOF fermented with and without inducers could not fulfill the requirements to be considered as LOF in terms of its N, P, and K contents. However, the K content in the LOF decomposed by yeast for 60D (1.68%) was closer to the standard.

Similarly, the micronutrient analytical results showed insufficient contents to meet plant requirements. The normal concentration range of Mn needed in plants is typically from 20 to 300 mg/kg (Brouder et al., 2003), Fe (50 to 250 mg/kg) (Goos & Johnson, 2000), Zn (20 to 100 mg/kg) (Slaton et al., 2005), and Cu (8 to 20 mg/kg (Tilley, 2021).

CONCLUSION

Results of the current study presented that fermentation of unmarketable vegetable leachates for a longer period significantly improved the pH, EC, macro-, and micro-nutrient contents of the produced LOF. Results of the macro- and micro-nutrient analyses for the LOF derived from unmarketable vegetables through fermentation for 60D using yeast as an inducer showed the highest nitrogen (0.95%), phosphorus (0.31%), potassium (1.68%), copper (0.23 ppm), and manganese (9.03 ppm). Moreover, this treatment resulted in optimum pH and EC values for the growth and development of plants. Results also showed that the concentrations

of the nutrients in the LOF increased with the advancement in the fermentation period; as the fermentation period increased, the concentrations of macro- and micro-nutrients consistently enhanced. Despite the significant improvements in quality with the addition of inducers and increased fermentation period, the contents of nutrients in the produced LOF did not meet the quality standards set for LOF by Agriculture Regulation No.28/Regulation of the Minister of Agriculture/OT.140/2/200. Considering the benefits of environmental management, soil health, reduction in chemical fertilizers, and costs incurred by smallholder farmers, further study with an increased ratio of inducers to unmarketable vegetable leachates and fermentation period are recommended to enhance the quality of LOF.

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REFERENCES

Aisyah, S., Sunarlim, N., & Solfan, B. (2011). Pengaruh urine sapi terfermentasi dengan dosis dan interval pemberian yang berbeda terhadap pertumbuhan sawi (*Brassica juncea* L.) [The effect of fermented cow urine with different doses and intervals on the growth of mustard greens (*Brassica juncea* L.)]. *Jurnal Agroteknologi*, *2*(1), 1-5. https://doi.org/10.24014/ja.v2i1.127

Armstrong, W. M. (1961). Distribution of potassium in baker's yeast. *Nature*, *192*, 65–66. https://doi.org/10.1038/192065b0

Ayuningtias, L. (2014). *Pengaruh aerasi dan penambahan silika dengan pemeraman yang berbeda terhadap kandungan N, P, dan K pupuk cair unit gas bio* [The effect of aeration and addition of silica with different ripening on the N, P, and K content of liquid fertilizers of bio gas units] [Unpublished Doctoral dissertation]. Universitas Brawijaya.

Balemi, T., & Negisho, K. (2012). Management of soil phosphorus and plant adaptation mechanisms to phosphorus stress for sustainable crop production: A review. *Journal of Soil Science and Plant Nutrition*, *12*(3), 547–562. https://doi.org/10.4067/S0718-95162012005000015

Brouder, S. M., Bongen, A. S., Eck, K. J., & Hawkins, S. E. (2003). *Manganese deficiencies in Indiana soils*. Purdue Univiversity Cooperative Extension Service. Agronomy Guide. https://www.agry.purdue.edu/ext/pubs/ay-276-W.pdf

Bunyan, J. (2022, May 7). As fertiliser prices go up, farmers seek cash subsidy to offset costs. *Malay Mail*. https://www.malaymail.com/news/malaysia/2022/05/07/as-fertiliser-prices-go-up-farmers-seek-cash-subsidy-to-offset-costs/2057519

Campbell, W. R., & Hanna, M. I. (1937). The determination of nitrogen by modified Kjeldhal Methods. *Journal of Biological Chemistry*, *119*(1), 1–7. https://doi.org/10.1016/S0021-9258(18)74426-8

da Silva, A. J. N., da Silva. R. A., da Silva Santos, J., de Medeiros, J. C. S., de Carvalho, F. G., da Silva, V. N., de Oliveira, C. J., de Araújo, A. C., da Silva, L. E. S. F., & Junior, J. G. (2015). Soil chemical properties and growth of sunflower (*Helianthus annuus* L.) as affected by the application of organic fertilizers and inoculation with arbuscular mycorrhizal fungi. *Revista Brasileira de Ciencia do Solo*, *39*, 151–161. https://doi.org/10.1590/01000683rbcs20150194

Deore, G. B., Limaye, A. S., Shinde, B. M., & Laware, S. L. (2010). Effect of novel organic liquid fertilizer on growth and yield in chilli (*Capsicum annum* L.). *Asian Journal of Experimental Biological Sciences*, *2010*, 15-19.

Duarte, C., Guerra, M., Daniel, P., Camelo, A. L., & Yommi, A. (2009). Quality changes of highbush blueberries fruit stored in CA with different CO_2 levels. *Journal of Food Science*, *74*(4), S154–S159. https://doi.org/10.1111/j.1750-3841.2009.01118.x

Food and Agriculture Organization of the United Nations. (2019). *The state of food and agriculture: Moving forward on food loss and waste reduction*. FAO. https://www.fao.org/3/ca6030en/ca6030en.pdf

Ginandjar, S., Frasetya, B., Nugraha, W., & Subandi, M. (2019). The effect of liquid organic fertilizer of vegetable waste and planting media on growth and yield of strawberry (*Fragaria* spp.) earlibrite cultivar. In *IOP Conference Series: Earth and Environmental Science* (Vol. 334, No.1, p.012033). IOP Publishing. https://doi.org/10.1088/1755-1315/334/1/012033

Goos, R. J., & Johnson, B. E. (2000). A comparison of three methods for reducing iron-deficiency chlorosis in soybean. *Agronomy Journal*, *92*(6), 1135–1139. https://doi.org/10.2134/agronj2000.9261135x

Hamalová, M., Hodslavská, J., Janoš, P., & Kanický, V. (1997). Determination of phosphorus, potassium, and magnesium in fertilizers by inductively coupled plasma–atomic emission spectroscopy and comparison with other techniques. *Journal of AOAC International*, *80*(6), 1151–1155. https://doi.org/10.1093/jaoac/80.6.1151

Hastuti, B., Astuti, R. K., & Hadi, S. (2022). Effect of fermentation time and sugar concentration on the quality characteristic of organic fertilizer from cattle and rabbit manure using vinnase media. *Moroccan Journal of Chemistry*, *10*(3), 387-395. https://doi.org/10.48317/imist.prsm/morjchem-v10i3.32666

Hazri, H. (2022, April 2). Food security a matter of national security. *New Straits Times*. https://www.nst.com.my/opinion/columnists/2022/04/785343/food-security-matter-national-security

Hidayati, Y. A., Kurnani, T. B. A., Marlina, E. T., & Harlia, E. (2011). Kualitas pupuk cair hasil pengolahan feses sapi potong menggunakan *Saccharomyces cereviceae* [Liquid fertilizer quality produced by beef cattle feces fermentation using *Saccharomyces cereviceae*]. *Jurnal Ilmu Ternak*, *11*(2), 104–107.

Ibrahim, M., Tasi'u, Y. R., Umma, M., & Dangora I. I. (2014). The effects of inorganic fertilizer on soil characteristics and production of egg plant (*Solanum melongena* L.) in Warawa area of Kano State. *Standard Research Journal of Agricultural Sciences*, *2*(8), 129–135.

Kala, D. R., Rosenani, A. B., Fauziah, C. I., Ahmad, S. H., Radziah, O., & Rosazlin, A. (2011). Commercial organic fertilizers and their labeling in Malaysia. *Malaysian Journal of Soil Science*, *15*, 147–157.

Mohd Kamaruddin, M. I. S., Abdul Mutalib, A., Ismail, Z., Ab-Latif, Z., & Che Man, S. I. (2021). Effects of chicken manure and shrimp paste on chili pepper (*Capsicum frustescens*) growth. *Asian Journal of Vocational Education and Humanities*, *2*(2), 22–26. https://doi.org/10.53797/ajvah.v2I2.4.2021

Lal, R., Kimble, J. M., Follett, R. F., & Stewart, B. A. (Eds.). (1997). *Soil processes and the carbon cycle* (Vol. 11). CRC Press.

Leghari, S. J., Wahocho, N. A., Laghari, G. M., HafeezLaghari, A., MustafaBhabhan, G., HussainTalpur, K., & Lashari, A. A. (2016). Role of nitrogen for plant growth and development: A review. *Advances in Environmental Biology*, *10*(9), 209–218.

Lesik, M. M. N. N., Dadi, O., Andira, G., & Laban, S. (2019). Nutrient analysis of liquid organic fertilizer from agricultural waste and rumen liquid. In *IOP Conference Series: Earth and Environmental Science*, (Vol 343, No. 1, p. 012178). IOP Publishing. https://doi.org/10.1088/1755-1315/343/1/012178

Maicas, S. (2020). The role of yeasts in fermentation processes. *Microorganisms*, *8*(8), 1142. https://doi.org/10.3390/microorganisms8081142

Martínez-Alcántara, B., Martínez-Cuenca, M. R., Bermejo, A., Legaz, F., & Quiñones, A. (2016). Liquid organic fertilizers for sustainable agriculture: Nutrient uptake of organic versus mineral fertilizers in citrus trees. *PLOS One*, *11*(10), e0161619. https://doi.org/10.1371/journal.pone.0161619

Mohd Zaini, N. S., Idris, H., Yaacob, J. S., Wan-Mohtar, W. A. A. Q. I., Samsudin, N. I. P., Abdul Sukor, A. S., Lim, E. J., & Abd Rahim, M. H. (2022). The potential of fermented food from Southeast Asia as biofertiliser. *Horticulturae*, *8*(2), 102. https://doi.org/10.3390/horticulturae8020102

Murphy, H. F (1968). *A report on the fertility status and other data on some soils of Ethiopia*. http://www.sciepub.com/reference/98585

Murphy, J., & Riley, J. P. (1962). A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*, *27*, 31–36. https://doi.org/10.1016/S0003-2670(00)88444-5

National Bureau of Agricultural Commodity and Food Standards. (2005) *Thai Agricultural Standard TAS 9503-2005: Compost*. ACFS. https://tas2go.acfs.go.th/upload_standard/47_en.pdf

Nhu, N. T. H., Chuen, N. L., & Riddech, N. (2018). The effects bio-fertilizer and liquid organic fertilizer on the growth of vegetables in the pot experiment. *Chiang Mai Journal of Science*, *45*(3), 1257–1273.

O'Connor, A. (2007, June 12). The claim: Brown sugar is healthier than white sugar. *The New York Times*. https://www.nytimes.com/2007/06/12/health/nutrition/12real.html

Pangaribuan, D. H., Sarno., Hendarto, K., Priyanto., Darma, A. K., & Aprillia, T. (2019). Liquid organic fertilizer from plant extracts improves the growth, yield, and quality of sweet corn (*Zea mays* L. var. Saccharata). *Pertanika Journal Tropical Agricultural Science*, *42*(3), 1157–1166.

Phibunwatthanawong, T., & Riddech, N. (2019). Liquid organic fertilizer production for growing vegetables under hydroponic condition. *International Journal of Recycling of Organic Waste in Agriculture*, *8*, 369–380. https://doi.org/10.1007/s40093-019-0257-7

Refilda., Pranesa, T. O., Emil, S., & Indrawati. (2018). Utilization of fermented ngapi nut peel (*Pithecellobium jiringa* Prain) as natural fertilizer and pesticide on tomatoes (*Solanum lycopersicum* Mill) plant. In *Journal of Physics: Conference Series* (Vol. 1116, No. 4, p. 042029. IOP Publishing. https://doi.org/10.1088/1742-6596/1116/4/042029

Santi, S. S. (2008). Kajian pemanfaatan limbah untuk pupuk cair organik dengan proses fermentasi [Study of waste utilization for organic liquid fertilizer with fermentation process]. *Jurnal Teknik Kimia*, *4*(2), 335-340. https://doi.org/10.33005/tekkim.v4i2.133

Slaton, N. A., Gbur, E. E., Wilson, C. E., & Norman, R. J. (2005). Rice response to granular zinc sources varying in water-soluble zinc. *Soil Science Society of America Journal*, *69*(2), 443–452. https://doi.org/10.2136/SSSAJ2005.0443

Tan, L. M. (2015). *Production of fertilizer using food wastes of vegetables and fruits* [Unpublished Doctoral dissertation]. Universiti Malaysia Sarawak.

Team Phactual. (2019, April 8). *Magnesium rich foods*. https://www.phactual.com/fnen/magnesium-rich-foods/

Tilley, N. (2021, April 7). *Copper and soil- How copper affects plants*. Gardening Know How. https://www.gardeningknowhow.com/garden-how-to/soil-fertilizers/copper-for-the-garden.htm

Ullah, A., Mushtaq, A., Qamar, R. A., & Ali, Z. U. (2019). Extraction of soil conditioners from food waste. *Science International*, *31*(2), 347–354.

United States Department of Agriculture. (2019). *Leavening agents, yeast, baker's, active dry*. USDA. https://fdc.nal.usda.gov/fdc-app.html#/food-details/175043/nutrients

Wahida, W., & Suryaningsih, N. (2016). Analisis kandungan unsur hara pupuk organik cair dari

limbah rumah tangga di Kabupaten Merauke [Analysis of nutrient content of liquid organic fertilizer from household waste in Merauke Regency]. *Agricola*, *6*(1), 23–30. https://doi.org/10.35724/AG.V6I1.398

Walker, G. M., & Stewart, G. G. (2016). *Saccharomyces cerevisiae* in the production of fermented beverages. *Beverages*, *2*(4), 30. https://doi.org/10.3390/beverages2040030

Wang, M., Zheng, Q., Shen, Q., & Guo, S. (2013). The critical role of potassium in plant stress response. *International Journal of Molecular Sciences*, *14*(4), 7370-7390. https://doi.org/10.3390/ijms14047370

Zhang, L., Sun, X., Tian, Y., & Gong, X. (2013). Effects of brown sugar and calcium superphosphate on the secondary fermentation of green waste. *Bioresource Technology*, *131*, 68–75. https://doi.org/10.1016/j.biortech.2012.10.059

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Developmental Toxicity of Zinc Oxide Nanoparticles on the Early Life Stage of Java Medaka (*Oryzias javanicus* Bleeker, 1856)

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ABSTRACT

With a high likelihood of being discharged into aquatic habitats, zinc oxide nanoparticles have been widely employed in a variety of industrial and commercial goods. Concerns over their effects on the environment and human health have grown. This study evaluated the developmental toxicity of zinc oxide nanoparticles **(**ZnO NPs) on the embryo Java medaka (*Oryzias javanicus*). With three replicates for each treatment group, the Java medaka embryos were subject to various concentrations of ZnO NPs (10, 25, 50, 100, and 150 µg/L). The heartbeat of treated embryos was increased compared to the control group at 5-, 8-, and 11-days post-exposure (dpe). However, the hatching and mortality of embryos decreased when the concentrations of ZnO NPs increased. Meanwhile, deformities such as low pigmentation, edema (yolk sac and pericardial edema), and spinal deformities were observed in the embryo and larva during the exposure time. Compared to previous studies, ZnO NPs show severe toxicity to selected endpoints at lower concentrations in the embryos of Java medaka.

Keywords: Early life stage, Medaka, nanoparticles, ZnO NPs

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INTRODUCTION

Developing new techniques for manufacturing goods and using these novel nanoparticles (NPs) has advanced in the USA, Europe, and Japan following the identification of carbon-60 fullerenes (C_{60}), carbon nanotubes, and quantum dots. The United States National Nanotechnology Initiatives defined NPs as substances with at least one dimension falling within the range of 1 and 100 nm (Kashiwada, 2006), and these particles can be nano-films, nano-wires and nano-tubes or NPs, while the application of these materials is known as nanotechnology (Handy et al., 2008; Patibandla et al., 2018). Different types of NPs could be categorized based on their structural constituents. Carbon-based NPs, like carbon nanotubes and carbon-black, fall into one category. Inorganic NPs, including aluminum (Al), bismuth (Bi), cobalt (Co), zinc oxide (ZnO), and copper oxide (CuO), form another category. Organic-based NPs, for example, dendrimers, micelles, liposomes, and polymer NPs, constitute a separate category. Additionally, composite-based NPs are another type that can be categorized based on their structural constituents (Amin et al., 2021). The hazards and advantages of these new materials have been extensively debated. The advantages of NPs are huge, and their benefits are still being studied.

At room temperature, these materials possess a wide band-gap semiconductor characterized by a band-gap energy of 3.3 eV (Sabir et al., 2014), excellent stability, resistance to corrosion, and the ability to catalyze photosynthesis (Hao et al., 2013). These materials are also non-migratory, fluorescent, piezoelectric, capable of absorbing light and scattering ultraviolet light (Li et al., 2018), diverse nanostructures (Bai et al., 2010). Despite having numerous applications, ZnO NPs are the third most widely produced nanoparticles worldwide, with annual production falling behind only silicon dioxide NPs (SiO_2 NPs) and titanium dioxide NPs (TiO_2 NPs) (García-Gómez et al., 2020; Rajput et al., 2018). These nanoparticles are frequently combined to produce sunscreens to ensure enhanced protection against ultraviolet (UV) radiation. However, ZnO NP usage may overtake TiO_2 NPs shortly by being able to absorb both UV-A and UV-B radiation; it provides enhanced protection and increased opacity (Wong et al., 2010). The use of ZnO NPs in cosmetics, medication delivery systems, therapies, and biosensors has been incorporated into human's daily lives. Several studies have been reported on the possible toxicity of ZnO NPs to aquatic environments; the key emphasis has been on acute toxicity studies of aquatic species. At the same time, the chronic toxicity studies of ZnO NPs were surprisingly scarce. Meanwhile, most of those studies focus on the early life stage of zebrafish because of their well-known biology and short life span. For instance, Bai et al. (2010) reported that the embryos of zebrafish dead after exposing to ZnO NPs at 50 and 100 mg/L, retarded the hatching at 1-25 mg/L, and caused abnormality after the 96-hour post-fertilization (hpf) exposure.

The aquatic invertebrate species *Daphnia magna* and *Thamnocephalus platyurus* have both been involved in ZnO NP toxicity studies, and comparable effective concentration (EC_{50}) and lethal concentration (LC_{50}) values were reported in the literature for them (Blinova et al., 2010; Heinlaan et al., 2008; Zhu et al., 2009). For instance, 3.20 and 0.18 mg/L were reported as 48-hr LC_{50} values for *D. magna* and *T. platyurus* (Blinova et al., 2010; Heinlaan et al., 2008). Meanwhile, for the exact NPs, the 48-hr EC_{50} was 2.60 mg/L for *D. magna,* and the 24-hr LC_{50} was 0.14 for *T. platyurus* (Blinova et al., 2010). Although the toxicity of ZnO NPs was reported in different studies on some living organisms in their habitat, a comprehensive deficit assessment in evaluating the toxicity of ZnO NPs and their long-term effect at environmentally relevant concentrations still exists.

According to widespread consensus, the growing fish embryo or larvae are the optimal phase in the lifespan of a bony fish because they are specifically susceptible to low levels of environmental pollution (Bai et al., 2010; Wu et al., 2010). Meanwhile, following unique characteristics associated with Java medaka (*O. javanicus*), such as their excellent osmotic adaptation to a wide range of salinity from freshwater and brackish water to salt water, their small size, transparent body, and their short life cycle, these fish were chosen as a test organism instead of other model organisms. Moreover, Java medaka is essential to estuarine ecosystems, providing a feeder fish for commercially, recreationally, and scientifically valuable species like the mudskipper. As a top dweller, the Java medaka is also exposed to pollutants introduced to the aquatic ecosystem by atmospheric deposition. Furthermore, the partial-life test of medaka fish has been frequently used to determine the developmental impact of various substances or to evaluate compounds derived from complicated environmental mixtures (Wu et al., 2010). Hence, this study was carried out to ascertain the developmental toxicity of ZnO NPs in the embryonic stage of the Java medaka.

MATERIALS AND METHODS

Java Medaka Culture and Embryo Selection

Java medaka breeding groups were collected from the estuary region of the Sepang River in Selangor, Malaysia. Then, they were maintained in overflow containers and fed fresh *Artemia nauplii* (brine shrimp) larvae three times daily. They were maintained under 14 hr light and 10 dark periods at 26°C. Prior to the experiment, newly fertilized cluster eggs were collected directly from the female body and washed several times to eliminate any remaining substances on the surface of the egg. Healthy embryos at less than 8 hpf were then selected for subsequent experiments.

Zinc Oxide NPs Stock Preparation and Characterization

Sigma-Aldrich (USA) supplied the uncoated ZnO NPs with a stated particle size of less than 50 nm and more than 97% purity.

Stirring was used to produce a stock solution of ZnO NPs (10 mg/L), which was then diluted to exposure concentrations. Embryos were induced to five distinct exposure levels of ZnO NPs (10, 25, 50, 100, and 150 µg/L). In our earlier work, the characterization of ZnO NPs for size in powder form using an X-ray diffraction spectrometer (XRD), shape or morphology using transmission electron microscopy (TEM), and size distribution and surface charge using dynamic light scattering (DLS) was already reported (Amin et al., 2021).

Embryo Bioassays

The exposure protocol was modified from the guidelines of the Organization for Economic Co-operation and Development (OECD) (2013) for testing chemicals through the fish: early life stage toxicity test. A single embryo at the blastula stage was placed in each of the test wells of a 24-well multi-plate. Each of the twenty wells contained 2 ml of ZnO NPs test solution, while the remaining four contained 2 ml of water per well. A total of 150 embryos had been utilized in the exposure groups, and 30 embryos were used in the control group for the triplicate experiment.

Based on the distinctive characteristics of the early life stage of *Oryzias latipes* (Japanese medaka), the embryonic process of Java medaka was observed (Iwamatsu & Kobayashi, 2002). At 5-, 8-, and 11-days post fertilization (dpf), embryos' heartbeats were counted over a 15-s (in three replicates) to estimate heart rate. A stereo microscope (Olympus Corporation, Japan) equipped with a digital camera was used to inspect the abnormalities in embryos and newly hatched larvae beginning at 11 dpf (also known as 1-day post-hatch [dph]) and continuing until the final day of the experiment, hatching numbers were measured daily. Unhatched embryos were no longer monitored after 20 days because they were regarded as hatching failures.

Statistical Analysis

One-way analysis of variance (ANOVA) was used with a 95% confidence interval to compare the heart rate at 5, 8, and 11 dpe, mortality, and hatching rates between the control and treatment groups by using GraphPad Prism (version 8.0.2) for Windows, which is developed by GraphPad Software (USA). The significance of the test results was determined at a P-value < 0.05. Additionally, the Shapiro-Wilk test was performed to assess the normal distribution of the data. The data are expressed as the mean ± standard deviation (SD).

RESULTS

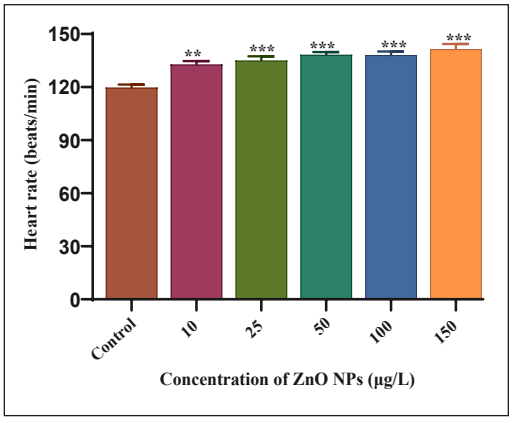
During the embryonic stage of Java medaka, the toxic effects produced by ZnO NPs on developmental endpoints were found to be dependent on the concentration.

The heart rate (y-axis) of embryos exposed to ZnO NP concentration (x-axis) at 5, 8, and 11 dpe is shown in Figure 1. Compared to the control, there was a significant concentration-related increase in the heart rate of exposed embryos to ZnO NPs. For instance, in contrast to control, at 5 dpe to 10 µg/L (132.8 ± 3.27 heart beats

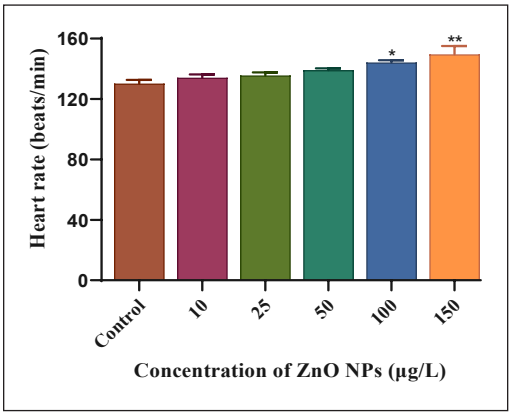
per minute (HBpM), $P < 0.03$), 25 µg/L (135.06 ± 4.00 HBpM, $P < 0.001$), 50 µg/L (138.30 ± 2.52 HBpM, $P < 0.001$), 100 µg/L (138.13 ± 3.40 HBpM, $P < 0.001$), and 150 µg/L (141.46 ± 5.00 HBpM, $P < 0.001$) showed significantly higher HBpM (Figure 1a). However, at 8 dpe to 100 µg/L (144.24 ± 2.61, $P < 0.01$) and 150 µg/L (149.63 ± 9.52 HBpM, $P < 0.03$), and 11 dpe to 10 µg/L (160.09 ± 1.85 HBpM, $P < 0.01$), 50 µg/L (161.18 ± 1.11 HBpM, $P < 0.01$), and 100 µg/L (160.9 ± 2.28 HBpM, $P < 0.01$) showed significantly higher HBpM compared to control group (Figures 1b and 1c).

From 11 dpe, the hatching of embryos was recorded. Hatching of embryos was started in control at 13 dpe with 30% of hatchability. With increasing concentrations of ZnO nanoparticles in the treatment groups, there was a reduction in the hatching rate of embryos. Figure 2 shows that the percentage of hatching success of Java medaka's embryos treated to ZnO NPs was inversely related. None of the individual embryos hatched when exposed to 150 µg/L at the end of the experiment (21 dpe). The lowest hatching success was observed at 100 µg/L with 63.33%, significantly ($P < 0.01$) lower than control at 100%. The percentage of hatching success of exposed embryos in treatment groups was decreased from 96.66, 90, 83.33, 63.33, and 0%. Meanwhile, some embryos failed to hatch after exposure to ZnO NPs.

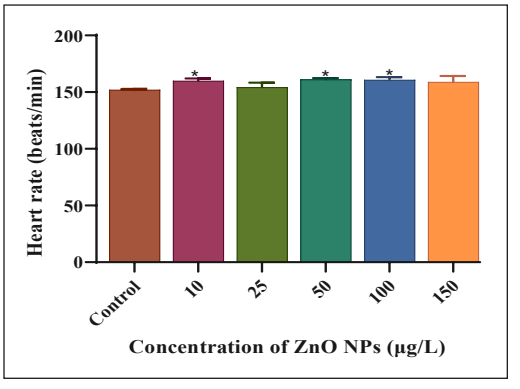
Throughout the experiment, no death embryos were recorded within the control group. However, concentration-dependent mortality was observed in treatment groups.



(a)



(b)



(c)

Figure 1. Heart rate of Java medaka's embryos at (a) 5-, (b) 8-, and (c) 11-days post-exposure to different concentrations of zinc oxide nanoparticles (ZnO NPs) *Note*. Data expressed as means ± SD with n = 3. *, **, *** shows significant difference at $P < 0.05$, $P < 0.01$, and $P < 0.001$ between control and treatment groups

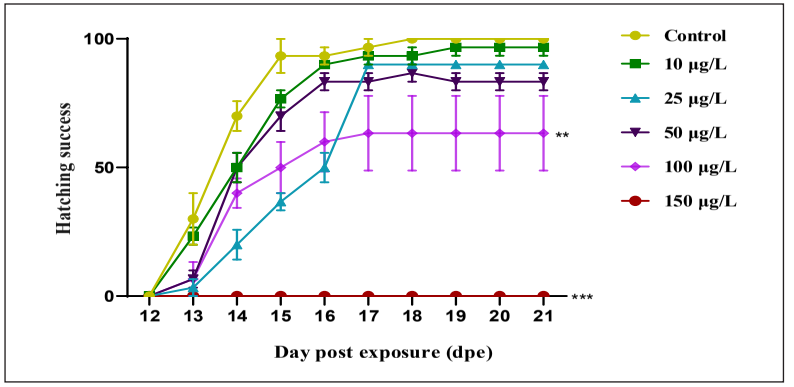


Figure 2. The hatching percentage of Java medaka's embryos exposed to different concentrations of zinc oxide nanoparticles, with n = 3

Note. **, *** shows a significant difference at $P < 0.01$ and $P < 0.001$, respectively, between control and treatment groups

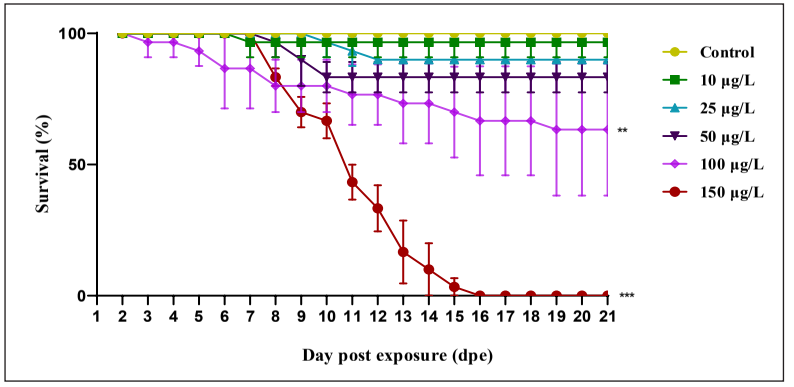


Figure 3. The survival rate of Java medaka's embryo exposed to different concentrations of zinc oxide nanoparticles, with n = 3

Note. *, **, *** shows a significant difference at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, between control and treatment groups

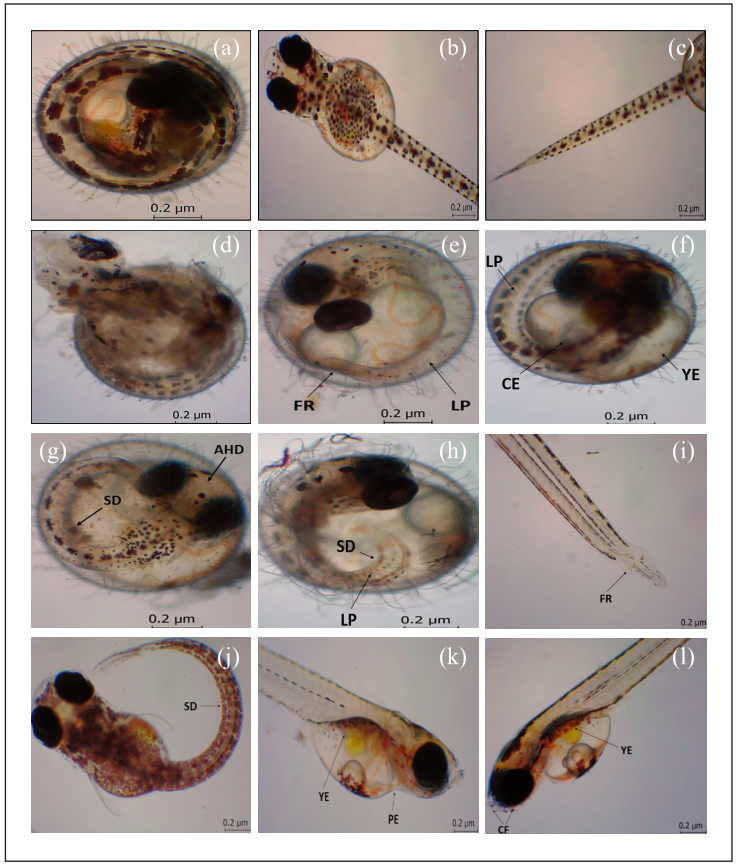


Figure 4. Abnormalities of Java medaka's embryos exposed to different concentrations of zinc oxide nanoparticles (ZnO NPs). (a, b, and c) control group; (d) failed hatching at 100 µg/L ZnO NPs; (e, j, and k) 25 µg/L ZnO NPs; (f and l) 50 µg/L ZnO NPs; (h) 100 µg/L ZnO NPs; (i) 10 µg/L ZnO NPs

Note. LP = Low pigmentation; FR = Fin rot; SD = Spinal deformities; CE = Cranial oedema; YE = Yolk sac oedema; CF = Craniofacial deformities

In contrast to control, significantly ($P < 0.001$) higher mortality was observed at 150 µg/L (100%) and 100 µg/L (36.66%) at the end of the experiment. However, a low mortality rate was observed at 10 µg/L (3.33%) and 25 µg/L (10.00%) (Figure 3).

The control embryos were observed to be normal (Figures 4a, 4b, and 4c). ZnO NPs-treated Java medaka embryos underwent numerous morphogenesis malformations during the embryonic exposure experiment, and these deformities depended on the exposure concentration. Observed abnormalities included low pigmentation, fin rot, spinal deformities, cranial edema, yolk sac edema, abnormal head deformities, abnormal pigmentation, lateral body curvature, hatching failure, and death of Java medaka embryos. However, after hatching in treatment groups, tail malformation, spinal deformities, edema, and short lifespan of juveniles were observed in post-hatched juveniles (Figure 4). Furthermore, edema (yolk sac and heart edema) and low pigmentation were frequently observed in treated embryos.

DISCUSSION

Acute toxicity studies of aquatic species have received the most attention in the research that has been published regarding the possible harmful impacts of ZnO NPs on aquatic ecosystems. Meanwhile, the long-term toxicity studies of ZnO NPs were surprisingly scarce. Previous research has found that ZnO NPs pose serious risks and have greater toxicity than other NPs in the aqueous ecosystem. Several developmental markers were evaluated in this study to investigate the potential developmental toxicity of ZnO NPs in the embryonic stage of Java medaka. The findings showed that ZnO NPs were hazardous to the endpoints throughout the embryo's development and induced abnormalities in the embryo and larvae. It is the first instance in which the developmental toxicity of ZnO NPs in Java medaka's embryos has been documented. Furthermore, it is noteworthy that in this study, we exposed Java medaka embryos for 21 days, significantly longer than the average freshwater fish embryo growth period of 5 days for zebrafish. Prolonged exposure may have consequences beyond the acute ones and may be useful in understanding the mechanism of ZnO NP toxicity.

The early-life stage of medaka bioassays is a sensitive laboratory model for assessing the impacts of contaminants (Wu & Zhou, 2012). Significant increases in toxicity occurred when medaka embryos were subjected to 10–150 µg/L of ZnO NPs. Our result was in accordance with previous studies—for example, P.-J. Chen et al. (2013) demonstrated that the toxicity of ZnO NPs increased by increasing exposure concentration on *O. latipes* (Japanese medaka) embryos. Meanwhile, the ZnO NPs killed zebrafish embryos at 50 and 100 mg/L, retarded hatching at 1-25 mg/L, and caused malformation after 96 hpf exposure. However, at exposure levels of 0.10 and 1 mg/L, ZnO NPs did not hinder the growth of *Dunaliella tertiolecta*. The lowest concentration at which an effect

was observed was 0.50 and 3 mg/L, and the EC_{50} after 96 hr was determined to be 2.42 and 4.45 mg/L for ZnO NPs with sizes of 100 and 200 nm, respectively (Hou et al., 2018). Studies have also demonstrated that the toxicity of ZnO NPs on bacteria (Sirelkhatim et al., 2015), algae (Suman et al., 2015), invertebrates (Ates et al., 2013; Khoshnood et al., 2016; Xiao et al., 2015), and vertebrates (Fernández et al., 2013; Murthy et al., 2022) are also influenced by their concentration. In our study, higher toxicity was observed in lower concentrations of ZnO NPs in the early life stage of Java medaka compared to other studies that involved fish as model organisms (Table 1).

Our studies show that the heart rate of Java medaka at the early life stage typically rises as they grow up. Concentration-dependent increases in heart rate were observed in exposed embryos of Java medaka. Increases in heart rate after exposure to ZnO NPs were also mentioned in the literature. Cong et al. (2017) demonstrated that the heart rate of *Oryzias melastigma* (marine medaka) exposed embryos increased by increasing the concentration of ZnO NPs. In contrast, Wu et al. (2010) conducted a study where the embryos of

Table 1
Comparison of zinc oxide nanoparticles (ZnO NPs) concentrations in toxicological studies on different species of fish

Species	Life stage	Concentration of ZnO NPs	Duration of exposure (Days)	References
Java medaka (*Oryzias javanicus*)	Embryo	10, 25, 50, 100, and 150 µg/L	21	Current study
Java medaka (*Oryzias javanicus*)	Embryo	0.10, 0.25, 0.50, 1, 5, and 10 mg/L	4	Amin et al. (2021)
Marine medaka (*Oryzias melastigma*)	Embryo	0.01, 0.1, 1, and 10 mg/L	20	Cong et al. (2017)
Zebrafish (*Danio rerio*)	Embryo	1, 5, 10, 25, 50, and 100 mg/L	4	Bai et al. (2010)
Zebrafish (*Danio rerio*)	Embryo	1, 5, 10, 20, 50, and 100 mg/L	4	Zhao et al. (2013)
Zebrafish (*Danio rerio*)	Embryo and larva	0.1, 0.5, 1, 5, 10, 50, and 100 mg/L	4	Zhu et al. (2009)
Carp (*Cyprinus carpio*)	Juvenile	50 mg/L	30	Hao et al. (2013)
Rainbow trout (*Oncorhynchus mykiss*)	Adult	1, 10, and 100 mg/L	4	Taherian et al. (2019)
Lobeo rohita	Adult	2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 55 mg/L	4	Aziz et al. (2020)
Common carp (*Cyprinus carpio*)	Adult	0.382, 0.573, and 1.146 mg/L	4	Rajkumar et al. (2022)
Tilapia (*Oreochromis niloticus*)	Adult	1 and 10 mg/L	14	Kaya et al. (2016)

Japanese medaka were exposed to silver nanoparticles. They discovered a notable increase in heart rate among the exposed embryos, with the intensity of the increase being directly related to the concentration of exposure (ranging from 100 to 400 µg/L).

However, the zebrafish embryos showed a concentration-dependent decrease in heart rate after exposure to silver NPs in the study of Asharani et al. (2008), leading them to conclude that the cardiac cycle was disrupted by increasing concentration of silver NPs. Similarly, Cong et al. (2017) and Wu et al. (2010) suggested that the effects of ZnO and silver NPs on the embryos could also cause an increased heart rate. Moreover, NPs are also suspected of interacting with the endothelium of the arteries, having an immediate impact on the atherosclerotic plaque, or serving as a location for the development of thrombi and myocardial infarctions (A. Peters et al., 2001; Gatti et al., 2004; Khandoga et al., 2004).

Through fish embryogenesis, hatching is considered to be an important stage. In the current study, rising ZnO NP concentrations impacted Java medaka hatching. Concentration-dependent hatching rate inhibition for ZnO NPs was also reported on marine medaka (Cong et al., 2017), zebrafish (Bai et al., 2010; T.-H. Chen et al., 2014; Zhao et al., 2013; Zhu et al., 2009), and yellow stripe goby (Li et al., 2018). The blocking of the chorion pore by the adsorption of ZnO NPs limits oxygen and nutrient supply, which could affect the activities of the proteolytic enzyme. The enzyme known as proteolytic is released by the growing embryo's glandular cells during the normal hatching of teleost embryos, which then consumes the chorion (Bai et al., 2010; Cong et al., 2017). Then, P.-J. Chen et al. (2013) trapped the iron NPs inside the chorion and reported that the transport membrane of oxygen (O_2) was affected, which finally delayed the hatching of Japanese medaka. He further mentioned that due to hypoxia-induced by stabilized nanoscale zerovalent iron (CMS-nZVI), the hatching of Japanese medaka was retarded.

An increase in exposed embryo mortality was observed compared to the control. The increase in mortality after exposure to ZnO NPs on the embryo of yellow stripe goby (*Mugilogobius chaulae*) (Li et al., 2018), zebra fish embryos-larva (Bai et al., 2010; Zhao et al., 2013), Java medaka (Amin et al., 2021), marine medaka (Cong et al., 2017) were also reported in the literature. In the current study, numerous deficits have been detected at the embryonic and larval stages after exposure to ZnO NPs, along with embryo death. The malformations include low pigmentation, fin rot, spinal deformities, cranial edema, yolk sac edema, abnormal head deformities, pigmentation, lateral body curvature, and tail malformations. Among all the observed morphological abnormalities, the presence of edema (specifically pericardial and yolk sac) and low pigmentation were frequently observed abnormalities across all the treatment groups. Similarly, Cong et al. (2017) showed that ZnO NP exposure causes fin rot, spinal abnormalities, craniofacial malformations, and edema (yolk sac) in the early life stage of marine medaka. In a study

by Wu et al. (2010), abnormalities such as tail, spinal cord flexure, and truncation were observed after exposing Japanese medaka to silver NPs.

Additionally, tail abnormalities, spinal deformities, and edema were observed after exposing different fish species' embryos to ZnO NPs (Bai et al., 2010; Li et al., 2018; Zhu et al., 2009). According to a recent investigation, tiny particles might be taken up by the medaka chorion and then transferred into the yolk sac and body, raising the possibility that they could remain persistent during the embryo's growth (Wu et al., 2010). Once iron NPs are ingested by the chorion, they may begin redox cycling iron due to pH differences between the chorion's exterior and interior or between different organs, which accelerates the production of internal reactive oxygen species (ROS) and results in oxidative damage and developmental toxicity (P.-J. Chen et al. (2013). Additionally, earlier research has shown that several *Vibrio polysaccharide* gene (*VPS*) mutations, such as the *vps18* mutant in zebrafish and the *vps11* mutation in medaka fish, may decrease pigmentation and result in the most severe abnormalities (Golling et al., 2002; Yu et al., 2006). Because of their size and physicochemical characteristics, nanoparticles may interact differently with physiologically important macromolecules like DNA, as suggested by Balbus et al. (2007) and the abnormalities observed in the current study.

Therefore, more research is required to ascertain if ZnO NPs can suppress or influence the expression of vps genes. Furthermore, silver NPs (Ag NPs) caused spinal cord deformities associated with detectable hemostasis (coagulated blood) in Japanese medaka (Wu et al., 2010). The proper development of the embryonic stage is hampered by hemostasis because it limits the number of nutrients transported by the blood from the yolk to the embryonic organs or tissues. Therefore, yolk sac edema abnormalities were more observed in the current study. Meanwhile, different studies also observed edema during the embryonic stage of fathead minnow (Laban et al., 2010), zebrafish (Lee et al., 2007; Zhao et al., 2013), and Japanese medaka (Wu & Zhou, 2012) when they were exposed to different NPs. Disturbance in osmoregulation caused by toxicants is the common reason for edema (Kiener et al., 2008). According to L. E. Peters et al. (2007), osmoregulation and the disturbed circulatory system were thought to be responsible for several malformations observed in medaka. The disturbance of the circulatory system and osmoregulation by ZnO NPs can be correlated with these abnormalities (Bai et al., 2010; Li et al., 2018; Zhu et al., 2009).

Java Medaka (*O. javanicus*) as a Nano-ecotoxicology Model

With findings acquired in a matter of days, this experiment indicates that Java medaka embryos may be employed as high productivity, high efficiency, economical, and highly responsive to the wide range of toxicants for examining the toxicity of NPs. Several organisms that study the effects

of NPs have a limited understanding of the genotoxic or metabolic effects due to the range of tests that may be performed or the model used. The zebrafish test organism has already been used in several nano-ecotoxicological studies (Bai et al., 2010; Xiong et al., 2011; Zhu et al., 2008). However, the model organism utilized in this work has several benefits, particularly for examining how NPs affect the environment. Java medaka shares many advantages with the well-known model organism Japanese medaka (*O. latipes*), such as their distribution, availability all year round, small size (3–4 cm), brief life span, and life cycle, rapid growth rate, readily identifiable, and easily cultured under laboratory conditions. Another notable feature of Java medaka is their sizable, see-through eggs and embryos, enabling convenient observation of their developmental stages (Amal et al., 2018, 2019; Salleh et al., 2017; Wittbrodt et al., 2002; Woo & Yum et, 2001).

Eggs fertilization and development occur externally. Daily, Java medaka produces a range of 30 to 50 eggs, with a potential total of 3,000 eggs during the mating season. The spawning event lasts less than one minute and closely regulates light cycles. Identifying and observing sexually active females of Java medaka is easy due to the presence of attachment filaments that secure the eggs to their bodies. Furthermore, the Java medaka is an ideal test organism as it reaches sexual maturity within a mere 100 days from hatching. It is much easier to collect eggs for subsequent research at the exact time of spawning, especially for those investigations involving extremely early stages of embryo formation up until later stages (Imai et al., 2007; Ismail & Yusof et al., 2011; Wittbrodt et al., 2001).

CONCLUSION

To the best of our knowledge, this study reported the developmental toxicity of ZnO NPs in the initial growth phase of Java medaka for the first time. Heart rate, hatching, mortality, and deformities of embryos were criteria to assist the developmental toxicity of ZnO NPs on the embryonic development of Java medaka. ZnO NPs show concentration-dependent toxicity to all developmental endpoints. The results demonstrate that Java medaka embryos show more sensitivity compared to the well-known model organism zebrafish to low concentrations of ZnO NPs, showing the potential of Java medaka embryos as an in vivo study to demonstrate the bioavailability and toxicity of NPs. The present study highlights tasks focused on the environmental-related concentration of NPs on fish embryos to manage their ecological risk.

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REFERENCES

Amal, M. N. A., Ismail, A., Saad, M. Z., Yasin, I. S. M., Nasruddin, N. S., Mastor, S. S., Rahman, M. H. A., & Mohamad, N. (2019). Study on *Streptococcus agalactiae* infection in Java medaka (*Oryzias javanicus* Bleeker, 1854) model. *Microbial Pathogenesis*, *131*, 47-52. https://doi.org/10.1016/j.micpath.2019.03.034

Amal, M. N. A., Zarif, S. T., Suhaiba, M. S., Aidil, M. R. M., Shaqinah, N. N., Zamri-Saad, M., & Ismail, A. (2018). The effects of fish gender on susceptibility to acute *Streptococcus agalactiae* infection in Java medaka *Oryzias javanicus*. *Microbial Pathogenesis*, *114*, 251-254. https://doi.org/10.1016/j.micpath.2017.11.069

Amin, N., Zulkifli, S. Z., Azmai, M. N. A., & Ismail, A. (2021). Toxicity of zinc oxide nanoparticles on the embryo of Java Medaka (*Oryzias javanicus* Bleeker, 1854): A comparative study. *Animals*, *11*(8), 2170. https://doi.org/10.3390/ani11082170

Asharani, P. V., Wu, Y. L., Gong, Z., & Valiyaveettil, S. (2008). Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology*, *19*(25), 255102. https://doi.org/10.1088/0957-4484/19/25/255102

Ates, M., Daniels, J., Arslan, Z., Farah, I. O., & Rivera, H. F. (2013). Comparative evaluation of impact of Zn and ZnO nanoparticles on brine shrimp (*Artemia salina*) larvae: Effects of particle size and solubility on toxicity. *Environmental Science: Processes and Impacts*, *15*(1), 225–233. https://doi.org/10.1039/C2EM30540B

Aziz, S., Abdullah, S., Abbas, K., & Zia, M. A. (2020). Effects of engineered zinc oxide nanoparticles on freshwater fish, *Labeo rohita*: Characterization of ZnO nanoparticles, acute toxicity, and oxidative stress. *Pakistan Veterinary Journal*, *40*(4), 479-483. https://doi.org/10.29261/pakvetj/2020.030

Bai, W., Zhang, Z., Tian, W., He, X., Ma, Y., Zhao, Y., & Chai, Z. (2010). Toxicity of zinc oxide nanoparticles to zebrafish embryo: A physicochemical study of toxicity mechanism. *Journal of Nanoparticle Research*, *12*, 1645-1654. https://doi.org/10.1007/s11051-009-9740-9

Balbus, J. M., Maynard, A. D., Colvin, V. L., Castranova, V., Daston, G. P., Denison, R. A., Dreher, K. L., Goering, P. L., Goldberg, A. M., Kulinowski, K. M., Monteiro-Riviere, N. A., Oberdörster, G., Omenn, G. S., Pinkerton, K. E., Ramos, K. S., Rest, K. M., Sass, J. B., Silbergeld, E. K., & Wong, B. A. (2007). Meeting report: Hazard assessment for nanoparticles—Report from an interdisciplinary workshop. *Environmental Health Perspectives*, *115*(11), 1654–1659. https://doi.org/10.1289/ehp.10327

Blinova, I., Ivask, A., Heinlaan, M., Mortimer, M., & Kahru, A. (2010). Ecotoxicity of nanoparticles of CuO and ZnO in natural water. *Environmental Pollution*, *158*(1), 41-47. https://doi.org/10.1016/j.envpol.2009.08.017

Chen, P.-J., Wu, W.-L., & Wu, K. C.-W. (2013). The zerovalent iron nanoparticle causes higher developmental toxicity than its oxidation products in early life stages of medaka fish. *Water Research*, *47*(12), 3899–3909. https://doi.org/10.1016/j.watres.2012.12.043

Chen, T.-H., Lin, C.-C., & Meng, P.-J. (2014). Zinc oxide nanoparticles alter hatching and larval locomotor activity in zebrafish (*Danio rerio*). *Journal of Hazardous Materials*, *277*, 134-140. https://doi.org/10.1016/j.jhazmat.2013.12.030

Cong, Y., Jin, F., Wang, J., & Mu, J. (2017). The embryotoxicity of ZnO nanoparticles to marine medaka, *Oryzias melastigma*. *Aquatic Toxicology*, *185*, 11–18. https://doi.org/10.1016/j.aquatox.2017.01.006

Fernández, D., García-Gómez, C., & Babín, M. (2013). *In vitro* evaluation of cellular responses induced by ZnO nanoparticles, zinc ions and bulk ZnO in fish cells. *Science of the Total*

Environment, *452–453*, 262–274. https://doi.org/10.1016/j.scitotenv.2013.02.079

García-Gómez, C., García-Gutiérrez, S., Obrador, A., & Fernández, M. D. (2020). Study of Zn availability, uptake, and effects on earthworms of zinc oxide nanoparticle *versus* bulk applied to two agricultural soils: Acidic and calcareous. *Chemosphere*, *239*, 124814. https://doi.org/10.1016/j.chemosphere.2019.124814

Gatti, A. M., Montanari, S., Monari, E., Gambarelli, A., Capitani, F., & Parisini, B. (2004). Detection of micro- and nano-sized biocompatible particles in the blood. *Journal of Materials Science: Materials in Medicine*, *15*, 469–472. https://doi.org/10.1023/B:JMSM.0000021122.49966.6d

Golling, G., Amsterdam, A., Sun, Z., Antonelli, M., Maldonado, E., Chen, W., Burgess, S., Haldi, M., Artzt, K., Farrington, S., Lin, S.-Y., Nissen, R. M., & Hopkins, N. (2002). Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. *Nature Genetics*, *31*, 135–140. https://doi.org/10.1038/ng896

Handy, R. D., von der Kammer, F., Lead, J. R., Hassellöv, M., Owen, R., & Crane, M. (2008). The ecotoxicology and chemistry of manufactured nanoparticles. *Ecotoxicology*, *17*, 287-314. https://doi.org/10.1007/s10646-008-0199-8

Hao, L., Chen, L., Hao, J., & Zhong, N. (2013). Bioaccumulation and sub-acute toxicity of zinc oxide nanoparticles in juvenile carp (*Cyprinus carpio*): A comparative study with its bulk counterparts. *Ecotoxicology and Environmental Safety*, *91*, 52-60. https://doi.org/10.1016/j.ecoenv.2013.01.007

Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.-C., & Kahru, A. (2008). Toxicity of nanosized and bulk ZnO, CuO, and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Chemosphere*, *71*(7), 1308-1316. https://doi.org/10.1016/j.chemosphere.2007.11.047

Hou, J., Wu, Y., Li, X., Wei, B., Li, S., & Wang, X. (2018). Toxic effects of different types of zinc oxide nanoparticles on algae, plants, invertebrates, vertebrates, and microorganisms. *Chemosphere*, *193*, 852–860. https://doi.org/10.1016/j.chemosphere.2017.11.077

Imai, S., Koyama, J., & Fujii, K. (2007). Effects of estrone on full life cycle of Java medaka (*Oryzias javanicus*), a new marine test fish. *Environmental Toxicology and Chemistry, 26*(4), 726-731. https://doi.org/10.1897/05-539R2.1

Ismail, A., & Yusof, S. (2011). Effect of mercury and cadmium on early life stages of Java medaka (*Oryzias javanicus*): A potential tropical test fish. *Marine Pollution Bulletin*, *63*(5-12), 347-349. https://doi.org/10.1016/j.marpolbul.2011.02.014

Iwamatsu, T., & Kobayashi, H. (2002). Electron microscopic observations of karyogamy in the fish egg. *Development, Growth and Differentiation*, *44*(5), 357-363. https://doi.org/10.1046/j.1440-169X.2002.00649.x

Kashiwada, S. (2006). Distribution of nanoparticles in the see-through medaka (*Oryzias latipes*). *Environmental Health Perspectives*, *114*(11), 1697–1702. https://doi.org/10.1289/ehp.9209

Kaya, H., Aydin, F., Gürkan, M., Yılmaz, S., Ates, M., Demir, V., & Arslan, Z. (2016). A comparative toxicity study between small and large size zinc oxide nanoparticles in tilapia (*Oreochromis niloticus*): Organ pathologies, osmoregulatory responses, and immunological parameters. *Chemosphere*, *144*, 571-582. https://doi.org/10.1016/j.chemosphere.2015.09.024

Khandoga, A., Stampfl, A., Takenaka, S., Schulz, H., Radykewicz, R., Kreyling, W., & Krombach, F. (2004). Ultrafine particles exert prothrombotic but not inflammatory effects on the hepatic microcirculation in healthy mice *in vivo*.

Circulation, *109*(10), 1320–1325. https://doi.org/10.1161/01.CIR.0000118524.62298.E8

Khoshnood, R., Jaafarzadeh, N., Jamili, S., Farshchi, P., & Taghavi, L. (2016). Acute toxicity of TiO_2, CuO and ZnO nanoparticles in brine shrimp, *Artemia franciscana*. *Iranian Journal of Fisheries Sciences*, *16*(4), 1287–1296.

Kiener, T. K., Selptsova-Friedrich, I., & Hunziker, W. (2008). *Tjp3/ZO-3* is critical for epidermal barrier function in zebrafish embryos. *Developmental Biology*, *316*(1), 36–49. https://doi.org/10.1016/j.ydbio.2007.12.047

Laban, G., Nies, L. F., Turco, R. F., Bickham, J. W., & Sepúlveda, M. S. (2010). The effects of silver nanoparticles on fathead minnow (*Pimephales promelas*) embryos. *Ecotoxicology*, *19*, 185-195. https://doi.org/10.1007/s10646-009-0404-4

Lee, K. J., Nallathamby, P. D., Browning, L. M., Osgood, C. J., & Xu, X.-H. N. (2007). *In vivo* imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. *ACS Nano*, *1*(2), 133–143. https://doi.org/10.1021/nn700048y

Li, J., Chen, Z., Huang, R., Miao, Z., Cai, L., & Du, Q. (2018). Toxicity assessment and histopathological analysis of nano-ZnO against marine fish (*Mugilogobius chulae*) embryos. *Journal of Environmental Sciences*, *73*, 78-88. https://doi.org/10.1016/j.jes.2018.01.015

Murthy, M, K., Mohanty, C, S., Swain, P., & Pattanayak, R. (2022). Assessment of toxicity in the freshwater tadpole *Polypedates maculatus* exposed to silver and zinc oxide nanoparticles: A multi-biomarker approach. *Chemosphere*, *293*, 133511, https://doi.org/10.1016/j.chemosphere.2021.133511

Organization for Economic Co-operation and Development. (2013). *Test No. 210: Fish, early-life stage toxicity test*. OECD. https://www.oecd-ilibrary.org/docserver/9789264203785-en.pdf?expires=1715742309&id=id&accname=guest&checksum=DBDC65B79C5369E61BACCAD90C9B798C

Patibandla, S., Zhang, Y., Tohari, A. M., Gu, P., Reilly, J., Chen, Y., & Shu, X. (2018). Comparative analysis of the toxicity of gold nanoparticles in zebrafish. *Journal of Applied Toxicology*, *38*(8), 1153-1161. https://doi.org/10.1002/jat.3628

Peters, A., Dockery, D. W., Muller, J. E., & Mittleman, M. A. (2001). Increased particulate air pollution and the triggering of myocardial infarction. *Circulation*, *103*(23), 2810–2815. https://doi.org/10.1161/01.CIR.103.23.2810

Peters, L. E., MacKinnon, M., Van Meer, T., van den Heuvel, M. R., & Dixon, D. G. (2007). Effects of oil sands process-affected waters and naphthenic acids on yellow perch (*Perca flavescens*) and Japanese medaka (*Orizias latipes*) embryonic development. *Chemosphere*, *67*(11), 2177–2183. https://doi.org/10.1016/j.chemosphere.2006.12.034

Rajkumar, K. S., Sivagaami, P., Ramkumar, A., Murugadas, A., Srinivasan, V., Arun, S., Kumar, P. S., & Thirumurugan, R. (2022). Bio-functionalized zinc oxide nanoparticles: Potential toxicity impact on freshwater fish *Cyprinus carpio*. *Chemosphere*, *290*, 133220. https://doi.org/10.1016/j.chemosphere.2021.133220

Rajput, V. D., Minkina, T. M., Behal, A., Sushkova, S. N., Mandzhieva, S., Singh, R., Gorovtsov, A., Tsitsuashvili, V. S., Purvis, W. O., Ghazaryan, K. A., & Movsesyan, H. S. (2018). Effects of zinc-oxide nanoparticles on soil, plants, animals, and soil organisms: A review. *Environmental Nanotechnology, Monitoring and Management*, *9*, 76-84. https://doi.org/10.1016/j.enmm.2017.12.006

Sabir, S., Arshad, M., & Chaudhari, S. K. (2014). Zinc oxide nanoparticles for revolutionizing agriculture: Synthesis and applications. *The Scientific World Journal*, *2014*, 925494. https://doi.org/10.1155/2014/925494

Salleh, A. F. M., Amal, M. N. A., Nasruddin, N. S., Zulkifli, S. Z., Yusuff, F. M., Ibrahim, W. N. W., & Ismail, A. (2017). Water pH effects on survival, reproductive performances, and ultrastructure of gonads, gills, and skins of the Java medaka (*Oryzias javanicus*). *Turkish Journal of Veterinary and Animal Sciences*, *41*(4), 471-481. https://doi.org/10.3906/vet-1701-9

Sirelkhatim, A., Mahmud, S., Seeni, A., Kaus, N. H. M., Ann, L. C., Bakhori, S. K. M., Hasan, H., & Mohamad, D. (2015). Review on zinc oxide nanoparticles: Antibacterial activity and toxicity mechanism. *Nano-Micro Letters*, *7*, 219–242. https://doi.org/10.1007/s40820-015-0040-x

Suman, T. Y., Radhika Rajasree, S. R., & Kirubagaran, R. (2015). Evaluation of zinc oxide nanoparticles toxicity on marine algae *Chlorella vulgaris* through flow cytometric, cytotoxicity and oxidative stress analysis. *Ecotoxicology and Environmental Safety*, *113*, 23–30. https://doi.org/10.1016/j.ecoenv.2014.11.015

Taherian, S. M. R., Hosseini, S. A., Jafari, A., Etminan, A., & Birjandi, M. (2019). Acute toxicity of zinc oxide nanoparticles from *Satureja hortensis* on rainbow trout (*Oncorhynchus mykiss*). *Turkish Journal of Fisheries and Aquatic Sciences*, *20*(6), 481-489. https://doi.org/10.4194/1303-2712-v20_6_06

Wittbrodt, J., Shima, A., & Schartl, M. (2002). Medaka - A model organism from the far east. *Nature Reviews Genetics*, *3*, 53-64. https://doi.org/10.1038/nrg704

Wong, S. W. Y., Leung, P. T. Y, Djurišić, A. B., & Leung, K. M. Y. (2010). Toxicities of nano zinc oxide to five marine organisms: influences of aggregate size and ion solubility. *Analytical and Bioanalytical Chemistry*, *396*, 609-618. https://doi.org/10.1007/s00216-009-3249-z

Woo, S., & Yum, S. (2011). Transcriptional response of marine medaka (*Oryzias javanicus*) on exposure to toxaphene. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, *153*(3), 355-361. https://doi.org/10.1016/j.cbpc.2010.12.006

Wu, Y., & Zhou, Q. (2012). Dose- and time-related changes in aerobic metabolism, chorionic disruption, and oxidative stress in embryonic medaka (*Oryzias latipes*): Underlying mechanisms for silver nanoparticle developmental toxicity. *Aquatic Toxicology*, *124–125*, 238–246. https://doi.org/10.1016/j.aquatox.2012.08.009

Wu, Y., Zhou, Q., Li, H., Liu, W., Wang, T., & Jiang, G. (2010). Effects of silver nanoparticles on the development and histopathology biomarkers of Japanese medaka (*Oryzias latipes*) using the partial-life test. *Aquatic Toxicology*, *100*(2), 160-167. https://doi.org/10.1016/j.aquatox.2009.11.014

Xiao, Y., Vijver, M. G., Chen, G., & Peijnenburg, W. J. G. M. (2015). Toxicity and accumulation of Cu and ZnO nanoparticles in *Daphnia magna*. *Environmental Science and Technology*, *49*(7), 4657–4664. https://doi.org/10.1021/acs.est.5b00538

Xiong, D., Fang, T., Yu, L., Sima, X., & Zhu, W. (2011). Effects of nano-scale TiO_2, ZnO, and their bulk counterparts on zebrafish: Acute toxicity, oxidative stress and oxidative damage. *Science of the Total Environment*, *409*(8), 1444-1452. https://doi.org/10.1016/j.scitotenv.2011.01.015

Yu, J.-F., Fukamachi, S., Mitani, H., Hori, H., & Kanamori, A. (2006). Reduced expression of *vps11* causes less pigmentation in medaka, *Oryzias latipes*. *Pigment Cell Research*, *19*(6), 628–634. https://doi.org/10.1111/j.1600-0749.2006.00346.x

Zhao, X., Wang, S., Wu, Y., You, H., & Lv, L. (2013). Acute ZnO nanoparticles exposure induces developmental toxicity, oxidative stress, and DNA damage in embryo-larval zebrafish.

Aquatic Toxicology, *136-137*, 49-59. https://doi.org/10.1016/j.aquatox.2013.03.019

Zhu, X., Wang, J., Zhang, X., Chang, Y., & Chen, Y. (2009). The impact of ZnO nanoparticle aggregates on the embryonic development of zebrafish (*Danio rerio*). *Nanotechnology*, *20*(19), 195103. https://doi.org/10.1088/0957-4484/20/19/195103

Zhu, X., Zhu, L., Duan, Z., Qi, R., Li, Y., & Lang, Y. (2008). Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to zebrafish (*Danio rerio*) early developmental stage. *Journal of Environmental Science and Health, Part A*, *43*(3), 278-284. https://doi.org/10.1080/10934520701792779

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